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# Effect of Weak Acids on Amino Acid Transport by *Penicillium chrysogenum*: Evidence for a Proton or Charge Gradient as the Driving Force

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A variety of weak acids at and below their $pK_a$ are potent inhibitors of transport in *Penicillium chrysogenum*. The effective compounds include sorbate, benzoate, and propionate (common antifungal agents), indoleacetate (a plant hormone), acetylsalicylate (aspirin), hexachlorophene, and a yellow pigment produced by the mycelia under nutrient-deficient conditions, as well as the classical uncouplers 2,4-dinitrophenol, *p*-nitrophenol, and azide. The results suggest that a proton gradient or charge gradient is involved in energizing membrane transport in *P. chrysogenum*. The unionized form of the weak acids could discharge the gradient by diffusing through the membrane and ionizing when they reach an interior compartment of higher pH. Experiments with 2,4-dinitrophenol and *p*-nitrophenol established that the ionized species are not absorbed by the mycelium to any great extent. The transport inhibitors also caused a decrease in cellular adenosine 5'-triphosphate (ATP) levels, but there was no constant correlation between inhibition of transport and suppression of cellular ATP. A decrease in aeration of the mycelial suspension had the same effect on transport and ATP levels as the addition of a weak organic acid. The effects on transport rates and ATP levels were reversible. The instantaneous inhibition of [$^{14}$C]L-leucine transport by $NH_4^+$ (and vice-versa) in nitrogen-starved mycelia at pH values of 7 or below can be explained by competition for a common energy-coupling system. The inhibition is not observed in carbon-starved mycelia in which the $NH_4^+$ transport system is absent or inactive (but the general amino acid transport is fully active), or in iodoacetate-treated mycelia in which the $NH_4^+$ transport system has been differentially inactivated. At pH values greater than 7.0, $NH_3$ and $HPO_4^{2-}$ inhibit transport, presumably by discharging the membrane proton or charge gradient. Aniline counteracts the inhibitory effect of $NH_3$ and $HPO_4^{2-}$, possibly by acting as a proton reservoir or buffer within the membrane.

During the past several years we have characterized a number of membrane transport systems of the filamentous fungus *Penicillium chrysogenum* (Table 8 of reference 14). Most of these systems develop in response to a specific nutrient limitation, are pH, temperature, and energy dependent, are stereo specific, display saturation kinetics, and are subject to feedback inhibition ("transinhibition") by their transported substrate or a closely related metabolite. During the course of our studies we observed a number of phenomena that we could not adequately explain. These phenomena included (i) marked differences in transport activity between seemingly identical cultures, or the same culture over short periods of time, (ii) irreproducible effects of certain metabolic inhibitors, (iii) dramatic effects of different buffers on the transport activity of cells at a given pH, and (iv) interactions between transport substrates (or substrates and other compounds) which could not be explained by competition for a common membrane carrier or by transinhibition. In this paper we attempt to summarize and explain some of the above phenomena in terms of the energetics of membrane transport in *P. chrysogenum*.

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## MATERIALS AND METHODS

The experiments described in this paper were carried out with *P. chrysogenum*, wild-type strain PS-75. The cultivation techniques and permease assays were described in detail in earlier papers (2, 19). Transport rates are reported on a cell dry weight basis. The standard assay buffers used in the present study were 0.05 M citrate (pH 3.2 and pH 4.5), 0.05 M phosphate (pH 6.0 and pH 7.0), and 0.05 M tris(hydroxymethyl)aminomethane (Tris; pH 8.0). In some experiments, 0.05 M 2-(*N*-morpholino)-ethanesulfonic acid (MES; pH 6.0), 2-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7.0), and *N*-tris(hydromethyl)methyl - 2 - aminopropanesulfonic acid (TAPS; pH 8.0) were used. In order to determine intracellular adenosine 5'-triphosphate (ATP) levels, 100 mg (wet weight) of mycelium was extracted in 10 ml of 66% aqueous ethanol at 80 C for 6 min. The extract was cooled, filtered, and diluted appropriately for (ATP) determination by the Firefly lantern extract method of Johnson (15; Sigma, FLE-50). Light emission was measured on a Beckman LS 230 scintillation counter after the coincidence circuit was turned off.

## RESULTS

**Effect of aeration.** On a number of occasions, we have been frustrated by unexplained increases or decreases in the specific transport activity of a batch of mycelium over a short period of time. These changes often made it difficult to evaluate the effects of potential inhibitors and to obtain clear-cut kinetic data. To compensate for the variability, it was often necessary to run control uptakes after every second or third measurement. We believe that the variation results from small changes in the degree of aeration of the mycelial suspension. For example, the increasing surface-to-volume ratio of a shaken culture as samples are removed is sufficient to stimulate transport. A suspension of 20 g (wet weight) of mycelium per liter of buffer equilibrated in a 2-liter Erlenmeyer flask will often yield mycelium with significantly lower transport rates than those observed for a suspension of 2 g (wet weight) of mycelium per 100 ml of buffer in a 500-ml Erlenmeyer flask on the same shaker. The importance of constant aeration is illustrated in Fig. 1. In most publications describing transport experiments with microorganisms, aeration conditions are not mentioned. It is possible that variations in aeration have no effect on transport by microorganisms that can obtain energy through anaerobic metabolism. It is also possible that oxygen is never a limiting factor in the dilute suspensions of bacteria or yeast used for transport experiments.

