Leakage during Seed Imbibition

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ABSTRACT

When pea embryos are placed in water solutes leak from them at a rate which declines rapidly at first and then more slowly. Embryos can be dried down over calcium chloride and will then leak as before when returned to water. Similar results were obtained with Ricinus seeds after removal of the testa.

Pea embryos that have first been allowed to imbibe some water through a small part of their surface (by placing them on damp filter-paper) leak relatively slowly when subsequently immersed in water; the greater the initial imbibition the slower the subsequent leakage. Likewise, embryos taken from peas that were harvested when succulent and tender show only slow leakage.

It is proposed that as seeds dry out in the course of development cell membranes lose their integrity. When such dry seeds are allowed to imbibe water there is a short period, before membrane integrity is re-established, during which solutes can leak out of the cells.

INTRODUCTION

When seeds are set to germinate the imbibition of water is accompanied by a leakage of substances, notably sugars but including also a variety of organic and amino acids. The extent of this leakage, as determined in laboratory tests, is positively correlated with pre-emergence mortality under field conditions (Bradnock and Matthews, 1970). There is evidence to suggest that the substances leaking into the soil from seeds stimulate the growth of fungal pathogens (Flentje and Saksena, 1964; Schroth and Cook, 1964). Seeds that have been cracked (Schroth and Cook, loc. cit.; Flentje and Saksena, loc. cit.) or scarified (McDonough and Chadwick, 1970) leak more than intact seeds, while complete removal of the testa from pea seeds leaving only the embryo doubles the amount of leakage (Larson, 1968).

In time-course studies Larson (loc. cit.) followed the leakage of sugar, amino acid, Kjeldahl nitrogen, and total dry weight over a 24-h period from pea seeds from which the testa had been removed. In each case leakage was faster in the first 2 h than in any subsequent 2-h period. The present experiments take these observations as a starting-point; the object has been to investigate more fully the time course of leakage under various conditions in the hope of gaining some insight into the mechanism of leakage.

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MATERIALS AND METHODS

Peas, *Pisum sativum* var. Meteor and castor beans *Ricinus communis* var. Gibsoni were supplied by Sutton's seeds. The testa was removed from the pea seeds with pointed forceps; for castor beans a razor blade was used. Care was taken to minimize damage to the underlying tissues (pea embryo or castor bean endosperm), any severely damaged material being rejected. Slight scoring of the pea cotyledons could not be avoided but a preliminary comparison of the leakage of electrolytes revealed little difference between peas prepared in this way and peas that were first soaked in water, the loosened testa gently pulled off and the embryos then dried down in a desiccator; the slight scoring of the cotyledons was therefore considered to be of little importance.

For some experiments pea embryos were first preimbibed before being immersed in water for leakage studies. The object of this preimbibition was to allow the embryos to take up water from a source that made contact with only a small part of the surface of the embryo and likewise only permitted leakage from a small part of the surface. In one procedure embryos were placed for 2-45 h with the root-shoot axis uppermost on moist filter-paper using the method described by Orphanos and Heydecker (1968). Alternatively embryos were placed individually into depressions (loaded with 0.05, 0.1, or 0.2 ml of water) on a standard spot tile; the tiles were placed in closed boxes for 24 h in the laboratory.

For leakage experiments, 50 pea embryos or 25 castor bean seeds minus testa were placed in a 100-ml beaker and 50 ml water added. The beaker was placed in an incubator at 20 °C for the required period of time. The supernatant liquid was then decanted through a small funnel into a measuring cylinder, a fresh 50 ml of water added and the beaker returned to 20 °C. The supernatant was made up to 50 ml in preparation for analysis.

Conductivity was measured with a Lock conductivity bridge Type BC 1. Potassium was determined by flame photometry and carbohydrates (as mg glucose) by the anthrone method of Loewus (1952). For protein determinations 20 ml of 20 per cent trichloracetic acid were added to 20 ml supernatant, the mixture cooled on ice for 30 min, and centrifuged at 3000 g for 15 min. The protein in the resulting precipitate was assayed according to Lowry, Rosebrough, Farr, and Randall (1951). To decide whether solute and solvent were both imbibed by peas, 50 pea embryos were immersed in 50 ml M sucrose and small samples taken from time to time for determination of refractive index and hence sucrose concentration.

