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Differential Leakage of Intracellular Substances from Imbibing Soybean Seeds[1]

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ABSTRACT

Leakage of electrolytes, substances absorbing UV light, and enzymic activities from imbibing soybean (*Glycine max* [L.] Merr.) seeds were compared to determine the extent that passive diffusion and cellular rupture contribute to each. Imbibing seeds with testae removed had average Arrhenius energies of activation (5 to 25°C) of 3.0 and 15.8 kilocalories per mole, respectively, for the leakage of electrolytes and embryo malate dehydrogenase activity. Leakage of embryo enzymes from imbibing seeds was dependent on loss of testa integrity and subsequent loss of cellular membrane integrity or inability to seal preexisting membrane discontinuities. These data suggest that electrolyte leakage from imbibing seeds is primarily by passive diffusion, whereas the diffusion of intracellular macromolecules is primarily dependent on physiological phenomena affecting membrane integrity. Kinetic data and examination of the composition of seed leachates indicated that the leakage of substances absorbing UV light during imbibition is due to both passive diffusion of low molecular weight solutes and macromolecules released from ruptured cells.

Many factors have been evaluated as indicators of seed vigor and germinability (2). Among these factors, one of the most positively correlated with low seedling vigor during germination is the leakage of various intracellular substances from imbibing seeds (3, 11, 15, 20, 30). It has been proposed that the reason for this correlation is that seed pathogen growth may be enhanced by the presence of seed leachates (23). However, recent studies with imbibing legume seeds suggest that some loss of vigor may be due to the destruction of seed tissues and the creation of infection sites by massive cellular rupture which can occur during imbibition (7, 9, 11).

Various methods have been employed to measure leakage from imbibing seeds, often with little apparent concern for the process or processes which contribute to the factor being measured. The most common measurements of seed leakage in steep water are conductivity of electrolytes (1, 3, 7, 14, 16, 19, 22, 24, 25, 27, 30) and UV light absorbance at various wavelengths (5, 7, 17, 19). Measurements of seed steep water macromolecules such as enzymic activities or protein have less commonly been used (1, 7, 24). Knowing that at least two processes could contribute to the leakage of substances measured in the aforementioned assays (*i.e.* diffusion of low mol wt substances through cell membranes during membrane and cellular hydration [23] and release and diffusion of cellular contents of ruptured embryo and or testa cells [7, 9]), one might wonder how each of these processes is reflected in the various assays for seed leakage during imbibition and what differences one would see in the kinetics of leakage of various seed components. If the assays used for seed leakage do not correlate with one another under most experimental conditions they should not be used interchangeably, as they have in the past, to study the leakage processes.

In this study, we utilized imbibing soybean seeds to examine commonly used seed leakage assays to gain insight on the contribution of passive diffusion through membranes and cellular rupture (preexisting or induced) to each measurement. Seeds with various degrees of testa integrity were used because testa integrity is a primary factor in controlling legume seed leakage and cellular damage during germination (7, 11, 12, 21, 24). Effects of temperature were studied to gain insight into the energetics of the leakage of various seed components during imbibition and effects of ψ_w[2] were studied because of previous studies which indicate a ψ_w effect on soybean seed leakage and seedling vigor (28, 29). Our findings indicate that passive diffusion of low mol wt solutes and membrane rupture-dependent diffusion of macromolecules during seed imbibition are quite different kinetically and that the contribution of each process is variable among the methods used for measuring seed leaching.

MATERIALS AND METHODS

Plant Material. Seeds of *Glycine max* (L.) Merr. cv Wells with a moisture content of about 6.5%, as determined previously (8), were used in all experiments 3 to 10 months after seed harvest. Germination ranged from 92 to 98%. Testae were removed from seeds as previously (7) for temperature and ψ_w studies. Seed lots used contained approximately equal numbers of seeds with and without epidermal cracking, an inherited trait (26) manifested by splitting of the epidermis, but having no visible effect on other testa cell layers (*cf.* Carlson [6] for details of testa anatomy). Seeds with cracks through all testa cell layers were removed from seed lots used.