**Effect of uncouplers and weak acids.** 2,4-Dinitrophenol (DNP), azide, and (to a lesser extent) *p*-nitrophenol (PNP) have been routinely used to establish the "energy dependence" of membrane transport systems. In our own studies, we noticed that DNP frequently gave variable results. The variability was traced to a marked pH effect on the inhibitory action of DNP within the pH optimum range of the transport systems studied (generally pH 5.5 to 7.0). The results are shown in line 1 of Table 1. The results are consistent with the idea that the unionized form of DNP is the true inhibitor of transport (9) and prompted us to examine the effects of a number of other weak acids on transport. The data are summarized in the rest of Table 1. Essentially identical results were obtained for the sulfate (4) and ammonium ion (10) transport systems. The results were surprising in that quite a few weak acids, not usually considered uncouplers, were potent inhibitors of transport at pH values at and below their $pK_a$ value. There is no reason to believe that the weak acids act by competing

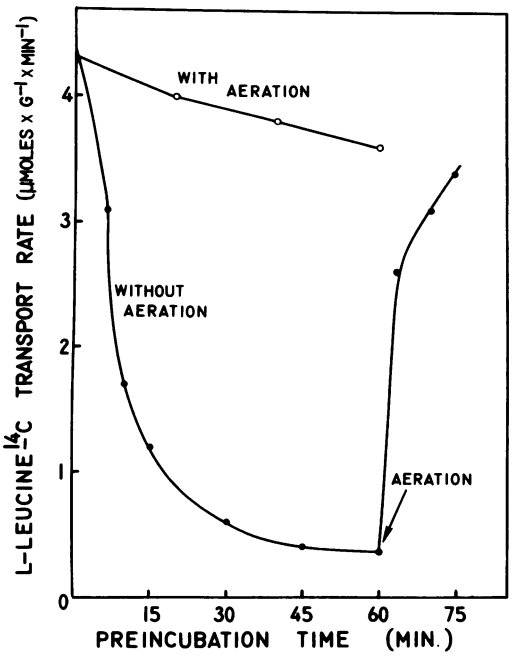


FIG. 1. *Effect of aeration on [$^{14}$C]L-leucine transport. A suspension of nitrogen-starved mycelium (2 g wet weight/100 ml) in 0.05 M phosphate buffer, pH 7.0, was divided into two parts. One part was incubated at room temperature with vigorous aeration on a rotary shaker. The other part was left open to the air but unshaken. Periodically, [$^{14}$C]L-leucine transport was measured at an initial substrate concentration of 2 × 10$^{-5}$ M. After 60 min, the unaerated suspension was placed on the shaker.*

TABLE 1. *Effect of weak acids on the transport of [$^{14}$C]L-leucine at different pH values*[a]

| Addition ($10^{-3}$ M) | $pK_a$ | Inhibition of transport (%) | | | | |
|---|---|---|---|---|---|---|
| | | pH 3.2 | pH 4.5 | pH 6.0 | pH 7.0 | pH 8.0 |
| DNP | 4 | >99 | >99 | 87 | 10 | +6[b] |
| PNP | 7 | 99 | 97 | 98 | 95 | 8 |
| Phenol | 10 | 33 | 0 | 7 | 0 | 0 |
| Benzoate | 4.2 | 97 | 89 | +34 | +21 | +2 |
| Sorbate | 4.9 | 97 | 82 | +17 | +10 | +7 |
| Propionate | 4.9 | 80 | 23 | +5 | 0 | 18 |
| Acetylsalicylate | 3.5 | 97 | 19 | 14 | +7 | +8 |
| Phenylacetate | 4.3 | >99 | 83 | 0 | +12 | +9 |
| Acetate | 4.8 | 84 | 16 | 0 | +7 | 0 |
| Butyrate | 4.8 | 94 | 26 | +14 | +11 | +14 |
| Indoleacetate | 4.8 | 96 | 22 | +8 | +5 | +14 |
| Azide | 4.7 | >99 | >99 | >99 | 96 | 25 |
| Hexachlorophene | 8[c] | >99 | >99 | >99 | >99 | >99 |
| Compound X[d] | | 75 | 23 | +17 | +15 | +10 |

[a] Nitrogen-starved mycelia were preincubated under standard assay conditions with the potential inhibitor at the pH values shown. After 15 min, $2 \times 10^{-5}$ M [$^{14}$C]L-leucine ($10^6$ counts per min per μmole) was added. Transport rates were established in the usual way (2, 19) from at least three samples taken over a 60-s period. The control transport rate was 30% of maximal at pH 3.2. At pH 8.0, transport was 60% of maximal. (The optimum pH range is 5.5 to 7.0). The values shown are relative to the control values at the given pH (ca. 2 to 6 μmol per g per min). The buffers used are described in Materials and Methods.

[b] Plus (+) indicates stimulation.

[c] Estimated.

