Plant Physiol. (1981) 67, 449–456
0032-0889/81/67/0449/08/$00.50/0

Role of the Testa in Preventing Cellular Rupture During Imbibition of Legume Seeds[1]

Received for publication May 27, 1980 and in revised form September 18, 1980

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ABSTRACT

Studies with the seeds of soybean, navy bean, pea, and peanut were made to determine the extent of leakage of intracellular enzymes during imbibition. Embryos with intact testae from all four species were found to leak detectable activities of either intracellular enzymes of the cytosol (glucose-6-phosphate dehydrogenase) or enzymes found in both the cytosol and organelles (malate dehydrogenase, glutamate dehydrogenase, glutamate oxaloacetate transaminase, and NADP-isocitrate dehydrogenase) after 6 hours imbibition at 25 C. Pea and peanut embryos with testae leaked considerably lower levels of activity for these enzymes than did those of soybean and bean. Leakage of mitochondrial marker enzymes (fumarase, cytochrome *c* oxidase, and adenylate kinase) was not detected from embryos with testae, suggesting that a differential diffusion of intracellular components out of cells occurred. Soybean and bean embryos without testae leaked high, and proportionally (per cent dry seed basis) similar, levels of all cytosol, cytosol-organelle, and mitochondrial marker enzymes and protein during imbibition, indicating that cell membranes were not differential to leakage and that they had ruptured. Pea and peanut embryos without testae leaked detectable activities of all cytosol and cytosol-organelle enzymes, although fumarase was the only detectable mitochondrial marker enzyme leaked, suggesting that some degree of differential leakage may have occurred in these species. The outermost layers of embryo cells of seeds without testae of all four species absorbed and sequestered the nonpermeating pigment Evan's blue after 5 to 15 minutes imbibition, indicating that membranes had ruptured. This occurred to a much lesser extent in seeds with intact testae. Both soybean and bean embryos without testae were observed to disintegrate during imbibition, whereas those of pea and peanut did not. These data indicate that seeds of certain legumes are susceptible to cellular rupture during imbibition when seed coats are damaged or missing.

In the development of the legume seed, the testa appears to function in interconverting amino acids and sugars supplied by the phloem to the developing embryo (19, 35, 36) and in preventing injury by differentiating into a sclerified integument as the embryo matures (27). It has also been proposed that the testa protects seeds against "leakage" of intracellular substances during imbibition (32). This function has been suggested to be of great importance in the initial stages of germination of legume seeds in that many substances which leak from seeds may offer a substrate for potential pathogens (32). Past studies have demonstrated that electrolytes, sugars, amino acids, organic acids, and proteins are released from seeds during imbibition (1, 6, 14, 18, 23, 29, 33, 34) and that, with removal of the testa from seeds, the "leakage phenomenon" is enhanced (14, 32). The amount of leakage during imbibition has been shown to correlate negatively with viability in studies with seeds of soybean (6, 39), pea (14, 24), bean (17), and peanut (1) and has suggested to some that the leaked substances may, in some way, decrease viability. Another study has shown that removal of the testa of pea seeds results in death of the outer layers of cotyledonary cells during imbibition (25). The question arises: does the testa protect against leakage or is the leakage only a symptom of a fundamental dysfunction which can occur in imbibition?

Two hypotheses have been promoted to explain the mechanism(s) of leakage of solutes during the imbibition of seeds. Larson (14) has suggested that cell membranes are ruptured during the initial phases of imbibition. Simon (31, 33) has proposed that the membranes of dry seeds are formed into hexagonal plates with pores formed in the areas of the phospholipid heads through which low-molecular weight solutes can leak from cells by passive diffusion during initial stages of membrane hydration (*e.g.* before phospholipids form typical bilayer membranes). Recently Powell and Matthews (25) have suggested that, in peas, cellular rupture and leakage through membranes may both occur when the testae are removed from seeds. To date, there has been a paucity of data that any macromolecules could move through the cell membrane during imbibition. Here, we have examined the leakage of imbibing seeds with and without testae for the presence of cytoplasmic, organelle, and organelle marker enzymes which would not pass through small membrane pores but which could only pass through very large membrane discontinuities or which would be the result of membrane rupture. In this way, we have tested both of the aforementioned hypotheses in a more definitive manner than has been hitherto reported.

MATERIALS AND METHODS

Plant Material. Seeds of soybean [*Glycine max* (L.) Merr. cv. Wells], pea (*Pisum sativum* L. var. Alaska), navy bean (*Phaseolus vulgaris* L. var. Sanilac), and peanut (*Arachis hypogea* L. var. Mammoth Virginia) were fully mature and used in reported experiments within 3 to 6 months after maturation. Moisture contents of seeds were determined by oven-drying seeds at 70 C for 5 days and were found to be 6.82, 4.85, 4.52, and 2.42% for soybeans, navy beans, peas, and peanuts, respectively. Germination ranged from 76% with peanuts to 96% with soybeans and peas at 25 C. In studies with dead seeds, the oven-dried seeds were used. Testae were removed from seeds by carefully prying off pieces with a razor blade. Seeds were handled during testa removal with sterile latex gloves.

Monitoring Seed Leakage. Leakage from seeds was monitored continuously during imbibition by placing 5 to 10 seeds in an open chamber containing 400 ml double-distilled H_2O at 25 C which was pumped through a cuvette in a Pye Unicam SP8-100 double-beam spectrophotometer, which recorded A at either A_{240}

[1] This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and the Wisconsin Crop Improvement Association.

or A_{280}, and back into the imbibition chamber. A probe containing a conductivity bridge (Beckman solubridge SD-SE) was inserted into the imbibition chamber so that measurement of electrolytes could be made. Aeration of the imbibition chamber was accomplished by returning the H_2O from the spectrophotometer at about 5 cm above the liquid level of imbibition chamber.

