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Energetics of Amino Acid Uptake by *Vicia faba* Leaf Tissues[1]

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ABSTRACT

The uptake of [U-^{14}C]threonine and of (α-^{14}C]aminoisobutyrate (α-AIB) by *Vicia faba* leaf discs is strongly pH dependent (optimum: pH 4.0) and exhibits biphasic saturation kinetics. Kinetics of α-AIB uptake at different pH values indicate that acidic pH values decrease the K_m of the carriers while the maximal velocity remains nearly unaffected. Similar results were obtained for both system 1 (from 0.5 to 5 millimolar) and system 2 (from 20 to 100 millimolar).

After addition of amino acids to a medium containing leaf fragments, alkalinizations depending both on the amino acid added and on its concentration have been recorded.

The effects of compounds which increase (fusicoccin) or decrease (uncouplers, ATPase inhibitors, high KCl concentrations) the protonmotive force were studied both on the acidification of the medium and on amino acid uptake by the tissues. There is a close relationship between the time required for the effect of these compounds on the acidification and that needed for inhibition of uptake.

Studies with thiol inhibitors show that 0.1 millimolar *N*-ethylmaleimide preferentially inhibits uptake by the mesophyll whereas 0.1 millimolar parachloromercuribenzenesulfonate affects rather uptake by the veins.

New evidence was found which added to the electrophysiological data already supporting the occurrence of proton amino acid symport in leaf tissues, particularly in the veins.

Mature leaves export sugars (mainly as sucrose) but also a broad spectrum of amino acids which may be translocated in substantial amounts. Although the mechanism of phloem loading of sucrose has been studied in some detail (7–10, 15, 16), little is known about the uptake of amino acid in leaf cells and particularly in phloem (3, 12, 25). Until now, most of the evidence suggesting that amino acids are co-transported with protons (and/or ions) comes from electrophysiological studies. For example, a transient depolarization of the membrane potential is recorded upon addition of amino acids to *Lemna gibba* fronds (14) and *Avena* coleoptiles (13, 18). In the present paper, we have further tested the amino acid proton symport hypothesis by studying the effects of external pH and of various effectors of the protonmotive force on amino acid uptake and by recording proton fluxes linked to amino acid transport. *Vicia faba* (broadbean) leaves were selected because the lower epidermis can be removed easily, resulting in excellent contact and exchange between the apoplast and the incubation medium. The heterogeneity of the tissues under investigation necessitated measurements of net uptake of label as well as study of its distribution by autoradiography. In several cases, autoradiographs showed that the mesophyll and the veins exhibited a different sensitivity. α-AIB[2] and threonine were used because preliminary studies revealed only a slight metabolism of these compounds in our material.

MATERIALS AND METHODS

Plant Material. Non-nodulated broadbean (*Vicia faba* cv Aguadulce), were grown under conditions already described (10). The experiments were performed on plants possessing four mature bifoliolate leaves.

Preparation of Leaf Discs. Leaf discs (12-mm diameter) were punched with a cork borer from the third and the fourth mature leaves, after the lower epidermis had been stripped off. The mean dry weight of one disc was 2.2 mg. The discs (15 for each set) were floated for various times (see figures) on a preincubation medium (10 ml) containing 250 mM mannitol (as an osmoticum), 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$ (reference medium), and a buffer. The buffers used were: 10 mM Na citrate, 20 mM di-Na phosphate for pH 3.0 to 7.0, 20 mM Mes for pH 5.0 to 6.0, 20 mM Hepes for pH 7.0 and 20 mM Tricine for pH 8.0 and 8.8. In some experiments, an inhibitor was added; the solutions were contained in Petri dishes and continuously stirred on a reciprocal shaker (60 shakes/min). The temperature was 20 ± 1°C and the light intensity was 10 w m^{-2}.

Uptake Experiments. Uptake experiments were performed in the light (10 w m^{-2}) inasmuch as light promotes uptake of amino acids by our material (12). After preincubation, the discs were transferred to the incubation medium for various times (see "Results"). The composition of the incubation medium was the same as that of the preincubation solution except for ^{14}C-labeled amino acid. At the end of incubation, the tissues were rinsed 3 times (3 min each) in a medium containing unlabeled amino acid at the same concentration as the incubation medium. The discs were harvested, lyophilized, exposed on NS 2T films (Kodak), and combusted in an oxidizer (Oxymat IN 4102, Intertechnique). The radioactivity was counted by liquid scintillation spectrometry (Intertechnique SL 33).

