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The Mechanism of Amino Acid Efflux from Seed Coats of Developing Pea Seeds as Revealed by Uptake Experiments

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The uptake of amino acids by excised seed coat halves of developing seeds of pea (*Pisum sativum* L.) was characterized. The influx of L-valine and L-glutamic acid was proportional to their external concentration, with coefficients of proportionality (k) of 11.0 and 7.1 μmol g^{-1} fresh weight min^{-1} M^{-1}, respectively. The influx of L-lysine could be analyzed into a component with linear kinetics (k = 8.1 μmol g^{-1} fresh weight min^{-1} M^{-1}) and one with saturation kinetics (Michaelis constant = 6.5 mM), but the latter may have resulted from the mutual interaction between the influx of the cationic lysine and the membrane potential. The influx of the amino acids was not affected by 10 μM carbonylcyanide *m*-chlorophenylhydrazone, but was inhibited by about 50% in the presence of 2.5 mM *p*-chloromercuribenzene sulfonic acid. Conservative estimates of the permeability coefficients of the plasma membrane of seed coat parenchyma cells for lysine, glutamic acid, and several neutral amino acids were all in the range of 4×10^{-7} cm s^{-1} to 9×10^{-7} cm s^{-1}, which is 4 to 5 orders of magnitude greater than those reported for artificial lipid bilayers. It is concluded that nonselective pores constitute a pathway in the plasma membrane for passive transport of amino acids. It is argued that this pathway is also used for the efflux of endogenous amino acids, the process by which nitrogen becomes available for the embryo.

Embryos of developing legume seeds receive their nutrients from the surrounding seed coat. Extensive studies have shown that essentially all organic nutrients arrive in the seed coat by way of the phloem (Pate, 1980; Peoples and Gifford, 1990). Phloem unloading in the seed coat is currently considered to be symplasmic (Offler and Patrick, 1984, 1993; Grusak and Minchin, 1988; Offler et al., 1989). Because the embryo is symplasmically isolated from the seed coat, the nutrients eventually have to be transferred from the seed coat symplasm into the apoplasm before they can be taken up by the embryo.

Sugars and amino acids are the major organic nutrients supplied by the seed coat to the developing embryo. In "empty" seed coat experiments, at a stage at which the RWC of the cotyledons was 75%, the pea (*Pisum sativum* L.) seed coat released Suc and Glc in a ratio of 12:1 hexose equivalents (De Jong and Wolswinkel 1995), and a mixture of some 20 amino acids, in which Gln (25 mol%), Ala (20 mol%), and Thr (15 mol%) were the three principal components (Lanfermeijer et al., 1992). At this stage of development, the initial efflux of sugars was approximately 1.5 μmol seed $coat^{-1}$ h^{-1}, and that of amino acids approximately 2.5 μmol seed $coat^{-1}$ h^{-1} (de Jong and Wolswinkel, 1995). It can be estimated that these effluxes, measured under experimental conditions, exceed the requirement of the embryo in planta by 2- and 8-fold, respectively (Lanfermeijer et al., 1992; de Jong et al., 1996).

Remarkably, the initial efflux of sugars as well as of amino acids has been recorded to be precisely the same for attached and detached seed coats (de Jong and Wolswinkel, 1995), which is strong evidence that the solutes are released from the seed coat parenchyma rather than directly from the phloem strands in the seed coat. This corroborates the contention that phloem-imported assimilates are unloaded symplasmically, and that the final step in the process by which nutrients become available for the embryo is their transport across the plasma membrane of seed-coat parenchyma cells.

The mechanism by which sugars and amino acids are released by seed coats is not fully understood. Release of Suc by seed coats of *Vicia faba* and *Phaseolus vulgaris* has been suggested to be a H^+/Suc antiport (Fiew and Patrick, 1993; Walker et al., 1995). By contrast, we obtained evidence that the release of Suc, as well as of amino acids from pea seed coats, is a passive, facilitated membrane-transport process (de Jong and Wolswinkel, 1995). The latter view was supported by uptake experiments that revealed that Suc can be transported across the plasma membrane of seed coat parenchyma by a passive, facilitated mechanism. In addition, the process of Suc uptake by the seed coat appeared to have much in common with the process of Suc release from the seed coat symplasm (de Jong et al., 1996).