Imbibition Conditions. Temperature and ψ_w experiments (Figs. 1–3) were with three replications about 2 g seed in 100 ml double-distilled H_2O or osmoticum per replication for each treatment. Steep water was stirred gently before recording conductivity measurements. Aliquots (0.2 ml) of steep water were removed every 15 to 20 min and placed on ice for enzyme assays. Enzyme assays were within 2 h from sampling time. Temperature was maintained by immersing beakers with seeds into a thermo-regulated water bath. E_a values were calculated as before (8). Polyethylene glycol 8,000 (Fisher Scientific Co.) was used to adjust ψ_w. Verification of ψ_w was with a Wescor 5100C vapor pressure osmometer.

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[2] Abbreviations: ψ, water potential; E_a, Arrhenius energy of activation; GOT, glutamate oxaloacetate transaminase (EC 2.6.1.1); MDH, NAD-malate dehydrogenase (EC 1.1.1.37); NADP-ICDH, NADP-isocitrate dehydrogenase (EC 1.1.1.42); GDH, NAD-glutamate dehydrogenase (EC 1.4.1.3); AK, adenylate kinase (EC 2.7.4.3).

Experiments with seeds with and without epidermal cracking (Figs. 4 and 5; Table I) were with three seed lots (5.0–5.2 g) for each time point and for each testa type. At sampling times seeds were removed, quickly blotted with tissue paper, and immediately weighed. Steep water was placed on ice for measurement of leakage components. In some studies, testae were removed from seeds at 3, 4.5, or 6 h after imbibition and embryos were rinsed for 10 to 15 s in 2 ml of distilled H_2O.

Leakage Measurements. Conductivity, A_{260}, A_{280}, and enzyme activities were assayed as before (7). Protein determinations were by the method of Bradford (4). Measurement of the percentage of leakage absorbing UV light at A_{260} and A_{280} due to protein and nucleic acids was made by saturating leachates, which had been measured for A_{260} and A_{280}, with $(NH_4)_2SO_4$, centrifuging at 1,200*g* for 20 min, and measuring supernatants for A_{260} and A_{280}. A_{260} and A_{280} measurements for $(NH_4)_2SO_4$-saturated water were used as a reference in a double-beam spectrophotometer. Leachates between the testa and embryo of imbibing seeds were assayed by inserting a No. 27.5 hypodermic needle through the testa near the hilum, and removing the fluid with a hypodermic syringe after 0.5 to 1 h of imbibition. In some seeds, there is a space between the testa and embryo on either side of the hilum which has a volume of between 1 and 10 μl between 0.5 and 1 h of imbibition. Care was taken to avoid piercing the embryo. The fluid from 10 to 30 seeds was pooled and 10 μl was used for each assay for GOT.

RESULTS AND DISCUSSION

Temperature Effects. Over the initial 150 min of imbibition, the effect of temperature on the leakage of MDH activity from imbibing soybean seeds without testae was much greater than on the leakage of electrolytes (Figs. 1 and 2). Temperatures above 25°C were not used for calculations of Q_{10} and E_a values because MDH lost activity when incubated at such temperatures (data not shown). The average Q_{10} and E_a for electrolyte leakage between 4.2 and 24.5°C over 150 min were 1.19 and 3.0 kcal/mol (calculated from Fig. 2), respectively. These values suggest that electrolyte leakage is primarily due to passive diffusion (14). Similar Q_{10} and E_a values have been found for electrolyte leakage from imbibing soybean cotyledons (14) and for the leakage of substances absorbing light at 254 nm from imbibing radish seeds with testae and sugar pine seeds with testae removed (17). In contrast, the average Q_{10} and E_a for the leakage of MDH over the same temperature range and time period were 2.46 and 15.0 kcal/mol, respectively, suggesting that the leakage of intracellular macromolecules from imbibing seeds is primarily due to a physiological or biochemical process or processes. Temperature effects on the leakage of MDH were not due to temperature effects on enzyme stability or enzyme activation. No appreciable loss or gain of MDH activity was detected in leakage samples over several hours of incubation at temperatures from 0 to 25°C (data not shown). The lack of linearity of Arrhenius plots (Fig. 2) for electrolyte and MDH leakage suggests that more than one factor limits or contributes to the leakage of both in the initial 150 min of imbibition. It should be noted that the temperature at which the greatest rate of MDH leakage was found, 25°C, is also the temperature at which 'Wells' soybeans have the greatest seedling vigor (10).