Radiochemicals. [U-^{14}C]Threonine (228 mCi/mmol) and [α-^{14}C]aminoisobutyrate (56 mCi/mmol) were from Amersham France (Versailles).

pH Measurements. One g of mature leaf fragments (3 to 6 cm^2 each, cut with a razor blade) without lower epidermis was floated onto 20 ml of the following medium (reference medium: 250 mM mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$; initial pH: 6.0) and shaken from time to time. After 1 h, the fragments were transferred to 20 ml of the same medium and the pH of the solution was monitored with the RTS 622 or RTS 822 pH recording unit (Radiometer, Copenhagen) (for details see Ref. 10). When the pH of the medium had reached 4.8 (about 5 h after the preparation of fragments), a definite concentration of amino acid adjusted at pH

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[2] Abbreviations: α-AIB, α-aminoisobutyrate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DES, diethylstilbestrol; DNP, 2,4-dinitrophenol; FC, fusicoccin; NEM, *N*-ethylmaleimide; PCMBS, parachloromercuribenzenesulfonate.

4.8 was added. This pH was selected because it is near the optimal pH value for amino acid uptake. In most cases, the volume of the solution added did not exceed 1% of the initial volume.

RESULTS

General Properties of Amino Acid Uptake. Experiments reported elsewhere (11) show that the uptake of both [α-^{14}C]AIB and [^{14}C]threonine is linear with time during at least 3 h at pH 4.0 and 6.0. Autoradiographs performed after short incubations did not show any shift between the appearance of label in the veins and in the mesophyll; whatever the duration of uptake, the radioactivity present in the veins is always greater than that found in the mesophyll. Despite this preferential accumulation in the veins, the mesophyll contains more label after incubation of tissues with ^{14}C-amino acids than after incubation with [^{14}C]sucrose (compare, for example, Fig. 2 below with Fig. 5 of Ref. 9). Concentration dependence of both [^{14}C]threonine and [α-^{14}C]AIB uptake exhibits two saturable phases and this led us to select two concentration ranges when studying the effect of pH on uptake as reported below.

Effect of pH on Amino Acid Uptake. The amino acid uptake markedly depended on the external pH and showed an optimum at pH 4.0 for the two amino acids studied (Fig. 1). The autoradiographs (Fig. 2) showed that both the mesophyll and the veins were involved in the increase in uptake at acidic pH (6.0 to 4.0). Contrarily, at pH 3.5, the discrepancy between the veins and the mesophyll was strongly enhanced, since a heavy label was clearly seen in the minor veins, while the label of the mesophyll was strongly inhibited (see, for example, threonine in Fig. 2).

In *Beta* (16) or *Vicia* (9) leaf discs, the proton concentration of the external medium affects the K_m of the sucrose carrier without marked effect on the V_{max}. In *Vicia*, this effect is clearly seen only with the high affinity carrier (9). Concerning amino acid uptake, acidic pH values apparently increase the V_{max} of the threonine carrier in the haustorium of *Polytrichum* (5). Fig. 3 shows that in our material, acidic pH values decreased the K_m of the carrier for α-AIB whereas the V_{max} was only slightly affected between pH 4.0 and 7.0. These phenomena were observed both at low (Fig. 3A) or high (Fig. 3B) α-AIB concentrations.

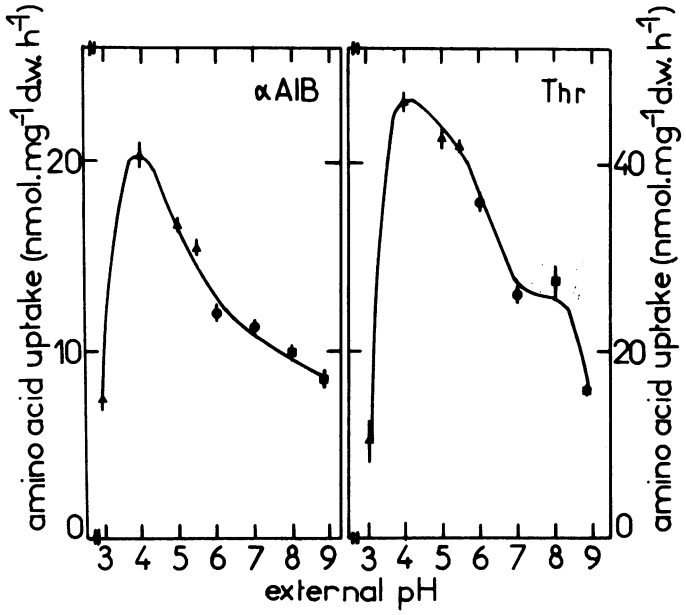

FIG. 1. Effect of pH on uptake of amino acids. The tissues were preincubated for 1 h on the reference medium buffered at the required pH and then incubated for 1 h at the same pH with 5 mM ^{14}C-amino acids (1.5 μCi in 10 ml). Each point is the mean of five triplicates ± SE. (▲), 10 mM Na citrate + 20 mM di-Na phosphate; (●), 20 mM Mes; (■), 20 mM Tricine.