Here we report the uptake of exogenously supplied amino acids by isolated pea seed coats. From the measured amino acid influxes, information is deduced about the permeability of the plasma membrane of seed coat parenchyma cells. Furthermore, the characteristics of amino acid uptake are compared with those reported for the release of endogenous amino acids from pea seed coats (de Jong and Wolswinkel, 1995).

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Abbreviations: ACPC, 1-aminocyclopentane-1-carboxylic acid; AIB, 2-aminoisobutyric acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; PCMBS, *p*-chloromercuribenzene sulfonic acid; RWC, relative water content; $t_{1/2}$, half-life.

MATERIALS AND METHODS

Pea (*Pisum sativum* L. cv Marzia) plants were grown in a growth chamber as described previously (de Jong and Wolswinkel, 1995), except that no flowers were removed from the plants. The seeds used were at the final stage of seed filling and the embryos had a RWC of 57 ± 2%. The seed coats were collected and prepared as described previously (de Jong et al., 1996).

Uptake Experiments

The uptake of ^{14}C-labeled amino acids was determined as described for the uptake of sugars (de Jong et al., 1996). Briefly, 10 seed coat halves were incubated in 5 mL of standard medium (0.5 mM $CaCl_2$, 400 mM mannitol, 2 mM Mes buffer, adjusted to pH 5.5 with KOH), supplied with 3.6 kBq of either U-^{14}C-labeled L-Val (10.1 GBq mmol^{-1}), L-Glu (10.4 GBq mmol^{-1}), L-Lys (12.2 GBq mmol^{-1}), AIB (1.97 GBq mmol^{-1}), ACPC (2.18 GBq mmol^{-1}), L-Ala (370 MBq mmol^{-1}), L-Thr (370 MBq mmol^{-1}), or L-Arg (12.3 GBq mmol^{-1}), and the desired amount of unlabeled amino acid. After the uptake period, the seed coat halves were rinsed five times with 10 mL of demineralized water. To further exclude free-space uptake, the "initial" influxes were computed from the uptake between 5 and 20 min of incubation. Influxes were expressed on the basis of fresh weight. The concentration of ^{14}C (applied ^{14}C-amino acid and its possible metabolic products) in the seed coat tissue was calculated, taking into account that an average seed coat weighs 92 mg (fresh weight) and that the RWC of the seed coat is 80% (Lanfermeijer et al., 1992). The concentration dependence of the Lys influx was evaluated by fitting various rate equations, as described by Borstlap and Schuurmans (1988).

RESULTS

Time Course of Amino Acid Uptake

Uptake of ^{14}C-labeled L-Val, L-Glu, and L-Lys by seed coats was determined at a very low concentration (<0.1 μM) and at 10 mM and 100 mM. The time course of the relative uptake, i.e. the ratio of the concentration of label in the seed coat to the concentration of label in the medium, $[^{14}C]_i/[^{14}C]_o$, is shown in Figure 1.

The uptake of L-Val proceeded rather slowly (Fig. 1A). Even after 1 h of incubation its concentration in the seed coat was still lower than the external concentration. Up to 30 min of incubation, the relative uptake of Val was identical at the three widely different external concentrations, implying that the amount of Val taken up by the seed coat was directly proportional to its concentration in the medium. During a prolonged incubation, further uptake of [^{14}C]Val was seen only when the amino acid was supplied at the submicromolar concentration (Fig. 2A). This sustained uptake is probably the result of metabolic conversion, such as incorporation into proteins, since it was not observed for the nonprotein amino acids AIB and ACPC (Fig. 2B). The course of the relative uptake of these amino acids, supplied at submicromolar concentrations, was identical to that of L-Val at an external concentration of 100 mM. In these cases the uptake attained a plateau when the internal concentration was about 0.6 times the external concentration, indicating that only 60% of the tissue volume was accessible to the exogenously supplied amino acids. The uptake of L-Glu was somewhat slower, but otherwise it was essentially the same as for L-Val (Fig. 1B).

