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Amino Acid Transport into Cultured Tobacco Cells

II. EFFECT OF CALCIUM[1]

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ABSTRACT

The effects of calcium ions on lysine transport into cultured Wisconsin-38 tobacco cells were examined. In the presence of calcium, lysine was transported at a relatively low rate for 30 to 40 minutes followed by a period of increasing rates and subsequent stabilization at a higher rate after 2 to 3 hours. In the absence of calcium, transport was uniformly low.

Time-dependent stimulation of lysine transport rate was observed after the cells had been preincubated in calcium-containing media. Similar treatments also resulted in the stimulated uptake of a variety of other amino acids, organic compounds, and sulfate. The stimulation of lysine uptake was apparently not due to nutrient starvation.

Lysine transport was not stimulated in a time-dependent fashion by K^+, La^{3+}, Mg^{2+}, or Mn^{2+}. Cells with stimulated transport rates continued to exhibit high rates when washed with calcium-containing media followed by transport in calcium-containing media. All other cation wash treatments were inhibitory, although magnesium treatments resulted in partial preservation of stimulated transport rates. Cycloheximide inhibited the calcium/time-dependent stimulation of lysine transport and caused the stimulated rate to decay.

The initial experimental treatments or the culture conditions may represent some form of shock that alters the membrane transport mechanism, thus reducing transport. The observed calcium/time-dependent stimulation may require protein synthesis and represents damage repair.

The effects of "washing" and "aging" of experimental plant material prior to metabolic studies have been long recognized. Although the physiological bases for these phenomena have been the subject of recent reviews, they are not well-understood (29, 30). One commonly occurring requirement, however, is for divalent cations, particularly Ca^{2+}, to reduce washing or aging effects and to achieve optimum responses (29, 30).

Several recent studies have addressed the effects of aging and washing on membrane transport in a variety of plant tissues as well as in cell cultures (1, 18, 19, 25, 26). Experimental treatments that represent either osmotic or monovalent cationic shock (*i.e.* the absence of sufficient calcium) result in decreased rates of transport and an increased loss of protein from the cells (1, 18, 19). Similar experiments have resulted in the characterization of periplasmic proteins involved in membrane transport in bacterial systems (10, 20).

In studies on Ser transport, Smith (26) has demonstrated several roles for Ca^{2+}; these include the time-dependent stimulation of uptake, the maintenance of high transport rates, and the retention of transported material. The stimulation of transport may be due to an increased protonmotive force available for symport (26) or to direct effects on the carrier *per se* (15).

The study presented here was initiated when Ca^{2+}-dependent changes in the initial rates of lysine transport were observed. Here, we reported the effects of washing and calcium on the transport of lysine and several other compounds.

MATERIALS AND METHODS

Tobacco cell [*Nicotiana tabacum* cv. Wisconsin-38 (W-38)] were cultured in Gamborg's B-5 liquid medium (pH 5.5) (5) on a rotary shaker (80 rpm) maintained at 23 C in the dark. The major components of B-5 are 1 mM $CaCl_2$, $MgSO_4$, NaH_2PO_4, $(NH_4)_2SO_4$, 25 mM KNO_3, 20 g/l sucrose, and 1 mg/l 2,4-dichlorophenoxyacetic acid. Cells were harvested for transport experiments at midlog phase. L-[U-^{14}C]Lysine, D-[U-^{14}C]mannitol, [2-^{14}C]uracil, [2-^{14}C]thymidine, and $Na_2{}^{35}SO_4$ were obtained from ICN. L-[U-^{14}C]Alanine, L-[U-^{14}C]aspartate, L-[U-^{14}C]arginine, and L-[U-^{14}C]leucine were obtained from Schwarz/Mann. L-[U-^{14}C]-Cysteine, L-[U-^{14}C]malic acid, D-[U-^{14}C]glucose, 3-P-[U-^{14}C]glyceric acid, and [U-^{14}C]acetic acid were obtained from Amersham. Aquasol-2 scintillation fluid was supplied by New England Nuclear. All other analytical grade chemicals were obtained from commercial suppliers.