We did not observe decreases in the E_a for the leakage of electrolytes from imbibing soybean seeds without testae at temperatures below 20°C as did Leopold (14). We attribute the differences between our respective studies to differences in initial seed moisture content, cultivar used, time interval used to calculate leakage rates, and embryo tissues used. We found that after 150 min of imbibition, values for total electrolytes or MDH activity were very reproducible (compare 24.5°C values in Fig. 1 with 25°C, $\psi_w = 0$ values in Fig. 3), in contrast to the extremely variable

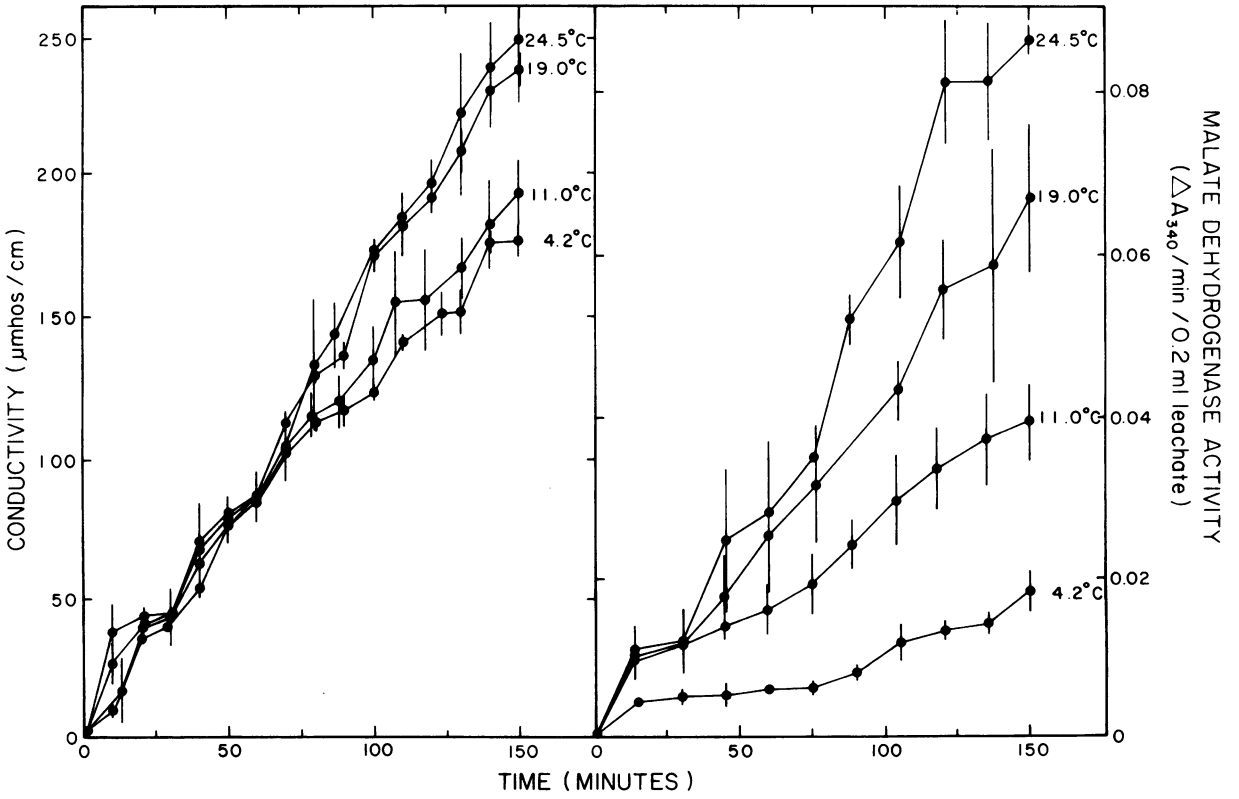

FIG. 1. Effect of temperature on leaching of electrolytes and MDH activity from imbibing 'Wells' soybean seeds with testae removed. Each data point represents the mean ± SD of three replications of about 2.0 g seeds in 100 ml double-distilled H_2O. Electrolyte and MDH measurements were from the same experiments.

