RESPIRATORY VALUES
DURING EMBRYONIC DEVELOPMENT
OF *GINKGO biloba* L.

PROCUREMENT AND ASSESSMENT OF RESPIRATORY VALUES PERTINENT TO EARLY EMBRYONIC DEVELOPMENT OF *GINKGO biloba* L.

By

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SCOPE AND CONTENTS:

An approach is developed for determining respiratory values of ovules and embryos of Ginkgo biloba L. An analysis of respiratory drift is supplemented by secondary experiments on factors influencing respiration as affecting growth.

PREFACE

This work was undertaken as part of a program of experimental embryology on some selected Gymnosperms. Ginkgo biloba L., generally regarded as among the most primitive of the Gymnosperms, was selected for study because of mechanical convenience mainly, and not for phylogenetic reasons. Nevertheless, its primitiveness was also thought to be of significance because post-zygotic ontogeny in this form is simple, and organ initiation is not deferred or complicated by the secondary imposition of an elaborate suspensorial system which the higher Gymnosperms possess.

There is now support for the view that post-zygotic ontogenetic pattern can be somewhat manipulated by modification of environment. (15) Yet the ultimate explanation for this is not available. Probably the main reason for this is that no one has yet devised an approach to the understanding of change in the immediate environment of the very young embryo; indeed that there is change in the normal environment is really only an assumption. The establishment of validity for this assumption is of concern in the present work.

I would like to express thanks and sincere appreciation to Dr. N. W. Radforth, Professor of Botany, McMaster University, for his guidance and enthusiastic suggestions.

Also, I am indebted to McMaster University for awarding me a Research Assistantship without which this work would not have been undertaken.

TABLE OF CONTENTS

INTRODUCTION

A scarcity of knowledge about fundamental mechanisms of growth control on small groups of cells of the higher plants suggests the empirical purpose of this investigation.

It has long been known that wherever new protoplasm is produced, high respiratory and growth rates go hand in hand. In spite of this commonly known fact there is surprisingly little evidence linking respiration with growth. (2). However, two sets of experiments in particular do much to convince us that a close correlation between respiratory rate and growth exists. Firstly, in an extensive study on Avena coleoptiles Bonner reports a 40 and 30 per cent stimulation of growth and respiration respectively. (2). Nickel observed that O_2 consumption was stimulated 40 to 60 per cent while growth attained with this, is 20 per cent over control. These experiments were carried out with varying concentrations of growth hormones. In yet another experiment O_2 consumption increased by 20 per cent and greatest growth was also 20 per cent above control. (1).

Using a wide range of plants, Stiles and Leach have made determinations of the changes in the respiratory quotient (R. Q.) during the early stages in the germination of seeds which contain different food reserves, for example, starch, hemicellulose, and fatty oil. (16). They found that in each species examined, the quotient changed during development. These changes were attributed to the fact that different food reserves were used at different stages in development. Thus with maize, which contains little sugar, much starch and some fat, they found that

the quotient fell from an initial value of unity to 0.75 and later rose to unity once more. It was suggested that initially sugar acted as the respirable substrate, that fat was oxidized later and finally that starch was consumed. Similar experiments were done by F. N. Craig on Lupinus albus, by Leach on wheat, by Merry and Goddard on barley grain and seedlings, and by Mayer on lettuce seeds (3,4,10,13,12) Their investigations explain the significance of respiratory quotient. They do not specify for stages far in advance of the germination period.

W. O. James describes experimentation with germinating seedlings of the grass family which follows normal growth and respiratory rates as the embryos develop. (7). The present work bears some resemblance to that of James in that considerations of respiratory values pertinent to embryonic environment are also of concern. However, this investigation focuses on early, rather than late, development of the embryos and here no fundamental approach for respiratory comparisons has been established. Although experiments on respiratory drift are to be anticipated, the main theme will of necessity be on the development of method that will lead to an understanding of biological principles at present out of reach.

In a literature survey on plant embryo growth N. K. Ziebur summarizes by saying that the field is relatively underdeveloped. (19) "A number of workers have had a variety of results with different materials, and it is difficult to draw from their data any conclusions as to the important factors which in general influence the growth of plant embryos." In this work Ginkgo biloba L. is used in the hope of elucidating implications which play a rôle in early growth phenomena. Specifically, these implications have resolved into the following several questions, the first

of which became primary as an objective in this work with the others secondary but nevertheless significant as subsidiary aims.

a. Is it possible to establish an approach whereby assessment of change in the immediate environment of the very young embryo can be achieved? Respiratory activity is used as an indication of change in the immediate environment because, of all factors, it is perhaps simplest to assess, and is usually intimately associated with other biological events.

b. If such an approach reveals valid and pertinent results, what part of the total respiration of the intact gametophyte is contributed by the embryo (sporophyte)?

c. What is the effect of wounding parental tissues relative to respiratory values, should these be obtainable?

d. Do individual gametophytes under identical circumstances vary with respect to gaseous exchange?

e. Do light conditions significantly affect respiratory values?

With the terms of investigation designated, appropriate preparation of material for experimental purposes had to be considered.

MATERIALS AND PROCEDURES

Collection and Storage

On the basis of earlier experience in an investigation with different purpose (Radforth 1936), ovules were gathered in Mount Pleasant Cemetery, Toronto, between mid-August and mid-October 1957. (14). Following picking, the ovules were immediately transferred to cold storage at 2°C. Some 1000 ripe ovules were collected on Oct. 17 from a tree on Markland Street, Hamilton. These could be brought down by vigorous shaking of the tree rather than by a laborious hand-picking procedure. A third group was obtained indirectly from China, having been imported by a Toronto merchant. This last group of seeds had been air dried.

Dissection

The objectives direct attention to a precise locus of activity, the region containing the specific area of activity in which parental (gametophytic) and embryonic (sporophytic) tissues are situated side by side. Therefore, isolation of all but these two kinds of tissue was a first requirement. In some cases, when wounding of gametophytic tissue or emphasis on post-zygotic mass was contemplated, further dissection was required. In all cases the procedure of preparation first entailed separating the female gametophyte from all three layers of the integument. Care was taken to remove the inner fleshy coat which upon ripening becomes a tightly clinging brown membrane enveloping the soft gametophyte. The gametophytes were then ready for the next step in the procedure, the respiratory analysis.

Methods of Gas Analysis

Experimentation on respiratory analysis was carried out with the use of a standard Bronwill Warburg apparatus. Two methods of operating this apparatus are described in the literature. The first one is called the indirect method of Warburg, the second the direct method. (18). The former depends upon differential gas absorption because of contrasting amounts of medium in correlated vessels, the latter affords direct measurement of oxygen loss. Both methods as well as a third one have been used. Presumably no detailed explanation of the first two is necessary as they are well known and widely accepted. The third method is the writer's adaptation based on the principle of the direct method with this difference that, instead of having two vessels with two different tissue samples, one containing alkali, the other having no absorptive agent, only one mass of tissue with one vessel is used. This mass is first measured for O_2 consumption plus CO_2 liberation with no alkali in the vessel then for O_2 consumption only, with alkali in the same vessel. In no case does the alkali touch the tissue.

Methods Pertaining to Experiments on Drift

The group of ovules collected for this purpose was collected on October 17, '57 and kept in cold storage until January 7, '58, when the first drift experiment began. A second experiment was started on February 18, '58. The material of this experiment differed in no way from that of the first, except in time in cold storage. The second experiment was designed to check the first.

To provide material for each experiment a large group of ovules was placed in vermiculite and earth, and kept in a greenhouse at room

temperature. On each day of a test measurement two ovules were removed from this group and analysed with the direct method.

After completion of the gas analysis the tissues were fixed in Lavdovsky's fluid, for three hours, under continuous shaking. (5). The pieces of tissue were then removed and placed in 70% alcohol for storage. This made possible an accumulation for handling material on a larger and thus more efficient scale. Butyl alcohol was used for the dehydration process. The material was embedded in tissue mat and sectioned to a thickness of 12 to 20 μ. The sections were fixed to slides using 1% aqueous albumen. (9) A combination of fast green in 95% ethanol and borax carmine, also in 95% ethanol, was used to stain cell walls and nuclei respectively. Following a rinse in absolute alcohol the sections were permanently mounted in Euparal.

Methods Used to Isolate Special Implicated Factors

One objective calls for separation of the proembryo from the parental food mass. To achieve this 4 oblique incisions were made to remove the two archegonia with their immediate surroundings en bloc. The total mass thus removed from the gametophyte measured a little less than 4 by 3 by 3 mm.

For the excision of more advanced embryos, the gametophyte was carefully incised along the median plane, to a depth of 3 to 4 mm. The two halves were then grasped and forced apart by hand, exposing the embryo which separated from the surrounding tissue with no adherence of the latter.

Experimentation concerning another factor, wound respiration, was initiated by cutting two gametophytes longitudinally and placing the cut

surfaces of both halves of each exposed in their vessels.

To test for natural variation, two analyses were made in close succession under identical conditions. This procedure was repeated a number of times on gametophytes in various stages of development.

In order to assess for influence of light, two 100 watt bulbs were suspended directly above the constant temperature bath. The distance between bulb and tissue amounted to 4 inches. This situation contributed the maximum of light; to obtain the minimum, the experiment was repeated in the dark.