Collection of Protein and Enzymes from Leakage and Seeds. Routine collection of leaked protein and enzymes from seeds was by placing 20 (soybeans, beans, peas) or 4 to 5 (peanuts) seeds in 80 ml double-distilled H_2O for 6 h, straining the leakage through a 177-μm Teflon mesh, and then precipitating proteins from 25-ml aliquots with 100% saturating ammonium sulfate at 0 C. Precipitates were pelleted at 20,000g for 30 min and resuspended in 2.0 ml suspension buffer [100 mM Hepes (pH 7.5), 10 mM β-mercaptoethanol]. Some experiments were conducted with 0.02% (w/v) NaN_3 in the H_2O used for imbibition as a check against bacterial contamination during the course of experimentation. Testae of seeds (60 for soybean, pea, and bean; 16 for peanut) were placed in 25 ml double-distilled H_2O for 6 h, after which 20 ml H_2O containing leakage was treated as for whole seeds. Time-course studies were conducted by collecting leakage after 1, 2, 4, and 6 h imbibition and completely replacing H_2O for imbibition after each collection. To check for the possibility of ammonium sulfate denaturation of enzymes, some samples of leakage were concentrated from 80 ml to about 2 ml with a vacuum molecular filter apparatus (Millipore Immersible CX with an exclusion limit of 10,000 daltons) and enzyme assays were conducted with the concentrated samples. At the end of each 6-h imbibition, seeds and seed particles were homogenized in 40 ml suspension buffer with a MSE homogenizer (Measuring and Scientific Equipment, London, UK) set at highest speed and chilled to 0 C. Homogenates were centrifuged at 20,000g and supernatants used for enzyme assays. Dry seeds were ground in a mortar and pestle (near 0 C) with 10 ml suspension buffer/g seeds and prepared for enzyme assays as with imbibed seeds.

Enzyme Assays. All dehydrogenase and dehydrogenase-linked enzyme assays were conducted spectrophotometrically measuring the reduction of $NAD(P)^+$ or the oxidation of NAD(P)H at 340 nm with a Pye Unicam SP8-100 double-beam spectrophotometer at 30 C. Except for the assay buffer (100 mM Hepes), assays are as previously described for GDH[2] and MDH (7), NADP-ICDH and G6P-DH (8), and GOT (9). Assays for AK were coupled to G6P-DH and reaction mixtures (2 ml) contained, in order of addition and in final concentration: 75 mM Hepes (pH 7.5), 0.1 ml enzyme preparation, 0.25 mM $NADP^+$, 5 mM $MgCl_2$, 12.75 units G6P-DH plus 25 units hexokinase (mixed yeast enzymes, Sigma), and 15 mM glucose. Endogenous activity was subtracted (−glucose reduction of $NADP^+$) and reactions were linear for at least 10 min. Fumarase assays were conducted by coupling malate production to the production of oxaloacetic acid with malic enzyme. Fumarase reaction mixtures (3 ml) contained, by order of addition: 80 mM Hepes (pH 7.5), 5 mM $NADP^+$, 0.1 ml enzyme preparation, 10 mM $MgCl_2$, 0.8 units NADP-malic enzyme (from chicken liver, Sigma), and 1 mM fumarate (Fischer Scientific Co.). (We have found that fumarate from certain sources is contaminated with malate and will give apparent fumarase activity without the addition of fumarase to reaction mixtures.) Reactions were linear for up to 20 min. Cyt c oxidase assays were by monitoring Cyt c oxidation at 550 nm with reaction mixtures containing, in order of addition: 90 mM Hepes (pH 7.5), 60 μM reduced Cyt c (type VI from horse heart (Sigma), reduced with ascorbate and dialyzed with 1,000 volumes H_2O for 18 h at 5 C; A_{550}/A_{565} = >20), and 50 μl enzyme preparation to a final volume of 1.0 ml. Reactions were completely inhibited by 10 mM KCN. Rates were calculated for the first 2 min reaction. Protein was determined by the method of Bradford (4).

[2] Abbreviations: GDH: glutamate dehydrogenase (EC 1.4.1.3); MDH: malate dehydrogenase (EC 1.1.1.37); NADP-ICDH: NADP-isocitrate dehydrogenase (EC 1.1.1.42); G6P-DH: glucose-6-phosphate dehydrogenase (EC 1.1.1.49); GOT: glutamate oxaloacetate transaminase (EC 2.6.1.1); AK: adenylate kinase (EC 2.7.4.3). SDH: succinate dehydrogenase (EC 1.3.99.1).

RESULTS AND DISCUSSION

PRELIMINARY OBSERVATIONS OF IMBIBITION AND LEAKAGE

During the first 5 to 15 min hydration of a soybean embryo without a testa, the cotyledons separate due to the more rapid hydration of the abaxial sides (hemispherical "outside" surfaces) of the cotyledons than the adaxial sides (flat "inside" facing surfaces), which are initially not in contact with H_2O (Fig. 1). There is greater hydration at the abaxial longitudinal ends of the cotyledons, resulting in these two ends bending toward each other and the two cotyledons being pushed away from each other. Also, the edges of each cotyledon joining the adaxial and abaxial surfaces hydrate very rapidly forming a continuous circular swollen ridge of tissue. These ridges of tissue aid in pushing the cotyledons away from each other. The actual separation of the cotyledons takes 1 to 5 s, with a point of attachment remaining at the axis end of the embryo. Due to pressure resulting from the two abaxial longitudinal surfaces hydrating very rapidly, the tissues of the inner adaxial surfaces are compressed and distorted, forming ridges perpendicular to the longitudinal plane of the embryo.