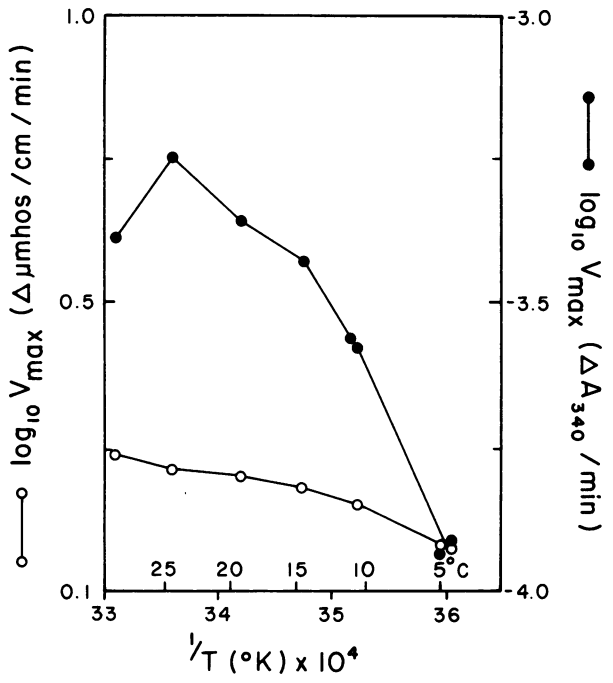

FIG. 2. Arrhenius plots of electrolyte (○) and malate dehydrogenase activity (●) leakage from imbibing 'Wells' soybean seeds with testae removed. Experiments were as in Figure 1 with each data point representing the mean of three experiments. Leakage rates were determined from the initial 150 min of imbibition.

rates observed in the initial 0 to 30 min of leakage. We often, but not always, observed an initial burst of leakage, followed by a lag and then a second burst of leakage, suggesting that either two differing tissues or two differing processes, or both influence leakage. Variation in observed initial rates of leakage may have been due in part to various degrees of adherence of dead and crushed endosperm debris to the seed embryo (*cf.* 6) among experimental seed lots. Dead seed tissues may leach electrolytes, substances absorbing UV light, and macromolecules, including active enzymes, at much higher rates than live tissues (7, 14, 17). The biphasic leakage pattern that we often observed is also discernable in other studies with imbibing soybean seeds with a low moisture content, such as we used, but not with seeds with high moisture contents (19). We used seeds with a moisture content of 6.5% because this level of moisture is normal for commercially purchased seed lots.

Effects of Steep Water ψ_w. Rates of leakage of electrolytes from imbibing seeds without testae decreased as steep water ψ_w decreased (Fig. 3). However, the magnitude of decrease in rate of electrolyte leakage decreased markedly as ψ_w was progressively decreased to −3.0 bars. In contrast, the rate of leakage of MDH activity appeared to decrease in response to decreasing steep water ψ_w in two distinct phases, an initial large decrease between 0 and −0.5 bars and a second decrease between −1.0 and −3.0 bars. These data suggest that the leakage of macromolecules may be the result of at least two phenomena which are differentially affected by steep water ψ_w.

Testa and Cellular Rupture. If the legume seed testa is an impenetrable barrier to the diffusion of intracellular macromolecules from embryo cells ruptured during imbibition or before imbibition, it is apparent that one simply cannot measure cellular rupture in legume seeds by assaying for such substances in steep water. However, if embryo cellular rupture occurs largely or only during imbibition in seeds without testae or with fractured testae, this point is irrelevant. To determine the degree of embryo cellular rupture in imbibing soybean seeds with testae, we examined the fluid which accumulates between the testa and embryo of some seeds during imbibition and rinses of embryos after testae removal at 3 to 6 h imbibition (Table I). We assayed GOT because it is in high activity in embryos and is absent in testae of soybean seeds (7); hence, its presence would not indicate testa cellular rupture, but only embryo cellular rupture in seeds with testae. The low levels of GOT activity (less than 1% of the total activity leached from seeds without testae in the same period of time, *cf.* 7) in fluid between the testae and embryos of imbibing seeds and the lack of detectable activity in rinses from embryos with testae (data not shown) suggest that little cellular rupture occurs until soybean seed embryo tissues are exposed directly to water. However, one could also speculate that the seeds with testae do have embryo cellular ruptures and that the presence of the testa allows ruptures to close during imbibition. Ruptures in human erythrocytes can be closed by adjusting cell environment (13). If this is occurring in imbibing soybean embryo cells of seeds with testae, it must be occurring rapidly, for little or no GOT activity is leaching from imbibing embryos either early or late during imbibition (Table I). Also, the net effect of such a phenomenon on seed vigor and germination would probably be nil in that no permanent damage is sustained.