RESULTS

The time course of leakage

Fig. 1A shows the time course of leakage from pea seeds minus testa as determined by the rate of increase in the conductivity of the water in which they are immersed, and as seen by the rate of loss of potassium. The two curves are very similar, the rate in each declining rapidly during the first hour of imbibition and then more slowly. Fig. 1B records the rate of leakage of sugar and of protein in the same experiment, again characterized by a rapid initial fall. The quite marked increase in the rate of leakage of sugar and protein between 4 and 8 h is reflected in the smaller increases in the curves for electrolyte leakage. The flat surfaces of the two pea cotyledons are closely pressed together in the intact seed and of course stay this way when the testa is removed; however, after about 4 h immersion in water they begin to move apart so that the flat surfaces become wetted for the first time, and it may be that the progressive wetting of these surfaces, like the initial wetting of the convex surfaces, is responsible for a short period of rapid leakage.

In view of the over-all similarity of the four curves in Fig. 1 it seemed unnecessary to repeat all the measurements to assess leakage in subsequent experiments; because they are so rapid and simple, conductivity determinations were used as the measure of leakage in most of the remaining experiments.
In the experiment of Fig. 1 the first measurements, taken at quarter-hourly intervals, already show a rapid decline in the rate of leakage. To establish the time course over the initial period more clearly, conductivity was measured at 1-min intervals for 15 min and then less frequently (Fig. 2). It is clear that leakage occurs most rapidly in the first minute of imbibition. It could be argued that some of the material appearing in the water in the first few minutes had been deposited on the surface of the embryo from the nucellus or endosperm during seed maturation. To test this possibility the embryos used in the experiment of Fig. 2 were dried in a desiccator over calcium chloride and the experiment repeated. The results, also presented in Fig. 2, again show a rapid initial leakage. It must be concluded that leakage starts as soon as dry embryos begin to imbibe, and that electrolyte is leaking from within the embryos.

Fig. 3 illustrates a comparable experiment with castor bean seeds minus testa. Once again, and despite the morphological and biochemical differences between the seeds of *Ricinus* and those of *Pisum*, leakage follows a rapidly declining pattern.

The progress of electrolyte leakage was compared with the time course of imbibition (Fig. 4A). Imbibition is complete after about 8 h by which time leakage has declined to a very low rate. The first quarter-hour is characterized by rapid leakage and rapid water uptake (Fig. 4B).

**Leakage following preimbibition**

Fig. 4A presents a comparison of leakage and imbibition by dry embryos with others that had already taken up 7·2 g water from damp filter-paper. It will be seen that these preimbibed embryos finally take up slightly more water than the dry ones but they never leak as rapidly as the dry embryos. Fig. 5 sets out the results of a series of such experiments in which embryos were preimbibed to various degrees by placing them either on damp filter-paper for the requisite period or with the appropriate amount of water in a depression on a spot tile. It is clear that the more water the embryos take up during the preimbibition period, the less leakage there is when they are later immersed in water.

The rapid decline in leakage rate that is observed when dry embryos are immersed in water (Fig. 4A) is also related to the progressive uptake of water. Thus, seeds immersed for 1·5 h take up 4 g water, and leak at a rate of approximately 175 μMho/h. Sectioning of the embryos showed that only the outer cell layers became hydrated after 1·5 h immersion in water. Embryos allowed to imbibe from damp paper for 9 h had a similar appearance; although only a small part of the embryo had been in direct contact with water, conduction from cell to cell had evidently been sufficient to hydrate the cells in the outer layers. Comparison of Figs. 4A and 5 shows that approximately the same extent of imbibition—and of leakage—is induced by 1·5 h immersion or 9 h preimbibition.

**The effect of drying on leakage**

In the experiments of Figs. 2 and 3, pea embryos or *Ricinus* seeds minus testa were allowed to imbibe, then dried over calcium chloride and set to imbibe
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Fig. 1. A. Time course of leakage of electrolytes and potassium from pea embryos immersed in water. B. Time course of leakage of sugar and protein.
FIG. 2. Time course of electrolyte leakage from pea embryos immersed in water (closed circles). After 60 min the embryos were removed from the water, dried over calcium chloride, and then returned to water (open circles).

FIG. 3. Time course of electrolyte leakage from Ricinus seeds after removal of testa. Three experiments are shown, the seeds being dried down over calcium chloride between experiments.