Transport Experiments. Cells (1.0 g) were harvested at midlog phase as previously reported (6, 8) washed with 100 ml of appropriate transport medium, and then 0.25 g cells were placed in 39 ml transport medium contained in a 125-ml Erlenmeyer flask fitted with a cotton stopper. The transport medium consisted of either B-5 without 2,4-D, 1 mM $CaCl_2$ (pH 5.5), or 10 mM Btp,[4] 2% sucrose, 1 mM $CaCl_2$ (pH 5.5). The cells then were preincubated for the specified times at 23 C in the dark on a rotary shaker (100 rpm). Transport was initiated by the addition of 1 ml labeled compound (0.5 μCi) at final concentration of 5×10^{-5} M. Transport was terminated by filtering the cells and washing with 50 ml of the appropriate unlabeled compound at 5×10^{-4} M. The weighed cells then were placed in 1 ml H_2O and 15 ml Aquasol-2, and the radioactivities were determined by liquid scintillation counting (8). The results represent the mean of at least two experiments each with four replicates, and error is expressed as standard deviation.

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[4] Abbreviations: Btp, bis-Tris-propane; CHM, cycloheximide.

RESULTS AND DISCUSSION

Initial experiments to determine the linearity of lysine transport with time in simple media systems (*i.e.* buffer, sucrose, and no Ca^{2+}) indicated that little or no net transport was occurring. Lysine uptake was difficult to distinguish from diffusion because the cells exhibited only a slight accumulation of labeled lysine over the medium concentration. The addition of 1 mM $CaCl_2$ to the transport medium caused changes in the rate of lysine transport (Figs. 1 and 2). In these experiments where lysine was added at zero time (no preincubation), the rate of uptake was constant for about 30 to 40 min (Fig. 1), followed by a period of increase and subsequent stabilization at a new higher rate after 2 to 3 h (Fig. 2). This phenomenon occurred in both the B-5 growth medium and the Btp transport medium (Figs. 1 and 2) and was dependent on the inclusion of calcium in the medium. The generally higher rates of transport in the presence of Btp medium *versus* B-5 medium (Fig. 1) are to be expected in light of the 25 mM KCl present in B-5 medium. Potassium ions have been shown to inhibit calcium-dependent stimulation of serine transport (26), as well as to reduce the plasmalemma electropotential either in the presence or absence of calcium (11). The higher rates of transport in Figure 2 *versus* Figure 3 (to be discussed below) are due to the way in which transport rate was determined. In Figure 2, the results represent average transport/h for the given time period, whereas the Figure 3 results represent the rates determined after a given period of preincubation. The rates in Figures 1 and 2 may also be reduced by substrate depletion from the medium and feedback inhibition (transinhibition) by transported amino acid (unpublished results). The increases in uptake rates are probably not due to nutrient starvation because the changes in rates were observed in both the B-5 growth medium and the Btp transport medium.

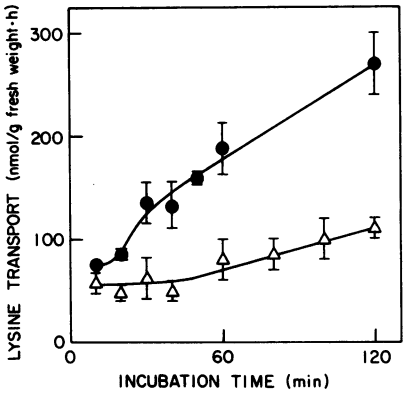

FIG. 1. Changes in lysine transport rate with incubation time. ●——●, 10 mM Btp, 1 mM $CaCl_2$, 2% sucrose (pH 5.5); △——△, B-5 growth medium including 1 mM $CaCl_2$ (see "Materials and Methods" for other constituents) (pH 5.5).

Several studies have observed amino acid transport over short time periods using a variety of media and techniques (2, 8, 12, 13). The results of such experiments may represent transport in plant material that was in a dynamic state. Plant scientists have long employed preincubation or aging treatments prior to performing a variety of physiological experiments (29); however, only a few studies using cell cultures (26, 28) have considered these aging phenomena.

Effect of Calcium on Lysine Transport. The time-dependent calcium stimulation of Ser, Cys, and sulfate transport has been observed in Xanthi tobacco cells (26). Wisconsin-38 tobacco cells exhibited stimulation of lysine transport in cells that were preincubated prior to transport in calcium-containing B-5 or Btp transport medium (Fig. 3). Maximum lysine uptake rate was observed after cells had been preincubated in the presence of calcium for 4 to 6 h (Fig. 3). In the absence of calcium, lysine transport rates were low (Fig. 3) and generally declined with preincubation time, resulting in little or no accumulation of lysine over the concentration in the medium. The similar results with B-5 and Btp, again, suggest that the preincubation effect did not result from a general increase in uptake due to starvation but resulted from a specific calcium effect(s).