RESULTS

Difficulties Characteristic to Plant Material

Fresh material of Ginkgo, ontogenetically appropriate, can be secured only once a year. This posed experimental difficulties, especially when it was discovered, as will be shown later, that apparently a long period of cold storage of the ovules had a markedly deleterious effect on their capacity to develop. Thus, experiments concerning drift could not be verified as equivalents because material identical to the original was not guaranteed. Indeed, it could be assumed that reproduction of the experiments on drift was done with material altered to an unknown extent by a longer time of storage.

Another difficulty was provided by a low percentage of fertilization in this locality. This lowered the uniformity of the material to the point at which each unit, composed of gametophyte with sporophyte initials, had to be treated on an individual basis.

Effectiveness of Storage

Cold storage at 2°C. proved satisfactory until some time in February. From a high of about 25% growth response, (determined by removing ovules to room temperature) in early January, a necessarily lower percentage of proembryos developed in proportion to age in cold storage until late February when none were found to respond to higher temperatures. These results are parallel with those of Hatano and Kano who found that, using a storage temperature of 5°C., embryos collected in October elongated until January of the next year. (6).

Of those ovules of Chinese origin that had been placed in moist earth at room temperature, 15% germinated. Thus it can be claimed that the ovules from China had been effectively preserved much longer, probably by partial dehydration. The gametophytes of this group were still turgid, completely filling the cavity within the hard seed coat. Those in which the gametophyte had shrunk to some fraction of its original volume were generally not capable of germination, although on dissection all sporophytes were found to be arrested at the same stage of development, namely that just before emergence of the sporophytic root. Germination occurred for a period of three months after addition of water; this indicates the wide variations in rate of growth that occur in Ginkgo.

Suitability of Methods of Gas Analysis

Experimentation using the indirect method of Warburg at first gave rise to unexplainably large variations in manometric readings, a situation that was apparently unrelated to cold storage effect (cf. loc. cit. p. 10, Fig. 4, p. 14). Later, a certain measure of stability in readings was obtained. However, though discrepancies were reduced the results were thought to have questionable validity as the following example will suggest. On Jan. 1, '58 an R. Q. of 6.1 was found and on Jan. 3, '58, an R. Q. of 7.1. In both instances the material had been removed from cold storage for a few hours before the gas analysis.

Determinations showing greater dependability were thus desirable.

It has been found that a difference in CO_2 concentration of the atmosphere used in respiration, provided the concentrations are low, has little effect on the rate of respiration. (18). This obviates any serious errors that might cling to the second, or direct, method. Analyses

based on the application of the direct method were considerable in number. They effectively revealed the occurrence of respiratory drift (cf. Figs. 1, 2, 3, 5 which show the results of direct method analyses).

A prime function of the methods employed is the Warburg apparatus itself. An expression of its accuracy is therefore desirable, and accordingly this is provided in the graph Fig. 4. To procure the graph, data derived from direct method applications for short time intervals have been used.

Slight inaccuracy was experienced with the direct method when it was used to determine the amount of variation between individuals (p.18). This led to the development and adoption of the third method with which such inaccuracies were avoided. Suitability of method was, of course, determined largely on the basis of preliminary results. Analyses to follow were undertaken largely to substantiate the choice of method, and to justify their application for expanded work in the future.

Experiments on Drift

The direct method, in nearly fifty applications, revealed results summarized in the graphs Figs. 1, 2, 3, 4, 5. It will be noted that, where O_2 uptake and CO_2 liberation were assessed in terms of drift, several months were involved (Figs. 1, 5, p.21). The data of Fig. 3, though of a separate set of experiments, are complementary to those in Fig. 1, in the sense that they follow the drift trend exhibited at the end of the time sequence for Fig. 1.

Fig. 4 shows that on the basis of the direct method, R. Q. values derived from data of Figs. 1 and 2 vary within limits of 0.7 and 1.4.

Results based on the application of the third method are now

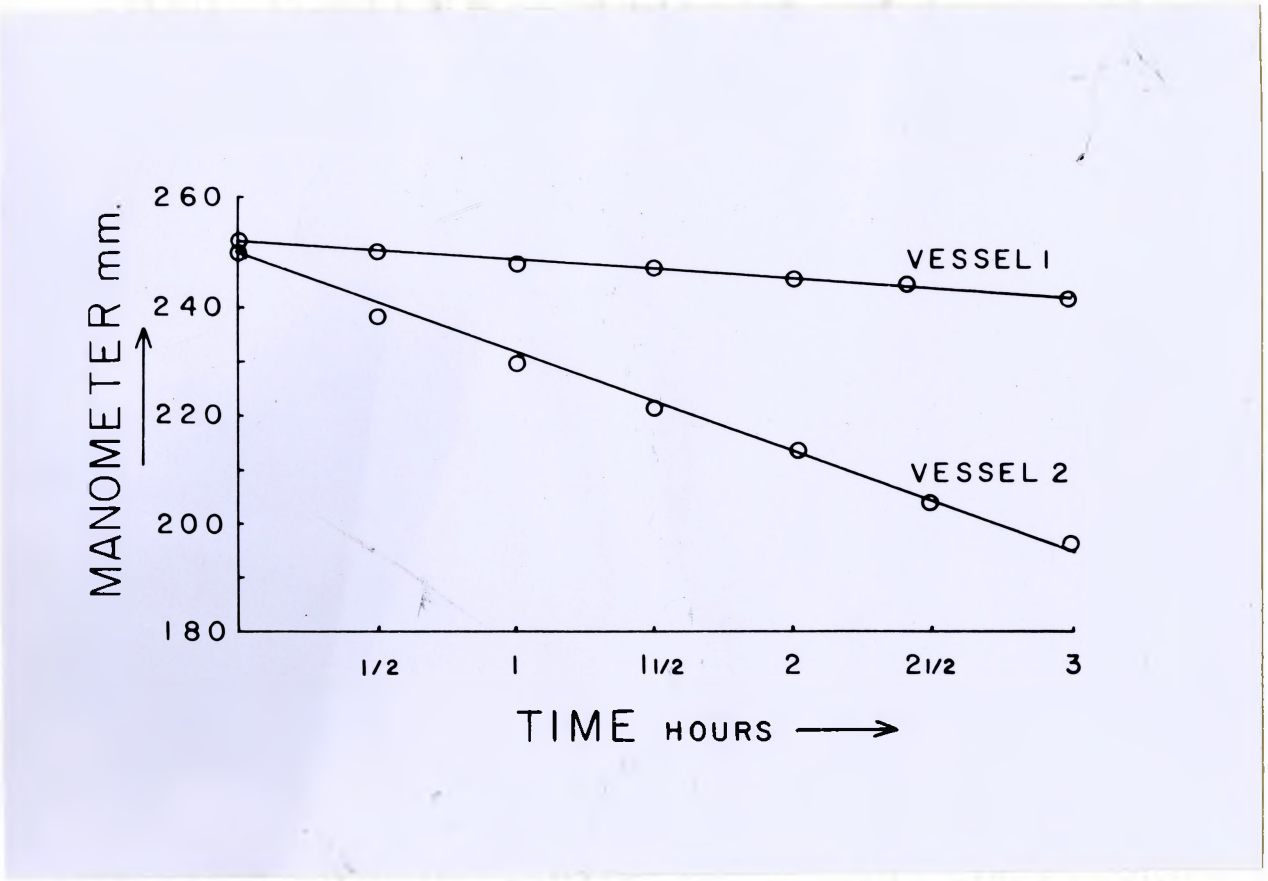

Fig. 1 Graph demonstrating consistency of performance of Warburg apparatus becoming evident when respiratory rate is constant.

Note: Average deviation for vessel 1 - 1%;
for vessel 2 - 3%.