[d] Compound X is a yellow, extracellular pigment produced by *P. chrysogenum* under nutrient-deficient conditions. The pigment was partially purified from culture filtrates and added to the assay medium at a concentration approximately the same as that found in the medium of mycelium nitrogen starved for 12 h.

with the substrate for a common binding site since (i) all three specific transport systems tested are affected in the same way, (ii) there are no structural resemblances between inhibitors and substrates, and (iii) the inhibition is not instantaneously reversed when the inhibitors are washed away. The inhibition, however, is slowly reversible. This is shown in Fig. 2 for DNP-treated mycelium. When the mycelium was washed before resuspension, the yellow color of adsorbed DNP disappeared. Similar results were obtained for mycelia preincubated with other weak acids, including PNP, azide, and benzoate. The inhibition by hexachlorophene, however, was not reversed. In fact, the mycelial pad of hexachlorophene-treated mycelium was gummy, suggesting that the cells were lysed. Preincubation with hexachlorophene at $10^{-5}$ M did not cause apparent lysis but inhibited transport by 53%. When $10^{-4}$ M hexachlorophene was added to the assay medium simultaneously with the substrate, transport was inhibited 97%. The general inhibition by weak acids strongly suggests that a proton or charge gradient is somehow involved in energizing membrane transport in *P. chrysogenum*. The unionized weak acids could discharge the gradient by passively diffusing into the lipophilic cell membrane and then ionizing when they reach an interior compartment of low proton concentration (not necessarily the cytoplasm; see reference 16). In the experiment reported in Table 1, the mycelia were preincubated with the weak acids for 15 min prior to measuring [$^{14}$C]L-leucine transport. Essentially identical results were obtained when the weak acids were added immediately before the substrate or during substrate transport (e.g., Fig. 4 of reference 1 or Fig. 6 of reference 4). The results shown in Table 2 confirm that the ionized species do not penetrate the cell membrane to any great extent.

**Effect of weak bases.** If weak acids discharge a proton gradient and thereby inhibit active transport, is it possible to stimulate transport by preincubating mycelia with weak lipophilic bases? Unfortunately, those bases which have the best combination of lipid solubility and $pK_a$ were unavailable (e.g., morphine). Pyridine and aniline stimulated transport slightly (ca. 5 to 15% from nonphosphate buffers), but the stimulation was seen only for amino acid and methylamine transport by nitrogen-starved mycelia. The lack of significant stimulation by aniline and pyridine is

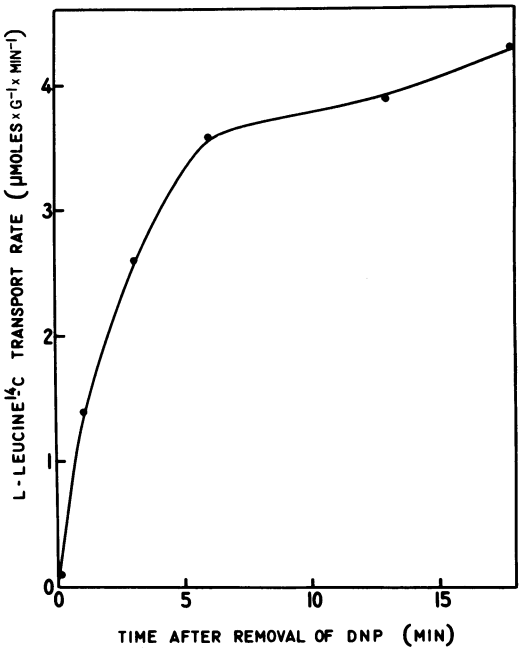


FIG. 2. *Reversal of DNP inhibition of transport. The mycelium was preincubated with $10^{-3}$ M DNP at pH 6.0 for 15 min. The mycelium was then filtered and washed rapidly and resuspended in fresh buffer. Transport of 2 × $10^{-5}$ M [$^{14}$C]L-leucine was measured periodically after resuspension.*

TABLE 2. *Effect of pH on uptake of DNP and PNP by mycelium*[a]

| Incubation buffer (0.05 M) | Mycelial DNP content (μmoles/g wet wt) | | Mycelial PNP content (μmoles/g wet wt) |
|---|---|---|---|
| | At $10^{-4}$ M DNP | At $10^{-3}$ M DNP | |
| Citrate, pH 3.5 | 3.1 | | |
| Citrate, pH 4.5 | 2.2 | 23.3 | |
| MES, pH 5.3 | 1.5 | 7.6 | 4.0 |
| MES, pH 6.2 | 0.3 | 2.9 | 2.9 |
| MOPS, pH 7.0 | 0.07 | 0.8 | 2.8 |
| Tris, pH 7.7 | 0.1 | 0.4 | 1.8 |
| TAPS, pH 9.2 | 0 | | 1.0 |

[a] Nitrogen-starved mycelia were incubated at a density of 2 g (wet weight) per 100 ml with $10^{-4}$ M or $10^{-3}$ M DNP or $10^{-3}$ M PNP in the buffers shown above. After exactly 1 min, a 10-ml sample was filtered rapidly with suction. The mycelial pad was extracted for 10 min with 10 ml of boiling water. The extract was then filtered and made up to 25 ml with 1 M KOH. The concentration of DNP or PNP in each extract was determined spectrophotometrically at 360 nm (DNP) or 400 nm (PNP). The absorbance was read against a blank extract prepared from mycelium which had not been exposed to any phenol.