A concentration of 1 mM $CaCl_2$ allowed maximum time-dependent stimulation of lysine transport, after either 4 or 8 h preincubation (Fig. 4). Transport in Btp medium was inhibited by 5 mM $CaCl_2$ preincubation. In agreement with these results, Maas *et al.* (18) have shown that higher concentrations of $CaCl_2$ were inhibitory to Pi uptake. These workers (18) suggest that $CaCl_2$ may cause a shock to uptake systems not unlike K^+ and Na^+ treatments at higher osmotic potentials.

The effects of $CaCl_2$ concentration on lysine transport were carried out with several buffer systems including B-5 growth medium, 10 mM Na citrate, and 10 mM Mes. As discussed above, the results with B-5 exhibited the same pattern as those with Btp (Fig. 4), whereas citrate and Mes each exhibited different patterns. Transport in the citrate buffer system was not inhibited by 5 mM $CaCl_2$. The lack of inhibition may be due to the formation of Ca-citrate complexes, thus lowering the free Ca^{2+} in the medium. Lysine uptake in Mes was stimulated with preincubation time but was inhibited by increasing $CaCl_2$ concentration. For example, the rate in 10 mM Mes without Ca^{2+} was 767 ± 41 nmol/g fresh

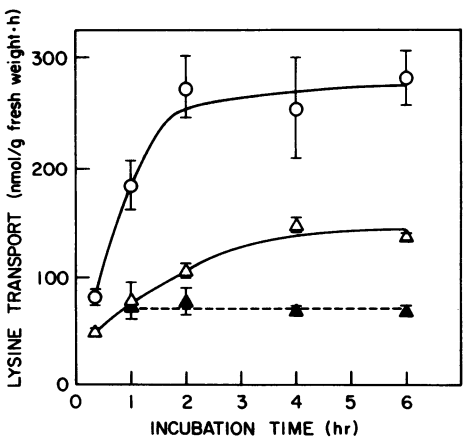

FIG. 2. Changes in lysine transport rate with incubation time. ○——○, 10 mM Btp, 1 mM $CaCl_2$, 2% sucrose (pH 5.5); △——△, B-5 including 1 mM $CaCl_2$ (pH 5.5); ▲- - -▲, B-5 medium minus $CaCl_2$.

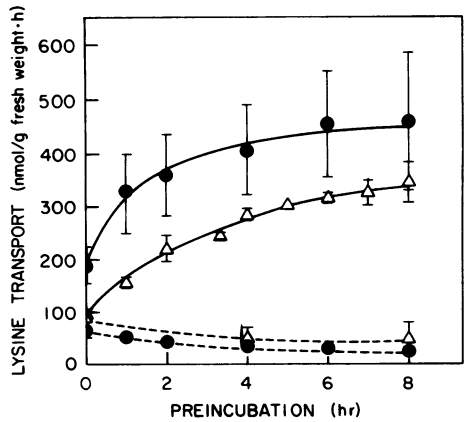

FIG. 3. Effect of preincubation on lysine transport rate. ●——●, 10 mM Btp, 1 mM $CaCl_2$, 2% sucrose (pH 5.5); ●- - -●, Btp medium minus $CaCl_2$; △——△, B-5 including 1 mM $CaCl_2$ (pH 5.5): △- - -△, B-5 medium minus $CaCl_2$. Transport was determined for 1 h.

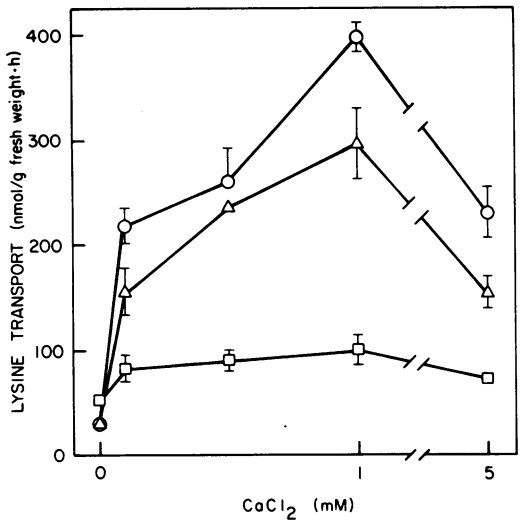

FIG. 4. Effect of calcium concentration on lysine transport rate. Medium was 10 mM Btp, 2% sucrose (pH 5.5) supplemented with appropriate $CaCl_2$ concentration. □——□, no preincubation; △——△, 4 h preincubation; ○——○, 8 hr preincubation. Transport was determined for 1 h.