A quite different pattern was found for L-Lys in that its relative uptake clearly depended on the external Lys concentration (Fig. 1C). At the lowest concentration used (56 nM), the label from [^{14}C]Lys was already accumulated by the seed coat within 10 min of incubation. When supplied at a concentration of 100 mM, however, the relative uptake of Lys proceeded similarly to that of Val and Glu, and reached a plateau when the ratio $[^{14}C]_i/[^{14}C]_o$ was about 0.6.

Concentration Dependence of Amino Acid Influx

The concentration dependence of the influx of L-Val and L-Glu can be described by a single parameter, the proportionality constant k, which is determined from the quotient of influx and external concentration, $v/[S]$ (Table I). Mean values of determinations at three different concentrations were 11.0 ± 0.5 μmol g^{-1} fresh weight min^{-1} M^{-1} for L-Val and 7.1 ± 0.3 μmol g^{-1} fresh weight min^{-1} M^{-1} for L-Glu. Similar values of k were found for some other neutral amino acids, including Ala and Thr, which are major components of the amino acid mixture released by pea seed coats (Lanfermeijer et al., 1992), and the nonprotein amino acids AIB and ACPC. The k values were all within the

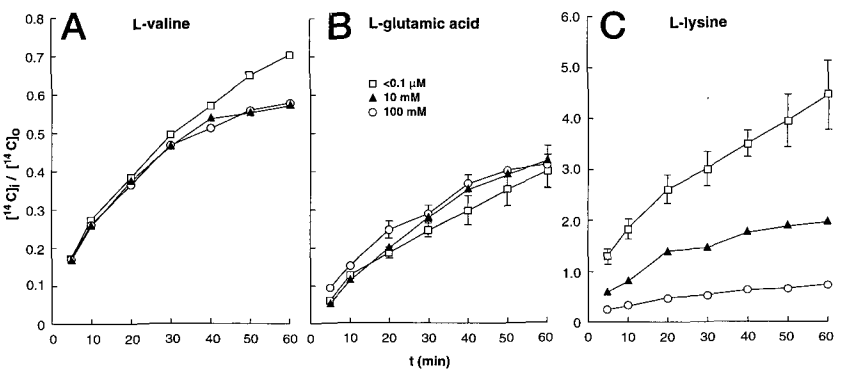

Figure 1. Time course of the uptake of ^{14}C-labeled L-Val (A), L-Glu (B), and L-Lys (C), expressed as the ratio of the concentration of label in the seed coat to the concentration of label in the medium ($[^{14}C]_i/[^{14}C]_o$). The initial concentrations of the amino acids in the medium were <0.1 μM (□), 10 mM (▲), and 100 mM (○). Concentrations < 0.1 μM were: L-Val, 74 nM; L-Glu, 81 nM; and L-Lys, 56 nM. Each point represents the mean ± SE of at least three experiments. Bars are not shown when smaller than the symbols.

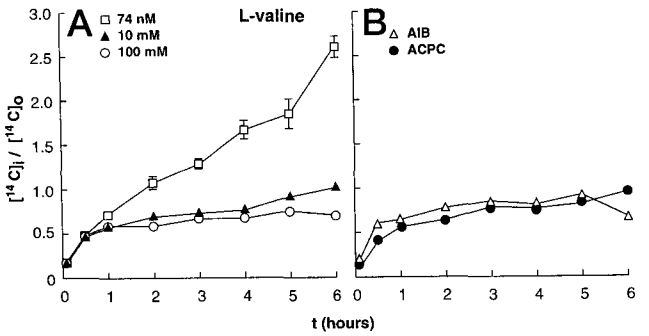

Figure 2. Time course of the uptake of ^{14}C-labeled L-Val (A), AIB (△), and ACPC (●) (B), expressed as the ratio of the concentration of label in the seed coat to the concentration of label in the medium ($[^{14}C]_i/[^{14}C]_o$). The initial concentrations of L-Val were 74 nM (□), 10 mM (▲), or 100 mM (○). The initial concentrations of AIB and ACPC were 264 and 337 nM, respectively.