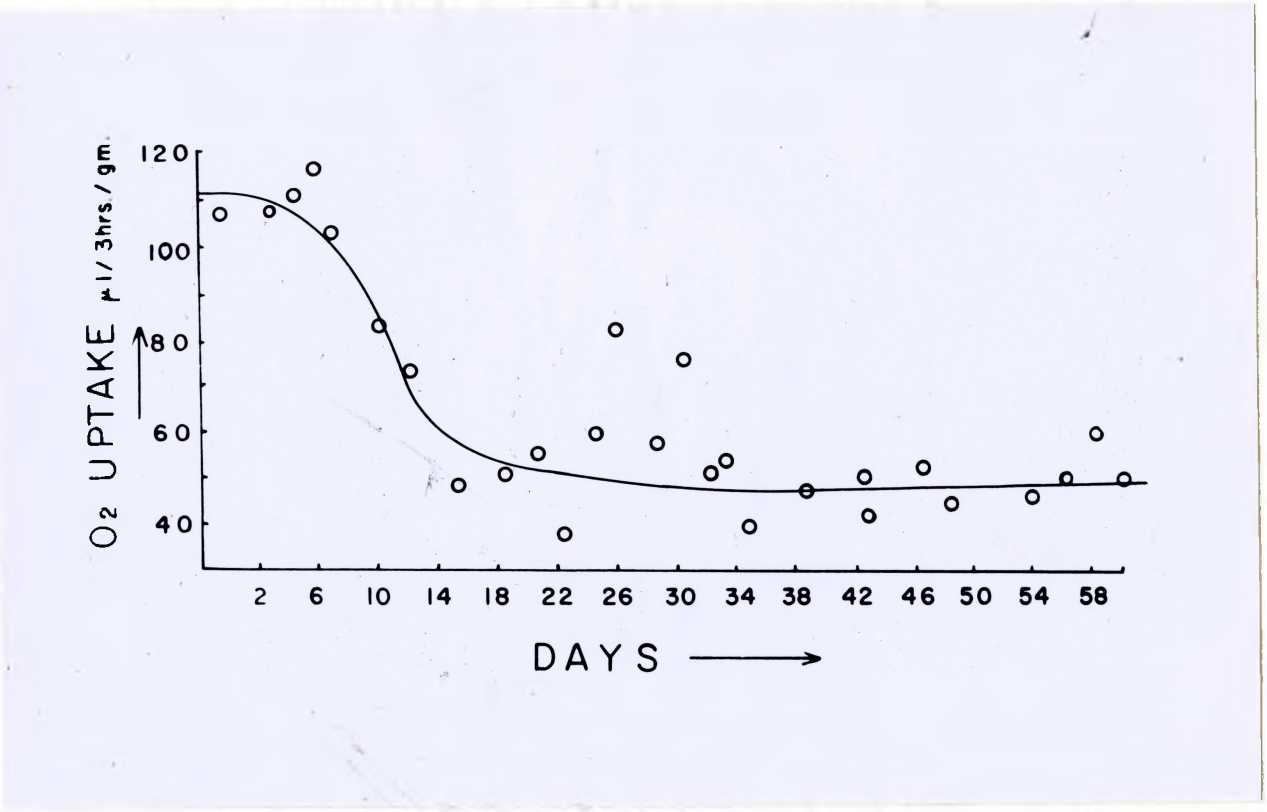

Fig. 2 Graph showing the drift of Oxygen consumption as determined by the direct method.
Experiment I - Jan. 11 - March 4, 1958.

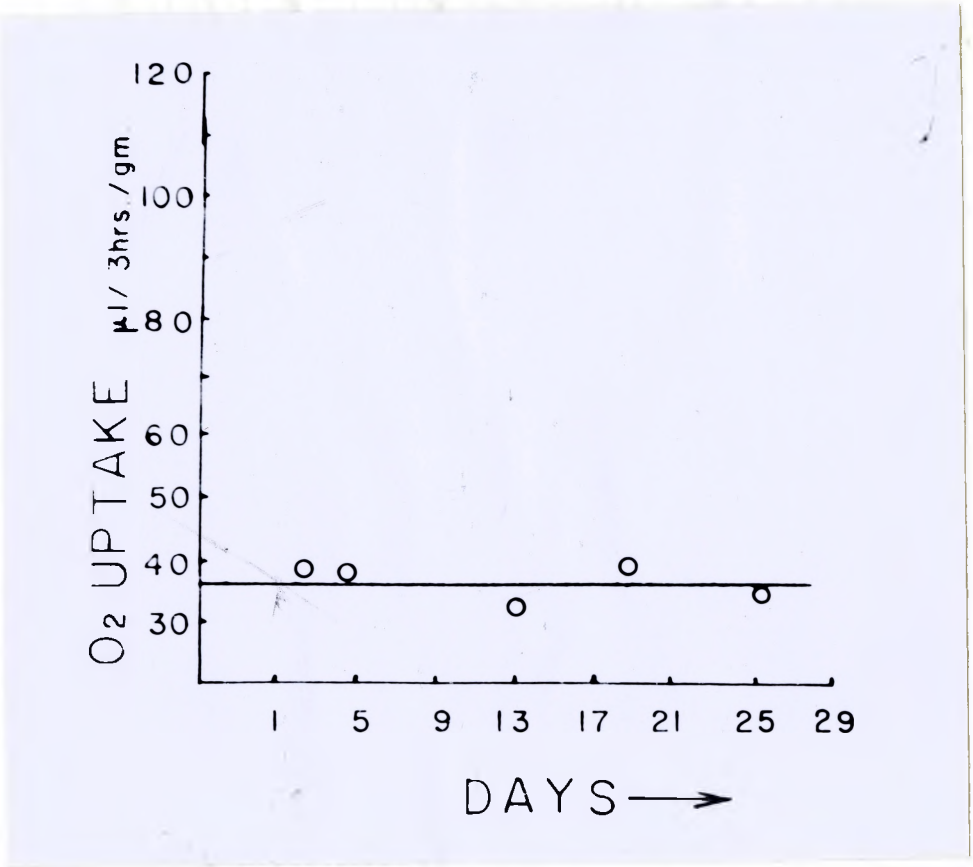

Fig. 3 Graph showing drift of O_2 consumption as determined by the direct method.
Experiment II - Feb. 18 - Mar. 14, 1958

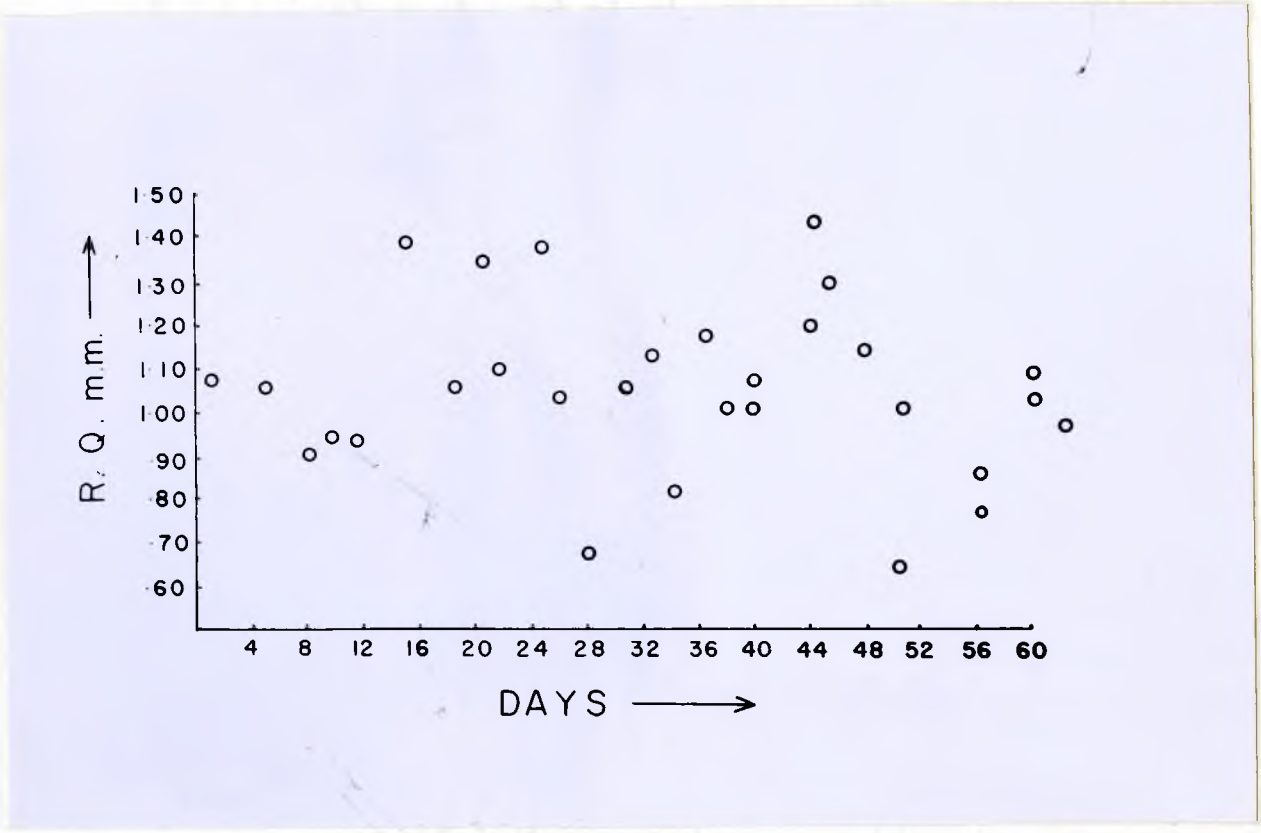

Fig. 4 Graph showing a pattern of the Drift of R.Q. as determined by the direct method.
Experiments I and II - Jan. 11 - March 14, 1958.

submitted. First among these are those obtained from the material of Chinese origin. The data shown are derived from a fruit that had been kept in moist earth at greenhouse temperatures for 18 days. The embryo was still completely enclosed in the gametophyte. On May 27, 1958 it measured 16 mm. in length including the cotyledons.

TABLE I

RATE OF RESPIRATION OF

	The Intact Gametophyte		The Embryo		The Gametophyte Only	
O_2	49	μl/3hrs./gm.	1269	μl/3 hrs./gm.	80	μl/3 hrs./gm.
CO_2	50	"	1375	"	98	"
R.Q.	1.03		1.08	"	1.22	

The reading in Table I for the intact gametophyte corresponds to those plotted on the graph Fig. 2. If extrapolated, it would fall in early April on the graph of experiment I, Fig. 2, agreeing with the constant level beginning in late April, and thus still present in April. It is to be understood that only the first column refers to the experiment on drift, the others will be referred to in another connection.

