

Uptake and accumulation of putrescine and its lethality in *Anacystis nidulans*

(polyamines/cyanobacteria/ion trapping)

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ABSTRACT The rate of uptake of putrescine by *Anacystis nidulans* has been shown to depend on the external pH and the extracellular concentration of putrescine. Accumulation of exogenous putrescine was also proportional to the concentration of putrescine in the medium, suggesting that putrescine uptake was not subject to cellular regulation. An equation was derived to test the hypothesis that putrescine accumulation was due to ion trapping. Comparison of the predicted and observed intracellular concentrations of putrescine under various conditions showed a close correlation in support of the hypothesis of ion trapping. Under conditions leading to cell death (e.g., 150 μ M putrescine, pH 9.8), the correlation did not hold as a result of leakage of accumulated putrescine.

It has been observed that exogenous putrescine is inhibitory to the photoautotrophic growth of *Anacystis nidulans* (1). The fact that exogenous putrescine was toxic at the concentration (0.15 mM) at which this normal metabolite is present within the cyanobacterium during exponential growth has led to additional studies of the mechanism of this effect. Uptake of putrescine and the other polyamines appears to be mediated by active transport in *Escherichia coli* (2) and in animal cells (3, 4), but nothing was known about polyamine transport in the cyanobacteria. *A. nidulans* is usually grown in a minimal medium that has a low buffering capacity, resulting in a large increase in the external pH during exponential growth (1). The cytoplasmic pH, however, remains relatively constant as the external pH is altered (5) and can be either acidic or basic relative to the external pH. The resultant pH gradient between the cytoplasm and the medium can cause the concentration of weak acids and bases (refs. 5 and 6; J. Gibson, unpublished results) which are permeable in the uncharged form but are impermeable when charged. This mechanism of accumulation (ion trapping or diffusion trapping) also accounts for the concentration of NH_3 in green algae (7) and bicarbonate in chloroplasts (8). An analysis of the kinetics of putrescine uptake and of the equilibrium concentration of accumulated putrescine within the cell as a function of the pH of the medium and external concentration of putrescine is consistent with the theory that the concentration of putrescine is due to diffusion of the neutral molecule and trapping of the charged ion.

MATERIALS AND METHODS

Growth and Viability of *A. nidulans*. *A. nidulans* 625 was obtained from the Indiana University Culture Collection (Bloomington, IN). Strain 625 was grown at 30°C with rotary shaking in Allen's medium containing 1% NaHCO_3 with illumination from the top with white light at 7–8 W/cm², as de-

scribed (1). Cell number was determined in a Petroff-Hauser microscopic counting chamber. Viability was measured by the single cell plating technique (9).

Uptake of Putrescine. Exponential phase cultures were harvested by centrifugation at 10,000 $\times g$ for 5 min, washed and resuspended in fresh growth medium containing 30 mM NaHCO_3 , and adjusted to the indicated pH values with NaOH. To equilibrate the cells to light and temperature conditions, we preincubated the cultures for 15 min in a 30°C water bath illuminated from above with two 20-W fluorescent light bulbs at a distance of 32 cm. Uptake was initiated by the addition of [¹⁴C]putrescine at the indicated concentrations with specific activities that ranged from 0.40 to 89.9 Ci/mol (1 Ci = 3.7 $\times 10^{10}$ becquerels). The initial rate of uptake was measured in a 5-ml culture of 8 $\times 10^7$ cells per ml by removing 0.25-ml samples at 1.0-min intervals for 5 min. The accumulation of putrescine was determined at lower cell concentrations (5 $\times 10^7$ cells per ml) to avoid exhaustion of exogenous putrescine. In both types of experiments, samples were filtered onto Millipore HA filters (0.45 μ m) to trap cells and washed three times with 5 ml of cold water. Filters were dried and radioactivity was measured via liquid scintillation in a Packard TriCarb 3390. In the experiments described in Tables 2 and 3 and in Fig. 3, the accumulation of putrescine was monitored by the filtering technique described above. When the accumulation of putrescine reached equilibrium, the internal concentration of putrescine was determined by the centrifugation technique described below for methylamine, with ³H₂O as a marker for the pellet volume (10) and [¹⁴C]sucrose as a marker for the extracellular space (8).

