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# Calcium Modulation of Polyamine Transport Is Lost in a Putrescine-Sensitive Mutant of *Neurospora crassa*[1]

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**Putrescine transport in *Neurospora* is saturable and concentrative in dilute buffers, but in the growth medium putrescine simply equilibrates across the cell membrane. We describe a mutant, *puu-1*, that can concentrate putrescine from the growth medium because the polyamine transport system has lost its normal sensitivity to $Ca^{2+}$. The wild type closely resembles the mutant if it is washed with citrate and ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid. The mutant phenotype also appears in the wild type after treatment with cycloheximide. The results suggest that putrescine uptake is normally regulated by an unstable $Ca^{2+}$-binding protein that restricts polyamine uptake. This protein is evidently distinct from the polyamine-binding function for uptake, which is normal in mutant and in cycloheximide-treated wild type cells. The *puu-1* mutation, stripping of $Ca^{2+}$, and cycloheximide treatment all cause an impairment of amino acid transport, indicating that other membrane transport functions rely upon the product of the *puu-1*$^+$ gene. Preliminary evidence suggests that the putrescine carrier is not the $Ca^{2+}$-sensitive, low-affinity $K^+$-transport system, but $K^+$ efflux does accompany putrescine uptake.** 

Most organisms take up polyamines (putrescine, spermidine, and spermine) by an active transport system, despite their ability to make these compounds internally. The system may allow adjustments to the polyamine pools of cells of multicellular organisms; it may serve polyamine catabolic pathways; and it may even allow accumulation of putrescine as an osmoticum.

*Neurospora crassa* makes polyamines, but does not use them as catabolic substrates. The organism has a polyamine transport system that takes up all three natural polyamines with moderate affinity ($K_m$'s: putrescine = 600 μM; spermidine = 240 μM; spermine = 70 μM) from a dilute buffer containing 20 mM $Na^+$ (1). In the growth medium, however, this system is inhibited by monovalent cations and by $Ca^{2+}$, and all transport of polyamines takes place by way of a nonsaturable, diffusional system that equilibrates polyamines across the cell membrane (1, 2). The concentrative uptake system appears only in $Ca^{2+}$-free buffers having low monovalent cation concentrations. We have in fact questioned whether the natural substrates of the system include the polyamines (1).

We describe here a mutant of *N. crassa, puu-1,* able to concentrate polyamines from low concentrations in the medium, owing to a loss of inhibition of the polyamine uptake system by cell-bound $Ca^{2+}$. The strain is intoxicated by polyamines in concentrations to which the wild type is indifferent. The results suggest that a component of the cell membrane protects *N. crassa* from absorbing toxic levels of polyamines—and perhaps other cations—from the environment. The accompanying paper (3) defines the discretionary capacity of *N. crassa* for polyamines and its intracellular location, using the *puu-1* and another putrescine-accumulating strain.

## MATERIALS AND METHODS

*Strains, media, growth, and genetic techniques.* The wild type strain, ORS6a, a strain containing the *puu-1* allele, GB1 (IC2296-1a) and (in two experiments) a strain carrying both the *puu-1* and the *aga* (arginaseless, allele UM906) mutations (IC2296-65a) were used in physiological work. GB1 is the only known *puu-1* allele. The *alcoy* stock was used to localize *puu-1* genetically in initial studies (4). Other mutant stocks bearing combinations of the following mutations were used in genetic mapping and other work: *spe-1* (PE4), *arg-2* (CD80), *cot-1* (C102), *inl* (89601), and the permease mutations *pmb, pmn,* and *pmg.* All genes are described in the compendium of *N. crassa* mutants of Perkins *et al.* (5). Strains carrying all these mutations are available from the Fungal Ge-

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Standard techniques and media were used for most microbiological and genetic work (6). Vogel's medium N was used for growth studies and stock maintenance. Westergaard's medium was used for genetic crosses. Exponential cultures were grown in boiling flasks, with forced aeration from a glass tube reaching to the bottom (6). Stationary growth was carried out in 10 ml Vogel's medium in 50-ml Erlenmeyer flasks at 32°C. In genetic analysis of the *puu-1* mutation, the sensitivity of the *puu-1* mutant to 5 mM putrescine in the medium was used to score the marker.

The concentrations of the main salts of Vogel's medium, which we tested individually for their effect on putrescine uptake are as follows: 8 mM $Na_3$ citrate, 25 mM $NH_4NO_3$, 37 mM $K_2HPO_4$, 0.8 mM $MgSO_4$, and 0.7 mM $CaCl_2$. Trace elements were added at the following concentrations ($\mu$M): Zn, 17; Fe, 2.6; Cu, 1.0; Mn, 0.3; Mo, 0.2.

*Metabolite uptake and determination.* Uptake and excretion of polyamines were measured as described previously (1, 2). Cells were washed gently on Miracloth (Calbiochem), without the compression of severe vacuum filtration when transferring cells from the growth medium to the buffer in which transport was measured. $^{14}C$-labeled polyamine uptake rates were measured at 25°C in normal or variously modified growth medium (Vogel's medium N), and in Mops–glucose[5] buffer, pH 7.2 (10 or 20 mM NaOH neutralized to pH 7.2 with 3-(*N*-morpholino)propanesulfonic acid, and 0.2% glucose). The buffer will be referred to as Mops buffer, and the $Na^+$ concentration will be specified in text, figures, and tables. Substrate saturation curves were performed with different concentrations of polyamine for 5 min (putrescine) or 15 min (spermidine) intervals, and $K_m$ and $V_{max}$ determinations were drawn from curves from which the nonsaturable component was subtracted (1). In this work, as in previous work (1), the range of variation of the rate of the nonsaturable transport is about twofold; that of the saturable system of wild type is about 50%, presumably owing to the variation in $Ca^{2+}$ remaining bound to the cells (this work).