On June 4, 1958, another gametophyte was analysed which contained a 25 mm. embryo. 10 mm. of the root protruded from the gametophyte. O_2 consumption was 288 μl/3 hrs./gm.; CO_2 liberation 250 μl/3 hrs./gm., and the R.Q. 0.87. This ovule had been in the greenhouse for 26 days.

After 81 days a surviving embryo from cold storage, 10.8 cm. long, showed an O_2 consumption of 338 μl/3 hrs./gm. for the whole mass of the seedling and gametophyte. CO_2 liberation occurred at the rate of 296 μl/3 hrs./gm. The R.Q. was 0.87. The gametophyte had shrunken to four-

fifths its original size, but it was quite intact and fully turgid, the tip of the stem had become green, and the seedling barely fitted in the 20 cc. vessel. This embryo was from the Markland ovules removed from cold storage on Feb. 18, 1958.

Effect of Implicated Factors

Embryo respiration as a single factor bearing on the total rate of gaseous exchange of the gametophyte is expressed on a comparative basis. From an experiment conducted on Feb. 4, 1958 with material from the same lot as that used in the first drift experiment, and which at this stage had been at greenhouse temperature for 26 days, the contribution of the embryo was derived thus:

TABLE II

RESPIRATORY RATE OF

	The Intact Gametophyte	The Embryo*	The Gametophyte Only
O_2 (1)	30.8 μl/3 hrs./gm.	1020 μl/3 hrs./gm.	61.5 μl/3 hrs./gm.
Weight (2)	1.733 gm.	0.035 gm.	1.383 gm.
Total Respiration (Product of 1 x 2)	53.4 units	35.8 units	85.2 units

The total respiration of the gametophyte plus embryo is 121.0 units. Of this, the respiration of the embryo is 29.6%. By weight the embryo contributes 2.5% of the total mass. The volume of the embryo at this stage, which is characterized by the formation of cell walls (cf. Plate 8), is actually not more than 2 mm^3.

* with adhering gametophytic remnants.

The direct method was used in this experiment; because of this the O_2 consumption datum is the only reliable one (cf. p. 26).

From the data of Table I, for which the third method was used, the contribution of a more advanced embryo may be assessed. To assist in this Table III was prepared.

TABLE III

RESPIRATORY RATE OF

	The Intact Gametophyte	The Embryo	The Gametophyte Only
O_2 (1)	49 µl/3 hrs./gm.	1296 µl/3 hrs./gm.	80 µl/3 hrs./gm.
Weight (2)	1.795 gm.	0.0555 gm.	1.746 gm.
Total Respiration (Product of 1 x 2)	88.0 units	70.5 units	139.5 units

The embryo contributes 34% of the respiration of the total after separation.

By weight the 13 mm. embryo, which separated completely and easily from the food mass, is 3.1% of the total.

The Effect of Wounding.

To assess the effect of wounding as an implication in the derivation of embryo respiration the first of the results of the experiments on separation (Table II p. 16) will be utilized because wound surfaces were produced with the separation of the embryonic mass. It is perhaps well to emphasize that, because the direct method was used, CO_2 and R.Q. figures were not as accurate as the O_2 figure (cf. p. 26).

The total respiration for the intact gametophyte is 45 units;

the total for the two wounded masses was 110 units, of which 83 were from the passive food mass, weighing 1.383 gm., and 27 from the active embryo with adjacent tissues, weighing only .035 gm. Thus, the increase in O_2 consumption due to wounding is 110-45, or 65 units. This is an increase of 145% over the original 45 units.

The following data are from a so-called "degenerate" gametophyte in which the tissue was intact, but which contained an unfertilized, degenerated egg cell. O_2 consumption was 28.8 μl/3 hrs./gm. CO_2 liberated was 110 μl/3 hrs./gm. and the R.Q. was 1.42. This constitutes an increase of 170% in O_2 consumption.

The third, and last of these experiments, concerns the dissection of a gametophyte done in order to remove the 16 mm. long embryo. The data from which the effect of wounding is derived are shown on p. 15. Because the third method was used, the CO_2 figure, and consequently the R.Q. figure, is as accurate as the O_2 figure. In O_2 consumption there is an increase of 163%, in CO_2 liberation, 195%, and in R.Q. an 18.5% increase.

The Variation Between Individual Gametophytes

All of the following results are based on the application of the direct method. The first of these data are derived from an analysis done on Feb. 12, 1958 with material from the same lot as that used in the first drift experiment. Column 1 consists of data from the first analysis, Column 2 of those from the succeeding one. There was no time lapse between the two.

The variation in O_2 consumption is 84%, in CO_2 liberation it is 8%, and in R.Q., 1.2%.

TABLE IV

	Column 1	Column 2
O_2 consumption	51.3 μl/3 hrs./gm.	47.2 μl/3 hrs./gm.
CO_2 liberation	41 "	37 "
R.Q.	0.80	0.79

The second experiment was done on May 1 with material from the same lot used above. The two ovules are both "degenerates". There is a time lapse of 12 hours between Columns 1 and 2, during which the gametophytes were kept in their respective vessels.

TABLE V

	Column 1	Column 2
O_2 consumption	132 μl/3 hrs./gm.	96 μl/3 hrs./gm.
CO_2 liberation	150 "	110 "
R. Q.	1.14	1.15

The variation in O_2 consumption is 27%, in CO_2 liberation it is also 27% and in R. Q. it is 1%.

The third experiment provides data obtained by reversing the gametophytes in their respective vessels, and then during the succeeding run. It was done on May 5, 1958 with material from the same lot as the above.

Because the direct method is used here the procedure of reversing gametophytes is theoretically equal to repeating the experiment without reversal (c f. p. 20).

TABLE VI

	Column 1	Column 2
O_2 consumption	76 μl/3 hrs./gm.	66 μl/3 hrs./gm.
CO_2 liberation	110 "	100 "
R.Q.	1.45	1.51

The variation in O_2 consumption is 13%. In CO_2 liberation 9% and in R. Q. 4%.

The Effect of Fertilization upon Respiration of the Gametophyte

The results given below (Table VII) were extracted from the data derived in the course of the experiments on drift. The material and methods pertaining to this experiment are therefore identical with those of the experiment on drift.

To appreciate the results, reference should be made to the fact that the writer has, for convenience, recognized six "stages" of development. Three represent pre-fertilization states and three are post-fertilized ones. The meaning attached to these stages is expressed later where ontogenetic classification is described (p. 38).

TABLE VII

Days at Greenhouse Temperature	Stage No.	Rate of O_2 Uptake	R.Q.
4	3 fertilized	83 μl/3 hrs./gm.	
	2 unfertilized	71 "	
20	5 fertilized	62.5 "	1.10
	1 unfertilized	51 "	1.35
42	5 fertilized	50 "	1.05
	1 unfertilized	37 "	1.41
64	5 fertilized	48 "	1.00
	1 unfertilized	31 "	1.51
85	25 mm. fertilized	288 "	0.87
	1 unfertilized	22.5 "	2.7