Within 10 to 30 min after the initiation of imbibition, strips of tissue on the joining edges connecting the abaxial and adaxial

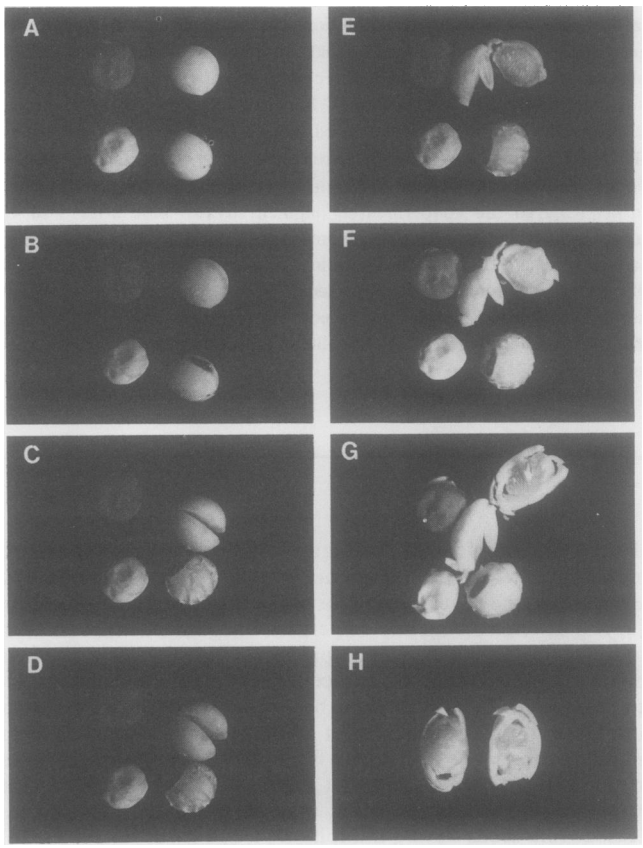

FIG. 1. Imbibition at 25 C of: A to G, soybeans without (top right) and with (bottom right) testae and peas without (top left) and with (bottom left) testae; H, soybean without testa. Times of imbibition: A, 45 s; B, 12 min; C, 16 min; D, 22 min; E, 25 min; F, 34 min; G, 1h; H, 1 h and 15 min.

surfaces of the cotyledons begin to split away from the cotyledons. At this time, the interior of the adaxial surfaces of the cotyledons have still not hydrated and there is no room for the rapid expansion of the tissues at the edges of the adaxial surfaces; therefore, the pressure of these fully hydrated "edge" tissues results in the displacement of large strips of tissue (1 to 7 mm in length). This process continues inward on the adaxial surfaces as new areas become the edge of the adaxial surface.

After 30 to 60 min imbibition, the water around the embryos becomes turbid and chalky in color, suggesting that cellular rupture has occurred. Microscopic observation (× 1,000) of the leakage reveals no particles larger than 1 to 2 μm in length and no identifiable cellular components, other than very small particles which may be protein bodies. These particles stain blue with Coomassie blue dye which binds with protein.

Experiments with three other soybean cultivars (data not shown) and 'Sanilac' bean embryos without testae indicated that they follow a similar pattern of hydration and splitting of tissues to that of 'Wells' soybeans during imbibition.

In contrast to soybeans and beans, pea (Fig. 1) and peanut embryos without testae imbibe H_2O uniformly with no separation of cotyledons or splitting of tissues. These findings are in agreement with those of Waggoner and Parlange (37) which demonstrate the uniform wetting of pea cotyledons during imbibiton.

After 5 to 15 min imbibition of seeds without testae in a 1% (w/v) solution of Evan's blue, cells in contact with the solution began to accumulate this nonpermeable dye. Uptake and retention of Evan's blue by plant cells has been shown to indicate that cellular membranes are not differentially permeable and that they have ruptured (13, 30). After 40 min imbibiton of seeds without testae, many cells bordering fissures in the cotyledons and axes stained heavily with Evan's blue (Fig. 2). After 2 h imbibition, Evan's blue had permeated three to four cell layers of axes and cotyledonary tissues. Pieces of tissue which had split from the main body of the cotyledons of soybeans (Fig. 1) and beans stained heavily, indicating that most all cells in these fragments were ruptured.

Embryo cells of seeds with intact testae (visual inspection) seldom stained when imbibed in the Evan's blue solution. After 2 h imbibition, testae could be removed and embryo cells had accumulated little or no dye. This suggests that rupture of cells does not occur after hydration. When we did find embryo cells of seeds with intact testae stained with Evan's blue, the staining was localized to an area adjacent to a split or fracture in the testae, which was not detectable before the onset of imbibition. The disintegration of tissues and rupture of cells of soybean embryos without testae during imbibition was reflected by large increases in A at A_{280} (Fig. 3) and conductivity (Fig. 4) of H_2O of imbibition. A rapid increase in A_{280} and conductivity occurs within the first 1 to 5 min imbibition followed by a marked reduction in rate of change for 5 to 20 min, and then a second rapid increase in rate of change occurs from 15 to 60 min after the beginning of imbibition, followed by a steady decline in rate for several hours. These data are consistent with those of Leopold and co-workers (5, 23) for the first 30 to 40 min imbibition of soybean cotyledons with a moisture content similar to that of the embryos we used. The studies with soybean cotyledons (5, 23) and others with pea seeds (34) indicate that low moisture content in seeds promotes leakage during imbibition. We chose the lowest seed moisture content for each species that would allow good germination. Imbibitional temperature affects leakage rates less drastically (5). We chose 25 C for imbibition because this temperature is optimal for germination of 'Wells' soybeans (11) and is at or near the temperature used in previous legume seed imbibitional studies (1, 23, 33, 34).