Table I. *Effect of Preincubation on Uptake of Amino Acids*

The transport medium was 10 mM Btp ± 1 mM $CaCl_2$, 2% sucrose (pH 5.5); amino acid concentration was 50 μM.

Amino Acid	$CaCl_2$	Uptake Following Preincubation Times		
		0 h	4 h	8 h
		nmol/g fresh wt·h		
Lys	−	134 ± 25	54 ± 19	59 ± 30
	+	217 ± 84	412 ± 86	440 ± 82
Ala	−	317 ± 44	428 ± 47	472 ± 87
	+	439 ± 53	749 ± 37	813 ± 115
Arg	−	587 ± 42	266 ± 13	147 ± 61
	+	905 ± 78	1058 ± 206	1075 ± 176
Asp	−	26 ± 4	38 ± 3	51 ± 28
	+	37 ± 8	88 ± 13	135 ± 23
Cys	−	179 ± 44	166 ± 93	108 ± 45
	+	335 ± 19	347 ± 14	588 ± 60
Leu	−	261 ± 44	225 ± 31	312 ± 141
	+	344 ± 14	768 ± 30	1113 ± 124

weight·h after preincubation, whereas the rate in Mes + 1 mM $CaCl_2$ was 476 ± 26 nmol/g fresh weight·h. The basis for this inhibition is presently unknown to us; however, interactions between "Good's" buffers and divalent metal cations have been reported recently (21).

The extent of calcium stimulated uptake phenomena was tested with a variety of amino acids. All of the amino acids tested, with the exception of Arg (under conditions tested), exhibited time-dependent increases in transport rates in the presence of calcium (Table I). In the absence of calcium, transport rates generally declined; however, some increase in Ala and Leu uptake was observed (Table I). The reason(s) for these increases is presently unknown, although the different responses may be due to subsequent metabolism of transported material.

It is probable that the results presented in Table I do not represent initial rate velocities for some of the test compounds because high transport rates may have significantly altered the medium substrate concentration. The position of the test concentration (50 μM) on the uptake saturation curve as well as the nutritional and growth status of the cells may also cloud this experiment (Ref. 26, unpublished data). We have since determined

Table II. *Effect of Preincubation on Uptake of Several Compounds*

Transport medium was as in Table I; solute concentration was 50 μM.

Compound	$CaCl_2$	Uptake Following Preincubation Times		
		0 h	4 h	8 h
		nmol/g fresh wt·h		
Acetate	−	264 ± 40	246 ± 54	252 ± 55
	+	327 ± 69	366 ± 54	374 ± 38
Glucose	−	4.2 ± 0.6	4.6 ± 0.4	4.2 ± 0.7
	+	4.6 ± 0.4	6.9 ± 0.8	8.4 ± 1.8
Malate	−	3.8 ± 0.5	2.8 ± 0.5	2.5 ± 0.5
	+	4.8 ± 0.5	6.0 ± 0.5	8.0 ± 0.0
Mannitol	−	2.4 ± 0.5	2.4 ± 0.4	2.3 ± 0.5
	+	1.6 ± 0.6	2.0 ± 0.9	2.3 ± 0.9
Glycerate-3-P	−	6.5 ± 0.5	2.8 ± 0.5	3.5 ± 0.5
	+	9.5 ± 1.0	18 ± 1.0	28 ± 1.0
Sulfate	−	1.3 ± 1.0	1.9 ± 0.7	2.9 ± 1.2
	+	1.2 ± 0.8	21 ± 14	36 ± 22
Thymidine	−	13 ± 0.9	16 ± 0.5	12 ± 2.0
	+	22 ± 2.0	31 ± 2.0	31 ± 2.0
Uracil	−	14 ± 2.0	11 ± 1.9	12 ± 1.8
	+	30 ± 11	47 ± 17	66 ± 11

that Arg transport, for example, is quite active and has an approximate K_m and V_{max} of 10^{-4} M and 8,000 nmol/g fresh weight·h, respectively. Accordingly, under more favorable conditions, we have found that Arg uptake exhibits a 2- to 3-fold increase in response to Ca^{2+} preincubation with a stimulation time course similar to that shown for lysine (unpublished data).