For most experiments, we measured transport activity with 1 mM putrescine over a 15-min interval. For the wild type in conditions where uptake was diffusional, uptake was not linear. Instead, internal and external putrescine equilibrated in about 7 min. This component of uptake also prevailed in the *puu-1* mutant. In comparison of strains, the diffusional component represented an unacceptably high "blank" (of about 0.2–0.6 nmol per minute per milligram dry weight) in 5-min assays where the saturable system had low activity. The longer interval of uptake rendered the active component more conspicuous and, after subtraction of the diffusional blank, more accurate. Data in the later experiments are reported in terms of nanomoles per milligram per 15 min, without subtracting the diffusional component.

We used doubling time (in minutes) and polyamine pool sizes (see Results) to calculate steady-state parameters of influx and efflux. The rationale was that of Karlin *et al.* (7), based simply on a growth equation ($A_x = A_0e^{kt}$), where $A_x$ and $A_0$ are the values at the end and beginning of an interval (1 min); $t$ is time in minutes; and $k$ is the growth constant (ln 2 divided by doubling time).

Uptake of $^{14}C$-labeled amino acids of known specific radioactivities was measured by methods similar to those used for polyamine uptake. Activities of three amino acid uptake systems were measured at 25°C for 2 min. The general system substrate was 1 mM [$^{14}C$]aminoisobutyric acid (8); the neutral system was measured with 1 mM [$^{3}H$]leucine in the presence of 1 mM arginine (9); and the basic system was measured with 0.1 mM [$^{14}C$]arginine in the presence of 0.1 mM leucine (10).

$K^+$, $Na^+$, $Ca^{2+}$, and $Mg^{2+}$ were determined by atomic absorption spectroscopy as described previously (11). Polyamine extraction and determination by high performance liquid chromatography were done as described previously (12). $K^+$ efflux was measured by loss of cellular $K^+$ in various conditions, including unbuffered water, to which certain transport substrates were added. Cells (2–6 mg dry weight equivalent) were collected on 5-$\mu$m membranes and extracted with trichloroacetic acid, and $K^+$ in the extract was determined after appropriate dilution.

Large molar excesses (e.g., 100-fold) of putrescine over spermidine prevailed in putrescine-containing media where small amounts of spermidine excretion might have occurred. Dansylation of spermidine would be compromised under these conditions. We therefore not only isolated polyamines from media on Dowex-50W columns (0.7 × 7.5 cm, 200–400 mesh, 8% crosslinking, $H^+$ form), but also separated putrescine and spermidine. Medium (2 ml) from a culture was applied to the column, then the column was washed with 15 ml 1.5 N HCl, and 6 ml of 3.25 N HCl. Putrescine was then eluted in 6 ml 3.5 N HCl; following this, spermidine was eluted in 6 ml 6 N HCl. Spermine (2 nmol) was added as an internal standard for spermidine, and emerged in the 6 N HCl fraction. There was virtually no overlap of putrescine with the other polyamines. Fractions were evaporated to dryness, redissolved in a small amount of $HClO_4$-EDTA buffer (12), and dansylated as usual.

G.R.B. and A.D.H. were largely responsible for the isolation and genetics of the *puu-1* mutant; R.H.D. and J.L.R. performed the remainder of the work.

## RESULTS

### *Genetics of the puu-1 Mutant*

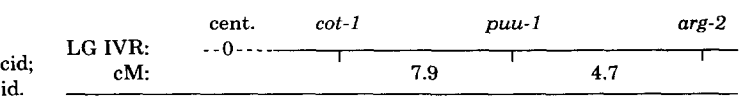
Strains carrying *spe-1* mutations lack ODC and require at least 0.4 mM putrescine for optimal growth (13). A strain carrying a *spe-1* mutation was plated (without mutagenesis) on medium containing 0.05 mM putrescine, which normally supports no growth. A single colony appeared and was isolated. It retained an absolute polyamine requirement. Growth of the variant was severely inhibited by 5 mM putrescine, which hardly impaired growth of the parent strain. Genetic work on the variant and its descendents defined a mutation at a new locus, *puu-1* (*pu*trescine *u*ptake). The mutation is unlinked to *spe-1*, which lies on Linkage Group V. Using the putrescine-sensitive phenotype to analyze crosses with *alcoy* and with *cot-1*, we localized the mutation to Linkage Group IV. A three-point cross showed that *puu-1* lay between *cot-1* and *arg-2* (Table I).

Additional mutants with the *puu-1* growth phenotype were selected after mutagenesis. The severity of the pu-

TABLE I

Progeny of the Cross *cot-1 puu-1 arg*$^+$ × *cot*$^+$ *puu*$^+$ *arg-2*

| Progeny genotype | | | Number |
|---|---|---|---|
| *cot-1* | *puu-1* | *arg*$^+$ | 108 |
| *cot*$^+$ | *puu*$^+$ | *arg-2* | 134 |
| *cot*$^+$ | *puu-1* | *arg*$^+$ | 1 |
| *cot-1* | *puu*$^+$ | *arg-2* | 21 |
| *cot-1* | *puu-1* | *arg-2* | 5 |
| *cot*$^+$ | *puu*$^+$ | *arg*$^+$ | 8 |
| *cot*$^+$ | *puu-1* | *arg-2* | 0 |
| *cot-1* | *puu*$^+$ | *arg*$^+$ | 0 |




[5] Abbreviations used: Mops, 3-(*N*-morpholino) propanesulfonic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether) *N,N'*-tetraacetic acid.

trescine uptake or inhibition phenotypes varied widely, and most suffered growth or morphological defects that made them difficult to work with. None was allelic to *puu-1*. Reversions of the *puu-1* mutant were similarly variable. Almost all were due to unlinked suppressor mutations, and none lacked the polyamine transport system. Limited genetic tests showed many reversion events to be non-allelic to one another. Because of the pleiotropy of *puu-1* and the diversity of mutations that duplicated or reversed its effect, further genetic studies were deferred in favor of characterizing the original mutant in detail.