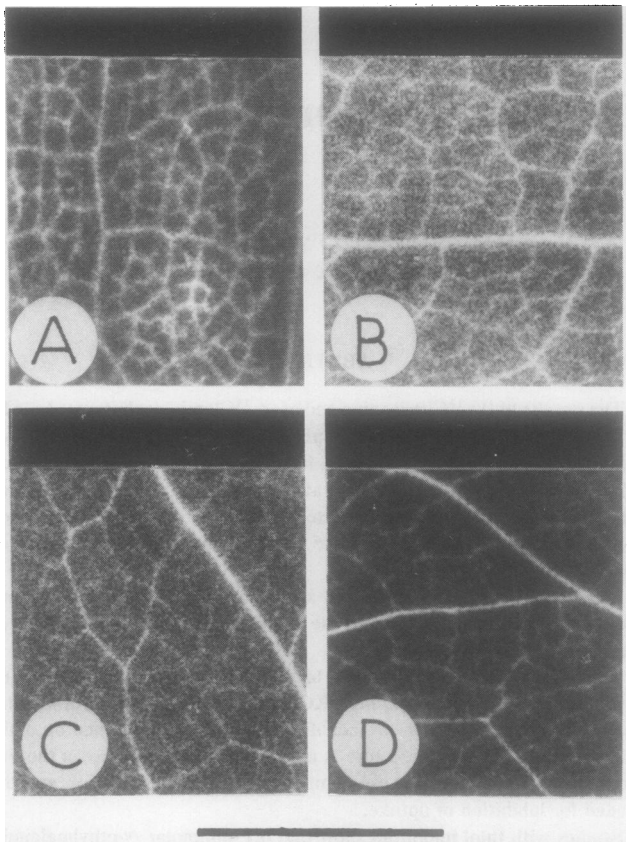

FIG. 2. Effect of pH on the uptake of [U-^{14}C]threonine by different tissues of the leaf. For experimental conditions, see the legend of Figure 1. A, pH 3.5; B, pH 4.0; C, pH 6.0; D, pH 8.0. Exposure time: 5 d. The scale bar stands for 5 mm and the top side of each picture shows the background of the film.

Proton Fluxes Associated with Amino Acids Uptake. Results like those presented in Figure 3 have been interpreted in terms of proton-substrate symport (9, 16). If this hypothesis is valid, one should also be able to detect proton fluxes associated with the uptake of substrate. The following experiments address these fluxes.

While the addition of threonine to a medium containing broadbean leaf tissues was followed by a marked alkalinization of the medium (Fig. 4A), only a decrease in the acidification rate can be observed upon addition of α-AIB (Fig. 4B). Since under similar conditions threonine was 2-fold more rapidly absorbed than α-AIB, the pH transient induced upon substrate addition seemed to be related to the rate of uptake of the amino acid added. When the medium contained no tissue, no pH change occurred (Fig. 4, C and D). The pH transients appeared 2 or 3 min after the addition of the amino acid and the amplitude of the alkalinization was maximal after 10 to 60 min. Between 0.5 and 10 mM, the higher the substrate concentration added, the stronger was the alkalinization induced (Fig. 4E). The autoradiographs of leaf tissues supplied with ^{14}C-amino acids under the same conditions as those used for recording the pH transient showed that the label was more concentrated in the veins than in the mesophyll (data not shown). It should be noted that pH transients induced by amino acid addition could be observed after short incubation times (less than 1 h after the preparation of the tissues) although they are less visible. This result is slightly different from that obtained with sucrose since the pH changes induced by this sugar