not surprising. In healthy, aerobic mycelia, it might be impossible to "overcharge" the gradient. On the other hand, it might be possible to demonstrate stimulation under some conditions where the presumed gradient would be partially discharged. Table 3 shows the only conditions we have found in which aniline significantly and reproducibly stimulates [$^{14}$C]L-leucine transport. It can be seen that $NH_4Cl$ and phosphate inhibit [$^{14}$C]L-leucine transport and that the inhibition increases markedly as the pH of the incubation medium increases. Aniline appears to stimulate transport by reversing the inhibition (Table 3 and Fig. 3). If we accept the hypothesis that a proton or charge gradient is involved in energizing [$^{14}$C]L-leucine transport, then the

TABLE 3. *Effect of aniline on [$^{14}$C]L-leucine transport*[a]

| Addition | [$^{14}$C]L-leucine transport rate[b] | | Stimulation[c] (%) |
|---|---|---|---|
| | Control | + Aniline | |
| Nitrogen-starved mycelium | | | |
| Citrate, pH 4.5 | 4.8 | 5.0 | 5 |
| MES, pH 6.0 | 4.6 | 5.1 | 11 |
| MOPS, pH 7.0 | 4.5 | 4.8 | 7 |
| TAPS, pH 8.0 | 3.9 | 4.2 | 8 |
| Citrate, pH 4.5 + $10^{-2}$ M $NH_4Cl$ | 2.5 | 2.8 | 12 |
| MES, pH 6.0 + $10^{-2}$ M $NH_4Cl$ | 1.12 | 1.65 | 47 |
| MOPS, pH 7.0 + $10^{-2}$ M $NH_4Cl$ | 0.85 | 1.52 | 79 |
| TAPS, pH 8.0 + $10^{-2}$ M $NH_4Cl$ | 0.14 | 0.17 | 20 |
| Phosphate, pH 6.0 | 3.9 | 5.0 | 28 |
| Phosphate, pH 7.0 | 2.6 | 4.5 | 73 |
| Phosphate, pH 8.0 | 1.2 | 3.3 | 175 |
| Carbon-starved mycelium | | | |
| TAPS, pH 8.0 | 3.4 | 3.9 | 15 |
| TAPS, pH 8.0 + $10^{-2}$ M $NH_4Cl$ | 1.7 | 2.5 | 47 |
| Phosphate, pH 8.0 | 2.7 | 3.5 | 31 |

[a] Nitrogen- or carbon-starved mycelia were preincubated under standard assay conditions in the buffer shown with or without 0.01 M aniline. After 15 min, 2 × $10^{-5}$ M [$^{14}$C]L-leucine ($10^6$ counts per min per μmole) was added. $NH_4Cl$ was added simultaneously with the labeled substrate.

[b] Expressed as micromoles of product formed per gram per minute.

[c] Stimulation was calculated by the following equation: $[V_{(+\text{ aniline})} - V_{(\text{control})}]/[V_{(\text{control})}] \times 100\%$.

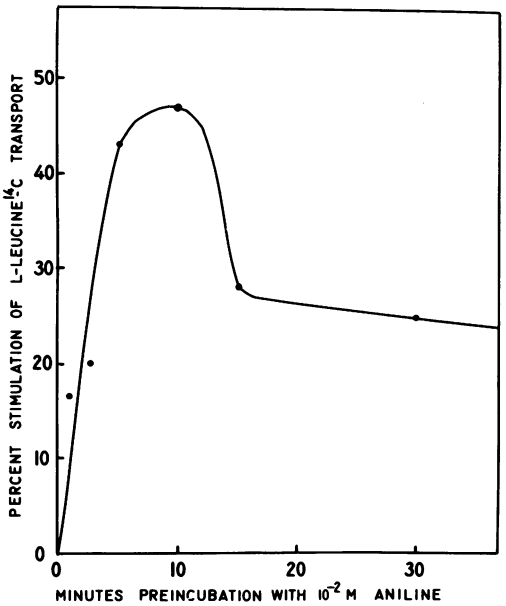


FIG. 3. *Nitrogen-starved mycelia were preincubated with $10^{-2}$ M aniline in 0.05 M phosphate buffer, pH 7. After the times indicated, the [$^{14}$C]L-leucine transport rate was measured in the same incubation medium.*

obvious conclusion is that $NH_3$ and $HPO_4^{2-}$ somehow ruin the gradient, and aniline prevents the collapse. The effects of $NH_4Cl$ were investigated in detail.