CO₂ Fixation. Exponential phase cultures were harvested, washed twice in growth medium lacking Na_2CO_3 , and resuspended in growth medium lacking Na_2CO_3 (pH 9.5) at a density of 8 $\times 10^7$ cells per ml. After 15 min of preincubation in an illuminated 30°C waterbath, $\text{NaH}^{14}\text{CO}_3$ was added to a final specific activity of 0.03 Ci/mol (11). At various times, 0.5-ml aliquots were removed, washed onto Millipore HA filters (0.45 μ m) to trap cells, and washed three times with 5 ml of ice-cold distilled H₂O. The filters were dried and radioactivity was determined in Liquifluor.

For studies on the energy requirement of putrescine uptake, carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) or dichlorophenyl dimethyl urea (DCMU) were added at the indicated concentrations 5 min prior to initiation of uptake. The initial rate of putrescine uptake and rate of CO₂ fixation were determined as described above.

Determination of Intracellular pH. Internal pH was cal-

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenyl hydrazone; DCMU, dichlorophenyl dimethyl urea.

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culated from the distribution of methylamine (10). Cells were concentrated to 5×10^8 cells per ml in 0.25 ml of growth medium and adjusted to the appropriate pH. The cells were then equilibrated at 30°C for 5 min in the presence of $^3\text{H}_2\text{O}$ (25 $\mu\text{Ci/ml}$). [^{14}C]Methylamine (1 $\mu\text{Ci/ml}$) was added and the mixture was incubated for 1 min. The suspensions were immediately transferred in small microfuge tubes (0.4 ml) in triplicate and centrifuged for 1 min in the light in a Beckman microfuge (model B) equipped with a transparent lid. Samples of 50 μl were taken from the supernatant and mixed with 0.5 ml of 5% perchloric acid. A slice from the middle of the pellet was taken (avoiding the pellet/supernatant interface) and mixed with 0.5 ml of perchloric acid. The samples were kept at 4°C for 12 hr to allow complete extraction of radioactivity and centrifuged (8000 $\times g$, 5 min, 4°C). Aliquots of 0.2 ml of each supernatant were mixed with 10 ml of 3a70 scintillation fluid and both ^{14}C and ^3H content was determined. [^{14}C]Sucrose served as a marker for the extracellular space (8).

Polyamine Assay. The procedures for dansylation of cell extracts and fluorimetric estimation of the dansyl polyamines after separation by thin-layer chromatography have been described (1).

Radioisotopes and Chemicals. [1,4- ^{14}C]Putrescine (89.9 Ci/mol), sodium [^{14}C]bicarbonate (9.3 Ci/mol), [^{14}C]methylamine (56.1 Ci/mol), [^{14}C]sucrose (6730 Ci/mol), $^3\text{H}_2\text{O}$ (18 Ci/mol), and Liquifluor were purchased from New England Nuclear. 3a70 scintillation fluid was purchased from Research Products International (Elk Grove Village, IL). Putrescine dihydrochloride was obtained from Calbiochem. CCCP was purchased from Sigma and DCMU was kindly donated by H. Lyman.

RESULTS AND DISCUSSION

Uptake of Putrescine. The initial rate of uptake was strongly dependent on the pH of the medium and the external concentration of putrescine. When [^{14}C]putrescine was added to cultures of *A. nidulans* at an external concentration of 15 μM , the rate of uptake increased as an exponential function of the increase in external pH in the range of 8.5–10.5 (Fig. 1). At pH 9.5, the rate of uptake increased linearly as a function of external putrescine in the range of 1–200 μM (Fig. 2).

The results given above suggest that uncharged putrescine diffuses freely across the cell membrane. The pK values of putrescine at 30°C are 9.0 and 10.5 (12); the pH dependence of putrescine uptake indicates that the concentration of the neutral molecule may have been rate limiting. The relation of uptake to putrescine concentration also supports the theory of diffusion. If putrescine uptake was facilitated, rate saturation would be expected to occur at high external concentrations of putrescine, but this was not observed. It is conceivable that saturation would occur at concentrations greater than 200 μM . However, it was not possible to extend the concentration range due to rapid cell death and leakage of putrescine caused by external concentrations of putrescine greater than 200 μM at pH 9.5. The initial rate of uptake of 1 μM putrescine was not affected by the addition of a 20-fold excess of structural analogs of putrescine, diaminopropane and diaminopentane (data not shown). This result could be interpreted either as facilitated transport, which is highly specific for putrescine, or simple diffusion of putrescine. The fact that the analogs themselves are rapidly taken up by these cells makes the notion of simple diffusion most probable.