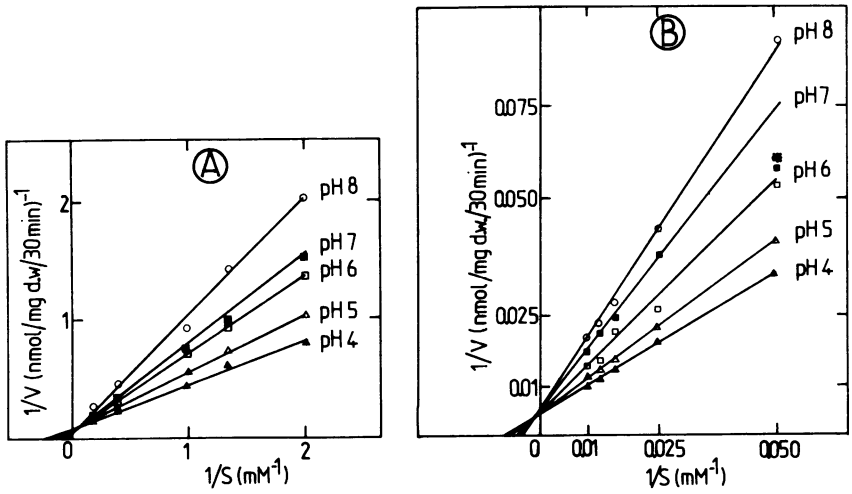

FIG. 3. Kinetics of α-AIB uptake at different pH values. The absorption was studied at 0.5, 0.75, 1.0, 2.5, and 5.0 (set I) and 20, 40, 60, 80, 100 mM (set II) amino acid. The discs were floated for 1 h on the reference medium buffered at the required pH and incubated for 30 min in the same solution with ^{14}C-amino acid. The label was 1 μCi/10 ml in set I and 4 μCi/10 ml in set II. Mannitol was added to keep the overall osmotic pressure at 250 mM. Low (A) and high (B) α-AIB concentrations. Each point is the mean of five sets of three discs. (*), not considered. For system 1, at pH 4.0, K_m = 6.4 mM and, pH 8.0, K_m = 52.3 mM; for system 2, at pH 4.0, K_m = 123 mM and, at pH 8.0, K_m = 434 mM.

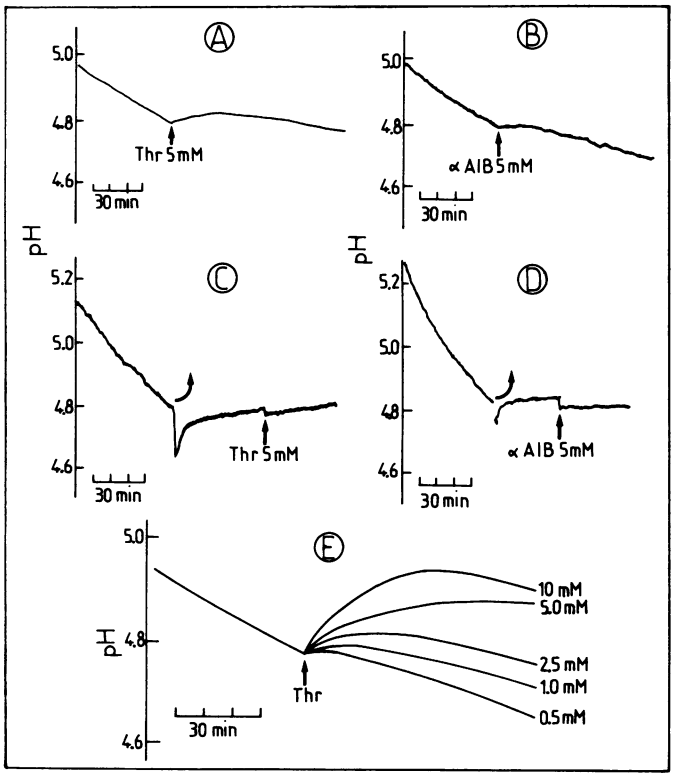

FIG. 4. The effect of amino acids additions on the pH of the medium. One g (fresh weight) of mature leaf fragments was incubated on 20 ml of reference medium. Threonine (Thr, A) or α-AIB (B) were added at the time indicated by the arrows. C and D, Control experiments where the tissues were removed from the medium (first arrow) prior to the addition of threonine (C) or α-AIB (D). E, Effect of increasing threonine concentrations.

are best detected after 2 h of preincubation with *Ricinus* cotyledons (19) and after 3 to 4 h with *Vicia* leaf tissues (8).

Action of Various Effectors of the Protonmotive Force on the Uptake of Amino Acids by the Leaf Tissues. The proton-amino acid symport predicts that the rates of uptake of α-AIB and threonine should depend on the protonmotive force. Thereby, this uptake should be sensitive to compounds affecting this force. We have studied the effect of substances which can increase (FC) or decrease (ATPase inhibitors, protonophores, high KCl concentrations, thiol-reacting inhibitors) the protonmotive force. FC rapidly stimulates the activity of the plasmalemma proton pump, thereby enhancing the transmembrane pH gradient and the membrane potential (22). An earlier paper (12), showed that FC promotes the uptake of amino acids by broadbean leaf discs, both in the light and in the dark. These results had been obtained after relatively long incubation times (3 h) in the presence of the phytotoxin. This stimulatory effect can be detected within 10 or 20 min after addition of 0.01 mM FC (Fig. 5). The stimulation of amino acids uptake by FC concerns both the mesophyll and the veins (12).

DNP and CCCP principally act as protonophores and therefore deplete the protonmotive force. These compounds strongly in-