A comparison among four legume species of imbibitional leakage of embryos without testae over a 6-h period revealed that soybeans and beans, the two species that fractured during imbibition, leaked considerably more substances absorbing at 260 nm and protein than did peas or peanuts, the two species which remained visually intact during imbibition (Fig. 5). A similar trend was found for MDH activity in leakage which is reflected as percentage of total MDH activity leaked from the seed. Differences in leakage at A_{280} were easily apparent between beans, peas, and peanuts. Soybeans leaked much higher levels of substances absorbing at A_{280} than did any other species. Inasmuch as soybean seeds had a much higher moisture content than the seeds of other species tested, these data could indicate that low moisture content affects soybeans more than the others during imbibition. Lowering the moisture content of soybeans to levels approaching those of the peas and peanuts used here will greatly increase leakage (23). On the other hand, these data could indicate that soybeans have more substances available for leakage than do other species. This raises a question: Is everything within the embryo cells available

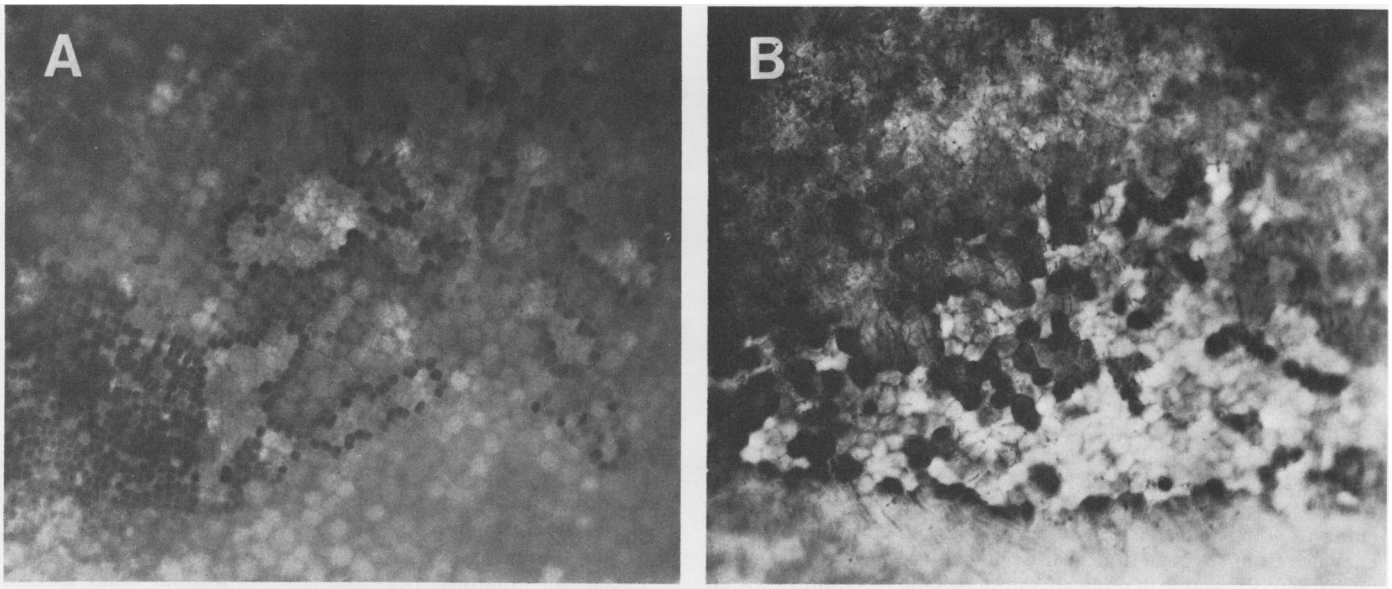

FIG. 2. Abaxial surfaces of soybean (A) and pea (B) cotyledons from seeds imbibed for 40 min at 25 C in a 1% (w/v) solution of Evan's blue. Photographs were taken at × 100 magnification. Cells which appear very dark are heavily stained with Evan's blue.

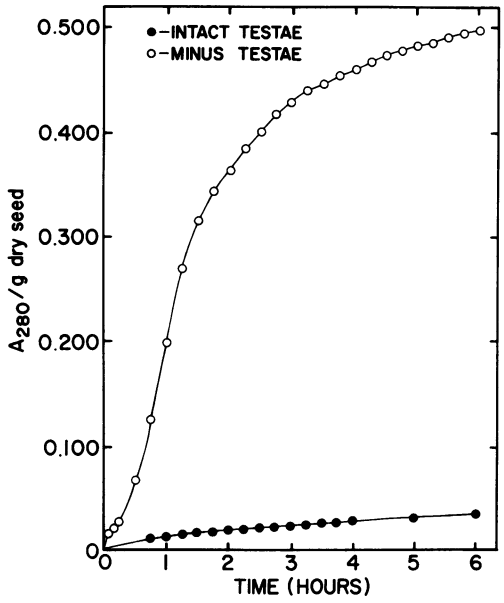

FIG. 3. Leakage of substances absorbing at 280 nm from soybean embryos with and without testae during imbibition at 25 C.