The Effect of Fertilization Upon Respiration of the Gametophyte

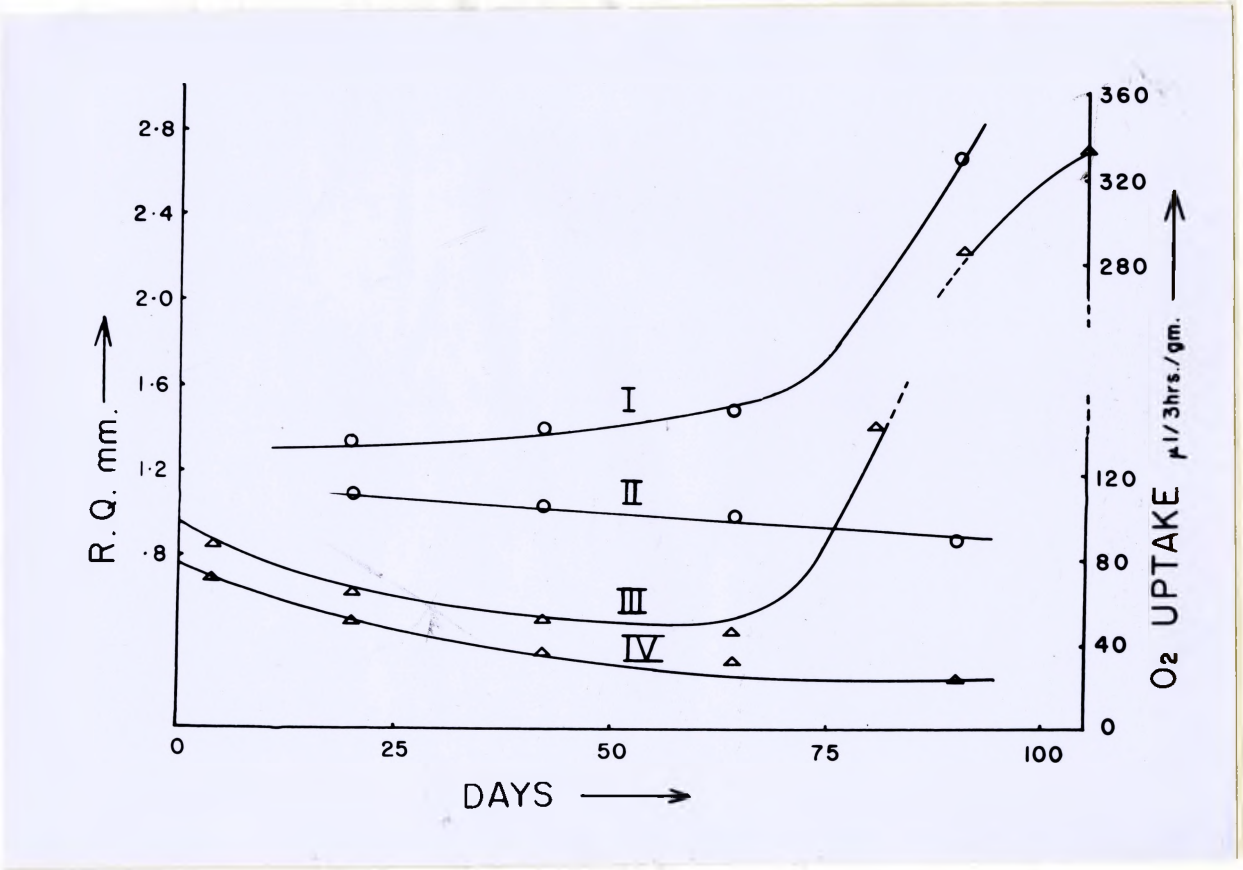

Fig. 5. Graph showing the effect of fertilization upon respiration of the gametophyte.

NOTE: Curve I showing slow increase of R.Q. with age of unfertilized ovule, after 60 days decay sets in rapidly.

Curve II showing slow decrease of R.Q. with growth of embryo.

Curve III showing drift of O_2 consumption accompanying development of the embryo.

Curve IV showing drift of O_2 consumption accompanying degeneration of the unfertilized gametophyte.

The last entry (Table VII) was obtained from Chinese material. After having been stored dry for an indefinite period this material was kept under the same greenhouse conditions as the other. The two ovules used here had been out of storage for 26 days, and were at a stage corresponding to 85 - 95 days of the Toronto material.

The data of Table VII are graphically represented in Fig. 5, p. 21, to facilitate further comparison.

The Effect of Varying Light Conditions

The data pertaining to the influence of light on the rate of respiration were obtained on Nov. 26, 1957, with two ovules from the group collected on Markland Street. At the time of the experiment they had been in cold storage for 40 days; they were removed from cold storage a few hours prior to the experiment.

Because this experiment was carried out with the indirect method, O_2 consumption data only will be used. In the light it is 120 μl/3 hrs./gm. of O_2. In the dark, 126 μl/3 hrs./gm.

The Ontogenetic Sequence

It will be appreciated that during experimentation, time sequence, not ontogenetic sequence was used as a reference basis. It was possible to reveal ontogenetic stage after gas analysis, but not before.

To give some indication of the various stages of development referred to in preceding results of respiratory analyses a number of microphotographs are used. They illustrate the system of classification.

Plates I - III refer to the development of the egg mass; in each case the larger picture is an enlargement of the smaller. The smaller photo shows the orientation of the egg mass and the larger indicates

structure detail. Fig. 1 is from fresh material collected in Toronto and fixed on Aug. 14, 1957. Fig. 2 is on Sept. 2; Fig. 3 on Sept. 21. There is a gradual enlargement of the archegonium, a solidification of the egg mass and finally the development of a large nucleus. Plates II and III represent stages 0 - 5 respectively, as referred to on page 20. All are from material used in the experiments on drift. Fig. 9 shows the degenerated egg mass lying close to the archegonial wall. Fig. 8 shows the more common and characteristic shrunken "crescent" of the degenerated egg mass. Fig. 7 shows the absence of egg cell and presence of fungal remains which follows the advanced disintegration of the egg mass. Fig. 10 shows the multinucleatic stage of development just preceding the earliest stage to possess cell walls as shown on Fig. 11. Figs. 12 and 13 are cross- and longi-tudinal sections of an embryo a few mm. in length. Fig. 14 is an enlargement of a part of the archegonium showing the greater part of a degenerated egg mass. Fig. 15 is a cross section through a young embryo of stage 5. These last two figures are from fresh material collected in Toronto on Sept. 30 and Oct. 8 respectively. A treatment dealing with stages beyond those dealt with here can be found in Lyon. (11).

DISCUSSION

When preliminary procedures had been developed, and analysis had commenced, there had been hopes that the ovules would have demonstrated uniform and consistent rates of development. That this was not the case may have been, in part, due to the experimentally imposed need to resort to cold storage. Also, if cold storage arrested rather than killed the normally advancing embryos, and if the imposed dormancy could be lifted promptly and regularly the convenience of the procedure would more than justify its use. Indeed this now has been the case to the extent that a qualitative evaluation of the drift of gas exchange could be made.

The hopes that development would be uniform, that is, simultaneous in all fertilized ovules have not been justified by the results. It will bear emphasis that in two cases sporophytes were 8 and 11 mm. long after 81 days of growth, in two others 3 and 5 mm. after the same time under identical circumstances. In view of these findings and others recorded in the results this work must rely for its conclusions on measurements based on the course of development of individual cases.

As observed in results (page 9) the method of partial dehydration for storage purposes is successful over periods of time which cannot be achieved by the cold storage method. However, for short periods of arrested development low temperature is the only alternative. It can completely arrest development at very early stages whereas dehydration allows for considerable initial growth at the expense of food and water

resources of the gametophytic mass. As all the Chinese embryos were in a relatively advanced stage of development with the cotyledons about 15 mm. long they could be used as collateral rather than direct evidence. They constituted the most advanced stages which could be handled with a Warburg.

The indirect method of Warburg was the first to be used because it was thought to offer possibility of greatest accuracy. The advantage it holds is that of measurement under atmospheric conditions which correspond closely to the normal. The main difficulty, it seems, concerned the difference in fluid level in the two manometric vessels used together in an experiment. Whereas the one gametophyte would have 9/10 of its surface exposed to the atmosphere in the vessel, the other would be immersed in fluid medium. On the other hand the difference in fluid level in the two vessels is the essential part of the indirect method and could, as such, not be eliminated. The resulting difference in aeration of the tissues is unavoidable. Shaking the vessels in their constant temperature bath in order to obtain better aeration did not remedy this situation.

Results obtained with the indirect method (page 9) which were unpredictable and erratic can be accounted for by postulating that the submerged condition causes partial anaerobions which, in turn, gives rise to very high R.Q. and low O_2 uptake. This is true only in the case of the vessel with the larger volume of liquid. In the other vessel the gametophyte had the bulk of its surface area above the level of the liquid and diffusion presumably took place at a sufficiently rapid rate. These results showing wide discrepancy, were at first rejected as being

due to erroneous technique. Later a corroboration of these data was found in the form of an experiment done by James and James which showed a parallel situation; (9). The data are obtained by submerging germinating barley seedlings which in aerobic conditions display an R. Q. close to unity. The resulting R.Q. was 7.0 in this case. This result offers both an explanation and a good support for the above postulate.

Repeated experiments have shown the direct method to be stable and reliable and well suited to the material. However, this method does not take into account the natural variation in respiratory rate among various masses of tissue. For this method we have had to make the assumption, as is done in the literature on this subject, that similar tissues under identical circumstances would show acceptably identical rates of respiration. This assumption was found to be invalid (cf. Table IV, VI, p. 19). The only manner in which this assumption can be avoided is by using only one piece of tissue firstly in a vessel containing KOH, secondly in a vessel containing no KOH. The rate of O_2 consumption as well as the rate of CO_2 liberation can thus be measured directly. This is followed in method three.

In the attempt to measure gaseous exchange of the embryo directly, a major difficulty was encountered. It was found that in order to separate the embryo from the gametophyte 4 incisions had to be made. The resulting piece of tissue had 4 wound surfaces and one intact surface, the apex of the gametophyte. Wound respiration, a result of the incisions, was very large and it obscured the precise respiratory effect of the embryo.

Although by weight the embryo with its immediate surroundings

amounted to no more than 2.5% of the total (p. 16) almost one third of the total respiration was carried on in this area. That this area is physiologically very different from the bulk of the gametophyte is thus evident. This is, of course, the case only if fertilization has occurred. Evidence of marked local physiological difference is strenthened by the visual observation that the area immediately surrounding the archegonia stays green long after the rest of the gametophyte has turned yellow.

The next experiment (p.17,Table III) is more precise in that the separation of the embryo from its normal environment is complete. Although the embryo has grown enormously (some 50 times the volume of a dividing zygote (Pl. III, Fig. 10), the mass of tissue is comparable to that where embryo separation was incomplete (supra). Again the small "active" part is observed to contribute one third of the total respiration, while it represents just over 3% of the total weight.