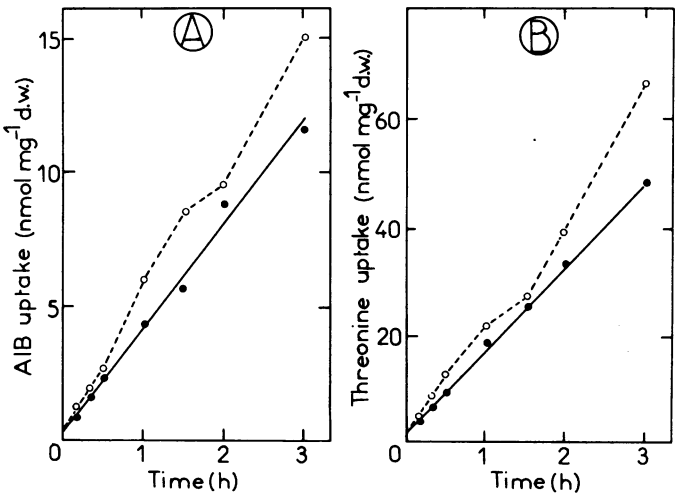

FIG. 5. Stimulation of [^{14}C]threonine and [α-^{14}C]AIB uptake by 0.01 mM fusicoccin. Leaf discs were preincubated without FC for 30 min on the reference medium buffered at pH 6.0 (citrate/phosphate) and incubated on a similar solution containing 2.5 mM [^{14}C]threonine (2 μCi/20 ml) with (○) or without (●) 0.01 mM FC. Each point was obtained from five discs, and the experiments were repeated three other times with the same results.

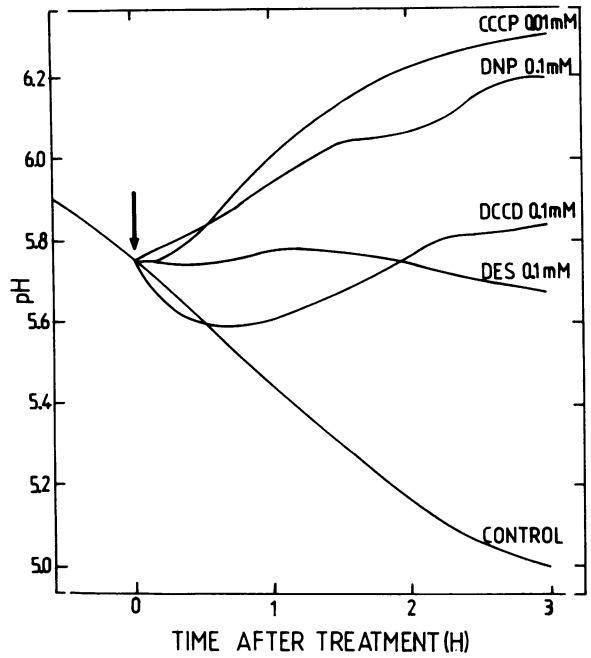

FIG. 6. Effects of uncouplers and ATPase inhibitors on the acidification of a solution (reference medium) containing *Vicia* leaf fragments without epidermis (0.5 g fresh weight in 20 ml of medium).

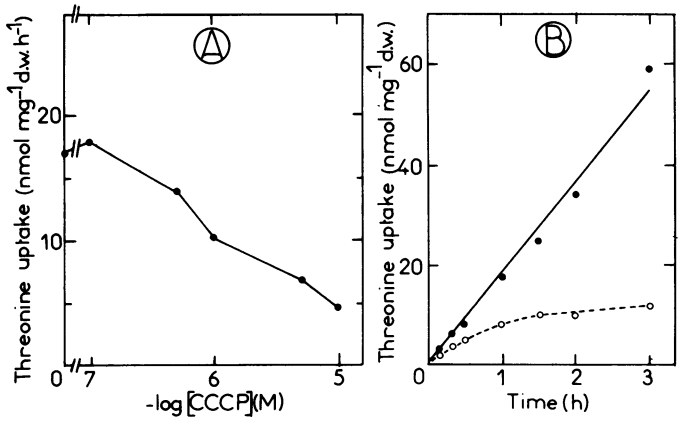

FIG. 7. Inhibition of [^{14}C]threonine uptake by CCCP. A, Effect of increasing concentrations of CCCP. The discs were preincubated (2 h) and incubated (1 h) on the reference medium at pH 6.0. The inhibitors were present during preincubation and incubation. Each point is the mean of three sets of five discs. B, Time course of inhibition of 2.5 mM [U-^{14}C] threonine uptake by 0.01 mM CCCP. After 30-min preincubation without the inhibitor, the discs were incubated for various times with (○) or without (●) 0.01 mM CCCP. Each point is the mean of five discs.

hibited H^+ efflux and induced an alkalinization of the medium containing broadbean leaf fragments (Fig. 6). At the same time, they also severely decreased the entry of amino acids in the tissues (an example is given in Fig. 7 with CCCP on threonine uptake). The inhibitory effect of these substances, which was detectable within 10 min (Fig. 7B) concerned the mesophyll and the veins (autoradiographs not shown).