A heterokaryon containing *arg-2 puu-1 inl*$^+$ and *arg*$^+$ *puu*$^+$ *inl* nuclei grew well in minimal medium supplemented with 5 mM putrescine. This indicated that the *puu-1* mutation was recessive to its wild type allele and thus represented a deficiency or loss of function.

### *Altered Transport Characteristics of the puu-1 Mutant*

Wild type *Neurospora* cannot concentrate polyamines from Vogel's medium, because the saturable polyamine transport system is inhibited (1, 2). Putrescine and spermidine equilibrate across the cell membrane by a diffusional mechanism (2). In the same medium, the *puu-1* strain displays saturable uptake of putrescine and spermidine (Fig. 1). This accounts for the origin of the *puu-1* mutation by the selection method we used. The final slopes of the lines in Fig. 1 revealed that the strains shared the diffusional component of uptake. The apparent $K_m$'s of the *puu-1* strain were about 3 mM for both substrates under these conditions. After a wash and resuspension in Mops buffer (20 mM $Na^+$), both strains displayed saturable polyamine uptake, with normal $K_m$'s for putrescine (0.3–0.7 mM) and spermidine (0.25 mM) (1); the $K_m$'s were lower in buffer owing to lack of competitive inhibition by salts in the medium. The $V_{max}$ of uptake by *puu-1* strains in Mops buffer was 2.5 to 8 times greater than that of the wild type. The putrescine uptake rate and $K_m$ of both strains were insensitive to pH variation between 5.2 and 8.2 (data not shown).

The putrescine pool of both Puu$^+$ and Puu$^-$ strains grown in minimal medium is approximately 0.8 to 1.0 nmol per milligram dry weight (1, 3). The wild type, grown in the presence of 1 mM putrescine, has a putrescine pool of about 2 to 3 nmol per milligram, or approximately 1 mM in cell water [2.5 μl per milligram dry weight (14)]. This bears out our previous finding that putrescine equilibrates diffusionally across the cell membrane (2). However, in 1 mM putrescine, the *puu-1* strain contains approximately 120 nmol putrescine per milligram, nominally about 48 mM, indicating considerable concentrative transport. The molar value is belied by the fact that most of the putrescine is in the vacuole (3). (If 1 mM spermidine is added to *puu-1* cells, the spermidine pool becomes twofold normal. Spermidine at higher concentrations does not seriously inhibit growth.) The difference between

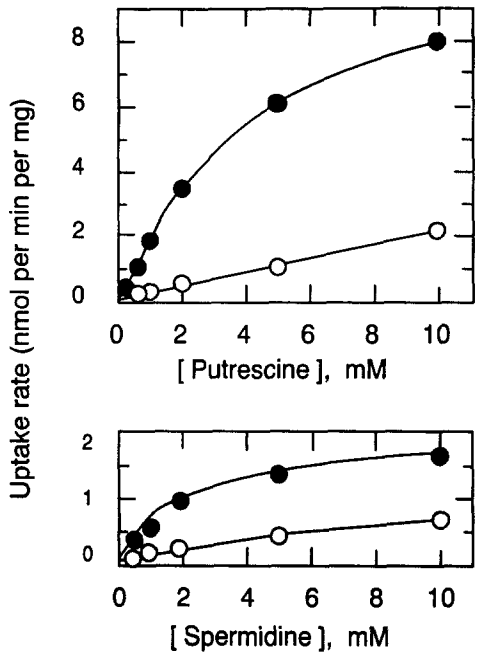



**FIG. 1.** Comparison of polyamine uptake by wild type and *puu-1* in Vogel's medium. Rate of uptake versus putrescine (top) or spermidine (bottom) concentration by wild type (open circles) and *puu-1* (closed circles), using a 5-min uptake period.

strains in their ability to accumulate putrescine is roughly correlated with the $V_{max}$ of the strains' saturable uptake systems (see below).

During steady-state growth of either strain in putrescine, the influx rate should be the same as the rate of efflux plus whatever is required to maintain polyamine pools during growth. If the high pool of putrescine in *puu-1* were primarily the result of impaired efflux, influx rates at steady-state would be normal or low. If the high pool was owing to a higher rate of influx, the influx rate at steady-state should remain high. A test of these expectations showed that *puu-1* uptake at steady-state was much higher than that of the wild type, indicating that influx is altered in the mutant (Fig. 2).

We also calculated the amount of putrescine uptake required to maintain the putrescine and spermidine pools of the two strains during growth in 1 mM putrescine (3), assuming no contribution from biosynthesis. This showed that the measured rate of uptake at steady state (0.11 and 0.5 nmol per minute per milligram for wild type and *puu-1*, respectively, from Fig. 2) roughly approximated the need (0.14 and 0.6 nmol per minute per milligram, respectively). Only a very small efflux was expected from wild type, and, indeed, little efflux from wild type can be seen upon transfer from 1 mM putrescine to minimal medium. A small, but definite efflux (0.1 nmol per minute per milligram) was actually seen for *puu-1* in a parallel experiment, in keeping with its higher intracellular putrescine concentration. Spermidine efflux was not detected

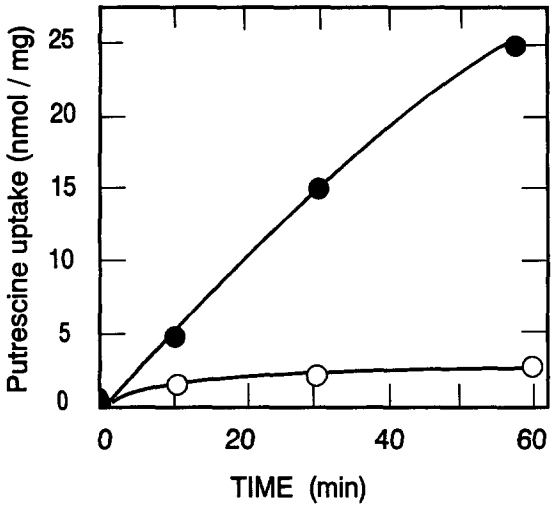



FIG. 2. Uptake of [$^{14}$C]putrescine by cultures during steady-state growth in the presence of 1 mM putrescine. Steady-state cultures of wild type (open circles) and *puu-1* (closed circles) strains growing in the presence of 1 mM putrescine were washed and introduced to fresh medium containing 1 mM putrescine. A small amount of [$^{14}$C]putrescine was added to each. Cells (of 2 ml culture) were then harvested at the times indicated and counted.

in either strain, either in the parent culture or in the minimal medium to which it was transferred. These calculations and observations show that the primary effect of the *puu-1* mutation is on influx, rather than efflux of putrescine.