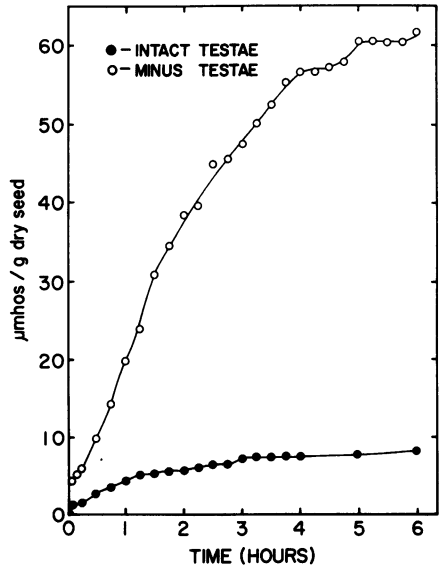

FIG. 4. Leakage of electrolytes from soybean embryos with and without testae during imbibition at 25 C.

for leakage, or are only certain substances leaked? If every intracellular component within the cells is leaked in the same proportion, it would suggest that leakage through cellular membranes is not differential and that leakage is through very large gaps in the plasmalemma.

ENZYME RELEASE DURING IMBIBITION

Embryos with Testae. Intracellular enzymes which are primarily from the cytosol [G6P-DH (8)] or from both cytosol and organelles [MDH (2), GDH (12), GOT (9), NADP-ICDH (3, 11)] are leaked with detectable activities during imbibition of soybean and bean embryos with testae (Table I). Levels of leaked activities for these enzymes from soybean and bean seeds with testae were found to be extremely variable in replicate samples. This variation appeared to be due to our inability to detect cracks in the testae of many seeds by visual inspection until after the onset of imbibition and the unequal number of seeds with cracked testae in replicates. Others have noted greater variability in H_2O uptake and solute leakage in studies utilizing soybean seeds with testae (5) and have used seeds without testae or seed fragments for studies on imbibition leakage (5, 23) and respiration (15). Soybean embryos with testae always leaked higher levels of cytosol and cytosol-organelle activities than the amount of protein that was leaked when all were calculated on a percentage dry-seed basis. The opposite was true for beans. The levels of activities of these enzymes which leaked during imbibition were proportionally nearly the same, except for G6P-DH which was higher in soybeans. In beans, the opposite was true with G6P-DH activity in leakage lower than activities for cytosol-organelle enzymes.

Pea seeds with testae leaked low levels of MDH, GOT, and NADP-ICDH activities. These were the three cytosol-organelle enzymes with the highest levels of activity in dry pea seeds (Table III). The other enzymes may have been leaked in insufficient quantity to be measurable. Peanuts with testae leaked only very low levels of MDH activity but quite high levels of NADP-ICDH activity. The NADP-ICDH activity may have come from the testae, which were very high in this enzyme activity.

Leakage of activities of mitochondrial marker enzymes Cyt *c* oxidase, fumarase, and AK (16, 26) was not detectable from any species tested with testae (Table II). This suggests that mitochondria do not leak from seeds with testae.

Heat-killed (70 C for 5 days) umimbibed soybean seeds with testae leaked much higher levels of protein and activities of enzymes than did live seeds (Tables I and II). This may have been due to cracking of testae during dehydration of seeds. In general, seeds with greater mass developed cracks more frequently than did small seeds. This again may have contributed to variation among replicate samples.

Unimbibed seeds heated to 100 C for 2 days also retained high levels of activity for enzymes assayed (MDH, G6P-DH, and NADP-ICDH), whereas seeds imbibed for 6 h and boiled at 100 C for 15 min had no recoverable activity for these enzymes (data not shown). This suggests that there is a differential effect of heat on enzymes in hydrated and dry seeds.

Embryos without Testae. Soybean and bean embryos without testae were found to leak high levels of all cytosol, cytosol-organelle, and mitochondrial enzymes assayed (Tables I and II). Proportionally (percentage of total activity before imbibition), leakage of soybean enzymes was nearly the same (about 5 to 6%) for MDH, GDH, G6P-DH, and Cyt *c* oxidase regardless of the method of concentration of leakage protein. Proportional levels of leaked protein were only slightly lower. Soybean GOT, NADP-ICDH, and AK activities were near the aforementioned levels when concentrated by vacuum filtration of the imbibitional leakage through molecular sieves. However, leaked activities of GOT and AK were considerably lower and NADP-ICDH activity was much higher than other activities on a percentage dry seed basis when these enzymes were concentrated from ammonium sulfate precipitations. This suggests that ammonium sulfate precipitation alters the activities of these enzymes. In contrast, soybean fumarase activity in the leakage was proportionally similar to that of other enzymes in the ammonium sulfate precipitation but was completely absent in samples concentrated with molecular filtration. Over-all, it appears that most soybean and bean enzymes and protein leaked in the same relative proportions from seeds lacking testae during imbibition.

Samples of leakage of seeds imbibed with sodium azide were not significantly different from those without it, suggesting that bacterial contamination does not contribute to recoverable enzyme activities in the leakage.

