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AMINO ACID PERMEABILITY OF PEA CHLOROPLASTS AS MEASURED BY OSMOTICALLY DETERMINED REFLECTION COEFFICIENTS

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SUMMARY

1. Chloroplasts were isolated in 2 min from *Pisum sativum* and then placed in external solutions varying in composition and osmotic pressure. The relative changes in chloroplast volume caused by the impermeable solute sucrose compared with equal concentrations of various amino acids were determined. This permitted the calculation of the reflection coefficients of the latter compounds by making use of a generalized version of the Boyle–Van 't Hoff relation.

2. The amino acids with their reflection coefficients in parentheses are as follows: (i) aliphatic amino acids: glycine (0.03), DL-alanine (0.01), DL-valine (0.35), L-norvaline (0.03), L-leucine (0.56) and L-isoleucine (0.33); (ii) hydroxy amino acids: DL-serine (0.04), DL-homoserine (0.03) and DL-threonine (0.02); (iii) aromatic amino acid: DL-phenylalanine (0.03); (iv) sulfur-containing amino acid: DL-methionine (0.04); (v) imino acid: L-proline (0.05).

3. The three amino acids (valine, leucine and isoleucine) with reflection coefficients differing appreciably from zero all have a methyl group attached to the backbone of the molecule. Such an increase in reflection coefficient attributable to methyl groups was also found for the following series of compounds: acetamide (0.52), *N*-methylacetamide (0.70), and *N*,*N*-dimethylacetamide (0.84).

4. The low values for the amino acid reflection coefficients suggest that the limiting membranes of pea chloroplasts are quite permeable to these compounds, a conclusion in harmony with various metabolic studies *in vivo* and *in vitro*.

INTRODUCTION

Amino acids are important products of photosynthetic CO_2 fixation[1–4] and can be incorporated into chloroplast proteins[5–7]. Studies with green plants exposed to $^{14}CO_2$ in the light have shown that initially the chloroplast proteins have a much higher specific activity than do cytoplasmic proteins[5,6]. Subsequently, the specific activity of cytoplasmic proteins increases while that of the chloroplast proteins decreases, suggesting a passage of amino acids out of the chloroplasts. ONGUN AND STOCKING[8] studied the labeling pattern in subcellular fractions following the feeding of [^{14}C]serine into tobacco petioles. They concluded that this amino acid easily passes from the cytoplasm into the chloroplasts. Similarly, AACH AND HEBER[9]

found that glycine, alanine, serine and aspartic acid readily leave spinach chloroplasts *in vivo*. Studies involving the incorporation of radioactive amino acids into proteins have been performed using chloroplasts isolated from tobacco[10–12], bean[11], spinach[13], and ennucleated cells of Acetabularia[14]. Leucine[10], glycine[11], valine[12,13], and phenylalanine[13] were all incorporated into the chloroplast proteins, strongly suggesting that these amino acids can pass across the membranes surrounding the chloroplasts. So far no data of a physicochemical nature have been presented on the permeability properties of chloroplast membranes to amino acids.

The volume of a membrane-bounded body such as a chloroplast changes in response to variations in the osmotic pressure of the external solution. This is a consequence of the properties of membranes, which generally allow water to move readily across them while certain solutes cannot. In particular, chloroplasts isolated from Nitella[15], poke weed[16], spinach[16–18] and pea[19] respond osmotically in sucrose and other solutions. Such osmometric behavior is usually described using the Boyle–Van 't Hoff relation[19,20]:

$$\pi^{\circ}(V - b) = RT \sum_{j} \varphi_j n_j^{\mathrm{i}} \qquad (1)$$

where π^0 is the osmotic pressure of the external solution, V is the volume of the chloroplast, b is the non-aqueous volume within V, φ_j is a correction factor, and n_j^{i} is the number of moles of species j within $V-b$ (R and T have their usual meanings of the gas constant and absolute temperature). Eqn. 1 is often adequate for describing osmotic responses when solutes do not cross membranes. However, in situations of general biological interest, solutes do cross membranes and this must be taken into account when considering the effect of external osmotic pressure on chloroplast volume. In fact, the osmotic response to a particular solute can be used to obtain information on the permeability properties of that substance, an approach used here to study the penetration of amino acids across the membranes surrounding pea chloroplasts.