The reason that putrescine efflux from *puu-1* was not much greater is that excess intracellular putrescine under these conditions is largely vacuolar (3). In the accompanying paper, we show the vacuolar accumulation of putrescine in *puu-1* is the result, not the cause, of putrescine accumulation in the cell itself.

### *Differential Sensitivity of Wild Type and puu-1 to Calcium*

Most cations of the medium inhibit putrescine uptake, the effect of $Ca^{2+}$ being the most severe (1). Unlike omissions of other cations, omission of $Ca^{2+}$ from the medium led to an increased putrescine uptake rate in the wild type and a slightly decreased uptake rate in *puu-1* (Fig. 3). Omission of $Ca^{2+}$ also allowed the wild type to accumulate a substantial putrescine pool from media containing 1 mM of the diamine (Fig. 4). Varying the $Ca^{2+}$ concentration of the medium reveals an extreme difference in the sensitivities of wild type and *puu-1* cells (Fig. 4). The data suggested that the competitive inhibition of putrescine uptake by $Ca^{2+}$ previously described for wild type (1) might be less strong in *puu-1*.

This idea was tested by testing washed cells of the two strains in Mops buffer for the sensitivity of putrescine uptake to $Ca^{2+}$. Unexpectedly, putrescine uptake was found to be equally sensitive in the two strains (Fig. 5), although they had different $V_{max}$ for transport. The discrepancy between the strains' sensitivities to $Ca^{2+}$ in Mops buffer (Fig. 5) and in growth medium (Figs. 3 and 4) was traced to citrate, a metal chelator present at 8 mM in the medium (Table II, Experiment I).

If $Ca^{2+}$ inhibited putrescine uptake solely by competitive inhibition (Fig. 5), citrate should mitigate the inhibition in both strains to the same degree by reducing the concentration of the free metal. As noted, this was not the case. The differential effect of citrate on the two strains with respect to $Ca^{2+}$ inhibition therefore suggested (i) that $Ca^{2+}$ had a second inhibitory action on wild type; (ii) that a high-affinity $Ca^{2+}$ binding site was involved, such that citrate was only weakly effective against added $Ca^{2+}$; and (iii) the *puu-1* mutant lacked sensitivity to this action of $Ca^{2+}$, which accounts for its ability to concentrate putrescine from the growth medium. Further experiments supported this scenario.

### *Cell-Bound Calcium Inhibits Putrescine Uptake Only in Wild Type*

$Na_3$ citrate stimulated putrescine uptake by the wild type, even in the absence of $Ca^{2+}$ (Table II). In the same experiment, $Na_3$ citrate inhibited uptake by *puu-1*, an effect exerted by the $Na^+$ counterion (NaCl had a similar effect). The stimulation of the wild type system by citrate (actually minimized by the $Na^+$ counterion) suggested that transport in the wild type was controlled by a chelatable factor firmly bound to the cell surface. $Ca^{2+}$ added back in the presence of citrate inhibited putrescine uptake only in the wild type (Table II). These data, like the effect of

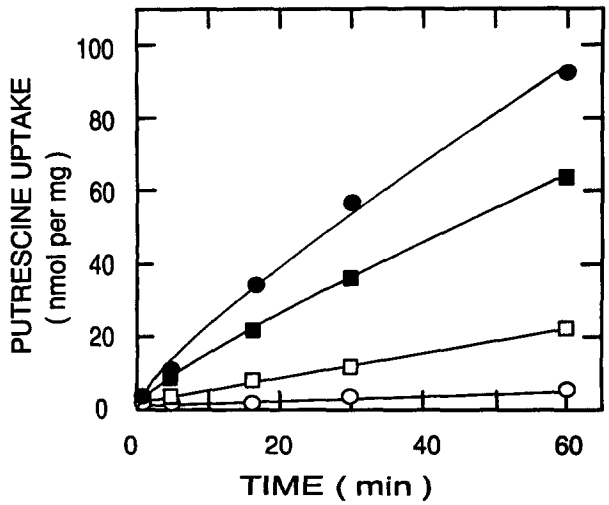



FIG. 3. Putrescine uptake from normal medium (circles) and medium lacking added $CaCl_2$ (squares). In this experiment the strains were grown in the normal or $Ca^{2+}$-deficient medium; virtually identical data came from cells grown in normal medium and merely tested under the two conditions. Pools of polyamines in the two strains were normal, and not affected by growth in the absence of $Ca^{2+}$. Wild type: open symbols; *puu-1*: closed symbols.

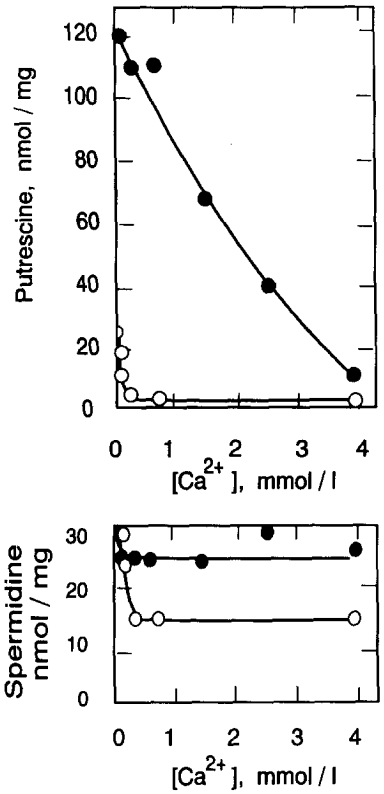



**FIG. 4.** Steady-state polyamine pools of mycelia grown in the presence of 1 mM putrescine and varying concentrations of $Ca^{2+}$. Wild type (open circles) and *puu-1* (closed circles) were harvested from cultures and putrescine (top) and spermidine (bottom) contents were determined.