range of 8 to 16 μmol g^{-1} fresh weight min^{-1} M^{-1}, and tended to be greater for smaller molecules (Table II). Much higher values of $v/[S]$ were found for the basic amino acids L-Arg and L-Lys (Tables I and II). In contrast to Val and Glu, the values of $v/[S]$ for L-Lys decreased from 52.5 μmol g^{-1} fresh weight min^{-1} M^{-1} at 56 nM to 12.8 μmol g^{-1} fresh weight min^{-1} M^{-1} at 100 mM (Table I), indicating the involvement of a saturable transport system. This was further verified by the results of a more detailed assessment of the concentration dependence of the Lys influx (Fig. 3). Curve fitting revealed that the influx of Lys could be formally analyzed into a saturable component (K_m = 6.5 ± 2.3 mM; V_{max} = 0.25 ± 0.09 μmol g^{-1} fresh weight min^{-1}) and a linear component with a constant of proportionality (k = 8.1 ± 1.6 μmol g^{-1} fresh weight min^{-1} M^{-1}) close to that for the uptake of neutral amino acids.

Table I. *Values of v/[S] for the uptake of L-Val, L-Glu, and L-Lys by pea seed coats at different external concentrations in the absence or presence of inhibitors*

Values given are means ± SE (n = 3–5). Note that 1 μmol g^{-1} fresh weight min^{-1} M^{-1} = 10^{-3} cm^3 fresh weight g^{-1} min^{-1}.

Amino Acid	v/[S]		
	No inhibitor	+CCCP	+PCMBS
	μmol g^{-1} fresh wt min^{-1} M^{-1}		
L-Val			
74 nM	11.7 ± 0.3	9.3 ± 0.7	4.7 ± 0.6
10 mM	10.9 ± 0.6	N.D.[a]	N.D.
100 mM	10.5 ± 1.2	12.8 ± 0.7	N.D.
L-Glu			
81 nM	6.6 ± 0.7	5.3 ± 0.2	N.D.
10 mM	6.8 ± 0.3	N.D.	N.D.
100 mM	8.0 ± 0.6	7.5 ± 0.6	N.D.
L-Lys			
56 nM	52.5 ± 5.0	57.9 ± 1.6	27.1 ± 0.6
10 mM	37.7 ± 1.9	N.D.	N.D.
100 mM	12.8 ± 1.5	13.9 ± 1.1	N.D.

[a] N.D., Not determined.

Table II. *Values of v/[S] for the uptake of several amino acids by pea seed coats*

Values given are from a single experiment. Note that 1 μmol g^{-1} fresh weight min^{-1} M^{-1} = 10^{-3} cm^3 g^{-1} fresh weight min^{-1}.

Amino Acid	Concentration	v/[S]
	μmol g^{-1} fresh wt min^{-1} M^{-1}	
L-Ala	1.86 μM	15.3
L-Thr	5.12 μM	13.6
AIB	264.0 nM	15.3
ACPC	337.0 nM	8.7
L-Arg	45.0 nM	32.1

Effects of CCCP and PCMBS on Amino Acid Uptake

The protonophore CCCP clearly reduced the uptake of Val and Glu (Fig. 4, A and B), but it inhibited the initial influx of these amino acids only slightly (Table I). Surprisingly, the uptake of Lys was not affected at all by CCCP (Fig. 4C). The sulfhydryl reagent PCMBS reduced the uptake of Val and Lys more strongly, and inhibited the initial influx of these amino acids by about 50% (Fig. 4, A and C; Table I).

DISCUSSION

Mechanism of the Influx of Neutral and Acidic Amino Acids

Because the outer surface of the seed coat has an impermeable covering (Offler and Patrick, 1984), it is to be expected that the release of solutes from isolated seed coats into a bathing medium occurs exclusively at the inner surface; this has been verified for the release of Suc (de Jong et al., 1996). We assume, therefore, that the uptake of amino acids also takes place exclusively at the inner surface of the seed coat.