Defective Testa Effects. Knowing that the testa does prevent permanent cellular rupture in soybean embryos during imbibition, one must assume that the presence of intracellular embryo enzymes in steep water would indicate lack or loss of testa integrity. To ascertain how loss or lack of testa integrity and subsequent cellular rupture are reflected in the commonly used seed imbibition assays, we compared the kinetics of the leakage of electrolytes, substances absorbing UV light, and GOT activity in small lots of soybean seeds with and without defective testae (*i.e.* de_1 trait, testa epidermal cracking) (Fig. 4). Seeds with the de_1 trait lose testa integrity and subsequently suffer cellular rupture more often than seeds with intact testae (11). The loss of testa integrity can easily be identified by the rapid hydration and ballooning of the testa away from the seed embryo during imbibition. Aboout 25% of seeds with the de_1 trait lost their testa integrity over the 6-h period of imbibition, as compared to less than 5% of seeds with intact testa. The sequential loss of testa integrity in seed lots is reflected by increasing leakage of GOT activity (Fig. 4D). Seeds with defective testa leached only very low levels of GOT activity over the first 2 h of imbibition. Rates of leakage increased sharply after 2 h and were linear for the next 4 h. Seeds with intact testae leached low levels of GOT activity between 3 and 6 h.

Leakage of many intracellular enzymes was much lower for seeds with intact testae after 6 h of imbibition (Table I); however, these differences were more or less pronounced depending on the enzyme assayed. Differences in the leakage of NADP-ICDH, GOT, and AK activity, all enzymes which are only detected in the seed embryo, are very great as compared to differences in the leakage of MDH, which is also leaked from the testa (7). MDH activity can be easily detected within a few minutes of imbibition (Fig. 5). The rapid and linear leakage of MDH from seeds with testae suggests to us that much MDH is leaking from the testa. These data suggest that if one is to monitor the leakage of an enzyme from seeds with testae it is important to know where the enzyme is located within the seed. We did not detect leakage of GDH activity from seeds with intact embryo. Differences in leakage of various enzymes from seeds with differing testae types were certainly due in part to differing amounts of testa rupturing. However, these differences could also be partially due to size of testa ruptures and cellular ruptures. The hole diameter in ruptured human erythrocyte cells and the average diameter of an enzyme within the cell will determine whether the enzyme will leak from the cell and at what rate it will leak if it does diffuse from the cell (13). An enzyme such as GDH, which is organellar, would require a large hole in the cell for it to leach from the cell while located