$Ca^{2+}$ omission from the medium, suggest that the citrate-chelatable factor originally bound to wild-type cells was $Ca^{2+}$.

Using *puu-1* and wild type strains, we compared the effect of a brief rinse of cells in 1 mM EGTA with a 2-min preincubation in 10 mM $Na_3$ citrate plus 10 mM EGTA, each followed by a wash in Mops buffer (Fig. 6). The brief rinse left the two strains with different uptake rates, and with opposite responses to added citrate (Fig. 6A, 0 $Ca^{2+}$), as seen before. The effect of $Ca^{2+}$ bore out previous experience: $Ca^{2+}$ inhibited both strains similarly, but citrate mitigated this effect only in the mutant (Fig. 6A). The strong chelator wash, however, rendered the wild type phenotype almost identical to that of the mutant (Fig. 6B). At 0 $Ca^{2+}$, the putrescine uptake rates were the same; $Na_3$ citrate inhibited the strains similarly (via the $Na^+$ counterion); and now added $Ca^{2+}$ inhibited *neither* strain when citrate was present.

This experiment illustrates the membrane-bound nature of the chelatable element and introduces another phenomenon: the lability of the $Ca^{2+}$-binding site. First, briefly rinsed wild type cells retain their low rate of transport, suggesting that the inhibitory $Ca^{2+}$ is still bound to the cells. The further inhibition of transport by added $Ca^{2+}$ is owing to the competitive inhibition common to the two strains (Fig. 5). Briefly rinsed wild type cells, tested in the presence of 10 mM citrate and the absence of $Ca^{2+}$, release cell-bound $Ca^{2+}$, and have an increased rate of uptake (Fig. 6A). However, when more than 0.5 mM $Ca^{2+}$ is also present, it inhibits, owing to the relative ineffectiveness of citrate in protecting the high-affinity binding site from $Ca^{2+}$. The latter site must be missing from *puu-1,* inasmuch as $Ca^{2+}$ inhibits the mutant only weakly in the parallel test (Fig. 6A).

Turning to cells washed in strong chelator, freed of the wash medium and tested in Mops buffer, we found the wild type relieved of its endogenous inhibitor, and in the absence of $Ca^{2+}$ or citrate, its uptake rate is even higher than that of *puu-1.* Moreover, it can no longer be inhibited by $Ca^{2+}$ in the presence of citrate. The latter finding suggests that once $Ca^{2+}$ is definitively removed, its site of action is lost rapidly from wild type cells.

The calcium requirement for growth in stationary liquid culture was similar in the wild type and *puu-1,* and both strains achieved 18 to 24% of their maximal weight in $Ca^{2+}$-free medium containing 10 mM EGTA. Putrescine did not stimulate growth of $Ca^{2+}$-free cultures. The $Ca^{2+}$ content of *puu-1* mycelia grown in minimal medium was somewhat higher than in the wild type, but most of the excess could be removed with a brief rinse in 1 mM EGTA. About 10–13% of the intracellular $Ca^{2+}$ of both strains was found in vacuoles. The growth of both strains was inhibited similarly by Verapamil, a $Ca^{2+}$-blocker; trifluoperazine, which inhibits $Ca^{2+}$-binding proteins; and the $Ca^{2+}$ ionophore A23187, indicating no fundamental dif-

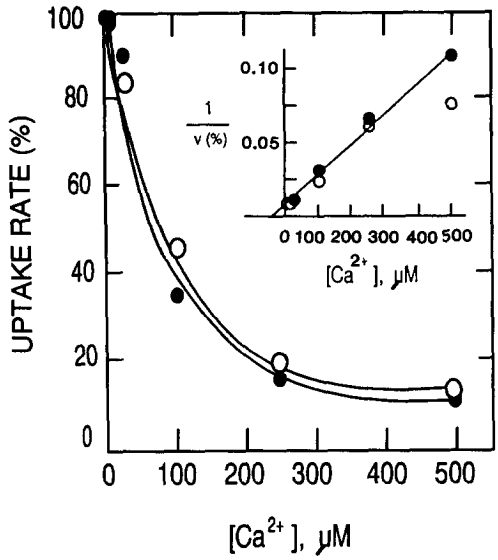



**FIG. 5.** Inhibition of putrescine uptake by $Ca^{2+}$ (as $CaCl_2$), measured after a brief rinse in Mops buffer (20 mM $Na^+$) plus 1 mM EGTA and tested in Mops buffer (20 mM $Na^+$). The absolute values for the saturable component of uptake of wild type (open circles) and *puu-1* strains (closed circles) at O $Ca^{2+}$ were 0.96 and 7.3 nmol putrescine per milligram per minute, respectively. Inset: reciprocal of uptake rate vs inhibitor concentration.