Results on page 20 give a survey of the effect of the presence of the embryo on the respiration of the gametophyte apart from any direct contributions it makes to the total respiration. The examples had been chosen at random, and with an effort to obtain intervals of approximately 20 days. It can be seen that in every case the fertilized ovule had a higher O_2 consumption than its unfertilized counterpart of the same age. This tendency becomes stronger as time passes, and it is very marked in the last example in which the fertilized ovule shows the high respiratory rate of the now dominant sporophyte and the unfertilized one the very low rate of a physiologically inactive tissue. It should be noted that there are as yet no visible signs of decay in the gametophyte. The R.Q.'s give an indication of the CO_2 production;

it decreases steadily in the fertilized ovules and increases in the un - fertilized ones.

Assuming this interpretation valid a method to separate fertilized ovules from unfertilized ones can be devised. Without dissection and consequent loss of the ovule in question one can ascertain whether an ovule is fertilized or not by measuring its R.Q.

The drift curve (Fig. 2) can be divided in a number of distinct stages:

First, there is a high initial average rate of the gametophytic tissue as a whole. The first 10 days out of cold storage show an O_2 consumption of approximately 110 μl/3 hrs./gm. This includes the contribution from the embryo.

Secondly, the average rate drops sharply in the course of 10 days to a new level of 50 μl/3 hrs./gm.

Thirdly, this rate of about 50 μl/3 hrs./gm. for the intact ovule is maintained for some 45 days. At the end of this period, at which time the lot of ovules in the greenhouse had been out of cold storage for 65 days, all of the gametophyte, including the area surrounding the archegonia, had turned yellowish but cell walls, turgor, and starch were intact.

The fourth and last stage is characterized by a sustained and spectacularly rapid increase in O_2 consumption and CO_2 liberation. The R.Q. changes little, but tends to decrease. The great rise in activity is directly attributable to the growing embryo which at the beginning of this stage contributes about half of the total exchange (cf. Tables II, III) and gradually takes over the larger part of respiratory

activity. Example one on page 15, still belongs to stage three but the next example, in which the embryo has increased from 16 to 25 mm. total length, shows a radical change. This is thought to be due to the emergence of the root with a subsequent rise in O_2 availability rather than to a mere increase in bulk of the embryo.

The third example (page 15) affords a comparison with the foregoing observation. The O_2 consumption of the larger embryo is 50 units higher, illustrating a trend towards a higher O_2 consumption coupled with growth of the sporophyte. The R.Q. has remained constant. The greatest increase in oxygen requirement is thus not found to be associated with the production of chlorophyl of the young plant, but with the emergence from the gametophyte. Oxygen is especially essential in growth in length. The cell enlargement which must take place requires intense respiratory metabolism during the vacuolation phase of cell growth during which oxygen requirements are highest. (cf. also 17, p.430.) Also, no doubt the increased surface area resulted in increased oxygen availability.

The early stages of embryonic development up to the stage in which the presence of the embryonic root can be recognized by a small protuberance at the archegonial end of the gametophyte, cannot be identified without sectioning. The embryo is destroyed in the process. A solution to the problem will be offered after discussion on the effect of the embryo on the respiration of the gametophyte.

In an examination of the first of the three examples of wound respiration (pages 17,18) an increase of 145% in O_2 consumption is observed to occur with a wound area of about 90 $mm.^2$, the total of 5 wound

areas resulting from the resection of the embryonic mass. In the second instance there was an increase of 170% in O_2 consumption with a wound area of about 120 mm^2. Finally, there was an increase of 163% in O_2 consumption with a wound area of about 120 mm^2. The latter two cases involved a longitudinal splitting of the gametophyte. In the second example the gametophyte did not contain an embryo, and in the third there was a 13 mm. long embryo. The initial rates of O_2 consumption of the intact gametophytes were 30.8, 49, and 29 $\mu l/3$ hrs./gm., for examples one, two, and three respectively. This seems to indicate that wound respiration is proportional to area rather than to the initial rate of respiration. The smaller the mass the greater the relative effect of wound respiration; in the case of these small bits of tissue 5 out of 6 surfaces would be wound areas, and the effect of wound respiration closes over the normal embryonic respiration.

Table IV, page 19, contains results typical of the variation in readings that occurs when two experiments using the same material are done in close succession. Between experiments the conditions are in no way altered and the tissues are undisturbed. The result shows an appreciable 8% variance in O_2 consumption presumably due to an increased time in the manometric vessel away from greenhouse conditions. There is also a very small R.Q. change of 1.2%. This last difference lies within the limits of accuracy of the direct method.

Table V shows the change in rates that can occur when gametophytes are left in the apparatus for some time. The ovules used in this experiment were in a stage on the curve leading towards lower O_2 consumption and higher R.Q. (Table VI). This tendency is apparently given added

impetus by unduly long confinement in a vessel.

Table VI contains the data which suggest to me that the assumption made when using the direct method is not valid even in those cases in which the two pieces of tissue, necessary in any one experiment, are apparently similar. In this case later sectioning showed that both gametophytes contained degenerated egg cells and that otherwise the tissues were identical. The procedure of this experiment was designed to disclose the accuracy achieved with the direct method. The difference in reading here is 13%. This is significantly more than the 8% difference found in Table IV. On theoretical grounds, the accuracy achieved with the third method is of the same order as that achieved in Table IV, and thus it is significantly greater than that achieved by the direct method.

Inquiry into the influence of light as possibly related to the obvious presence of chlorophyll in the female gametophyte showed no significant difference between gaseous exchange in the light from that in the dark. The fact that the fruit turns yellow throughout and that the developing sporophyte is yellowish white may have some bearing on this situation. At any rate it would appear that the photosynthetic reaction as it is usually understood is not sufficiently effective to lower O_2 uptake.

Discussion cannot be considered completed without some consideration of R. Q. values.

The expected R. Q. Resulting from the complete combustion of a carbohydrate, any carbohydrate, is exactly unity (8). That of a protein, depending on the form of its product, is broken down to approximately

unity, and that of a fat is 0.7. The R.Q. usually does not follow a simple course during metabolism since it is a complex of a number of simultaneous reactions. In the case of Ginkgo, with a typical starchy seed, the bulk of the substrate is formed by carbohydrates, but presumably there is some utilisation of fats. With the help of Sudan III as a fat stain (and Potassium Iodide as a starch stain) fat was shown to be present in the inner seed coat. It was not desirable by this method in the storage tissue of the gametophyte. The latter gave a positive reaction to Potassium Iodide, indicating the presence of starch. Accepting the negative reaction for fat in the gametophyte, R.Q., in the range 0.7 to 1.0 cannot very well be explained by stating that mainly fats were broken down. Thomas, in experiments with germinating peas with testas intact (R.Q. 2.8 - 4.2) and with testas off (R.Q. 1.5 - 2.4) attributes high R.Q. to a partially anaerobic carbohydrate metabolism. (17) In senescent apples, respiring in air R.Q.'s greater than 1.3 indicate that anaerobic respiration has occurred, possibly owing to incipiant cell disorganization.

This hypothesis supports the disclosure of very high R.Q.'s in submerged gametophytes. It also may explain a gradual increase in R.Q. with age of unfertilized gametophytes. In the case of the latter it is possibly due to tissue degeneration with a resulting breakdown of protoplasmic streaming.

SUMMARY AND CONCLUSIONS

Data on respiratory values found in the course of development of the entire endogenous content of the female sporangium of Ginkgo or of its components can now be obtained with reasonable accuracy. They constitute an approach to the interpretation of change in the immediate environment of the young embryo. The Warburg apparatus is adequately suitable to derive these data. The direct method of Warburg was used essentially, but it was found necessary to adapt this method to formulate a third one, entailing the use of only one mass of tissue. It afforded greater accuracy because individual gametophytes under identical circumstances did not prove to have the same rates of respiration. Also in establishing drift pattern the direct method is applicable only in the early part of the ontogeny. Later, when the embryo passes stage 5, respiratory individuality between gametophytic masses becomes pronounced, and necessitates the use of the third method.

Because of the various experimental difficulties it was difficult to judge the respiratory role of the embryo alone. It approximated just less than one third of the total in the proembryonic stage, just over one third in the 10 mm. stage and over half at the emergence of the embryonic root.

It would appear to be unrealistic to assess total environment by reference to the respiratory response of the embryo alone for it was clear that the gametophyte contributed as well. Depending upon whether fertilization took place, the gametophyte may, in medium to late phases,

make a constant or a decreasing contribution to respiratory values. During early development of the embryo the decided drop in O_2 consumption is primarily attributable to the gametophyte.

One of the difficulties encountered in the interpretation of partial respiratory contribution resulted from the disruptive effect of wounding. A beginning towards overcoming this essentially technical difficulty was made by the measurement of change in respiratory values due to wounding of particular areas.

The probable influence of temperature and humidity was avoided by standardizing conditions. The only physical factor empirically investigated was light; it was found to have no detectable influence on respiratory rate.

One of the more significant contributions following the establishment of the approach to total environmental assessment, was the disclosure of characteristic respiratory drift over a period of 4 months. A pattern likely related to growth rates is established by the data on the rate of O_2 consumption. This is high in the proembryonic stage, seeks a constant level during the development from 2 - 15 mm., rises sharply at the emergence of the sporophytic root, and finally rises slowly in the course of development to a 10 cm. size. The fact that individual gametophytes under identical circumstances differ with respect to gaseous exchange values does not prevent the procurement of this characteristic curve because the difference is such that it occurs within a range that does not alter the pattern of the curve.