**Effect of $NH_4^+$ on [$^{14}$C]L-leucine transport.** We first noticed that $NH_4^+$ inhibited the general amino acid transport system several years ago (3). There seemed to be two explanations for this phenomenon: (i) $NH_4^+$ competes with amino acids for a common carrier or binding protein, or (ii) $NH_4^+$ is transported into the mycelium very rapidly where it is converted to amino acid transinhibitors of the general amino acid system. Subsequent studies established that nitrogen starvation does indeed derepress or deinhibit a specific $NH_4^+$ transport system with a very low $K_m$ for $NH_4^+$ (10). However, it did not seem likely that transinhibition could account for the dramatic instantaneous effect of $NH_4^+$. Some typical results are shown in Table 4. The effect of methylammonium ion is also shown. Methylammonium is a substrate of the $NH_4^+$ transport system (10). The reciprocal experiments gave similar results. For example, L-leucine at $2 \times 10^{-5}$ to $2 \times 10^{-4}$ M inhibited the transport of $2 \times 10^{-5}$ M [$^{14}$C]methylammonium 43 to 48% at pH values from 4.5 to 7.0. The fact that the mutual inhibitions were relatively insensitive to inhibitor concentration after an initial marked effect suggested that we were not dealing with a simple case of competition for a common carrier or binding protein. The inhibition of [$^{14}$C]L-leucine transport by $NH_4+$ is evident at normal assay pH values (5.5 to 7.0) only if the $NH_4^+$ transport system is also present. Thus, $10^{-2}$ M $NH_4^+$ has virtually no effect on [$^{14}$C]L-leucine transport by carbon-starved mycelium at pH <7. Carbon-starved mycelia are fully derepressed and deinhibited for the general amino acid transport system (19), yet they possess extremely low $NH_4^+$ transport activity. The obvious conclusion is that $NH_4^+$ must be transported to exert its instantaneous inhibitory effect. This conclusion is supported by the results shown in Table 5. It can be seen that iodoacetate or iodoacetamide almost completely inhibits the $NH_4^+$ transport system (assayed with [*methyl-$^{14}$C*]ammonium), but the system transporting [$^{14}$C]L-leucine retains 30% of its uninhibited activity. The residual activity is insensitive to $NH_4^+$. Figure 4 shows the time course of $NH_4^+$ inhibition of [$^{14}$C]L-leucine transport. The inhibition by $NH_4^+$ is instantaneous, but decreases with time, then increases again. The decrease in inhibition during the first 15 min cannot be explained by depletion of $NH_4^+$ from the incubation medium. (At $10^{-2}$ M $NH_4^+$, less than 3% would have been removed from the medium within 15 min.) We interpret the results in the following way. Leucine and $NH_4^+$ are transported into the mycelium by two distinct systems. There is little or no cross-reaction of the two substrates with each other's carrier or binding proteins.

TABLE 4. *Inhibition of [$^{14}$C]L-leucine transport by $NH_4^+$ and methylamine*[a]

| Inhibitor concn (M) | Inhibition of [$^{14}$C]L-leucine transport (%) | | |
|---|---|---|---|
| | I = $NH_4^+$ | | I = Methylammonium ([L-leucine] = $2 \times 10^{-5}$ M) |
| | [L-leucine] = $2 \times 10^{-5}$ M | [L-leucine] = $10^{-4}$ M | |
| $10^{-6}$ | 58 | 45 | 7 |
| $10^{-5}$ | 61 | 59 | 18 |
| $10^{-4}$ | 73 | 71 | 24 |
| $10^{-3}$ | 74 | 73 | 52 |
| $10^{-2}$ | 88 | 84 | 75 |

[a] [$^{14}$C]L-leucine transport by nitrogen-starved mycelium was assayed under standard conditions in 0.05 M phosphate buffer, pH 7.0. The inhibitor and substrate were added simultaneously. [$^{14}$C]L-leucine transport rates were determined from samples taken 20 s after adding the labeled substrate plus inhibitor.

TABLE 5. *Inhibition of [$^{14}$C]L-leucine transport by $NH_4^+$ in the presence of iodoacetate and iodoacetamide*[a]

| Treatment | [$^{14}$C]methylamine transport rate[b] | [$^{14}$C]L-leucine transport rate[b] | |
|---|---|---|---|
| | | $-NH_4^+$ | $+NH_4^+$ |
| None (control) | 8.0 | 7.0 | 2.5 |
| + $10^{-2}$ M iodoacetate | 0.004 | 2.2 | 2.1 |
| + $10^{-2}$ M iodoacetamide | 0.003 | 2.5 | 2.2 |

[a] [$^{14}$C]L-leucine transport by nitrogen-starved mycelium was assayed under standard conditions in 0.05 M phosphate buffer, pH 7.0. The initial [$^{14}$C]L-leucine concentration was $2 \times 10^{-5}$ M. Iodoacetate or iodoacetamide ($10^{-2}$ M) were added 15 min before the addition of the labeled substrate. $NH_4Cl$ at $2 \times 10^{-4}$ M was added simultaneously with the labeled substrate.

[b] Results shown are expressed as micromoles per gram per minute.