Accumulation of Putrescine. The concentration of putrescine per ml of culture (5×10^7 cells per ml) that was accumulated by the cells with time is shown in Fig. 3. When putrescine was added at external concentrations of 50 μM or less, the cells

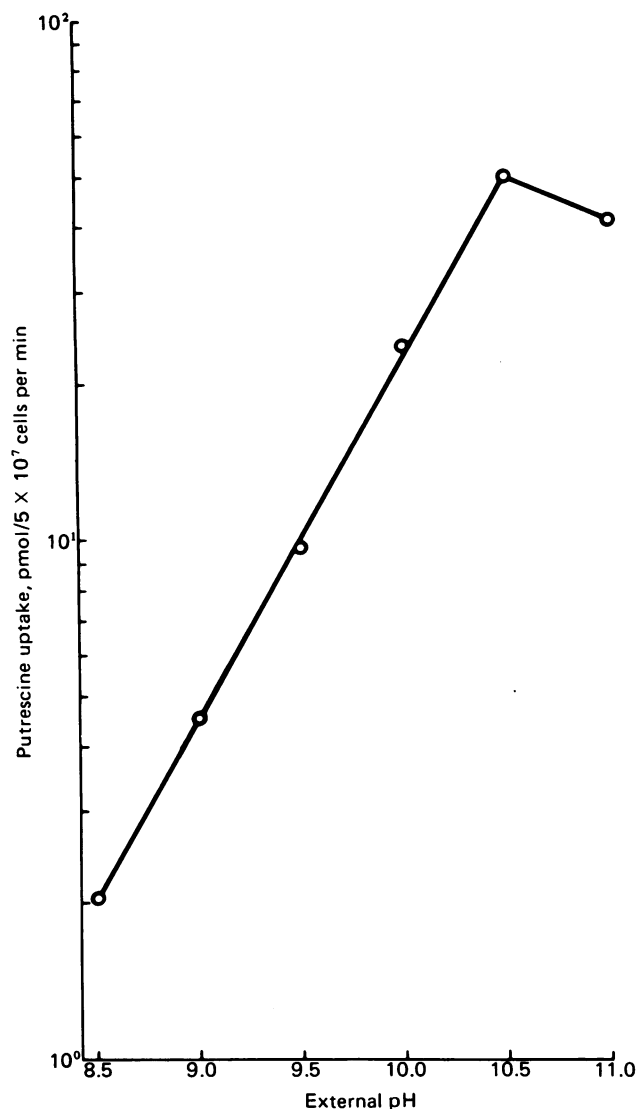


FIG. 1. Initial rate of putrescine uptake as a function of external pH. Exponential phase cells were washed and resuspended in growth medium in separate flasks which were adjusted to the indicated pH value. The initial rate of uptake was determined.

accumulated putrescine for 90 min. After that period, the amount of intracellular [^{14}C]putrescine per ml remained essentially constant. This was defined as the intracellular equilibrium concentration. The equilibrium level of putrescine within the cells increased proportionately with the concentration of putrescine added to the medium from 0.5 to 50 μM . When putrescine was added at 150 μM , however, the cells accumulated putrescine for a shorter period of time and did not reach an equilibrium state. This putrescine that had been accumulated initially was slowly lost from the cells.

Analysis of the intracellular [^{14}C]putrescine indicated that it had not been metabolized. Putrescine was added to cultures of *A. nidulans* at 1, 40, and 150 μM . At all concentrations, the intracellular putrescine that could be quantitated by the dansyl procedure was 98–100% of the radioactivity within the cell for at least 3 hr after the addition of putrescine.