Calcium treatments also caused time-dependent stimulation of uptake for several other compounds (Table II). Sulfate uptake was stimulated 10- to 15-fold. Smith has demonstrated that amount of time-dependent stimulation of sulfate uptake by calcium is dependent on the unstimulated rates of transport (26). The amount of stimulation is high when the unstimulated rate is low and *vice versa*. The internal sulfate pool is thought to regulate sulfate transport by a feedback mechanism (27).

The transport of glucose, malate, glycerate-3-P, and uracil was enhanced 2- to 3-fold, whereas thymidine uptake was only slightly stimulated (Table II). Acetate uptake was apparently not stimulated, although the fact that this compound is readily metabolized may make interpretation of uptake data difficult. Robinson and Mayo (24) reported that, in tobacco protoplasts, increased rates of transport were observed for Leu, uracil, UTP, glucose, and phosphate after preincubations in calcium-containing media (24). They also showed that small amounts of mannitol were transported, but this compound was not subject to calcium stimulation (24). Here, mannitol was not transported to any extent and did not exhibit calcium-stimulated uptake (Table II). The figure for mannitol (Table II) is similar to the 1.6 nmol/g fresh weight of lysine that was rapidly bound to W-38 cells and may be a measure of nonspecific binding or trapping in the free space and on the cell surface.

In the absence of calcium, the rates of transport remained relatively constant; however, small decreases in malate and glycerate-3-P uptake were noted after 8-h preincubations without calcium (Table II). The same treatment resulted in a small increase in sulfate transport (Table II). As in the case of Table I, the differential results in response to the absence of calcium may be due to the considerations discussed above.

A previous investigation (26) suggested that the time-dependent calcium stimulation of Ser transport was due to an increase in protonmotive force, which may subsequently drive transport at a higher rate via H^+ symport. Observations in our laboratory also

support the idea that lysine transport may be dependent on a proton gradient (6). It is unlikely that the same mechanism accounts for the uptake of all the compounds listed in Table II, although Higinbotham *et al.* (11) have shown that calcium treatments result in increased membrane potentials in *Avena* coleoptiles. They suggested that calcium may enhance a cation (H^+) pump (11). Rubinstein *et al.* (25) have demonstrated that osmotic shock causes a 60 to 70% decrease in transmembrane electropotential and a 70 to 90% inhibition of Leu transport in oat coleptiles.

At least a portion of the apparent calcium stimulation of transport appears to be due to decreasing efflux, thus increasing net transport. Cells preloaded with labeled lysine lost 2.6% of the internal labeled material when resuspended in medium containing Ca^{2+} (6). Alternatively, if the cells were resuspended in the absence of Ca^{2+}, 44% of the labeled material was lost during the same period (6). Calcium also prevented Ser efflux from preloaded tobacco cells (26).

Several other investigators have suggested that calcium and/or time-dependent stimulation of uptake may actually be recovery from some type of "damage" introduced during the experimental procedures (1, 18, 19, 28). The damage may be due to osmotic and/or ionic shock (1, 18, 19) or to changes in gas concentrations in the culture atmosphere (28). The recovery from osmotic or ionic shock requires relatively short time periods (4 to 6 h) (1, 18, 19), whereas recovery from gas shock requires a longer time (28). The experimental methods used here would introduce a gas shock according to the criteria of Thoiron *et al.* (28); however, the stimulation time periods here are more consistent with recovery from osmotic or ionic shock.

Effects of Other Cations. Tobacco cells were washed and preincubated in a variety of cation solutions to determine the specificity requirements for the stimulation phenomena. The data in Table III indicate that Ca^{2+} and Mg^{2+} resulted in a slight stimulation at zero time; however, Ca^{2+} was the only ion that caused time-dependent stimulation of transport. After 6 h preincubation, 5 mM magnesium appeared to preserve some transport activity at an intermediate level, when compared to the preincubation without any cation (Table III). Other ions either did not stimulate or were inhibitory of lysine transport (Table III). Smith (26) has shown that Mg^{2+} substitutes for Ca^{2+} in establishing high rates of Ser transport, whereas K^+ and La^{3+} inhibit Ca^{2+} stimulation. In a related experiment, we observed that cells from stationary cultures exhibited time-dependent stimulation of lysine transport in the presence of both Ca^{2+} and Mg^{2+}, although the degree of stimulation and the final stimulated rate in the presence of Ca^{2+} was much lower than in midlog cultures. Other experiments indicate that both the unstimulated and stimulated rates of lysine and Arg uptake vary with culture age (unpublished data). Similar results have been obtained for sulfate transport (26).