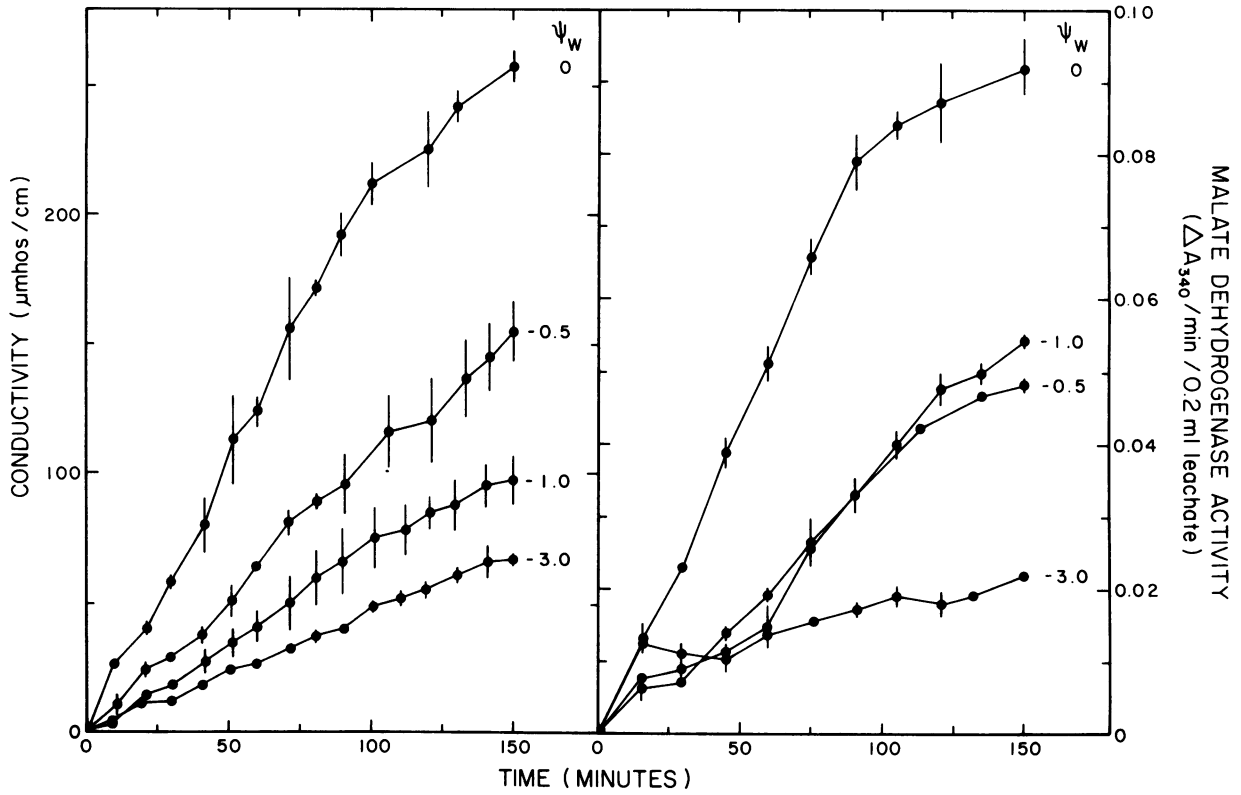

FIG. 3. Effect of steep water ψ_w on leaching of electrolytes and MDH activity from imbibing 'Wells' soybean seeds with testae removed. Experiments were as in Figure 1 except for ψ_w and temperature (25°C). ψ values are indicated in bars. Each data point represents the mean ± SD of three replications.

Table I. *Leachates from 'Wells' Soybean Seeds with and without Epidermal Cracking*

Seeds were imbibed at 25°C for 6 h, except in experiments measuring GOT activity between the testa and embryo, where leachate was removed between 30 min and 1 h of imbibition. Values for leachates between the testa and embryo are for fluid pooled from 10 to 30 seeds. Values for steep H_2O leachate are means ± SD of three replications of 5.0 to 5.2 g of seeds in 25 ml of double distilled H_2O.

	Intact Testae	Testae with Epidermal Cracking
Leachates between testa and embryo of intact seeds		
GOT (pmol/g dry seed wt·min)	8.3 ± 11.7	40.5 ± 46.5
Leachates in steep H_2O of seeds imbibed 6 h		
Protein ($\mu g\ g^{-1}$ dry seed)	150 ± 67	585 ± 235
A_{280} due to protein and nucleic acids (% of total A_{280})	36.0 ± 0.9	45.21 ± 3.5
A_{260} due to protein and nucleic acids (% of total A_{260})	32.6 ± 1.0	42.0 ± 3.6
	nmol/g dry seed wt·min	
Enzyme activities		
MDH	311 ± 255	1380 ± 76
NADP-ICDH	0.358 ± 0.621	5.82 ± 1.09
GDH	ND[a]	8.07 ± 3.88
AK	ND[a]	0.286 ± 0.495

[a] Not detected.

within mitochondria or plastids or would require ruptures in both organelles and plasmalemmae of sufficient size for diffusion through membranes.