The primary effect of DES and DCCD is to block the proton-pumping ATPases, particularly those located in the plasmalemma (2, 22). This blocking results in a rapid depletion of the proton-motive force (22). Preliminary experiments (11) have shown that, under our experimental conditions, 0.1 mM DES was more effective than 0.1 mM DCCD on threonine and on α-AIB uptake. DES lessened or stopped the rate of acidification of the medium after

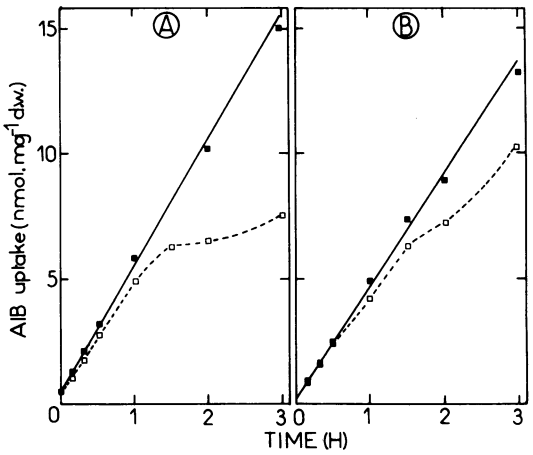

FIG. 8. Time course of inhibition of [α-^{14}C]AIB uptake by 0.1 mM DCCD (B) or 0.1 mM DES (A). The experimental schedule was similar to that described in the legend of Figure 7. Control (■); 0.1 mM DCCD or DES (□). Each point is the mean of five discs.

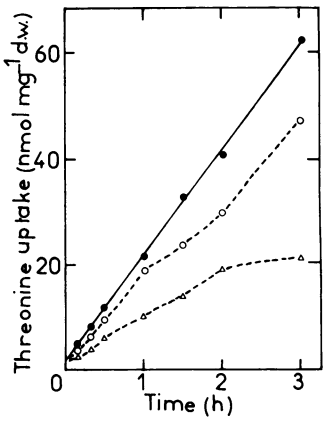

FIG. 9. Time course inhibition of [U-^{14}C]threonine uptake by high KCl concentrations. The experimental schedule was similar to that described in the legend of Figure 5. Control (●), 10 mM KCl (○), and 100 mM KCl (△).

3 to 5 min of lag phase (Fig. 6) while DCCD appeared inhibitory 20 to 40 min after it had been added to the bathing solution. At this time, DCCD could induce an alkalinization of the medium. As the uncouplers, the ATPase inhibitors DES and DCCD decreased amino acid uptake in the mesophyll and in the veins. The effect of DES on amino acid uptake was detectable within 10 min after the beginning of incubation (Fig. 8A), whereas the inhibition exerted by DCCD appeared only after 30 min (Fig. 8B).

Several workers have shown that high KCl concentrations can depolarize the membrane potential (13, 23, 24). This led us to study the effect of such treatments on amino acid uptake by our material. In the presence of 10 mM KCl, the uptake of [^{14}C] threonine was inhibited within 10 min (Fig. 9); this effect was more marked and faster in the presence of 100 mM KCl. Similar results have been obtained with α-AIB (data not shown). The inhibitory effect of KCl seemed more marked in the mesophyll than in the veins (Fig. 10).

We have studied the sensitivity of amino acid uptake to two thiol-reacting compounds, NEM and PCMBS. The plasma membrane is much more permeable to NEM than to PCMBS (16). In our material, 1 mM NEM rapidly inhibits the acidification of the medium by the tissues while 1 mM PCMBS exerts no marked effect on this process (10). The inhibitory effect of NEM on α-AIB uptake was detected at concentrations >0.01 mM, while PCMBS affected the uptake only at concentrations >0.1 mM (Fig.

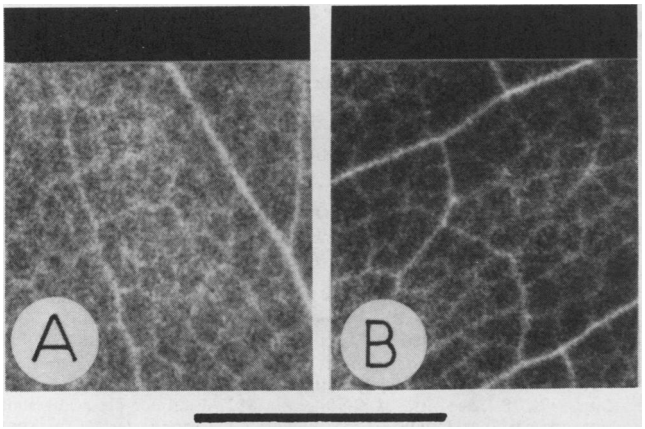

FIG. 10. Autoradiographs of leaf tissues incubated for 30 min in 2.5 mM [U-^{14}C]threonine without (A) or with 100 mM KCl (B). Exposure time: 12 days. The scale bar stands for 5 mm and the top side of each autoradiography shows the background of the film.