A suggestion of ontogeny of metabolic pathway can be made by inference on the basis of R.Q. drift. Because the change in R.Q. proceeds

uniformly in one direction the changes in metabolic pathways are thought to be relatively uncomplicated.

There are certain theoretical conclusions which appear to have attractive possibilities, especially in connection with future work on the culture of post-zygotic masses. One is that the submergence of the excised embryo in its nutrient medium would appear to be inadvisable. A second is that, when a nutrient medium is selected for such work, a liquid, not a solid, medium would provide optimum conditions. This choice might even be essential in promoting adequate growth response. A third is that, whatever nutrient medium is selected, it might best be chemically simple in accordance with the uncomplicated nature of the drift of R.Q. values. A fourth arises in view of the occurrence of two levels of O_2 consumption found in the gametophyte after its removal from cold storage. It is likely that one, either the higher or the lower, would provide a greater chance of success after transfer to the culture medium. Finally, contrasting values between respiratory rates of fertilized and unfertilized units suggest a means of determining whether fertilization has taken place. This might now enable us to prepare for isolation of the proembryonic mass without having to depend on prior dissection.

It seems reasonable to propose that a further application of the work relates to a principle that is quite fundamental, - the dependency for food of the sporophyte on gametophytic generation in higher plants. The results of the investigation suggest that the early embryonic mass is dependent but not on food alone. It also relies upon a physiological mechanism apparently existing between sporophyte and gametophyte. Examination of this appears to be easier now that an approach for assessing metabolic change has, at least in essence, been established.

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DESCRIPTION OF PLATES

PLATE I

Fig. 1 Showing an unfertilized egg as representing the youngest condition in development. x 70.

Fig. 2 Showing part of an unfertilized egg as it relates to the archegonial jacket. x 420.

Fig. 3 Showing an unfertilized egg, larger and denser than that in Fig. 1. x 70.

Fig. 4 Showing part of an unfertilized egg with cytoplasmic detail. x 420.

Fig. 5 Showing the position of an unfertilized egg with large nucleus and increased vacuolization. x38.

Fig. 6 Showing a part of the unfertilized egg (Fig. 5) containing structural detail of nucleus and dense cytoplasm. x 210.

PLATE II

Fig. 7 Showing the final condition of degeneration of the egg cell with fungal remains in the archegonial cavity. x 95. Stage 0.

Fig. 8 Showing the characteristic crescent, a remnant of the degenerated egg mass. x 95. Stage 1.

Fig. 9 Showing the degenerated egg mass distributed in a thin layer on the inner surface of the archegonial jacket. x 95. Stage 2.

PLATE III

Fig.10 Showing a proembryo in the multinucleate stage of development. x 95. Stage 3.

Fig.11 Showing a embryo in the earliest stage of cell wall formation x 95. Stage 4.

Fig. 12 Showing a crossection of a 7 mm. embryo. x 85. Stage 5.

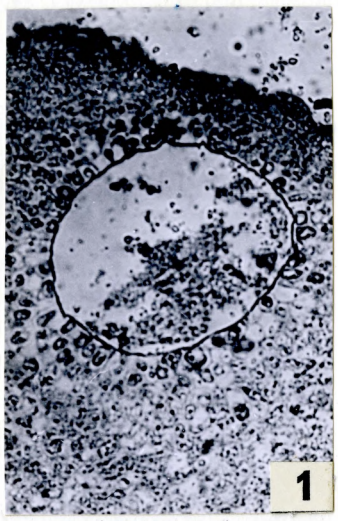
Fig.13 Showing a longitudinal section of a 7 mm. embryo. x 70.

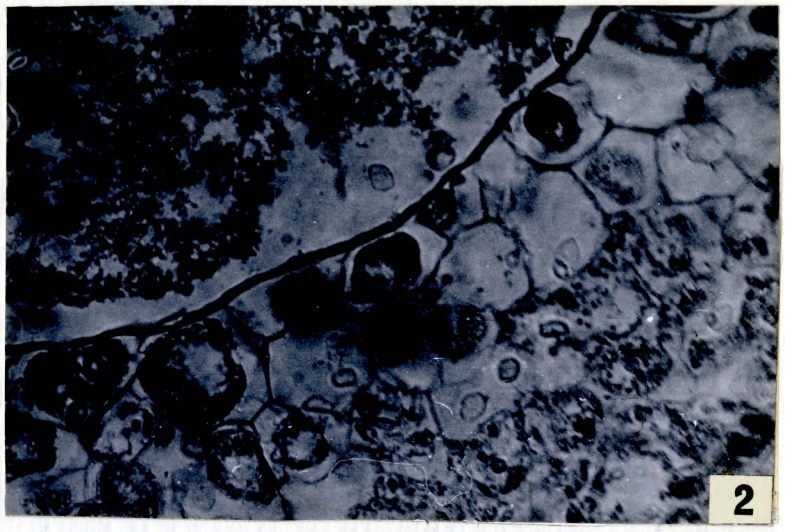
PLATE IV

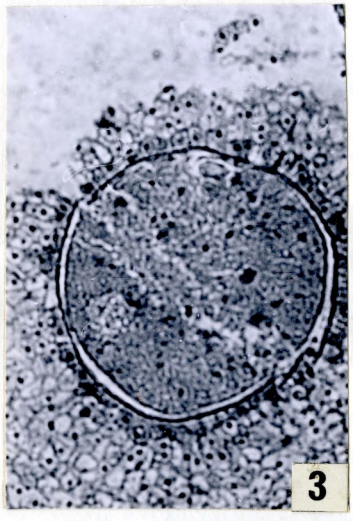
Fig.14 Showing a part of a degenerated egg mass to indicate structural difference with the surrounding tissue. x 300.

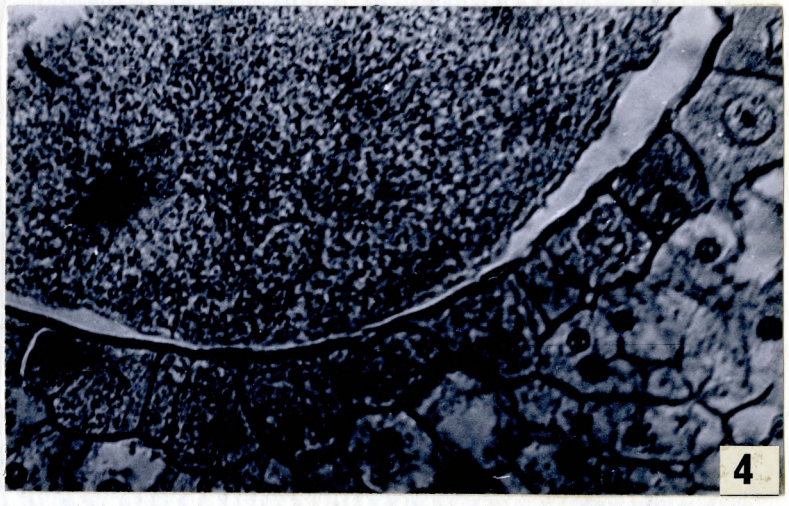
Fig.15 Showing an enlargement of a crossection of a 3 mm. embryo. x 475