However, the two systems compete for a limiting intramembrane "energy supply" (ATP, proton gradient, or whatever the driving force). The energy supply is not sufficient to translocate both substrates simultaneously at their individual control rates (ca 5 μmol per g per min at [L-leucine] $\simeq K_m$ [3] and ca. 10 μmol per g per min = $V_{max}$ for $NH_4^+$ at $[NH_4^+] = 10^5 \times K_m$ [10]). As the intracellular $NH_4^+$ pool builds up, the $NH_4^+$ transport system becomes transinhibited by $NH_4^+$ itself or a closely related metabolite, e.g., glutamine (10). The gradual transinhibition of the $NH_4^+$ system permits an increasing fraction of the translocation energy supply to be diverted to the system transporting L-leucine. By 15 min the $NH_4^+$ transport system is maximally transinhibited and [$^{14}$C]L-leucine transport can occur at near control rates. After longer times, the accumulated $NH_4^+$ promotes a build-up of amino acids intracellularly which transinhibit the general amino acid system causing the [$^{14}$C]L-leucine transport activity to decrease again.

**Effect of transport inhibitors on intracellular ATP levels.** Aerobic nitrogen-starved mycelia contain 1 to 2 μmol/g (wet weight) of ATP (ca. 5 to 10 μmol/g of dry weight) and transport [$^{14}$C]L-leucine at a rate of 4 to 6 μmol/g (dry weight) per min from $2 \times 10^{-5}$ M solutions. If ATP is involved in [$^{14}$C]L-leucine transport, we would expect to find some correlation between the specific transport activity of mycelia and intracellular ATP levels. The results for a wide variety of preincubation conditions are summarized in Fig. 5. There is no direct correlation. For example, benzoate can virtually abolish transport without having any appreciable effect on the ATP level. The inhibitors have been arbitrarily divided into two groups. Group I consists of the known inhibitors of mitochondrial ATP production: DNP, PNP, azide, cyanide, and hexachlorophene. Group II consists mainly of weak organic acids. In another experiment, we found that azide at $10^{-3}$ M and pH 7.0 decreased transport to 4% of the control rate within 7 s after its addition. At the same time, the ATP level dropped to 22% of the control. Over the next 2 min, the transport rate decreased to 1% of the control, while the ATP level leveled off at 13% of the control. Within 1 min after washing away the azide (the minimum operation time), transport increased to 76% of the original control value, while the ATP level increased back to 92% of the original value. Thus, it appears that the ATP turnover rate in *P. chrysogenum* is extremely rapid. An equally rapid rate has been reported for *Escherichia coli* (13).

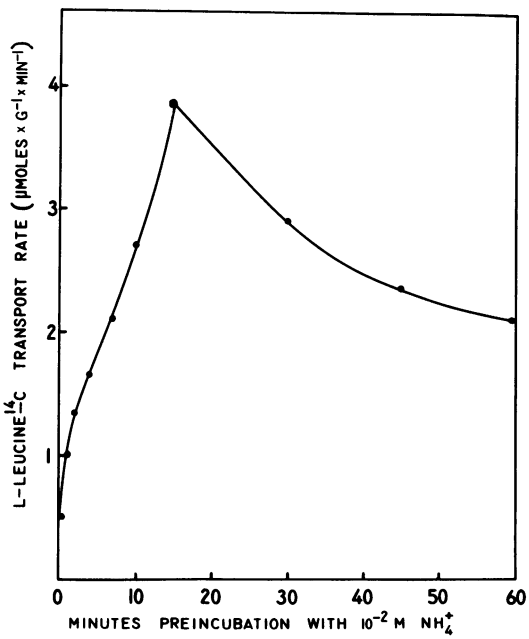


FIG. 4. *Effect of preincubation time with $NH_4^+$ on [$^{14}$C]L-leucine transport activity of nitrogen-starved mycelia. Mycelia were incubated in 0.02 M phosphate buffer, pH 7.0, containing $10^{-2}$ M $NH_4Cl$. Periodically, a sample of the suspension was removed and the [$^{14}$C]L-leucine transport rate was measured (without washing away the $NH_4Cl$). The [$^{14}$C]L-leucine concentration was $2 \times 10^{-5}$ M.*