TABLE II

Effects of Medium Ingredients on Putrescine Transport by Wild Type and *puu-1* Mycelia[a]

| Wash medium | Addition to Mops uptake medium | Putrescine uptake (nmol/mg/15 min) Wild type | *puu-1* |
|---|---|---|---|
| | Experiment I | | |
| Mops + 1 mM $Na_2$ EGTA | None | 27 | 166 |
| | 0.7 mM $CaCl_2$ | 3[b] | 4[b] |
| | 8 mM $Na_3$ citrate | 78 | 68 |
| | 0.7 mM $CaCl_2$ + 8 mM $Na_3$ citrate | 13 | 75 |
| | Experiment II | | |
| Mops + 1 mM $Na_2$ EGTA | None | 36 | 137 |
| | 10 mM $Na_3$ citrate | 64 | 60 |
| Mops + 10 mM $Na_3$ citrate + 10 mM $Na_2$ EGTA | None | 105 | 88 |
| | 10 mM $Na_3$ citrate | 39 | 33 |

[a] Uptake of putrescine from 1 mM putrescine. Cells were prepared by washing them gently and briefly in Mops buffer (20 mM $Na^+$) containing 1 mM $Na_2$ EGTA, or by incubating for 2 min in the Mops buffer (20 mM $Na^+$ with 10 mM $Na_3$ citrate and 10 mM $Na_2$ EGTA). In all cases, cells were then washed again in Mops buffer (10 mM $Na^+$) and tested therein, plus the indicated additions. The figures for Experiment II are also reported in Figs. 6A and 6B (0 $Ca^{2+}$).

[b] These values represent the basal, diffusional component of uptake.

ference between the two strains in $Ca^{2+}$ transport or metabolism.

### *$Na^+$-$K^+$ Exchange Remains $Ca^{2+}$-Sensitive in the puu-1 Mutant*

The exchange flux of $K^+$, and the exchange of $K^+$, $Na^+$, and $H^+$ is severely inhibited by $Ca^{2+}$, and $Ca^{2+}$ therefore contributes to the ability of *N. crassa* to maintain a high membrane potential (15). Because a number of unusual amines (e.g., Tris, choline, imidazole, triethylamine) are reported to stimulate $K^+$ efflux in $Ca^{2+}$-free buffers (15), we considered that the $K^+$ carrier might actually transport all of them, as well as putrescine. If this were the case, putrescine should not only promote $K^+$ efflux, but we would expect $K^+$ efflux promoted by $Na^+$ in the *puu-1* mutant to be $Ca^{2+}$-insensitive. Initial experiments showed that indeed substantial $K^+$ efflux followed the addition of 1 mM putrescine, both in the *puu-1* mutant, and in the wild type in $Ca^{2+}$-free buffer. $K^+$ efflux from *puu-1* cells was then measured after addition of 10 mM NaCl, 1 mM putrescine, or 1 mM arginine in the presence and absence of 0.025 mM $Ca^{2+}$. No other ionic solutes were added, and because citrate was not present, the concentration of $Ca^{2+}$ was lower in order to minimize the competitive inhibition it exerts upon putrescine uptake (see Fig. 5). The results (Table III) show that $Ca^{2+}$ greatly reduces $K^+$ efflux in the presence of NaCl, while $K^+$ efflux provoked by putrescine is almost indifferent to $Ca^{2+}$. (The small inhibition, about 14%, may result in part from competitive inhibition of $Ca^{2+}$ on putrescine entry.)

### *Other Characteristics of the puu-1 Mutant*

The *puu-1* strain is more sensitive than the wild type to paraquat, a poison that enters via the polyamine transport system in some mammalian cells (16). The wild type was unaffected by 10 μM paraquat, while *puu-1* was inhibited 64%. $La^{3+}$ ($10^{-3}$ M) inhibited growth of the *puu-1* mutant about 40%, whereas the wild type was not affected. We do not know whether $La^{3+}$ is transported into cells, nor whether it competes with polyamines in the transport process.

The *puu-1* mutant displays only 20 to 50% of the normal activity of the general amino acid uptake system and a

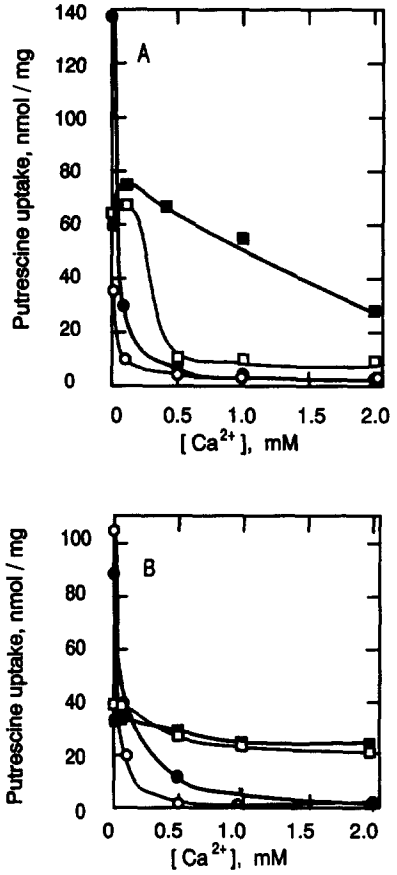



**FIG. 6.** Response of putrescine uptake by wild type and *puu-1* mycelia to citrate and to $CaCl_2$ after mild and strong chelator treatments. A, mycelia rinsed briefly in 1 mM EGTA before testing in Mops buffer (10 mM $Na^+$) with additives. B, mycelia incubated 2 min in 10 mM EGTA and 10 mM citrate, followed by thorough wash in Mops buffer before test of uptake in Mops (10 mM $Na^+$). Open symbols, wild type; closed symbols, *puu-1*. Circles, no citrate; squares, 10 mM $Na_3$ citrate during uptake test. The figures for 0 $Ca^{2+}$ in (A) and (B) are also reported in Table II, Experiment II.

TABLE III

Effects of $Ca^{2+}$ on $K^+$ Efflux from *puu-1* Mycelia following Addition of $NaCl_2$, Putrescine, and Arginine

| Addition[b] | $K^+$ efflux (%)[a] | |
|---|---|---|
| | No $Ca^{2+}$ | Plus 0.025 mM $Ca^{2+}$[c] |
| None | 5 | 13 |
| 10 mM NaCl | 50 | 18 |
| 1 mM putrescine 2HCl | 69 | 59 |
| 1 mM arginine HCl | 49 | 46 |

[a] The initial pool of $K^+$ was 317 nmol per milligram dry weight, nominally 127 mM.

[b] In all conditions, 0.2% glucose was present.