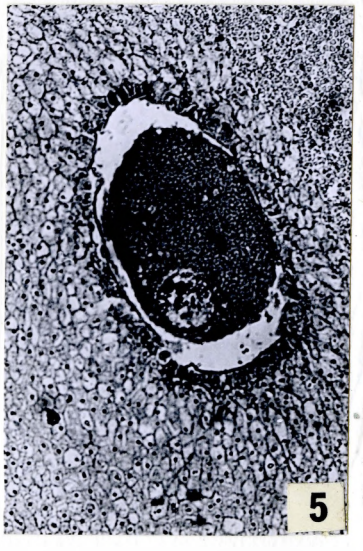
PLATE I

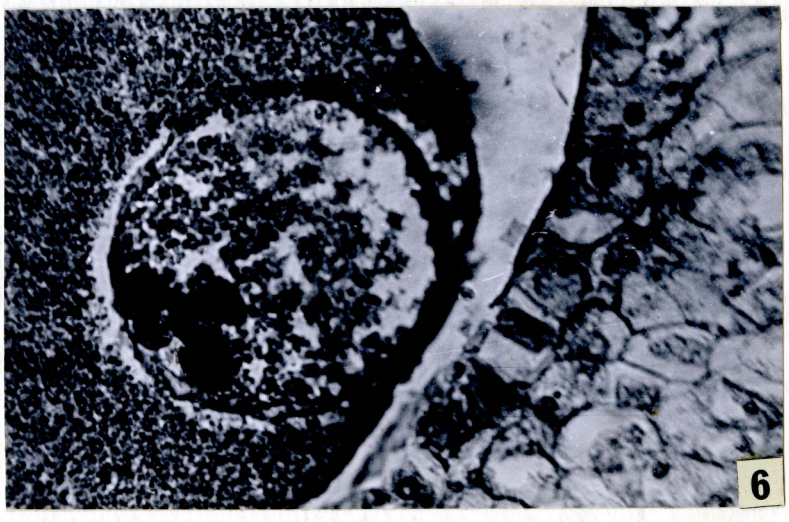












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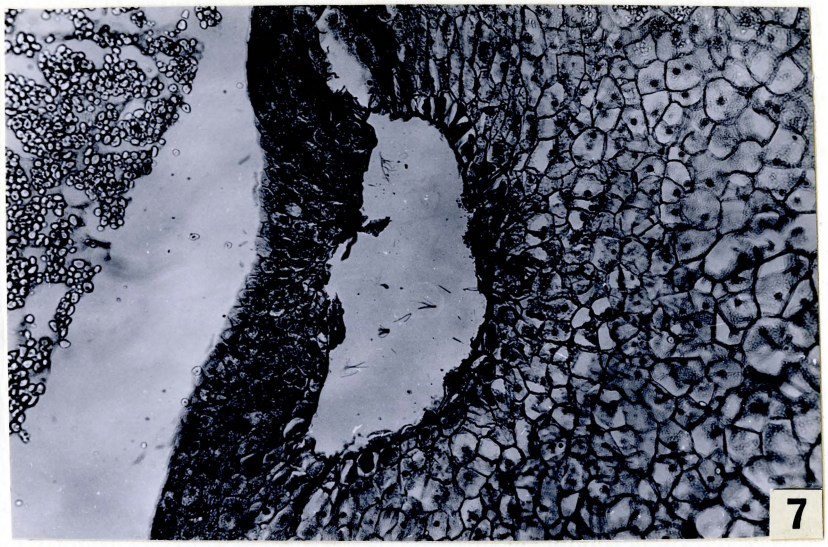


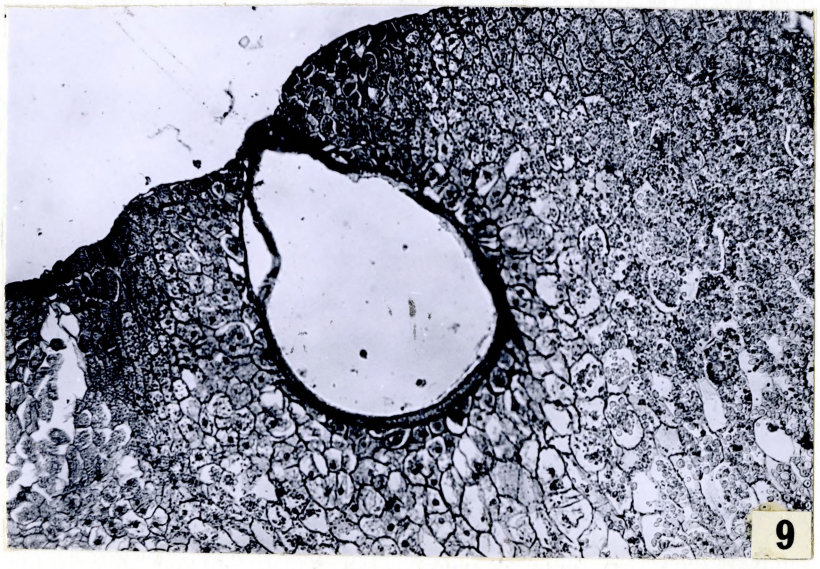
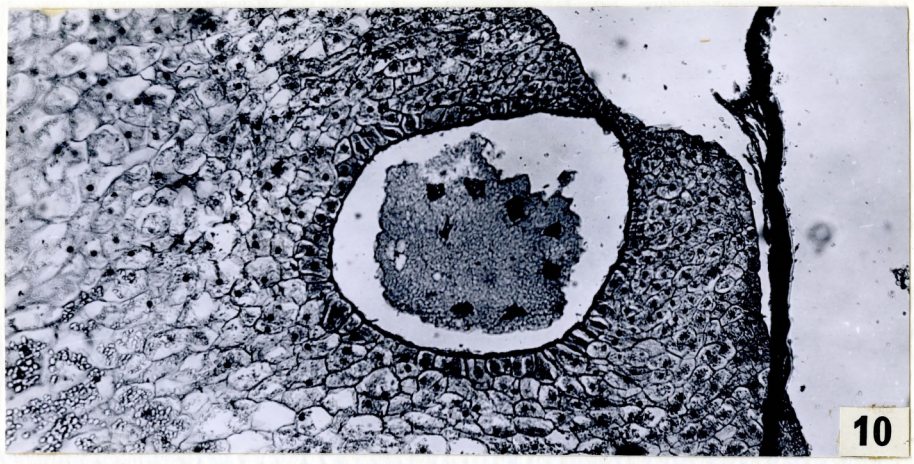
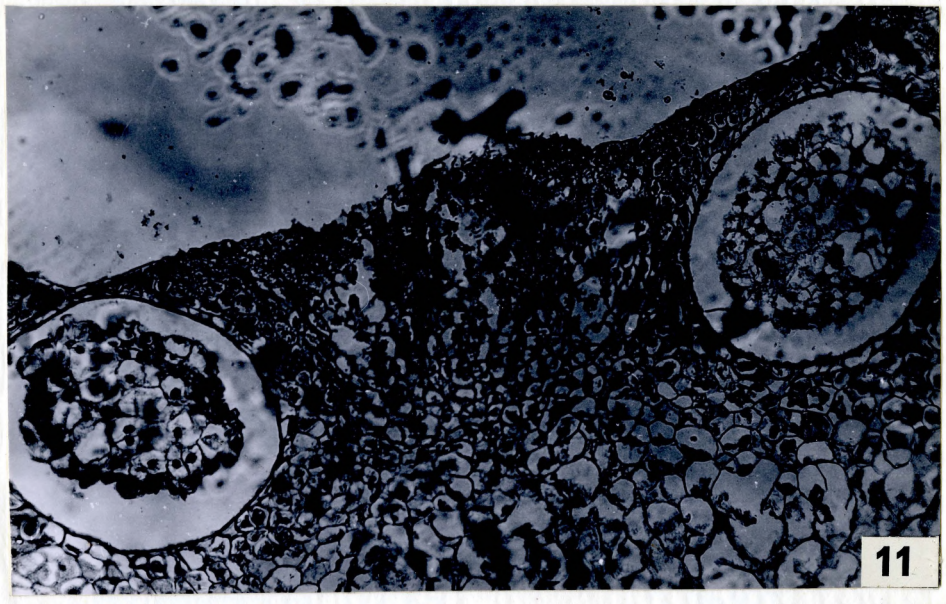
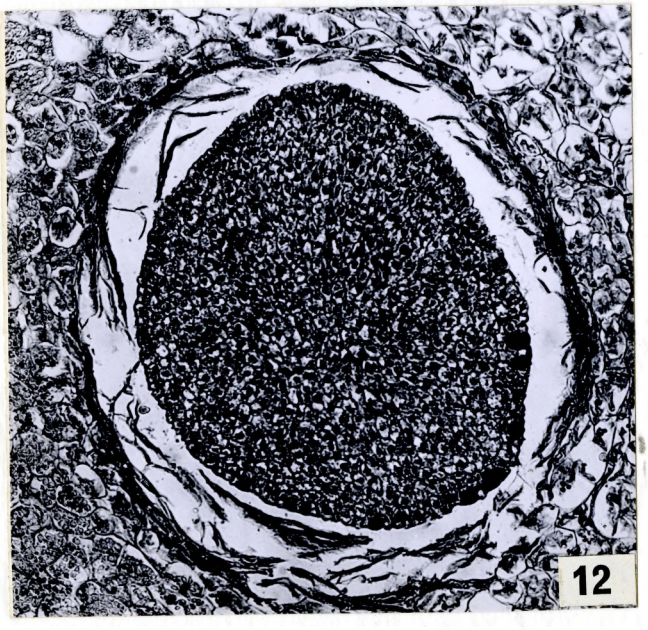


PLATE III







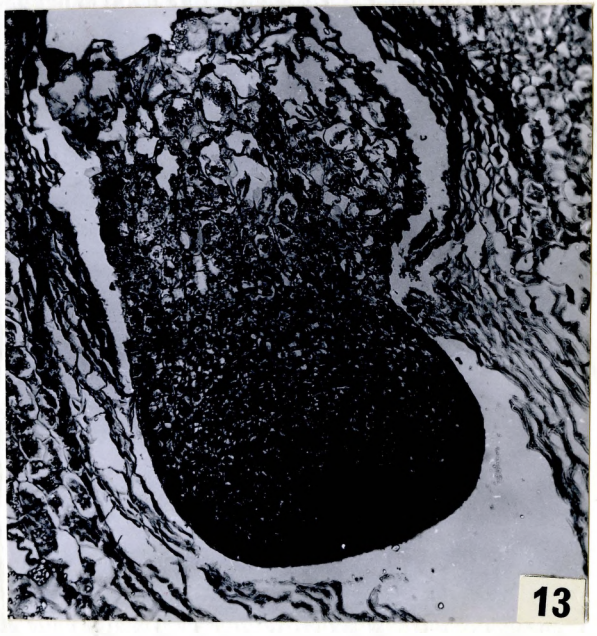


PLATE IV