Except for fumarase, peas and peanuts without testae leaked, on a percentage dry seed basis, considerably lower activities for enzymes and protein than did bean and soybean embryos (Tables

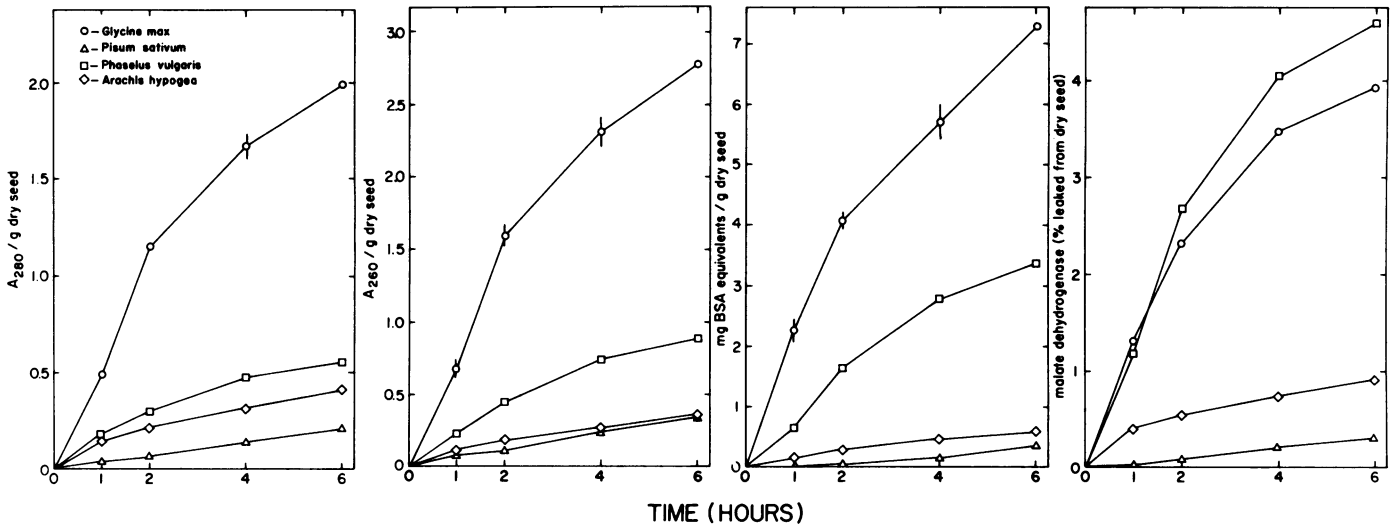

FIG. 5. Leakage of substances absorbing at 260 and 280 nm, protein, and malate dehydrogenase activity from embryos without testae of four legume species imbibing at 25 C. Each data point represents the mean ± SD of three separate experiments.

Table I. *Cytosol and Cytosol-organelle Enzyme Activities and Protein in Leakage from Embryos with Testae, Embryos without Testae, and Testae of Mature Legume Seeds after 6 h Imbibition at 25 C*

All leakage samples were concentrated from ammonium sulfate precipitations except where noted. Values in parentheses indicate per cent total activity in seeds before imbibition (see Table III). Mean values ± SD are for three separate experiments.

Legume	MDH	GDH	G6P-DH	GOT	NADP-ICDH	Protein
	nmol/g unimbibed seed or testae wt·min					*mg/g unimbibed weight*
G. max						
Embryos						
+ Testae	1,860 ± 1,770 (0.34)	8.9 ± 9.5 (0.25)	4.69 ± 6.49 (0.50)	27.4 ± 34.1 (0.29)	5.6 ± 7.6 (0.28)	0.550 ± 0.549 (0.19)
+ Testae (heat-killed)	14,460 ± 7,380 (2.62)	96.7 ± 42.3 (2.71)	15.0 ± 9.1 (1.61)	311 ± 94 (3.31)	27.7 ± 16.1 (1.38)	3.55 ± 2.47 (1.23)
− Testae	34,600 ± 500 (6.28)	189 ± 12 (5.29)	46.6 ± 0.8 (5.02)	132 ± 5 (1.41)	274 ± 37 (13.1)	14.2 ± 2.2 (4.91)
− Testae[a] (NaN_3)	34,200 ± 1,400 (6.21)	177 ± 13 (4.96)	46.2 ± 4.3 (4.97)	113 ± 40 (1.20)	349 ± 62 (17.4)	12.6 ± 1.6 (4.39)
− Testae[b]	34,100 (6.18)	181 (5.07)	47.0 (5.05)	430 (4.57)	145 (7.2)	10.6 (3.68)
Testae	4,574	146	ND[c]	ND	ND	
P. vulgaris						
Embryos						
+ Testae	1,220 ± 160 (0.47)	4.6 ± 0.8 (0.31)	1.00 ± 0.40 (0.16)	13.5 ± 2.3 (0.59)	8.9 ± 1.5 (0.32)	0.368 ± 0.088 (0.52)
− Testae	11,700 ± 1,400 (4.48)	73.2 ± 8.5 (4.88)	21.6 ± 2.35 (3.37)	139 ± 11.5 (6.04)	99.8 ± 10.7 (3.59)	4.539 ± 0.375 (6.41)
Testae	935	79	ND	ND	22.1	
P. sativum						
Embryos						
+ Testae	3 ± 4 (>0)	ND	ND	0.36 ± 0.31 (0.02)	1.8 ± 0.1 (0.04)	Trace (>0)
− Testae	2,500 ± 173 (0.90)	12.0 ± 2.9 (0.79)	2.32 ± 0.11 (0.36)	0.76 ± 0.68 (0.04)	33.6 ± 2.9 (0.74)	0.625 ± 0.081 (0.52)
Testae	94	1.4	ND	ND	ND	
A. hypogea						
Embryos						
+ Testae	0.5 ± 0.8 (>0)	ND	ND	ND	20.0 ± 3.5 (0.99)	0.083 ± 0.025 (0.07)
− Testae	2,480 ± 100 (1.01)	5.3 ± 1.4 (0.23)	34.1 ± 4.8 (0.80)	12.1 ± 1.37 (0.32)	40.8 ± 2.7 (2.02)	0.698 ± 0.088 (0.58)
Testae	ND	ND	ND	ND	349	

[a] 0.02% (w/v) NaN_3 added to imbibition water.