Table III. *Effects of Cation Preincubation on Lysine Transport*

Cells were washed and preincubated in Btp transport medium containing the appropriate cation. Transport was measured as described in text. All cations were present as chlorides. Concentrations were: $CaCl_2$, 1 mM; all others, 5 mM.

Cation	Lysine Transport Following Preincubation Times	
	0 h	6 h
	nmol/g fresh wt·h	
None	199 ± 7	47 ± 3
Ca^{2+}	325 ± 12	623 ± 58
K^+	210 ± 7	90 ± 5
La^{3+}	101 ± 11	61 ± 36
Mg^{2+}	264 ± 8	162 ± 32
Mn^{2+}	62 ± 7	11 ± 1

Table IV. *Effects of Cations on Lysine Transport in Calcium-preincubated Cells*

Cells were preincubated 6 h as described in text and then washed with 50 ml cation solution in Btp medium. Cells were subsequently incubated in Btp transport medium containing [^{14}C]lysine and the appropriate cation. Cations and concentrations were as in Table III.

Preincubation	Wash and Transport	Lysine Transport
		nmol/g fresh wt·h
−Ca	Control[a]	47 ± 3
−Ca	−Ca	67 ± 2
+Ca	Control[a]	775 ± 77
+Ca	−Ca	42 ± 2
+Ca	+Ca	666 ± 15
+Ca	+K	243 ± 16
+Ca	+La	181 ± 10
+Ca	+Mg	421 ± 10
+Ca	+Mn	43 ± 5

[a] No wash.

Cation Washes. Calcium-preincubated cells with high rates of transport continued to exhibit high rates after a 5-min (50-ml) wash in calcium-containing medium when transport was determined in the presence of Ca^{2+} (Table IV) (26). Cells that were washed with medium lacking a cation (minus Ca^{2+}) had low rates of lysine uptake (Table IV), similar to the rates in cells that received a 6-h minus Ca^{2+} preincubation treatment (Table III). Transport was reduced when the cells were treated with other cations (Table IV). Magnesium was effective in substituting for Ca^{2+} in the maintenance of only intermediate lysine transport rates (Table IV); however, Smith reported that Mg^{2+} could maintain high rates of Ser transport in Xanthi tobacco cells (26). Here, K^+ and La^{3+} resulted in about 70% reduction in lysine transport, whereas the Mn^{2+} treatment reduced transport to very low rates of uptake (Table IV). Other workers have shown that La^{3+} substitutes for Ca^{2+} in the maintenance of high Ser transport into Xanthi tobacco cells (26) and HCO_3^- influx into *Chara* (15). Smith (26) proposed two calcium sites that affected transport: (a) specific for Ca^{2+} required to increase the protonmotive force and (b) binding either Ca^{2+} or La^{3+} required to maintain the protonmotive force. Similarly, Lucas and Dainty (15) suggest that one calcium site affects the HCO_3^- transporter, whereas another calcium/lanthanum site is associated with membrane permeability. Both calcium (6, 26) and lanthanum (26) decrease efflux from preloaded cells. A site affecting general membrane permeability might be expected also to affect the magnitude of the protonmotive force.

The above data suggest that the removal of cells from B-5 medium and subsequent wash in the presence of Ca^{2+} with either B-5 or Btp transport medium must provide some shock effect. Smith (26) has demonstrated that Xanthi tobacco cells bind about 3.3 μmol Ca^{2+}/g fresh weight. With a culture fresh weight of 15 g, this figure would allow more than 60% of the calcium present in 80 ml B-5 culture medium (1 mM $CaCl_2$) to be bound by the cells. In addition, the medium macromolecules can potentially bind another 80 μmol (25); therefore, the free calcium content in the growth medium would be small. If the cells are starved for Ca^{2+} when harvested, the initial wash and transport treatments would have little effect on transport, regardless of Ca^{2+} treatments. Transport in these cells would be expected to be low, and the cells would require the Ca^{2+} preincubation period for maximum rates of uptake. These predictions are confirmed by the results in Figures 1 and 2 where similar rates of transport were observed in freshly harvested cells, independent of the Ca^{2+} treatment. Washed cells in calcium-containing medium would have adequate Ca^{2+} and high transport rates after preincubation treatments. An additional Ca^{2+} wash would have little effect on transport in preincubated cells, whereas a wash lacking Ca^{2+} would decrease transport

to the initial low transport rate of freshly harvested cells. The results in Table IV support these predictions and the hypothesis that the cells are in a calcium-starved environment in the growth medium.