[c] Concentration chosen, in the absence of citrate, to minimize competitive inhibition of putrescine transport.

less reproducible deficit in amino acid uptake by the basic and neutral systems. Amino acids do not compete seriously with polyamine transport by wild type (1). None of the mutations of the three amino acid uptake systems (*pmn, pmg,* or *pmb*) affects putrescine uptake, and none of them is closely linked to *puu-1*. Stripping of $Ca^{2+}$ from wild type cells by washing them with 10 mM citrate + 10 mM EGTA leads to a severe deficit (82%) in general amino acid transport when compared with cells rinsed in 1 mM EGTA. The data suggest that *puu-1* is pleiotropic, and not specifically affected in the transport of polyamines. However, it should be stressed that the mutation (and $Ca^{2+}$-depletion of the wild type) affects polyamine and amino acid transport in opposite directions.

*The Effect of Cycloheximide on Putrescine Transport*

Cycloheximide, added to wild type cultures in amounts sufficient to stop protein synthesis, induced the ability to concentrate putrescine from the growth medium. By 60 min after cycloheximide addition, the cells resembled *puu-1* (Fig. 7). Tests of the concentration-dependence of polyamine transport in cycloheximide-treated cells in Vogel's medium showed that they had gained activity for the saturable system (data not shown). The same treatment of *puu-1* cells, already displaying the saturable transport system in the growth medium, left their polyamine transport rate (Fig. 7) and pools unaltered. The effect of cycloheximide was through its effect on protein synthesis: mutants (*cyh-1* and *cyh-2*) resistant to cycloheximide by virtue of ribosomal alterations were unaffected by the drug (data not shown). After 10 min incubation in cycloheximide, the general amino acid uptake system of the wild type had declined to 54% of normal; the *puu-1* mutant was not tested.

## DISCUSSION

The *puu-1* mutation of *N. crassa* renders polyamine uptake insensitive to cell-bound $Ca^{2+}$ and thus imparts to cells the ability to concentrate polyamines from the $Ca^{2+}$-containing growth medium. The wild type strain, by contrast, can only equilibrate polyamines across the cell membrane by a nonsaturable process. The mutant differs in having a higher rate of polyamine uptake, not a higher affinity for substrate. Saturable putrescine uptake is accompanied by efflux of $K^+$, and both fluxes respond to the effects of cell-bound $Ca^{2+}$ (lower) and to the *puu-1* mutation (higher). The *puu-1* mutant is partially deficient in the activity of the general amino acid uptake system and perhaps of the neutral and basic systems as well. On minimal medium, however, $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ contents of *puu-1* cells are normal, as are the endogenously synthesized arginine and polyamine pools (3).

In some respects, the *puu-1* mutation resembles a mutation, *spsA1,* of *Aspergillus nidulans,* roughly characterized by Spathas *et al.* (17). The *spsA1* mutation makes the fungus sensitive to higher concentrations of spermidine, the carrier for which appears to be distinct from the putrescine carrier (18). Putrescine, in fact, did not inhibit the *spsA1* mutant.

$Ca^{2+}$ has two inhibitory actions on polyamine uptake by the wild type. The first is competitive inhibition of uptake (1). The *puu-1* mutant is as sensitive as the wild type to this action of $Ca^{2+}$. The second inhibitory action of $Ca^{2+}$ in the wild type was revealed by stripping the metal from cells by a strong chelator wash. After such a wash, the $V_{max}$ of the wild type polyamine uptake system increases to that of the mutant. The mutant shows no evidence of sensitivity to $Ca^{2+}$ at such a site. Study of

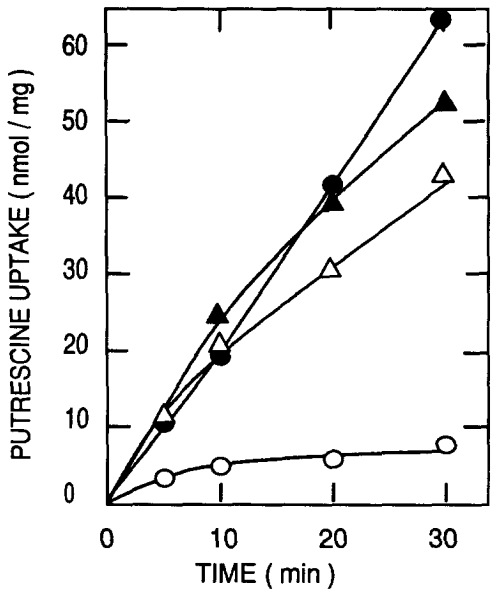



FIG. 7. Response of putrescine uptake to cycloheximide. Cells were grown in normal medium, and uptake was tested in the medium after adding 2 mM putrescine. Circles, untreated; triangles, incubated 60 min after addition of 10 μg cycloheximide per milliliter to the cultures. Open symbols, wild type; closed symbols, *puu-1 aga.*

cellular $Ca^{2+}$ content and localization, of the effect of $Ca^{2+}$-related antimetabolites, and of the growth requirement for $Ca^{2+}$ reveal no fundamental derangement of $Ca^{2+}$ metabolism in the mutant. We therefore infer the existence of a cell-surface protein that, when bound to $Ca^{2+}$, normally blocks polyamine uptake. This protein or function is labile, because sensitivity of polyamine uptake to $Ca^{2+}$ is lost upon definitive removal of $Ca^{2+}$, and upon incubating cells with cycloheximide. The observation that cycloheximide treatment and the removal of $Ca^{2+}$ do not affect polyamine uptake by the *puu-1* mutant shows that these treatments target the same system affected by the *puu-1* mutation.