[b] Leakage concentrated with molecular filter (12,000-dalton exclusion limit).

[c] ND, not detected.

I and II). Also, except for fumarase, pea and peanut embryos without testae either did not leak or leaked very low levels of mitochondrial marker enzymes (Table II), as compared to quite discernable levels of cytosol and cytosol-organelle enzymes (Table I). This suggests that mitochondrial marker enzymes are being differentially leaked in these species and that some degree of membrane integrity may exist in leaking cells. There is evidence that before imbibition of legume seeds, certain mitochondrial marker enzymes, such as SDH (26), are found in the cytosol and are incorporated into the mitochondria during imbibition (20). We did not measure SDH activity in leakage due to problems encountered with endogenous activity in our preparations. In general, isolated mitochondria become richer in protein, Cyt *c* oxidase, SDH, GDH, NADP-ICDH, MDH, and GOT during

Table II. *Mitochondrial Marker Enzyme Activities in Leakage of Legume Seeds with and without Testae after 6 h Imbibition at 25 C*

All leakage sample were concentrated from ammonium sulfate precipitations except where noted. Values in parentheses indicate the per cent total activity in seeds before imbibition (see Table III). Mean values ± SD are for three separate experiments.

Legume	Cyt *c* Oxidase	Fumarase	AK
	nmol/g unimbibed seed wt·min		
G. max embryos			
+ Testae	ND[a]	ND	ND
+ Testae (heat-killed)	6.46 ± 3.95 (2.47)	ND	34.4 ± 16 (0.76)
− Testae	16.86 ± 3.71 (6.44)	2.11 ± 0.21 (5.10)	186 ± 28 (3.37)
− Testae[b]	17.15 (6.55)	ND	3.45 (6.27)
P. vulgaris embryos			
+ Testae	ND	ND	ND
− Testae	13.25 ± 7.46 (3.73)	5.91 ± 2.25 (2.36)	89.7 ± 16 (2.00)
P. sativum embryos			
+ Testae	ND	ND	ND
− Testae	1.02 ± 0.22 (>0)	4.61 ± 0.23 (0.26)	ND
A. hypogea embryos			
+ Testae	ND	ND	ND
− Testae	ND	1.38 ± 0.17 (5.00)	ND

[a] ND, not detected.

[b] Leakage concentrated with molecular filters (12,000-dalton exclusion limit).

imbibition and germination (8, 9, 11, 21, 22). This may be due to incorporation of cytosol pools of these enzymes into mitochondria during hydration, *de novo* synthesis, or to an increase in structural integrity of mitochondria during hydration. The membranes of mitochondria from unimbibed or partially imbibed legume seeds are quite disorganized (28, 38), suggesting that any enzyme which is not in close association with the membranes of the unimbibed mitochondria may not be sequestered in the mitochondria and may leak from the seed as easily as a cytosol enzyme. This may be the case with fumarase in peas and peanuts here. In that Cyt *c* oxidase and AK activities are proportionally very low or absent as compared to cytosol or cytosol-organelle enzymes in the leakage of pea and peanut embryos without testae, we suggest that these two enzymes are either not available for leakage or that they are inactivated upon leaking. If they are in close association with mitochondria and discontinuities in the plasmalemmae are not sufficient for leakage of mitochondria, they would not be observed in leakage.

CHANGES IN EMBRYO ENZYME ACTIVITIES DURING IMBIBITION

Our previous studies with soybean embryos with testae have demonstrated that the activities of some enzymes, such as GDH, G6P-DH, and NADP-ICDH, decrease during the first few h imbibition (8) or under conditions of low temperature (11), which promotes leakage (5). With findings of high levels of leakage from soybeans and beans here (Tables I and II), we questioned whether the observed leakage of enzyme activities could account for these decreases in activity in the embryos. We found that, even to a greater extent than in our previous studies, recoverable embryo GDH, G6P-DH, and NADP-ICDH activities are dramatically decreased in seeds without testae during the first 6 h imbibition (Table III). Pea and peanut embryos without testae lost high levels of G6P-DH and NADP-ICDH activity over 6 h imbibition, but not of GDH activity. We also found that recoverable Cyt *c* oxidase and fumarase activities are much reduced in the embryos of all species tested after 6 h imbibition. In no case does the amount of enzyme activity recovered in the leakage for the aforementioned enzymes (Tables I and II) account for the decreases in the activities of these enzymes. This suggests that we are not recovering all activity that is leaked or that imbibition of seeds without testae can, in some unknown fashion, inactivate enzymes or render their activity unrecoverable by the methods we have used. If the loss of activities of these enzymes during imbibition is a true reflection of the *in vivo* situation, it would suggest that severe physiological dysfunctions are occurring. Changes in activity of embryo MDH, GOT, and AK over the 6-h period imbibition are negligible and can be explained by leakage or experimental error.