In carrot roots, cold NaCl treatment causes a shock effect that inhibits glucose and phosphate uptake and causes a release of protein from the cells (19); these workers conclude that the shock effect is due to monovalent cation effects on the plasmalemma. Amar and Reinhold (1) suggest that both monovalent cationic and osmotic shock may reduce transport. The above Na^+ shock can be prevented by additional Ca^{2+} or Mg^{2+} (19). The shocked tissue recovered the ability to transport in about 5 to 6 h when preincubated in the presence of Ca^{2+} (19). Osmotic shock was also reported to cause the inhibition of phosphate uptake in barley roots (18), α-aminoisobutyric acid uptake in bean leaf tissue (1), and the uptake of several compounds, including Leu, in oat coleoptiles (25). Incubation of shocked tissues in calcium-containing solutions results in recovery of transport in these studies (1, 18, 25). In addition, the osmotic shock causes the release of proteins from plant material (1, 18), similar to the shock effect seen in bacteria where such osmotic treatments result in the release of periplasmic proteins involved in membrane transport (10, 20). In leaf cells, the shock fluid decreased the time required to reestablish transport when included in the recovery medium; however, the shock fluid did not exhibit α-aminoisobutyric acid binding activity (1). We have observed that W-38 tobacco cells, which were labeled with [^{3}H]Leu during the preincubation period, lost 3 to 5 times more radioactive trichloroacetic acid-precipitable material when washed in the absence of Ca^{2+} than in the presence of Ca^{2+} (unpublished data). Calcium-preincubated cells with high rates of transport, when washed in the absence of Ca^{2+} (Table IV), exhibited low transport rates. This treatment may represent an ionic shock that results in the loss of a protein-involved transport. The 2- to 4-h preincubation required for the recovery of high transport rate suggests that the synthesis of new protein may be involved.

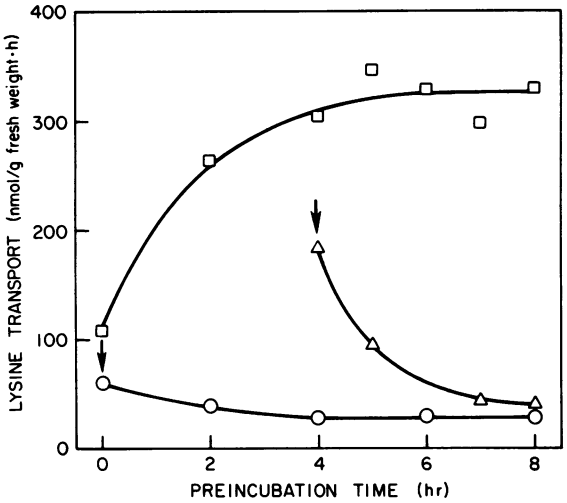

FIG. 5. Effect of CHM on time-dependent calcium stimulation of lysine transport. □——□, 10 mM Btp, 1 mM $CaCl_2$, 2% sucrose (pH 5.5). CHM (4 μg/ml) was added at zero time (○——○) or after 4-h preincubation (△——△). Transport was determined for 1 h.