The dependence of putrescine accumulation on the external concentration of putrescine suggests that putrescine uptake was not regulated by the cells. The above data, coupled with the knowledge that the internal pH of *A. nidulans* is acidic relative to the external pH (5), raised the possibility that the accumu-

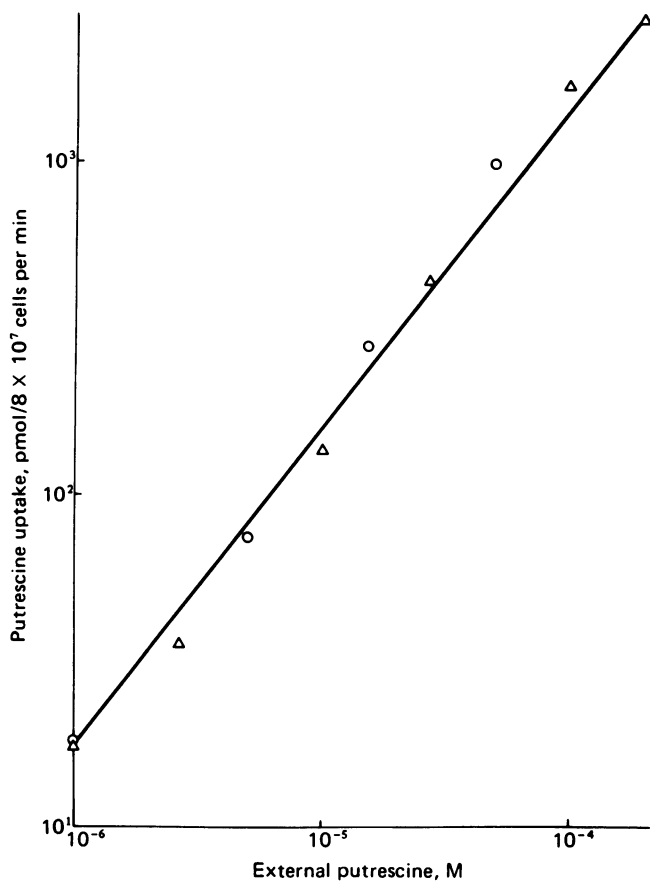


FIG. 2. Initial rate of uptake of putrescine at various external concentrations. Exponential phase cells of *A. nidulans* were washed and resuspended in growth medium (pH 9.5) in individual flasks at a cell density of 8×10^7 cells per ml. At zero time [^{14}C]putrescine was added at the indicated concentrations and time points were taken for 5 min at 1-min intervals. Triangles and circles represent different experiments.

lation of putrescine was due to ion trapping (diffusion trapping). According to this theory, the unprotonated molecules of putrescine would diffuse across the cell membrane and be protonated immediately inside the cell at the lower pH. The protonated molecules would then be trapped inside the cells because the cell membrane is essentially impermeable to charged molecules. The diffusion of the free base would continue until an equilibrium is reached, achieving an intracellular concentration of putrescine proportional to the external concentration of putrescine and the pH gradient across the cell membrane.

Effect of Inhibitors of Photosynthesis on Putrescine Uptake. If uptake and accumulation of putrescine are dependent upon the pH gradient, then disruption of the pH gradient by darkness or chemical inhibition of photosynthesis would be expected to result in a reduction of putrescine uptake. As shown in Table 1, factors that decrease photosynthesis also affected uptake of putrescine. The inhibition of photosynthesis was monitored by the ability of the cells to fix CO_2 . The presence of $10 \mu\text{M}$ putrescine had no effect on the rate of CO_2 fixation. In cells preincubated in the dark, CO_2 fixation was completely inhibited, as was putrescine uptake, although to a lesser extent. DCMU, an inhibitor of photosynthetic electron transport, and CCCP, an inhibitor of the photoproduction of ATP, reduced the rate of uptake of putrescine. These results indicate that disruption of energy production in *A. nidulans* results in decreased putrescine uptake.