To include the case of penetrating solutes, a rederivation of the conventional Boyle–Van 't Hoff relation is necessary. This derivation, which uses principles from irreversible thermodynamics, has been published elsewhere[20]. A form suitable for analyzing osmotic responses of pea chloroplasts[19] is:

$$\sum_{j} \sigma_j \pi_j^{\circ} = RT \frac{\sum_{j} \sigma_j \gamma_j^{\mathrm{i}} n_j^{\mathrm{i}}}{\bar{V}_{\mathrm{w}} n_{\mathrm{w}}^{\mathrm{i}}} \qquad (2)$$

where σ_j is the reflection coefficient of species j, π_j^0 is the osmotic pressure contributed by external species j, γ_j^{i} is the activity coefficient of internal species j, $\bar{V}_{\mathrm{w}}$ is the partial molal volume of water, and $n_{\mathrm{w}}^{\mathrm{i}}$ is the number of moles of water within the membrane-surrounded body. Reflection coefficients are parameters which characterize a particular solute crossing some specified membrane[20,21]. A species j which cannot cross a certain membrane has a σ_j of unity for that barrier. For instance, the reflection coefficient of pea chloroplast membranes for sucrose has been found to be 1.00 (ref. 19), an observation that will be exploited in the present studies. If the membrane does not distinguish or select between water and the solute j, then σ_j is zero. Reflection coefficients are useful for treating the many cases between the extremes of imper-

meability ($\sigma_j = 1$) and nonselectivity ($\sigma_j = 0$), such intermediate situations describing the penetration of amino acids into pea chloroplasts.

MATERIALS AND METHODS

Plant material and chloroplast isolation

Pisum sativum "Blue bantam" (W. Atlee Burpee Co., Riverside, Calif.) was grown at 20° in moist vermiculite, daylight fluorescent tubes providing 2000 lux for 12 h each day. On the 14th day, 15 g of leaves and stems from illuminated plants were ground for 10 sec while in a nylon bag[22] placed in a mortar containing 10 ml of 0.2 M sucrose buffered with 5 mM Tris–HCl buffer (pH 7.9). After grinding, the slurry was squeezed through the nylon bag, the whole cells and large debris remaining behind. The resulting homogenate was centrifuged for 60 sec at 1000 × *g* at 2°, the supernatant fluid was decanted, and the chloroplast pellet was resuspended by placing the tube on a vortex mixer. The entire isolation procedure required only 2 min (ref. 22) and yielded 95 % class I (intact) chloroplasts[23].

Packed weight measurement

After isolation, 0.2-ml aliquots of the resuspended chloroplasts containing approx. 300 μg chlorophyll were placed in 10 ml of specific solutions, which were present in centrifuge tubes preweighed to 0.05 mg. The tubes were then centrifuged for 3 min at 10000 × *g* at 0°, the supernatant fluid was decanted, the walls of the tube were carefully wiped dry, and the weight of the tube *plus* pellet was determined to within 0.05 mg. The known weight of the tube was subtracted in order to obtain the weight of the chloroplast pellet in the various solutions. The great experimental precision available for weight determinations compared with Coulter counter or optical methods for measuring chloroplast volume made the above technique suitable for the present studies. Experiments using light scattering by chloroplast suspensions indicated that the initial movement of water due to changes in the external osmotic pressure was complete within 1 sec, whereas a slow deteriorative swelling[23] took place if the chloroplasts were stored after isolation. Consequently, the centrifugation step for the packed weight determinations was begun immediately after adding the chloroplasts.