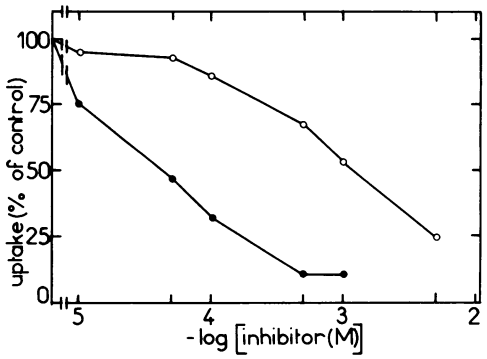

FIG. 11. Inhibition of [α-^{14}C]AIB (2.5 mM) uptake at pH 6.0 by various concentrations of NEM (●) and PCMBS (○). The preincubation (2 h) and the incubation (1 h) were performed with or without the inhibitor. Each point is the mean of five triplicates.

11). The inhibition exerted by these compounds on uptake could reach 85%. The autoradiographs (Fig. 12) show that the effects of various concentrations of these inhibitors were not the same in the veins compared to mesophyll. While 0.1 mM PCMBS decreased the radioactivity mainly in the veins, 0.1 mM NEM selectively affected the labeling of the mesophyll.

DISCUSSION

The accumulation of α-AIB and of threonine in the mesophyll and in the veins markedly depends on the pH and is optimal at pH 4.0. The stimulation of amino acid uptake by acidic pH values has been already noticed in various plant materials (27, 28). The optimal pH for α-AIB uptake is near 4.0 in *Hordeum vulgare* leaf tissues (26) and in the haustorium of *Polytrichum formosum* (5), and near 5.5 in *Avena sativa* coleoptiles (24). The stimulation of amino acid uptake by acidic pH values has sometimes been taken as an evidence in favor of an amino acid proton symport (24), but taken alone, this evidence is rather weak. The proton-substrate symport hypothesis relies on an interaction of protons with the substrate or its carrier and this should result in variations of the kinetic parameters describing the uptake when the pH is varied. The results presented in Figure 3 fit in well with this prediction, but they are not sufficient to demonstrate the symport. Provisionally, the kinetics of Figure 3 can be interpreted as competitive inhibition patterns which could be explained by OH^- inhibition or H^+ activation resulting in affinity changes of the carrier (9, 16). This interpretation still must be considered as tentative only

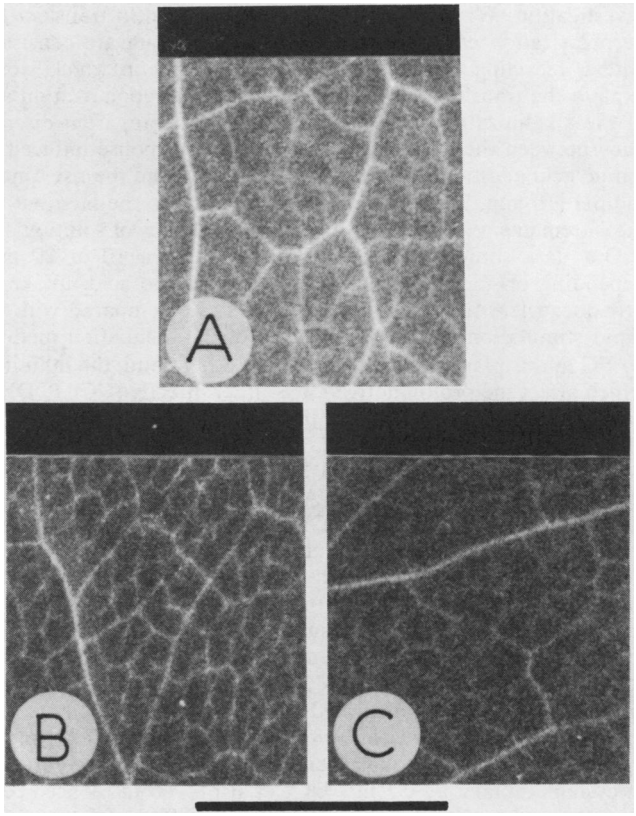

FIG. 12. Effect of NEM and PCMBS on the uptake of 2.5 mM [U-^{14}C] threonine. A, Control; B, 0.1 mM NEM; C, 0.1 mM PCMBS. Experimental details are in the legend of Figure 8. Exposure time: 6 d. The scale bar stands for 5 mm; background is shown in the top portion of each picture.

because (a) the parameters derived from Michaelis-Menten kinetics apply to enzymes in solution but are not necessarily valid for carriers embedded in a lipoidal structure; (b) the external pH may affect the activity of the carrier indirectly via a change in the surface charge density of the membrane which can alter local concentrations of the substrates (30); and (c) the pH measured in the bulk solution may be different from the pH in the cell wall. Further insight on the molecular mechanisms by which protons increase the activity of the carriers will be obtained only after isolation of these carriers and after their reinsertion in artificial lipid membranes. Our data are slightly different from those obtained on sucrose uptake by *Vicia faba* leaf. Amino acid (11) and sucrose (9) uptakes by this material are both apparently mediated by two kinetically different carriers. However, while the carrier operating at high (>5 mM) sucrose concentrations exhibits only little pH dependence (9), the carrier operating at high (20 to 100 mM) amino acid concentrations is still strongly sensitive to the external pH.