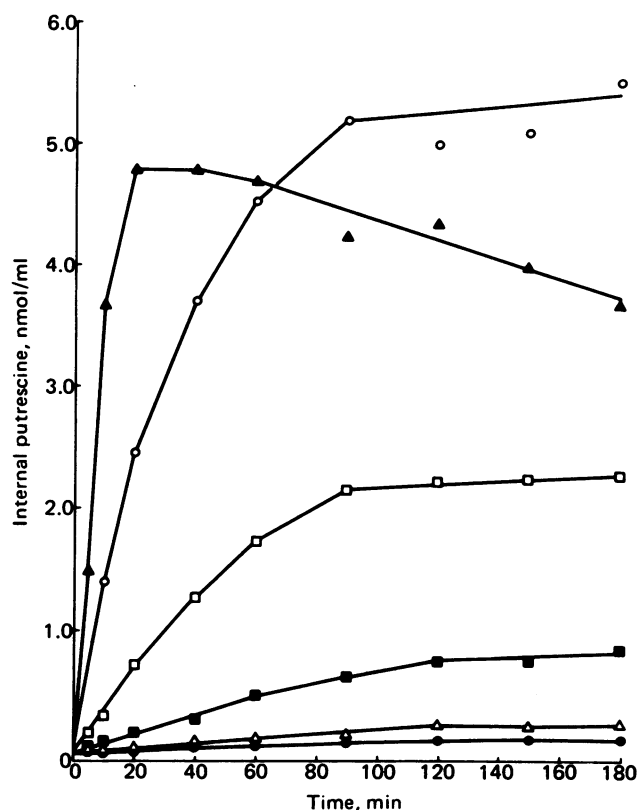


FIG. 3. Accumulation of putrescine in *A. nidulans*. Exponential phase cells were washed and resuspended in growth medium (pH 9.5). At zero time, [^{14}C]putrescine was added at the concentrations indicated: ●, $0.5 \mu\text{M}$; △, $1.0 \mu\text{M}$; ■, $5.0 \mu\text{M}$; □, $15.0 \mu\text{M}$; ○, $50 \mu\text{M}$; △, $150 \mu\text{M}$. At the indicated times, aliquots were removed and filtered.

Ion Trapping of Putrescine. To test the hypothesis of ion trapping, we derived an equation that could be used to predict the intracellular concentration of putrescine. The relative concentrations of the free base [B], the monoprotonated amine [BH^+], and the diprotonated amine [BH_2^{2+}] can be calculated from the pK values and the pH of the medium:

$$\text{pH} = \text{pK}_1 + \log \frac{[\text{BH}^+]}{[\text{BH}_2^{2+}]} \quad [1]$$

$$\text{pH} = \text{pK}_2 + \log \frac{[\text{B}]}{[\text{BH}^+]} \quad [2]$$

Table 1. Effect of inhibitors on CO_2 fixation and putrescine uptake

	Inhibition of CO_2 fixation, %	Inhibition of putrescine uptake, %
Control minus putrescine	0	—
Control plus putrescine	0	0
Absence of light	100	81
DCMU, 10^{-6} M	68	15
DCMU, 5×10^{-5} M	70	37
CCCP, 5×10^{-5} M	39	29
CCCP, 10^{-4} M	58	29

Exponential phase cells were washed and resuspended in growth medium (pH 9.5) and allowed to adjust to light conditions for 15 min. DCMU and CCCP were added 5 min prior to addition of $\text{NaH}^{14}\text{CO}_3$ and $10 \mu\text{M}$ [^3M]putrescine. Initial rate of uptake of putrescine and rate of CO_2 fixation were determined by sampling each flask for 5 min at 1-min intervals. The rate of CO_2 fixation in the control culture was $1.6 \text{ nmol/min per } 8 \times 10^7$ cells.

Table 2. Comparison of predicted and observed intracellular concentrations of putrescine at various external pH

pH _o	pH _i *	Intracellular putrescine, mM	
		Predicted†	Observed‡
9.00	7.49	0.51	0.53
9.25	7.51	0.08	1.04
9.50	7.58	1.60	1.56
9.75	7.62	2.48	2.58
10.00	7.65	3.69	3.74

Exponential phase cells of *A. nidulans* were washed, resuspended in growth medium at 5×10^7 cells per ml, and adjusted to the indicated pH; $1 \mu\text{M}$ [^{14}C]putrescine (89.9 Ci/mol) was added. The cultures were incubated in the light at 30°C until putrescine accumulation had reached equilibrium.

* Determined from the distribution of [^{14}C]methylamine between the medium and the sucrose-impermeable $^3\text{H}_2\text{O}$ space. $\text{pH}_i = \text{pH}_o + \log [\text{CH}_3\text{NH}_3^+]_o / [\text{CH}_3\text{NH}_3^+]_i$. The measurement was performed 150 min after addition of putrescine.

† $[\text{PUT}]_i = [\text{PUT}]_o [(1 - 10^{\text{pK}_1 - \text{pH}_o})(10^{\text{pK}_2 - \text{pH}_i}) + 1] / [(1 + 10^{\text{pK}_1 - \text{pH}_o})(10^{\text{pK}_2 - \text{pH}_o}) + 1]$.