Conversion of packed weight to mean chloroplast volume

The parameter of interest for analysis using the Boyle–Van 't Hoff relation is the chloroplast volume, whereas the above measurements gave the weight of the chloroplasts *plus* that of the supernatant fluid trapped in the interstices of the pellet. Certain of the pellets were therefore resuspended in the test solutions, whose densities had been measured with a 10-ml Weld pycnometer[24]. The density of the resuspended pellet was determined to within 10 μg/ml and the density of the original pellet was then calculated. Using the pellet density, packed weights could be converted to packed volumes (this conversion had very little effect on the relative values, *e.g.* the pellet densities for 50 mM of the various amino acids ranged only from 1.073 to 1.079 g/cm^3).

The chloroplast packed volumes calculated from the measured packed weights include a certain amount of "dead space" or interstitial fluid trapped in the pellet.

Previous experiments using [^{14}C]dextran indicated that the fraction of the pellet occupied by trapped supernatant fluid was 0.33 for chloroplasts isolated from plants in the light[23]. Using the same technique, it was found that the dead space in the pellet in the presence of amino acids averaged 99.9 ± 0.2 (S.E.)% of the value for the sucrose controls. Since the fraction of the pellet that was chloroplasts was therefore also the same under the various experimental conditions, the mean chloroplast volume is directly proportional to the packed volume.

Re-expression of the Boyle–Van 't Hoff relation

The experimental solutions used in these studies contained 0.2 M sucrose, 5 mM Tris–HCl buffer (pH 7.9) to which was added various concentrations (c_x) of a substance with an unknown reflection coefficient (σ_x). It would thus be convenient to express the extended version of the Boyle–Van 't Hoff relation (Eqn. 2) in terms of c_x and σ_x. For dilute aqueous solutions, the osmotic pressure of species j, π_j, can be replaced by RTc_j, where c_j is the concentration of species j. Thus the left-hand side of Eqn. 2 ($\sum_j \sigma_j \pi_j^0$) equals $\sigma_x RTc_x + \alpha$, where α is a constant representing the reflection coefficients times the osmotic pressures for 0.2 M sucrose, 5 mM Tris–HCl buffer (pH 7.9). The factor $\sum_j \sigma_j \gamma_j^i n_j^i$ on the right-hand side of Eqn. 2 can be replaced by the constant, β, which is determined by the contents of the chloroplasts. The quantity $\bar{V}_w n_w^i$ is the volume per mole of water times the number of moles of water in the chloroplasts. This volume of enclosed water is given by $V - b$ in the conventional Boyle–Van 't Hoff relation. Making these various substitutions and dividing on both sides by RT, Eqn. 2 can be rewritten:

$$\sigma_x c_x + \frac{\alpha}{RT} = \frac{\beta}{V - b} \tag{3}$$

Determination of reflection coefficients

Eqn. 3 is in a convenient form for analyzing osmotic responses of chloroplasts to solutes whose reflection coefficients are not known. However, first it is necessary to determine the magnitude of the constant b. To do this, various amounts of sucrose were added to increase c_x from 0.00 up to 0.20 M, and the packed volume, V, was determined in each case. The chloroplast packed volume obtained when c_x equaled 0 (*i.e.* for 0.2 M sucrose, 5 mM Tris–HCl buffer (pH 7.9)) was taken as the control. The best fit of the data to Eqn. 3 occurred for b equaling 57 % of the volume for the control. The relatively large value of b is due to the extensive membrane system and the large amount of protein in chloroplasts.

Now that the procedural details have been presented, the experimental approach will be briefly summarized. The packed weights of chloroplasts in various solutions were determined and then were converted to packed volumes using the measured densities. All solutions contained 0.2 M sucrose, 5 mM Tris–HCl buffer (pH 7.9), the chloroplast packed volume in this medium being used as a control. In the test solutions, various concentrations (c_x) of amino acids (purchased from Calbiochem, Los Angeles, Calif.) or additional sucrose were added and the chloroplast packing volumes, V, compared with the control. After subtracting b (57 % of the control volume), $1/(V - b)$ was plotted *versus* c_x, the slope of the straight line obtained being proportional to σ_x by Eqn. 3. Each data point given in the figures represents the average of three or more experiments.