CONCLUSIONS

Previous studies have indicated that, during legume seed imbibition, a variety of low-molecular weight substances are leaked from tissues (31, 32). Only one report has demonstrated that high-molecular weight substances (protein and catalase) are leaked from legume seeds (peanut) during imbibition (1); however, this study (1) indicated that more enzyme activity and protein leaches from peanuts with testae than without testae, suggesting that much of the leakage came from the testae or tissues associated with the testae (*e.g.* dried and dead endosperm tissues between the testae and cotyledons). Our data are directly contradictory to the aforementioned study in that we have found far more protein and enzyme activities to leak from peanut embryos without testae than those with testae (Tables I and II). Our data for soybean, bean, and pea embryos with and without testae are also contradictory to the aforementioned study (1). We unequivocally state that the testae of legume seeds inhibit the leakage of high-molecular weight intracellular substances during imbibition.

As to the mechanism of leakage during imbibition, our data strongly suggest that large discontinuities exist or are produced during imbibition of legume seeds. Soybean and bean seeds with testae leaked cytosol and cytosol-organelle enzymes profusely during imbibition, whereas pea and peanut seeds leaked some of these enzymes at low levels (Table I). No legume seeds with intact testae leaked detectable levels of any mitochondrial marker enzyme assayed over the course of imbibition (Table II). These data suggest that legume seeds with testae may leak intracellular substances which have high molecular weights [mol wt, ~70,000 for MDH (2) and ~300,000 for GDH (12)]. As to whether the discontinuities which allow the leakage of these high-molecular weight substances could be pores from the formation of phospholipids into hexagonal plates in the plasmalemma, as described by Simon (32), any argument would be equivocal. However, our data for soybean and bean seeds without testae suggest that mitochon-

Table III. *Enzyme Activities and Protein of Legume Embryos without Testae before and after Imbibition for 6 h at 25 C*

Values in parentheses indicate per cent total activity in seeds before imbibition. Mean values ± SD are for three separate experiments.

Legume	MDH		GDH		G6P-DH		GOT		NADP-ICDH		Cyt *c* Oxidase		Fumarase		AK		Protein	
	Dry seed	Imbibition	Dry seed	Imbibition	Dry seed	Imbibition	Dry seed	Imbibition	Dry seed	Imbibition	Dry seed	Imbibition	Dry seed	Imbibition	Dry seed	Imbibition	Dry seed	Imbibition
	μmol/g unimbibed seed wt·min																*mg/g dry seed*	
G. max	551	572 ± 70 (103.9)	3.57	1.44 ± 0.21 (40.3)	0.930	0.051 ± 0.012 (5.5)	9.42	8.71 ± 0.21 (92.5)	2.01	1.43 ± 0.17 (71.4)	0.262	0.0403 ± 0.018 (15.4)	0.0414	ND[a]	5.52	3.73 ± 0.61 (72.4)	288	259 ± 2.3
P. vulgaris	261	252 ± 64 (96.7)	1.50	0.84 ± 0.28 (55.8)	0.641	Trace (>0)	2.33	2.52 ± 0.49 (109.4)	2.78	1.52 ± 0.11 (54.7)	0.355	0.0132 ± 0.006 (3.7)	0.250	ND	4.49	4.44 ± 0.31 (98.8)	70.8	54.6 ± 5.7
P. sativum	284	298 ± 88 (104.9)	1.52	1.44 ± 0.10 (95.0)	0.644	0.178 ± 0.057 (27.6)	1.78	1.57 ± 0.04 (88.0)	4.54	2.94 ± 0.29 (64.8)	0.426	0.0252 ± 0.084 (12.6)	1.77	0.163 ± 0.014 (10.9)	4.53	4.26 ± 0.16 (93.9)	120.8	114 ± 3.12
A. hypogea	261	184 ± 18 (85.6)	2.29	2.41 ± 0.31 (109.0)	4.27	2.20 ± 0.79 (51.5)	3.84	4.57 ± 0.25 (118.8)	2.02	1.60 ± 0.46 (79.3)	0.200	0.0195 ± 0.002 (4.6)	0.0276	ND	7.65	6.97 ± 1.54 (91.1)	120.3	128 ± 6.03

[a] ND, not determined.

drial marker, cytosol, and cytosol-organelle enzyme activities and protein are leaked in roughly the same proportions as compared to dry seed levels. This indicates that, in these two species, under the conditions imposed in our studies, the plasmalemma is no barrier to the leakage of substances of various sizes. We conclude that the cells of viable soybean and bean embryos without testae can be ruptured by imbibition. However, this is probably true to a lesser or greater extent for other legume species or under differing experimental conditions. Much of the rupturing of cells of soybeans and bean embryos without testae may have been due to the splitting of tissues and pressure exerted on tissues due to unequal hydration of tissues during imbibition (Fig. 1).

The study presented here demonstrates that the testae of legume seeds act as protection against the rupture of embryo cells during imbibition of certain species such as soybean and bean. These findings suggest that caution should be applied in the interpretation of data from previous imbibitional studies conducted with legume seeds with testae removed or ground into particles and that future studies on seed imbibition should be designed with the knowledge that the seed coat serves an important function in protecting the embryo.

NOTE ADDED IN REVISION

Recent histological studies by Dunn *et al.* (10) have demonstrated that the entire cellular contents of peripheral cells of the hypocotyl-root axes are expelled through the plasmalemmae and cell walls during imbibition of seeds without testae. These studies indicate that the rupture of axes cells begins within 15 to 30 s after the onset of imbibition.

Acknowledgments—We thank Professor Earl T. Gritton, Professor Fredrick A. Bliss, and Roger K. Smith for providing seeds for the experiments. We also thank Professor Elwood A. Brickbauer for his support and interest in this project. Guy and Gil Richardson are gratefully acknowledged for their advice on printing the photographs.

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