‡ Calculated from the concentration of [^{14}C]putrescine in the sucrose-impermeable $^3\text{H}_2\text{O}$ space.

The total amine [A^{tot}] concentration is the sum of the three species, which can be expressed in terms of [B]:

$$[10^{\text{pK}_1 - \text{pH}}][10^{\text{pK}_2 - \text{pH}}][\text{B}] + [10^{\text{pK}_2 - \text{pH}}][\text{B}] + [\text{B}] = [\text{A}^{\text{tot}}]$$

or

$$[\text{B}] = \frac{[\text{A}^{\text{tot}}]}{[(1 + 10^{\text{pK}_1 - \text{pH}})(10^{\text{pK}_2 - \text{pH}}) + 1]}$$

According to the theory of ion trapping, the concentration of B inside the cell, $[\text{B}]_i$, is equal to the concentration of B outside the cells $[\text{B}]_o$. The total amine concentration inside the cells $[\text{A}^{\text{tot}}]_i$ can be defined as

$$[\text{A}^{\text{tot}}]_i = \frac{[\text{A}^{\text{tot}}]_o [(1 + 10^{\text{pK}_1 - \text{pH}_i})(10^{\text{pK}_2 - \text{pH}_i})]}{[(1 + 10^{\text{pK}_1 - \text{pH}_o})(10^{\text{pK}_2 - \text{pH}_o}) + 1]}$$

The theory of ion trapping was tested by comparing the predicted and observed intracellular concentrations of putrescine at equilibrium at various external pH values. Table 2 shows the effect of varying the external pH on the accumulation of putrescine. As the external pH was increased from 9.0 to 10.0, the concentration of the free base and the initial rate of uptake increased (Fig. 1). The internal concentration of putrescine at equilibrium also increased proportionately. The internal pH, calculated from the distribution of methylamine, was used to predict the equilibrium concentration of putrescine. As shown in Table 2, there was a close correlation between the predicted and observed values.

In an additional test, the pH was held constant and the external concentration of putrescine was varied. Table 3 shows the effect of the addition of various concentrations of putrescine at pH 9.5. The internal pH at zero time was 7.59. Low concentrations of putrescine (0.5–50 μM) had little effect on the intracellular pH and showed close correlation between the predicted and observed intracellular concentrations of putrescine. At external concentrations of 100 μM and greater, the observed internal concentration was less than that calculated. The cells did not reach a true equilibrium state (see Fig. 3). Putrescine was accumulated for 10–20 min, but the diamine then leaked out slowly. Under these conditions, the intracellular pH tended to increase. Note that the predicted intracellular concentrations of putrescine were based on the pH actually found; the discrepancy between the predicted and observed concentrations would be even greater if the calculations were based on a pH_i of 7.59.

Table 3. Comparison of predicted and observed intracellular concentrations of putrescine

[PUT] _o , μM	pH _i *	Intracellular putrescine, mM	
		Predicted†	Observed‡
0.5	7.59	0.76	0.77
1.0	7.59	1.53	1.56
2.0	7.59	3.20	3.53
5.0	7.59	7.55	7.86
10.0	7.60	15.8	16.9
20.0	7.60	31.5	33.1
50.0	7.62	71.9	67.5
100.0	7.65	157.6	86.9
150.0	7.98	236.4	74.5
200.0	7.70	315.2	14.4

Cells were treated as described in the legend of Table 2 except that all flasks were incubated at pH 9.5 and [^{14}C]putrescine was added at the concentrations indicated with specific activities ranging from 4.5 to 89.9 Ci/mol.

* Determined from methylamine distribution as in Table 2. Measurement performed 180 min after addition of putrescine. pH_i at zero time = 7.59.

† See Table 2.

‡ See Table 2.

The above data are consistent with the hypothesis that putrescine accumulation occurs by ion trapping at low putrescine concentrations. The lack of correlation at high external concentrations of putrescine can be explained by the toxicity of putrescine to *A. nidulans*.