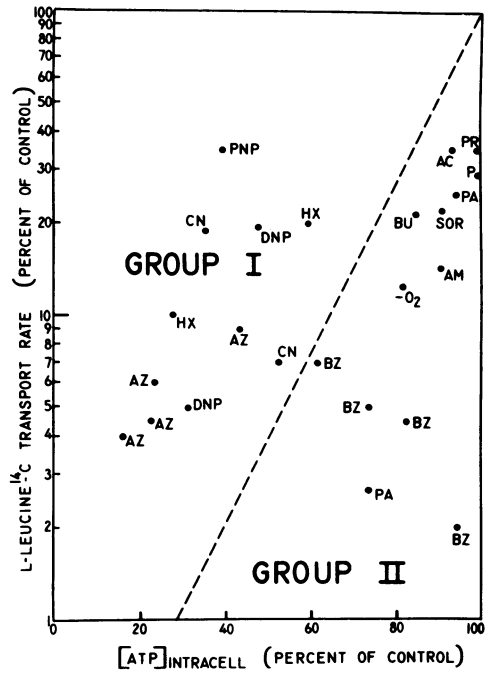


FIG. 5. *Effect of various inhibitors on the [$^{14}$C]L-leucine transport rate and the intracellular ATP levels in nitrogen-starved mycelia. Inhibitor concentrations ranged from $10^{-5}$ M to $10^{-3}$ M. Preincubation times ranged from 7 s to 15 min. The pH of the different incubation media ranged from 3.2 to 7.0. The control [$^{14}$C]L-leucine transport rates ranged from 2 to 6 μmol ($\times g^{-1} \times min^{-1}$). The control ATP levels ranged from 1 to 2 μmol/g (wet weight) of mycelium. Abbreviations: Ac, acetate; Pr, propionate; PA, phenylacetate; Bu, butyrate; Sor, sorbate; Bz, benzoate; PNP, p-nitrophenol; CN, cyanide; DNP, 2,4-dinitrophenol; Az, azide; Hx, hexachlorophene ($10^{-5}$ M); $-O_2$, no aeration; AM, ammonium chloride ($10^{-2}$ M, pH 7.0); P, phosphate (0.05 M, pH 8.0).*

## DISCUSSION

We have shown that a variety of weak organic acids inhibit membrane transport processes in *P. chrysogenum*. The active compounds include propionate, benzoate, and sorbate, which are common preservatives in packaged foods and are known to be effective only at low pH (17). Acetate at low pH has long been known to be toxic to microorganisms (20). Protocatechuic acid, a secondary plant metabolite, is believed to be an antimicrobial agent (7).

The results suggest that a proton or charge gradient is somehow involved in energizing amino acid transport in *P. chrysogenum*. However, our results cast no light on the exact nature, physical boundaries, or origin of the gradient. The gradient may arise from a pH difference and/or electrochemical potential between the cytoplasm and external medium, or a proton or charge difference between two compartments within the membrane, the "internal" compartment being more negative (or higher in $OH^-$) than the "external" compartment. Also, we do not attempt to explain how the gradient energizes transport. There are several reports of proton movements associated with active transport (8, 11, 12, 18, 21). Harold (11) recently reviewed the various models of ion current-coupled transport in microorganisms.

On the basis of its differential effect on ATP levels and transport, hexachlorophene is included in group I along with DNP, azide, and other classical uncouplers. Cammer and Moore (6) recently reported that hexachlorophene is an uncoupler of oxidative phosphorylation in rat liver mitochondria. It is noteworthy that only those compounds capable of inhibiting transport at pH 6 to 7 are also capable of decreasing cellular ATP levels. Compounds in this class (group I) include only the classical uncouplers and hexachlorophene. The pH of the cytoplasm of *P. chrysogenum* is about 6.0 (this is the pH of pure undiluted cell sap prepared with an X-press). Thus, it seems that group II weak acids are able to penetrate the plasma membrane from solutions of low pH and, thereby, destroy a proton or charge gradient used for nutrient transport; but once inside the cell, the fraction in the unionized form is too low to have an effect on the mitochondrial gradient used to generate ATP.

We believe that the inhibitory effect of $NH_4^+$ at pH values of 7.0 and below can be adequately explained by competition for a common energy source. The strong inhibition by $NH_4^+$ at pH 8.0 very likely results from the toxic effect of $NH_3$, which conceivably could wreck the proton or charge gradient by diffusing into an outer "proton-rich" compartment, ionizing, and then diffusing back into the medium. Thus, at pH 8.0, $10^{-2}$ M $NH_4^+$ inhibits [$^{14}$C]L-leucine transport by carbon-starved mycelia 50% even though the $NH_4^+$ transport system is essentially absent (Table 3). The $HPO_4^{2-}$ ion might do the same thing. Since the $pK_{a_2}$ of phosphate is about 7, some inhibition by phosphate is evident even at pH 6.0. The distinction between inhibition by "competition for a common energy source" and by "discharging a proton or charge gradient" may not be clear-cut since the normal energized transport of a substrate may involve concomitant discharge of a proton or charge

gradient. The abortive discharge of a proton gradient may be aided by the normal binding protein components of a transport system which facilitates the entry of $NH_3$ or $HPO_4^{2-}$ into the membrane. In this regard, it is noteworthy that (i) phosphate is more inhibitory at pH 8.0 to nitrogen-starved mycelia than to carbon-starved mycelia, (ii) the phosphate transport system is derepressed to a greater level in nitrogen-starved mycelia (*unpublished data*), although net phosphate uptake does not occur at pH 8.0 and above. Arsenate was much less inhibitory than phosphate at any given pH. Thus, the inhibition is not simply a result of nonspecific ionic strength effects. (The $pK_{a2}$ for arsenate is, in fact, slightly lower than $pK_{a_1}$ of phosphate.)

The stimulation of transport by aniline cannot be explained in an orthodox manner. Inhibition by a high concentration of a "non-physiological" compound is not unexpected, but activation of a physiological process is unusual. We can envision two ways in which aniline might counteract the inhibitory effects of $NH_3$ and $HPO_4^{2-}$. (i) The unionized amine diffuses into an inner membrane compartment where it builds up the $OH^-$ concentration by ionizing as a typical base, or (ii) the unionized amine diffuses into an outer proton-rich compartment where it is converted to the $R\text{-}NH_3^+$ form which acts as a proton reservoir or buffer. Suggestion (i) implies that, after ionization, the $R\text{-}NH_3^+$ form is excreted back to the medium or into the cytoplasm, whereas suggestion (ii) implies that the $R\text{-}NH_3^+$ form remains trapped in the proton-rich region. The fact that it takes about 10 min of preincubation with aniline before maximal stimulation occurs (or more precisely, maximal protection against $NH_3$ or $HPO_4^{2-}$) supports suggestion (ii). The 10-min period may represent the time required for electron transport processes to restore the protons captured by the ionization of aniline. We would expect process (i), which involves only diffusion and ionization, to be more rapid. (The inhibition by DNP, azide, and other weak acids is instantaneous.)