Unlike polyamine transport in *N. crassa*, removal of membrane-bound $Ca^{2+}$ *impairs* transport processes in many other organisms. In fact, it impairs amino acid transport even in *N. crassa*. Calcium chelators partially inactivate the transferrin receptor of rabbit reticulocytes (19) and the uptake of $\gamma$-aminobutryate in synaptosomes (20). $Ca^{2+}$ depletion slows *net* uptake of $K^+$ in corn root (21, 22); it diminishes lysine, arginine, sulfate, glucose, malate, glycerate-3-P, and uracil transport in tobacco cells (23, 24). Smith (25) used many of the same treatments we have used in his study of $Ca^{2+}$-stimulated serine uptake in tobacco cells. He proposed that $Ca^{2+}$ allows cells to maintain a high membrane potential, upon which serine transport and retention depends. Cells lose transport activity in the presence of $K^+$ (which depolarizes cells), but do so much more slowly if $CaCl_2$ is also present (25). Jones and Jennings (26) made the same general finding with respect to the growth of the fungus *Dendryphiella salina*. The point was made there that $Ca^{2+}$ reversed $Na^+$ inhibition of growth by preventing loss of internal $K^+$.

Why, then, is polyamine transport in *N. crassa* uniquely stimulated, rather than inhibited, by removal of $Ca^{2+}$ (and, by analogy, by the *puu-1* mutation)? Three component issues allow us to explore this matter: (i) What drives polyamine uptake? (ii) What is the polyamine carrier? (iii) What is the nature of the *puu-1*$^+$ product?

Inorganic ion transport in *N. crassa* has been studied in great detail in the laboratories of C. L. Slayman and C. W. Slayman. The membrane potential, established through the activity of the plasma-membrane $H^+$-ATPase (27), is the energy source for most secondary transport (28). The membrane potential drives, via $K^+$–$H^+$ symport (29), the establishment of a substantial $K^+$ gradient (30). (This requires extrusion of $2H^+$, mimicking a 1:1 $K^+$–$H^+$ exchange.) If $K^+$-replete cells are held in water, they retain $K^+$, but upon addition of $Na^+$, they readily exchange internal $K^+$ for the external cation (14). This has led the Slaymans (15) to analyze a fluent $K^+/Na^+/H^+$ exchange flux characteristic of *N. crassa*, depending upon the ions present in the cell and in the external medium. Significantly, $Ca^{2+}$ inhibits the $K^+/K^+$ exchange flux, but not the net $K^+$ accumulation, about 80%, with a concomitant increase of membrane potential [(31); summarized in Ref. (29)]. In other words, $Ca^{2+}$ makes the membrane less leaky, and energy otherwise dissipated is spared for improved transport of other solutes, as suggested above.

A substantial net efflux of $K^+$ occurs in our standard $Na^+$-Mops buffer, which contains no added $Ca^{2+}$. $Na^+$ entry presumably balances $K^+$ efflux to a large extent under this condition, as the Slayman laboratories have found (15). Addition of putrescine causes an even greater rate of $K^+$ efflux in the *puu-1* strain, concomitant with demonstrable entry of the amine. This behavior resembles previous observations that without $Ca^{2+}$, addition of imidazole, histidine, Tris, choline, ethanolamine, triethylamine, and $NH_4^+$ all caused loss of $K^+$ and $Na^+$ (15). Whether these compounds were taken up at the same time was not directly tested, but the membrane potential was not seriously affected (C. L. Slayman, personal communication).

If the effects of $Ca^{2+}$ on putrescine uptake are opposite to those on amino acids and, in other organisms, most other solutes, we must consider the possibility that putrescine can become part of the exchange flux involving $K^+$ and $Na^+$. In this sense putrescine transport as we have measured it here would not be immediately dependent upon membrane potential, but more directly dependent upon the transmembrane gradient of $K^+$. Energy is ultimately required to establish this gradient, but we have speculated previously that a residual $K^+$ gradient may account for energy-independent putrescine uptake (1).

We do not know the identity of the putrescine carrier. We feel that to *N. crassa*, putrescine is a peculiar substrate for transport into the cell. The fungus does not require external polyamines for growth, nor does it use them as C and N sources (1). The ability of *N. crassa* to take up polyamines is not an obvious advantage, and we have implied that the "polyamine transport system" may have evolved in relation to other needs entirely (1). Thus polyamine transport may be a gratuitous response to unusual circumstances—the absence of $Ca^{2+}$ and the presence of polyamine.

It is unlikely that the "polyamine transport system" is one of the $K^+$ carriers, of which there are high- and low-affinity forms in *N. crassa* and in yeast (32, 33). The data are too preliminary to exclude this possibility, but the *puu-1* mutant does retain $Ca^{2+}$ sensitivity of $K^+$ efflux upon $Na^+$ addition (as opposed to addition of putrescine). The fact that $K^+$, $Na^+$, and $NH_4^+$ all competitively inhibit putrescine transport; that both $K^+$ and putrescine influx are inhibited by $Ca^{2+}$, and that many amines, including putrescine, stimulate $K^+$ efflux may indicate only an indirect coupling of the various transport systems, and independent modulation of these systems by $Ca^{2+}$.

Cell-bound $Ca^{2+}$ is a prime influence on the rate of putrescine entry. This leads to consideration of the last question: what is the *puu-1* product? Impairment of the *puu-1*$^+$ product (by mutation, turnover, or removal of $Ca^{2+}$) increases putrescine uptake activity. Absence or

impairment of the *puu-1*$^+$ product renders the organism vulnerable to polyamine intoxication (3). Our results suggest that the *puu-1*$^+$ product is a $Ca^{2+}$-binding protein that imparts a beneficial impermeability of the plasma membrane to toxic materials (of which putrescine and $La^{3+}$ are two). The *puu-1* product also plays a significant role in the activity of amino acid and perhaps other transport systems. The protein could work by any of a number of means, from improving specificity of an unknown cation carrier by direct contact to changing the character of the bilayer in which this and other carriers are embedded. The pleiotropy of the *puu-1* mutation suggests a large scope of involvement with cation transport. Defining the character of the *puu-1* gene product will help us to discover its action, and perhaps the identity of the putrescine carrier and other carriers affected by the *puu-1* mutation.

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