FIG. 4. A. Time course of imbibition and electrolyte leakage from pea embryos immersed in water. One set of embryos was air-dry; the other had previously been allowed to imbibe water from moist filter-paper for 18 h. B. Time course of imbibition and electrolyte leakage from pea embryos during the first 4 h.

again; drying increased the rate of leakage. This observation was further pursued in an experiment in which embryos were immersed in water for 30 min, dried for a week or more over calcium chloride, then immersed for 30 min again and so on, making in all nine half-hour periods of imbibition. Leakage from these
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Fig. 5. Electrolyte leakage from pea embryos during the first 30 min of immersion in water. The experiment compares leakage from dry seeds with ones that had been allowed to imbibe some water beforehand (a) from damp filter-paper (closed circles), the figure beside each circle indicating the number of hours on filter-paper, or (b) from a drop of water, the size of the drop being shown beside the open circles.

Fig. 6. Electrolyte leakage from pea embryos immersed in water for 30 min, then dried over calcium chloride for a week or more, returned to water for 30 min, dried, and so on. A curve for leakage from pea embryos immersed continually for 4 h is shown for comparison.
embryos which were never allowed to imbibe much water was at all times more rapid than from embryos immersed for a continuous period of 4 h (Fig. 6).

The maturation of seeds in the pod provides a natural situation in which peas become progressively drier. Meteor peas were sown outdoors in May and harvested 3 months later. Some of the peas were then set aside to dry out in the laboratory. Samples were taken at the time of harvest and again after drying for determination of leakage rate and of fresh and dry weight. It will be seen from Table 1 that rapid leakage is a property of embryos from dry mature seeds—but not of the hydrated embryos harvested in August.

**Table 1. Leakage from Meteor peas**

<table>
<thead>
<tr>
<th>Date</th>
<th>Stage of development</th>
<th>Fresh wt of 50 seeds, g</th>
<th>Dry wt of 50 seeds, g</th>
<th>Conductivity after 30 min immersion of 50 embryos μMho/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 May 1971</td>
<td>Dry peas as sown</td>
<td>11·25</td>
<td>10·22</td>
<td>1830</td>
</tr>
<tr>
<td>18 Aug. 1971</td>
<td>Harvest: pods shrivelled; peas swollen, fresh green</td>
<td>23·90</td>
<td>11·81</td>
<td>140</td>
</tr>
<tr>
<td>14 Oct. 1971</td>
<td>Peas from above harvest stored 2 months in laboratory, until dry</td>
<td>12·03</td>
<td>11·05</td>
<td>1808</td>
</tr>
</tbody>
</table>

**Leakage and imbibition in M sucrose**

Embryos placed in M sucrose imbibe more slowly than embryos in water—and they leak somewhat less (Fig. 7). (As the presence of sucrose affects the conductivity of the solution, leakage was assessed in this experiment by measurements of potassium.) Embryos transferred from sucrose to water after 2 h imbibe very rapidly in the next 2 h, and leak a little faster than they did in the sucrose. The sucrose solution becomes more concentrated as imbibition proceeds.

**DISCUSSION**

The possibility that leakage is no more than a washing away of superficial residues can be discounted on the grounds that rapid leakage would not start again when embryos were returned to water after drying (Figs. 2, 3, 6); nor would pre-imbibition through a small part of the surface reduce leakage (Fig. 5). A second possibility, that leakage occurs from the tissue-free space or apoplast, is also rendered unlikely by the rapidity of the decline in leakage rate (Fig. 2) and the slow leakage following pre-imbibition (Fig. 5).

We are left with the likelihood that leakage is from within cells, i.e. from cytoplasm and vacuole. This accords with the wide variety of substances leaking out, but it runs counter to the conventional view that cellular contents are retained in situ by cell membranes. Larson (1968) proposes that cell membranes are ruptured by the rapid inrush of water as a seed imbibes, so allowing the leakage of cell contents, and, indeed, it is true that water uptake and leakage are both
rapid when seeds are first put in water (Fig. 4B). However, it is difficult on this view to see why leakage should slow down so soon; is membrane integrity re-established within minutes while water uptake is still rapid? Moreover, when

embryos first allowed to imbibe in M sucrose are later transferred to water (Fig. 7), the very rapid intake of water is not associated with a particularly fast rate of leakage.

An alternative hypothesis lays stress on the condition of membranes in dry seeds. Rapid leakage is always associated with the early stages of imbibition.