Obviously, the exogenously supplied amino acids entered the symplasm of the seed coat. This is indicated by the sustained uptake of Val when it was supplied at a submicromolar concentration (Fig. 2A) and by the inhibitory effect of PCMBS on the influx of Val (Fig. 4A; Table I).

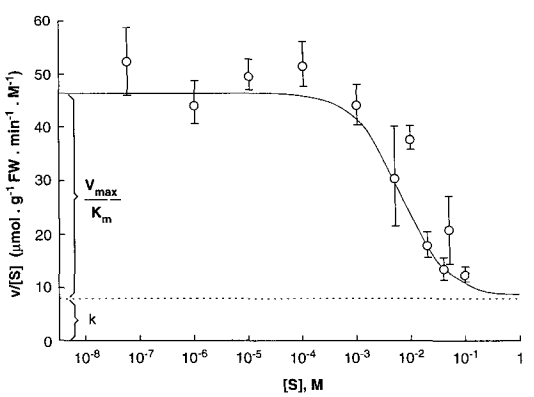

Figure 3. Concentration dependence of the influx of L-Lys. Each point represents the mean ± SE of at least three determinations. The curve drawn was computed from the parameter values for K_m, V_{max}, and k (see text), which were obtained by fitting the rate equation $v = V_{max}[S]/(K_m + [S]) + k[S]$ to the experimental data. Note that V_{max}/K_m = 38.4 μmol g^{-1} fresh weight min^{-1} M^{-1}. FW, Fresh weight.

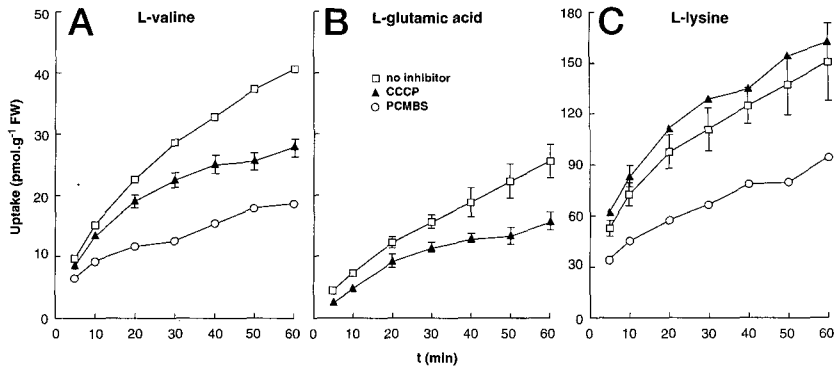

Figure 4. Uptake of ^{14}C-labeled L-Val (A), L-Glu (B), and L-Lys (C) in the absence of inhibitors (□), or in the presence of either 10 μM CCCP (▲) or 2.5 mM PCMBS (○). The initial concentrations of the amino acids in the medium were 74 nM for L-Val, 81 nM for L-Glu, and 56 nM for L-Lys. Each point represents the mean ± SE of at least two measurements. Bars are not shown when smaller than the symbols. FW, Fresh weight.

Furthermore, the concentration of label in the seed coat at equilibrium was about 0.6 times the external concentration (Fig. 2, A and B), which is clearly too high to be attributable to free-space uptake. As can be seen in Figures 1 and 2, the uptake of Glu, Val (10 and 100 mM, respectively), AIB, and ACPC proceeded with a $t_{1/2}$ of 20 to 40 min, which agrees very well with the $t_{1/2}$ of the fast component of amino acid efflux, as determined by Lanfermeijer et al. (1992). However, the slow component of amino acid efflux ($t_{1/2} > 3$ h) did not show up in the uptake experiments, which makes it questionable whether it represents the emptying of the vacuolar compartment, as has been suggested (Lanfermeijer et al., 1992).