Kinetics of electrolyte leakage from imbibing seeds with and without defective testae were similar (Fig. 4C) and resembled those for seed hydration (Fig. 6), suggesting that leakage of electrolytes is a function of seed hydration and is a passive process. The major difference between the two seed types was the slightly greater leakage from seeds with the de_1 trait between 3 and 6 h. The leakage kinetics of substances absorbing light at A_{260} and A_{280} were intermediate between those for electrolyte and GOT leakage (Fig. 4, A and B) suggesting that the leakage of these substances could be a reflection of both low mol wt compounds which would diffuse through a membrane and intracellular macromolecules released by cell rupture. This is supported by the finding that 32 to 46% of UV absorbtion by steep water after 6 h is due to precipitable proteins and nucleic acids (Table I).

These data strongly suggest that the measurement of embryo enzyme leakage during seed imbibition will indicate preexisting testa and embryo cellular damage and/or loss of testa integrity and subsequent cellular rupture during legume seed imbibition, whereas the measurement of electrolytes closely reflects the passive diffusion of solutes through seed membranes.

The large variation in the leakage of enzyme activities among replications of seeds with testae (Fig. 4; Table I) was also observed in our previous study (7). This variation appears to be due to differing numbers of seeds among replications losing testa integrity during imbibition and differing time requirements for the loss of testa integrity among individual seeds in replications. Such variability was not usually observed with imbibing seeds with testae removed (Figs. 1 and 3). Others have noted the greater variability in the leakage of solutes from imbibing soybean seeds with testae as compared to those without testae and have used this observation as a justification for using seeds with testae removed in imbibition studies (5).

CONCLUSIONS

Kinetic data presented here strongly suggest that the leakage of electrolytes from imbibing soybean seeds with or without testae is

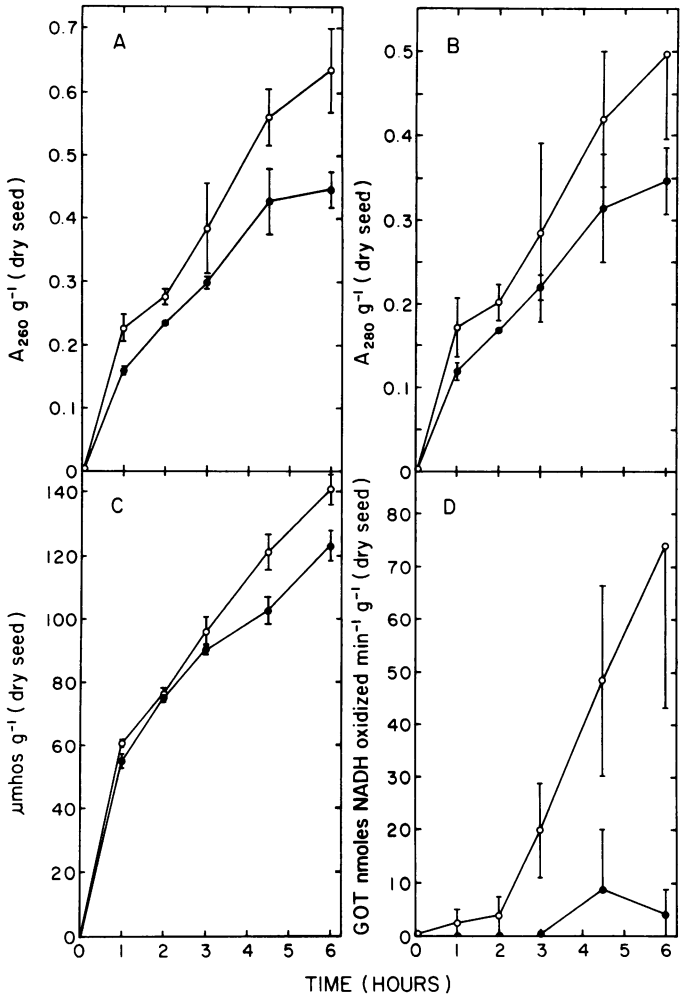

FIG. 4. Leachates in the steep water of imbibing 'Wells' soybean seeds with (○) and without (●) testa epidermal cracking. Leachates were from the same seeds as in Figure 6 and Table I. A_{260} (A), A_{280} (B), conductivity (C), and GOT activity (D) of steep H_2O were determined within 10 to 60 min of removal of seeds for imbibition H_2O. Each data point represents the mean ± SD of three replications of 5 to 5.2 g seeds in 25 ml of double-distilled H_2O at 25°C.