**Fig. 7.** Potassium leakage from pea embryos immersed in water or M sucrose. After 2 h one set of embryos was transferred from M sucrose to water (dotted lines). The upper graph shows the progressive increase in the concentration of the sucrose solution as imbibition proceeds.
As soon as the embryos absorb much water leakage slows down (Figs. 1, 2); it is slow too if embryos are preimbibed before being immersed in water (Fig. 5); or in developing seeds that are not yet air-dry (Table 1). Embryos can be made to leak rapidly again and again by drying them down after short periods of imbibition (Figs. 2, 3, 6).

These observations suggest a hypothesis, that the semi-permeable membranes (tonoplast and plasma membrane) which normally retain solutes within cells have lost their integrity in air-dry seeds; that is to say they do not act as retentive barriers when embryos are first placed in water. But as the cells imbibe water the membranes become re-established within minutes and then prevent further leakage (Fig. 2). The membranes can be made to progress through several such imbibition/drying cycles, allowing leakage each time they are dry (Figs. 2, 3, 6). The ease and rapidity with which the membranes can be switched from leaky to intact suggests that the change is a physical one, rather than a degradation of membrane constituents on drying with resynthesis on imbibition.

The very rapid initial leakage from pea embryos in water comes from the outermost cell layers, for sectioning reveals that only they are wetted in the first few minutes. As these cells imbibe, semi-permeable membranes would then become re-established and leakage would slow down rapidly. Deeper-seated cells would next become hydrated and leak for a period, but the long pathway of diffusion between such cells and the exterior would prevent the initial phase of rapid leakage continuing through the whole of the imbibition period. The electrolytes leaking out of the embryos originate therefore from the outermost cell layers. After several imbibition/drying cycles these cells have lost much of their original electrolyte content and the rate of leakage has then begun to fall (Figs. 3, 6).

Dry embryos can also imbibe when placed in M sucrose although more slowly than in water (Fig. 7). The predominant force responsible for imbibition is the very high matric potential of cell walls and cell contents. It is clear that sucrose solution could be imbibed as such by the cell walls; it might also be imbibed as such by the cell contents during the initial moments while they are still dry and leaky, but the amount of sucrose taken up in this way would be small and difficult to detect. Membrane integrity rapidly becomes re-established on hydration and thereafter water alone will enter the cell, sucrose being excluded. This has two consequences. On the one hand, it increases the concentration of the sucrose solution remaining within the cell walls. As membrane integrity is regained in one layer of cells after another the sucrose solution will become more and more concentrated, so lowering its water potential to the point where it effectively slows or even stops further imbibition (Fig. 7). In addition, as the solution becomes more concentrated it will become more viscous, which again reduces the rate at which it can penetrate further into the embryos. It is likely that the 18 per cent 20 M carbowax used by Perry and Harrison (1970) also delays imbibition by virtue of its high viscosity. A second consequence of the exclusion of sucrose from imbibed cells will be an increase in the concentration of sucrose in the supernatant solution (Fig. 7).
The hypothesis proposed here, that the membranes in dry embryos do not form continuous semi-permeable barriers around cells, is supported by recent biophysical studies. Although there is still considerable debate about the molecular architecture of membranes, there is no doubt that they contain phospholipids which are largely responsible for their permeability properties (van Deenen, 1965). There is now growing evidence from both in vitro studies and from work with membranes, that the arrangement of phospholipid molecules changes as the water content is lowered. Thus differential scanning calorimetry indicates that lecithin–water mixtures which contain less than 20 per cent water do not form ice on cooling to 0 °C. This ‘suggests a limiting value to the water content of a biological membrane beyond which it should not be possible to dry the membrane without disturbing severely its structure’ (Chapman, Williams, and Ladbrooke, 1967). X-ray diffraction studies of isolated membrane preparations also indicate that about 20 per cent water is ‘essential for the maintenance of lipoprotein association in the membrane’ (Finean, 1969). Brain phospholipid preparations are only lamellar in structure at water contents above 20 per cent; under drier conditions narrow water-filled channels penetrate through the lipid phase (Luzzati and Husson, 1962).

Air-dry seeds commonly contain less than 20 per cent water, and we regard the leakage that occurs when seeds or embryos are placed in water as evidence that the membrane structure has been ‘severely disturbed’.

LITERATURE CITED