Cameron and Le John (5) recently presented evidence implicating membrane-bound $Ca^{2+}$ in amino acid transport by the fungus *Achlya*. The specific role of $Ca^{2+}$ is unknown. The cation may contribute to a charge gradient, activate a membrane adenosine triphosphatase, or play a passive role in stabilizing a membrane component. The $HPO_4^{2-}$ ion is a chelator of $Ca^{2+}$. Thus, the inhibition by phosphate at high pH may result from the removal of $Ca^{2+}$ from a strategic location. The anilinium ion trapped within the membrane may act by mimicking $Ca^{2+}$.

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## LITERATURE CITED

1. Bellenger, N., P. Nissen, T. C. Wood, and I. H. Segel. 1968. Specificity and control of choline-*O*-sulfate transport in filamentous fungi. J. Bacteriol. **96**:1574-1585.
2. Benko, P. V., T. C. Wood, and I. H. Segel. 1967. Specificity and regulation of methionine transport in filamentous fungi. Arch. Biochem. Biophys. **122**:783-804.
3. Benko, P. V., T. C. Wood, and I. H. Segel. 1969. Multiplicity and regulation of amino acid transport in *Penicillium chrysogenum*. Arch. Biochem. Biophys. **129**:498-508.
4. Bradfield, G., P. Somerfield, T. Meyn, M. Holby, D. Babcock, D. Bradley, and I. H. Segel. 1970. Regulation of sulfate transport in filamentous fungi. Plant Physiol. **46**:720-727.
5. Cameron, L. E., and H. B. Le'John. 1972. On the involvement of calcium in amino acid transport and growth of the fungus *Achlya*. J. Biol. Chem., **247**:4729.
6. Cammer, W., and C. L. Moore, 1972. The effect of hexachlorophene on the respiration of brain and liver mitochondria. Biochem. Biophys. Res. Commun. **46**:1887-1894.
7. Doby, G. 1965. Plant biochemistry. Interscience Publishers, Budapest, p. 702.
8. Eddy, A. A., K. J. Indge, K. Backen, and J. A. Nowacki. 1970. Interactions between potassium ions and glycine transport in the yeast *Saccharomyces carlsbergensis*. Biochem. J. **120**:845-852.
9. Evans, W. R. 1971. The effect of cycloheximide on membrane transport in *Euglena*. J. Biol. Chem. **246**:6144-6151.
10. Hackette, S. L., G. E. Skye, C. Burton, and I. H. Segel. 1970. Characterization of an ammonium transport system in filamentous fungi with methylammonium-$^{14}C$ as the substrate. J. Biol. Chem. **245**:4241-4250.
11. Harold, F. M. 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. **36**:172-230.
12. Harold, F. M., and J. R. Baarda. 1968. Inhibition of membrane transport in *Streptococcus faecalis* by uncouplers of oxidative phosphorylation and its relationship to proton conduction. J. Bacteriol. **96**:2025-2034.
13. Holms, W. H., I. D. Hamilton, and A. G. Robertson. 1972. The rate of turnover of adenosine triphosphate pool of *Escherichia coli* growing aerobically in simple defined media. Arch. Mikrobiol. **83**:95-109.
14. Hunter, D. R. and I. H. Segel. 1971. Acidic and basic amino acid transport systems in *P. chrysogenum*. Arch. Biochem. Biophys. **144**:168-183.
15. Johnson, R. A., J. G. Hardman, A. E. Broadus, and E. W. Sutherland. 1970. Analysis of adenosine 3',5'-monophosphate with luciferase luminescence. Anal. Biochem. **35**:91-97.

16. Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev., Cambridge Phil. Soc. **41**:445–502.
17. Oka, S. 1964. Mechanism of antimicrobial effects of various food preservatives, p. 3–16. *In* N. Molin (ed.), Microbial inhibitors in food, 4th Int. Symp. Food Microbiol. Almquist and Wiksell, Stockholm.
18. Pavlasova, E., and F. M. Harold. 1969. Energy coupling in the transport of β-galactosides by *Escherichia coli*: effect of proton conductors. J. Bacteriol. **98**:198–204.
19. Syke, G. E., and I. H. Segel. 1970. Independent regulation of cysteine and cystine transport in *P. chrysogenum*. Arch. Biochem. Biophys. **138**:306–318.
20. Stanier, R. Y. 1963. The microbial world, (2nd ed.), p. 354. Prentice-Hall, Inc.
21. West, I. C. 1970. Lactose transport coupled to proton movements in *Escherichia coli*. Biochem. Biophys. Res. Comm. **41**:655–661.