The transient alkalinizations depending both on the nature of the amino acid added and on its concentration (Fig. 4) also strongly support the idea of a proton symport inasmuch as this mechanism predicts that a massive and sudden rise of the external substrate concentration will cause an influx of protons within the cells and a depolarization of the membrane potential due to the operation of the symport. As already noticed in the studies on the mechanisms of sugar (6, 8, 19, 21) and amino acid (4) uptake in various materials, these phenomena are transient. However, in some instances, long lasting (up to 48 h) alkalinizations have been recorded following the addition of amino acids to a medium containing *Lycopersicon* xylem parenchyma cells (29). Perfusing the hollow petiole of *Ricinus* with a solution containing 25 or 50 mM serine or alanine induces only a weak but long lasting decrease in the rate of acidification of the solution which normally occurs (1). In this context, it should be stressed that the amino acid-induced depolarization is always followed by a repolarization which occurs more or less rapidly depending on the species under

investigation. When the amino acid is removed, a transient hyperpolarization can be recorded (14). These data are consistent with a recycling of protons by the proton pump which would explain the transient nature of the membrane repolarization and of the alkalinization of external medium. The apparent discrepancy between the rise time of the electrical response induced by amino acid addition (less than 1 min (14, 18)) and the rise time of the pH (10 min, Fig. 3) response may be due to the fact that pH measurements were recorded from large volumes of solution.

Our data show that FC can rapidly (within 10 or 20 min, depending on the experiments) stimulate amino acid uptake by broadbean leaf tissues. This result has to be compared with the rapid stimulation of the acidification of the incubation medium by FC in this plant material (8). On the other hand, the inhibitors which affect the protonmotive force either directly (CCCP, DNP) or indirectly via a blocking of the proton pump (DES, DCCD) also inhibit amino acid uptake. The effect of these inhibitors is not strictly specific: CCCP and DNP do not act only as protonophores since they can inhibit ATPase activity by depleting the ATP pool; also, DES and DCCD, besides blocking the plasmalemma ATPase, can have side effects. Despite this lack of specificity, the important point is to underline the close relationship between the time required for the effect of these compounds on the protonmotive force (as measured by pH variations, Fig. 6) and that needed for inhibition of amino acid uptake (Figs. 7 and 8). Although several workers have already reported the stimulation exerted by FC on amino acid uptake (12, 13, 20, 24), little information concerning the effect of DES and DCCD on the uptake is available. Harrington and Smith (17) found no inhibition of cysteine uptake by 0.1 mM DCCD at pH 4.5 in tobacco cells. Unfortunately, in this later work, neither the effect of the inhibitor on the protonmotive force nor its relationship with the amino acid uptake has been studied. Our data clearly indicate a close relationship between the primary translocation (proton-pumping activity) and the secondary translocation (amino acid uptake).

Inasmuch as we assume that high KCl concentrations depolarize the transmembrane potential in *Vicia* as in other materials (13, 23, 24), the result in Figure 9 suggests that the electrical component of the protonmotive force is involved for the uptake of threonine and α-AIB. However, this result does not preclude that some part of the uptake is powered by the transmembrane pH gradient. The relative part played for solute uptake by the two components ($\Delta\psi$ and Δ pH) of the protonmotive force will only be elucidated by microelectrode measurements.

Taken together, our results strongly support the existence of an amino acid proton symport in the leaf and are complementary to the evidence already available, which mainly stemmed from electrophysiological measurements. While the proton sucrose symport seems to be mainly located in the phloem (8), the autoradiographs (Figs. 2, 10, and 12) clearly show that amino acids are taken up by the whole leaf, with a large part of the total uptake located in the parenchyma. The effect of various compounds on the loading of amino acids by the veins (see, for example, the autoradiographs showing the effect of FC on amino acid uptake in Ref. 12, Fig. 2) and the biochemical features of phloem make it likely that an amino acid proton symport is active not only in the mesophyll, but also in the veins. However, the results presented here show that the mesophyll and the conducting bundles exhibited a different sensitivity towards various treatments such as acidic pH values (Fig. 2), high KCl concentrations (Fig. 10), and thiol-reacting compounds (Fig. 12). Even if the mechanisms of uptake are the same in these tissues, they differ at least in their sensitivity to the environment. This differential sensitivity underlines that uptake by the mesophyll is not a necessary prerequisite for amino acid absorption by the veins, and that most of the label seen in the conducting bundles has been taken up directly from the apoplast. This differential sensitivity could be used to study the properties of amino acid uptake by the bundles with a minor contribution of the mesophyll cells.

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