Uptake of the neutral amino acids and Glu appears to be a passive, diffusion-like process because it was nonconcentrative, insensitive to CCCP, and displayed linear kinetics. Most likely, the uptake does not result from simple diffusion through the lipid bilayer of the membrane. The evidence for this is 2-fold. First, the inhibitory effect of PCMBS on the amino acid influx can be regarded as strong evidence for the involvement of a special component of the membrane. Second, it can be calculated that the permeability coefficient of the plasma membrane of seed coat parenchyma cells is much greater than expected for a lipid membrane. Permeability coefficients (P) can be estimated using the relationship $P = k/A$, where A is the plasma membrane area. The values of k determined for Glu, ACPC, Val, Thr, AIB, and Ala were in the range of 7.1×10^{-3} cm^3 g^{-1} fresh weight min^{-1} to 15.3×10^{-3} cm^3 g^{-1} fresh weight min^{-1} (Tables I and II). Adopting for A a value of 295 cm^2 g^{-1} fresh weight, which is probably an overestimate (de Jong et al., 1996), the permeability coefficients turn out to be in the range of 4.0×10^{-7} cm s^{-1} to 8.6×10^{-7} cm s^{-1}. This is 4 to 5 orders of magnitude greater than has been determined for the permeation of artificial lipid bilayers by the amino acids Gly, Ser, and Lys (Chakrabarti and Deamer, 1992).

Mechanism of the Influx of Basic Amino Acids

An important conclusion to be drawn from our experiments is that neutral and acidic amino acids can only be taken up passively by the pea seed coat, implying that transporters for active uptake, such as the H$^+$/amino acid symporters (Bush, 1993), are lacking in the seed coat parenchyma cells. This may not be true of the basic amino acids, because the saturable component of Lys uptake could represent a pathway for active transport. The calculated K_m of 6.5 mM, however, is much higher than the K_m values usually found for the uptake of L-Lys, which are either in the micromolar range or around 0.1 mM (see Heremans et al., 1997, and refs. therein). The uptake of Lys cannot be attributed to the activity of AAT1, a recently identified transporter for cationic amino acids, which has been suggested to play a role in supplying the developing seed with nitrogen (Frommer et al., 1995). In contrast to the "saturable component" of Lys uptake by seed coats, AAT1 has a high affinity for Lys (K_m = 35 μM), an affinity for L-Val and L-Glu, and sensitivity to CCCP.

Whether the saturable component represents the activity of a distinct transporter is uncertain because the complex kinetics may have resulted from the mutual interaction between the influx of the positively charged Lys and the membrane potential (Borst-Pauwels, 1993). Suppose that Lys and Val are transported across the plasma membrane by the same pathway. In the presence of a negative membrane potential the influx of the cationic Lys will be greatly enhanced. The membrane will become more depolarized, however, as the influx of the positively charged Lys increases. Thus, the linear kinetics may be apparent only at higher, depolarizing Lys concentrations, whereas at lower concentrations the relative influx of Lys, $v/[S]$, will increase because of the stimulatory effect of the negative membrane potential. As a result, the concentration dependency of the Lys influx will display an apparent saturable component (Fig. 3). The similar k values for Lys and the neutral amino acids and the observation that PCMBS inhibits the influx of Val and Lys to the same extent seem to favor this interpretation.

The finding that CCCP had no effect on the uptake of Lys need not be at variance with this explanation. It is true that partial membrane depolarizations have been recorded in *Riccia fluitans* after the addition of 10 μM CCCP (Felle and Bentrup, 1977), but it has also been reported that CCCP up to a concentration of 10 μM had no effect on the membrane potential of yeast cells (Beauvoit et al., 1991).

Mechanism of Amino Acid Efflux

At a developmental stage where the RWC of the cotyledons was 55%, the initial efflux of amino acids in experiments with "empty" seed coats has been determined to be