RESULTS

The osmotic responses of pea chloroplasts obtained upon varying the concentration of twelve different amino acids in the external solution are presented in Figs. 1–4. In each case, the osmotic response to sucrose is given as a control. The reflection coefficient of sucrose for the limiting membrane of pea chloroplasts is essentially unity[19]. Thus, the reflection coefficients for the amino acids can be determined by comparing the slopes of the osmotic responses they lead to compared with those for sucrose, the various values being summarized in Table I below. The amino acids whose reflection coefficients were measured needed to be water-soluble (which eliminated tyrosine, tryptophan and cystine). Furthermore, the amino acids selected must neither change the pH of the solution nor bring in additional ions (which eliminated aspartic acid, glutamic acid, arginine, histidine and lysine). This latter limitation is due to a large non-osmotic effect of ions including H^+ on the packing volume of chloroplasts, perhaps by affecting the amount of interstitial fluid trapped in the pellet.

Figs. 1–4 indicate that varying the external concentration of most of the amino

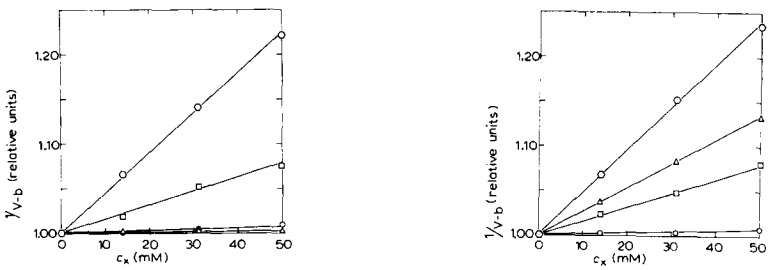


Fig. 1. Osmotic responses of pea chloroplasts to glycine (○), DL-alanine (△), DL-valine (□) and sucrose (◇). The abscissa, c_x, is the concentration of the various compounds added to chloroplasts suspended in 0.2 M sucrose, 5 mM Tris–HCl buffer (pH 7.9). The quantity $V-b$ represents the volume of osmotically responding water in chloroplasts of volume V. Thus an increase in the ordinate, $1/(V-b)$, indicates an efflux of water from the chloroplasts due to the increase in external osmotic pressure. The osmotic movement of water in response to various external concentrations of sucrose, whose reflection coefficient is 1.00 (ref. 19), is presented as a control.

Fig. 2. Osmotic responses of pea chloroplasts to L-norvaline (○), L-leucine (△), L-isoleucine (□) and sucrose (◇).

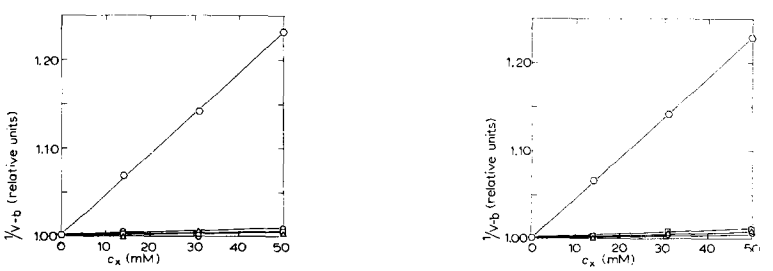


Fig. 3. Osmotic responses of pea chloroplasts to DL-serine (O), DL-homoserine (△), DL-threonine (□) and sucrose (◇).

Fig. 4. Osmotic responses of pea chloroplasts to DL-phenylalanine (O), DL-methionine (△), L-proline (□) and sucrose (◇).

acids tested does not lead to any appreciable osmotic volume changes of the chloroplasts. This means that the reflection coefficients of such amino acids interacting with the chloroplast membranes are close to zero, as appears to be the case for glycine and alanine (Fig. 1); norvaline (Fig. 2); serine, homoserine and threonine (Fig. 3); and phenylalanine, methionine and proline (Fig. 4). The exceptions having reflection coefficients substantially different from zero are valine (Fig. 1) and leucine and isoleucine (Fig. 2). As will be discussed below, these three amino acids all contain a methyl group branching off the otherwise straight chain of carbon atoms. This effect of branching methyl groups was further investigated using three compounds that differed only in the number of such groups attached to a nitrogen atom. As Fig. 5 indicates, the osmotic responses and consequently the reflection coefficients progressively increased in going from acetamide to *N*-methylacetamide to *N*,*N*-dimethylacetamide.