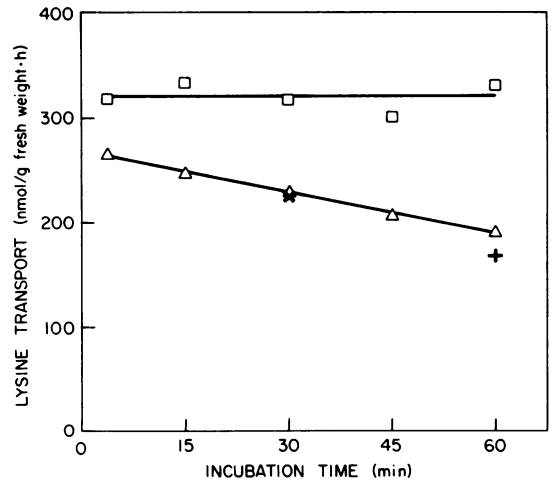

FIG. 6. Time course of CHM-induced decay of high rates of lysine transport. Cells were preincubated 4 h and transport was initiated at zero time. □——□, 10 mM Btp, 1 mM $CaCl_2$, 2% sucrose (pH 5.5) (control); △——△, CHM (4 μg/ml) added at zero time; ×, cells preincubated 30 min in CHM and washed with 100 ml control medium with transport for 30 min; +, cells preincubated 60 min in CHM and washed with 100 ml control medium with transport for 60 min.

Effect of Cycloheximide. CHM (4 μg/ml) inhibits protein synthesis in tobacco cells (9). CHM inhibited the Ca^{2+}/time-dependent stimulation of lysine transport (Fig. 5) and allowed the stimulated rate to decay (Figs. 5 and 6). Lysine transport (1-h incubation) was inhibited 40% when CHM was included in the transport medium (Figs. 5 and 6). The per cent inhibition was independent of the control transport rate; both stimulated and unstimulated rates of transport were inhibited by the same percentage (Fig. 5). The time course of CHM inhibition of stimulated lysine transport rates indicated that the inhibitor caused a linear decline in uptake rates from 5 to 60 min (Fig. 6). This curve does not, however, extrapolate to the control rate at very short exposure times, suggesting more than one mode of inhibition by CHM. Cells that had been pretreated with CHM for either 30 or 60 min and washed prior to transport measurements had rates that were similar to those observed when transport was determined in the presence of the inhibitor for similar time periods (Fig. 6). These results suggest that CHM does not compete for lysine uptake but causes some irreversible effect on transport during the time frame examined.

CHM inhibited Arg transport into Xanthi tobacco cells (23), whereas Leu transport into potato tuber tissue and barley roots was not affected (22). Both CHM and puromycin inhibited the time-dependent stimulation of solute transport in tobacco protoplasts (24). The results here indicate that CHM reduces lysine uptake and suggest that this compound may introduce artifacts into studies where protein synthesis is measured by labeled Leu incorporation into protein by reducing uptake. The inhibition by CHM is not due to increased efflux since preloaded cells lost only 2 to 5% of the total cellular-labeled material when resuspended in the presence of CHM.

The problems of using CHM as an inhibitor of protein synthesis have been addressed (3, 17); however, Lüttge *et al.* (16) demonstrated that CHM did not reduce respiratory O_2 uptake or ATP levels in barley roots. Both CHM and puromycin inhibited recovery of phosphate uptake in NaCl-shocked barley roots (18), and CHM inhibited recovery of glucose and phosphate uptake in carrot roots (19). These studies agree with the results here and suggest that the shock effect may be due to the removal of a protein involved in membrane transport. Alternatively, reduction of transport by inhibitors of protein synthesis may be due to transinhibition. In the case of amino acids, inhibition of protein synthesis would lead to elevated endogenous amino acid pools (4) that could potentially reduce uptake by feedback regulation (transinhibition). The phenomenon of transinhibition has been described in bacterial systems (14), and experiments in this laboratory suggest that the basic amino acid pools may regulate lysine transport (unpublished results).

CONCLUSIONS

The low transport rates observed here in freshly harvested cells may be the result of either monovalent cationic or osmotic shock, especially since the effective Ca^{2+} concentration in the culture flask was probably quite low. The subsequent recovery of high transport rates in the presence of Ca^{2+} suggests a time-dependent change in membrane properties that may require the synthesis of a protein involved in transport. The Ca^{2+} may be involved in the binding of such protein to the membrane or maintaining a membrane conformation that favors protein binding to maintain an intact transport system. This protein may be a transport system component common to the transport of several classes of solutes. If so, the protein may be involved in energy coupling to transport or some other energy-related function.

Future studies will examine the effects of other protein synthesis inhibitors on the preincubation phenomena and energy metabolism. The protein obtained from shocked cells will be concentrated and attempts will be made to reconstitute transport at high rates. Other studies will examine the membrane proteins synthesized during the preincubation period to determine if the Ca^{2+} stimulation of transport requires *de novo* protein synthesis.

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