1.43 μmol seed coat^{-1} h^{-1}, which is equivalent to 259 nmol g^{-1} fresh weight min^{-1} (Lanfermeijer et al., 1992). If this efflux occurred by the same pathway as the influx of exogenously supplied amino acids, the amino acid concentration, $[S]_i$, at the cytoplasmic face of the plasma membrane of seed coat parenchyma cells can be predicted from $v_{efflux} = k[S]_i$. With k in the range of 8 to 15 μmol g^{-1} fresh weight min^{-1} M^{-1}, it turns out that $[S]_i$ should be between 17 and 32 mM. This compares quite favorably with the average amino acid concentration in the seed coat at this stage of development, which in two different experiments has been determined to be 17 and 35 mM (Lanfermeijer et al., 1992). Hence, the rate by which endogenous amino acids are released from the seed coat symplast can be fully explained by the permeability of the plasma membrane of seed coat parenchyma as determined in uptake experiments. Additional evidence that the same pathway is involved is provided by the observation that both influx (Table I) and efflux (de Jong and Wolswinkel, 1995) were inhibited by about 50% in the presence of 2.5 mM PCMBS, whereas neither was affected by 10 μM CCCP.

It can be concluded that amino acids are transported across the plasma membrane of seed coat parenchyma cells exclusively by a passive mechanism. As a result, the net efflux will be determined by the amino acid concentration gradient across this membrane. The observation that in "empty" seed coat experiments the initial amino acid efflux exceeds the nitrogen requirement of the embryo by 4- to 8-fold is then simply explained by assuming that in vivo there is a simultaneous influx of amino acids into the seed coat from the apoplasmic space such that the net efflux matches the nitrogen demand of the embryo (Lanfermeijer et al., 1992).

It may be of interest to note that during the first phase of seed fill, the uptake of amino acids (L-Val) by the cotyledons of pea embryos is also exclusively by an unsaturable pathway (Lanfermeijer et al., 1990). This would imply that during a considerable period of seed development the transfer of amino acids from the seed coat symplasm to the symplasm of the cotyledons proceeds by passive transport through the plasma membranes of seed coat parenchyma and cotyledonary cells, respectively.

Transport of Sugars and Amino Acids through Nonselective Pores?

The characteristics of the uptake of amino acids by pea seed coats presented here are very similar to those described for the uptake of Suc (de Jong et al., 1996). Uptake was nonconcentrative, and about 60% of the seed coat tissue was accessible to the exogenously supplied solutes. Furthermore, the estimated permeability coefficients are of the same order of magnitude (Suc, 3.5×10^{-7} cm s^{-1}; amino acids, 4×10^{-7} cm s^{-1} to 9×10^{-7} cm s^{-1}), and their influx was partially inhibited by PCMBS but not by CCCP. Finally, the transport of Suc and amino acids displays linear kinetics, suggesting minimal interaction, if any, of the transported molecule with the transporter. The picture emerging from our studies, then, is that the plasma membrane of seed coat parenchyma has pores through which molecules as large as amino acids and Suc can diffuse freely. Such pores need not be restricted to a specialized organ such as the seed coat, but may have a more widespread occurrence in plant cells. Stanzel and co-workers (1988) estimated the permeability coefficient of the plasma membrane of cultured *Streptanthus tortuosus* cells for Suc, Glc, Fru, and sorbitol at 3.7×10^{-7} cm s^{-1}, surprisingly close to our estimates for Suc and amino acids. Additionally, the concentration dependence of the influx of various solutes into plant cells has often been found to contain a linear component that probably represents transport across the plasma membrane by facilitated diffusion or by diffusion through aqueous channels (see Borstlap, 1983, and refs. therein). Thus, it may be that the basal permeability of the plant plasma membrane is appreciably higher than that constrained by the diffusion through the lipid bilayer (Lieb and Stein, 1986). This raises the possibility that the nutrient-releasing function of the seed coat is gained not so much by the presence of specific exporters for Suc and amino acids, but rather by the absence of transporters that enable the cell to accumulate these solutes from the extracellular space.

Although the hypothesis of nonselective pores offers a ready explanation for the observed release of sugars and amino acids by seed coats, we realize that it does not fit easily with the current concept of the plasma membrane as a highly selective cell boundary. It is true that our measurements indicated a permeability of the plasma membrane of seed coat cells for Suc and amino acids exceeding that of artificial lipid membranes by several orders of magnitude, but it should be emphasized that this permeability is still very low. It could be that by the presence of only a very few nonselective pores the cell membrane becomes sufficiently permeable to Suc and amino acids to warrant the fluxes required to feed the embryo.

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