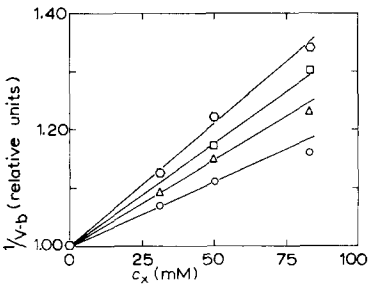


Fig. 5. Osmotic responses of pea chloroplasts to acetamide (O), *N*-methylacetamide (△), *N*,*N*-dimethylacetamide (□) and sucrose (◇).

DISCUSSION

The reflection coefficients of pea chloroplasts for various amino acids as determined from osmotic responses are summarized in Table I. Since the experimental approach was to add solutes to the external solution and then to see whether water moved out (a volume decrease), the reflection coefficients apply to the pair of membranes surrounding a chloroplast. One of the most striking features is the low value of the reflection coefficients, indicating that all of the amino acids tested can readily cross the membranes. For instance, the σ's for glycine, alanine, serine, threonine, phenylalanine, methionine and proline all lie between 0.01 and 0.05 (Table I). In contrast, it has been found that the reflection coefficients of glycine, alanine, serine and proline are all near unity for cellular membranes of the gallbladder epithelium[25]. This suggests that the molecular arrangement or type of lipids and proteins in chloroplast membranes is markedly different than is the case for gallbladder epithelial membranes. In particular, chloroplast membranes may contain channels in which the amino acids can readily diffuse from one side to the other. Such a condition would allow the amino acids produced as photosynthetic products to diffuse out of the chloroplasts into the surrounding cytoplasm where they could be used for cellular protein synthesis. On the other hand, if amino acids could easily cross cellular membranes, they would rapidly be lost from the cell.

The lack of appreciable osmotic responses by pea chloroplasts to most of the

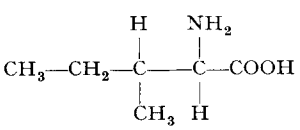
TABLE I

SUMMARY OF REFLECTION COEFFICIENTS DETERMINED FROM THE OSMOTIC RESPONSES OF PEA CHLOROPLASTS TO VARIOUS AMINO ACIDS COMPARED WITH THE OSMOTIC EFFECTS OF EQUAL CONCENTRATIONS OF SUCROSE ($\sigma = 1.00$) AS PRESENTED IN FIGS. 1–5

Amino acids	*Reflection coefficient*
Aliphatic amino acids	
Glycine	0.03
DL-Alanine	0.01
DL-Valine	0.35
L-Norvaline	0.03
L-Leucine	0.56
L-Isoleucine	0.33
Hydroxy amino acids	
DL-Serine	0.04
DL-Homoserine	0.03
DL-Threonine	0.02
Aromatic amino acid	
DL-Phenylalanine	0.03
Sulfur-containing amino acid	
DL-Methionine	0.04
Secondary amino (imino) acid	
L-Proline	0.05
Acetamide derivatives	
Acetamide	0.52
N-Methylacetamide	0.70
N,*N*-Dimethylacetamide	0.84

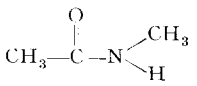
amino acids (Figs. 1–4) suggests that chloroplast membranes do not prevent the entry or exit of these compounds. This conclusion supports the observations of ONGUN AND STOCKING[8] who showed that serine readily enters tobacco chloroplasts *in vivo*. Likewise, AACH AND HEBER[9] presented compelling evidence for the rapid exit of glycine, alanine and serine from spinach chloroplasts in the plant cell. Moreover, the interpretation of our data is consistent with other experiments using ^{14}C-labeled amino acids both *in vivo*[5,10] and *in vitro*[10–14].