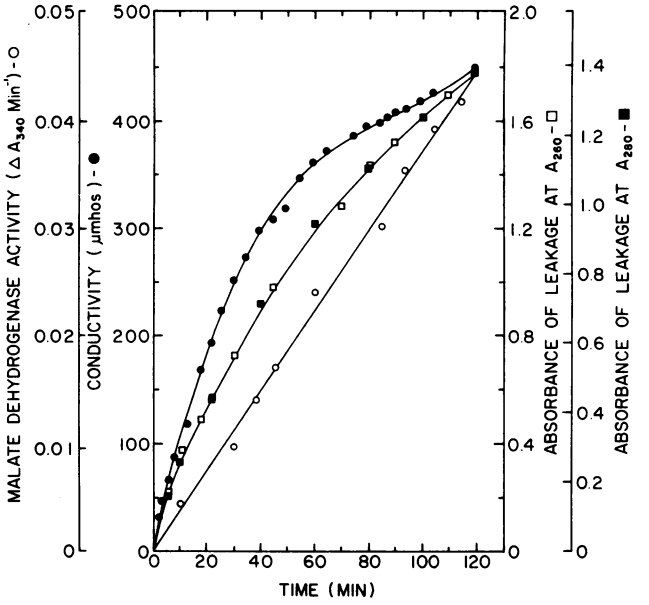

FIG. 5. Typical pattern of leaching of various seed components by imbibing 'Wells' soybean seeds. A normal population of seeds with and without testa epidermal cracking (200 g) was imbibed in 1 L of double-distilled H_2O at 25°C. Conductivity was measured continuously and 0.5-ml aliquots were removed routinely for A_{260}, A_{280}, and MDH activity measurements.

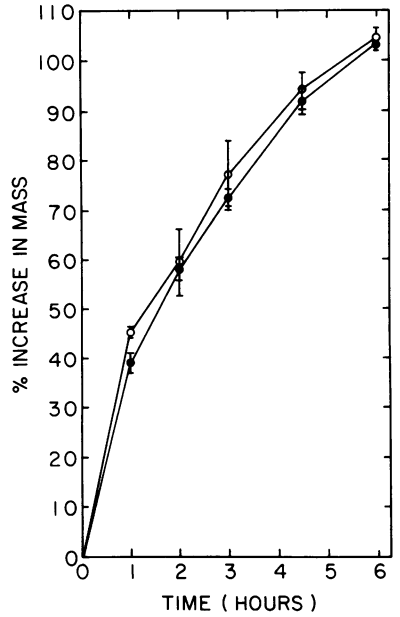

FIG. 6. Imbibitional increases in mass of 'Wells' soybean seeds with (○) and without (●) testa epidermal cracking. Each data point represents the mean ± SD of three replications. Seeds were the same as used in leachate studies (Fig. 4; Table I).

almost entirely by passive diffusion and is not necessarily an indication of cellular rupture. Studies with freeze-damaged tissues have also demonstrated that high levels of electrolyte leakage do not necessarily indicate cellular damage (18). The removal of the seed testa increases both the rates of electrolyte leakage and cellular rupture (7). The reason for increased electrolyte leakage may simply be due to removal of a barrier to diffusion. Increased cellular rupture after testa removal has little or no effect on electrolyte leakage (Fig. 1). Past studies which have attempted to show causative relationships between electrolyte leakage and cellular damage are not necessarily valid. In contrast, the leakage of embryo intracellular enzymes from imbibing seeds is due to a loss of testa integrity and cellular rupture, either preexisting or caused by imbibition. If seed embryo cells with testae do have preexisting ruptures, it is probable that they close early in seed hydration because little or no detectable intracellular embryo enzymes can be detected between the hydrating testa and embryo.

The use of seed steep water UV absorption measurements in imbibition studies should be discouraged because they reflect both passive diffusion of low mol wt substances and macromolecules from cellular ruptures (Fig. 4; Table I) and interpretation of data could be difficult. Data presented here strongly suggest that seed steep water electrolyte and enzymic activity measurements be used to measure passive diffusion and cellular rupture, respectively, in future seed imbibition studies.

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