Accumulation of Putrescine and Viability. Accumulation of putrescine to high internal concentrations results in a marked

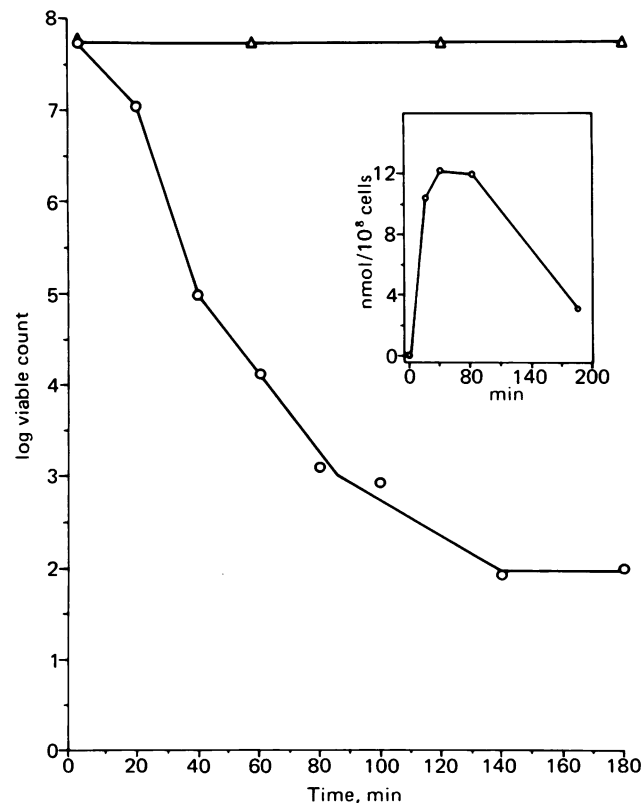


FIG. 4. Uptake of putrescine and viability of *A. nidulans*. Exponential phase cells were incubated in the presence (O) or absence (Δ) of 150 μM putrescine. Viability was determined. (Inset) Accumulation of intracellular putrescine.

and rapid loss of cell viability (Fig. 4). Exponential phase cells were incubated in the presence of 150 μ M putrescine (pH 9.8). The viability of the culture declined rapidly as putrescine was accumulated inside the cells (Fig. 4). The diamine was accumulated for 40 min and then lost from the cells. The viability declined for 100 min, leaving approximately 100 viable cells per ml. The survivors did not appear to be putrescine resistant. Attempts to obtain resistant mutants by standard replica plating techniques have been unsuccessful.

The fact that low levels of exogenous putrescine inhibit growth in *A. nidulans* can now be understood as a function of the considerable accumulation of putrescine within the cell. There can be an increase in the ratio of intracellular to extracellular putrescine of 500- to 3000-fold as a function of the pH generated during photoautotrophic growth. This increase appears to be due to the free diffusion of unchanged putrescine into the cell and its retention as the cationic form at the significantly lower intracellular pH. Rapidly generated high concentrations of intracellular putrescine lead to cell death. Data on the mechanism of putrescine toxicity will be presented in a subsequent communication.

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1. Ramakrishna, S., Guarino, L. & Cohen, S. S. (1978) *J. Bacteriol.* **134**, 744-750.
2. Tabor, C. W. & Tabor, H. (1966) *J. Biol. Chem.* **241**, 3714-3723.
3. Lajtha, A. & Shershen, H. (1974) *Arch. Biochem. Biophys.* **165**, 539-547.
4. Kano, K. & Oka, T. (1976) *J. Biol. Chem.* **251**, 2795-2800.
5. Falkner, G., Horner, F., Werdan, K. & Heldt, H. W. (1976) *Plant Physiol.* **58**, 717-718.
6. Padan, E. & Schuldiner, S. (1978) *J. Biol. Chem.* **253**, 3281-3286.
7. Shilo, M. & Shilo, M. (1962) *J. Gen. Microbiol.* **29**, 645-658.
8. Heldt, H. W. & Sauer, F. (1971) *Biochim. Biophys. Acta* **234**, 83-91.
9. Allen, M. M. (1968) *J. Phycol.* **4**, 1-4.
10. Padan, E., Zilberstein, D. & Rottenberg, H. (1976) *Eur. J. Biochem.* **63**, 533-541.
11. Ginzberg, D., Padan, E. & Shilo, M. (1968) *J. Virol.* **2**, 695-701.
12. Burtsch, C. R., Fernelius, W. C. & Block, B. P. (1958) *J. Am. Chem. Soc.* **62**, 444-450.