An interesting correlation observed in these studies of chloroplast reflection coefficients deals with the effect of methyl groups. Table I indicates that three of the amino acids had σ_x's appreciably greater than zero, *viz.* valine ($\sigma_x = 0.35$), leucine (0.56) and isoleucine (0.33). All three compounds contain a methyl group as a branch to the main backbone, *e.g.* isoleucine:



while leucine differs by having the methyl group on the γ-carbon and valine does not have the δ-carbon. Norvaline is a straight-chain amino acid which is isomeric with

valine. However, its reflection coefficient is only 0.03 (Table I), compared with 0.35 for valine. Thus, the branching of the carbon backbone apparently decreases the ease with which the molecules cross the chloroplast membranes. This point was further investigated using various methylated derivatives of acetamide, *e.g.* *N*-methylacetamide:



Upon addition of methyl groups in going from acetamide to *N*-methylacetamide to *N*,*N*-dimethylacetamide, the reflection coefficients for the pea chloroplast membranes increased from 0.52 to 0.70 to 0.84 (Table I), indicating a progressive decrease in permeability. Such a decrease in permeability to nonelectrolytes as methyl groups are added as branches has also been observed for the cellular membranes of gallbladder epithelium[25] and for Nitella internodal cells[26].

Reflection coefficients have been empirically related to permeability properties by DIAMOND AND WRIGHT[27]. They plotted the σ's of 52 nonelectrolytes measured by them using rabbit gallbladder epithelium against the permeability coefficients (P) of the same compounds determined by COLLANDER[26] for the alga *Nitella mucronata*. Except for a few substances, a decrease in reflection coefficient was correlated with an increase in permeability. For instance, a σ_j of 1.00 is consistent with a P_j of less than 10^{-7} cm/sec, while a reflection coefficient of 0.50 corresponds to a P_j of approx. 10^{-5} cm/sec, while a σ_j of 0.05 might be obtained for a substance having a permeability coefficient of $2 \cdot 10^{-4}$ cm/sec. The same relationship between σ_j and P_j will be assumed here in order to help indicate the possible physiological significance of the various reflection coefficients measured for pea chloroplasts. The chloroplasts will be approximated as spheres of radius 2 μ (pea chloroplasts have volumes near 30–35 μ^3, ref. 19). The time necessary for some species j having a permeability coefficient P_j to diffuse out of a membrane-bounded sphere of radius r into a large external solution initially devoid of that species is[26,28]:

$$t = \frac{r}{3P_j} \ln \frac{c_j^i(0)}{c_j^i(t)} \tag{4}$$

where $c_j^i(0)$ is the initial internal concentration of species j and $c_j^i(t)$ is the concentration remaining at some later time t. From Eqn. 4, the amount of time required for the internal concentration to decrease 90 % is $2.303\ r/3P_j$ ($\ln c_j^i(0)/c_j^i(t)$ then equals $\ln 100/10$ or 2.303). For chloroplasts having radii of 2 μ and for a solute with a P_j equaling 10^{-7} cm/sec (*e.g.* for $\sigma_j = 1.00$), t is $(2.303)(2 \times 10^{-4})/(3)(10^{-7})$ or 2000 sec which is about 0.5 h. On the other hand, a permeability coefficient of 10^{-5} cm/sec (*e.g.* for $\sigma_j = 0.50$) leads to a time of 20 sec, while if P equals $2 \cdot 10^{-4}$ cm/sec (*e.g.* for $\sigma_j = 0.05$), t is only 1 sec. Seven of the amino acids (glycine, alanine, serine, threonine, phenylalanine, methionine and proline) had reflection coefficients of 0.05 or smaller for pea chloroplasts (Table I). This would correspond to times of 1 sec or less for the concentration within the chloroplasts to decrease by 90 %. It is not the intention of these calculations to give exact times applying to the egress of photosynthetic products from chloroplasts *in vivo*. Rather they are presented to indicate that the small values of

the reflection coefficients for the amino acids are consistent with the ready passage of these substances across the chloroplast membranes.

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