

Penicillium chrysogenum Takes up the Penicillin G Precursor Phenylacetic Acid by Passive Diffusion

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***Penicillium chrysogenum* utilizes phenylacetic acid as a side chain precursor in penicillin G biosynthesis. During industrial production of penicillin G, phenylacetic acid is fed in small amounts to the medium to avoid toxic side effects. Phenylacetic acid is taken up from the medium and intracellularly coupled to 6-aminopenicillanic acid. To enter the fungal cell, phenylacetic acid has to pass the plasma membrane. The process via which phenylacetic acid crosses the plasma membrane was studied in mycelia and liposomes. Uptake of phenylacetic acid by mycelium was nonsaturable, and the initial velocity increased logarithmically with decreasing external pH. Studies with liposomes demonstrated a rapid passive flux of the protonated species through liposomal membranes. These results indicate that phenylacetic acid passes the plasma membrane via passive diffusion of the protonated species. The rate of phenylacetic acid uptake at an external concentration of 3 mM is at least 200-fold higher than the penicillin production rate in the Panlabs P2 strain. In this strain, uptake of phenylacetic acid is not the rate-limiting step in penicillin G biosynthesis.**

Penicillium chrysogenum is a filamentous fungus which is used for commercial production of penicillins. It is able to synthesize penicillins with specific hydrophobic side chains when the appropriate precursor is fed to the production medium (1, 4, 5, 12). PA appears to be the best precursor, but the phenyl group can be substituted or replaced by other ring systems (1). Benzoic acid derivatives cannot be utilized, and because only monosubstituted acetic acids are incorporated, an α -methylene group seems to be essential. The effectiveness of the side chain precursor appears to depend largely on its toxicity and its resistance to oxidation by *P. chrysogenum*. In commercial processes, only PA, POA, and allylmercaptoacetic acid have been used to produce penicillin G, penicillin V, and penicillin O, respectively (4). In the absence of an exogenous side chain precursor, *P. chrysogenum* produces mainly 6-aminopenicillanic acid (3, 20). Under these conditions, monosubstituted acetic acids present at low intracellular concentrations are used to form small amounts of penicillins like benzylpenicillin, 2-pentenylpenicillin, *n*-amylpenicillin, *n*-heptylpenicillin, *p*-hydroxybenzylpenicillin, and isopenicillin N (27).

Incorporation of the side chain precursor is carried out by an AT during the last step in the penicillin biosynthetic pathway (21, 26). This enzyme converts isopenicillin N into penicillin G by exchange of the α -amino adipyl moiety for PA. Prior to this transacylation reaction, PA has to be activated to phenylacetyl-CoA. Recent studies suggest that this activation takes place by a general acetyl-CoA synthetase, which is involved in the primary metabolism of *P. chrysogenum* (22). Electron-microscopic studies with specific antibodies demonstrated that AT is present solely in a specific microbody (25). Furthermore, AT contains at the C terminus a typical peroxisomal targeting sequence 1 (alanine-arginine-leucine). AT lacking the targeting signal was found to be localized in the cytosol or small

vacuoles, and transformants containing this mutant AT were able to produce penicillin (24). Acetyl-CoA synthetase seems to be an intracellular enzyme, yet its exact location is unknown. The intracellular localization of acetyl-CoA synthetase and AT implies that PA has to be taken up by *P. chrysogenum* before it can synthesize penicillin G.

Uptake of PA by *P. chrysogenum* can take place either through simple diffusion or via a specific transport system. PA is a weak acid, and the undissociated species might rapidly diffuse across the lipid bilayer (12, 31). Fernández-Cañón and coworkers (9, 10, 23, 29) presented evidence for the presence of a specific transport system for the uptake of PA by *P. chrysogenum*, and no passive influx of PA was detected at a moderate range of pH values, i.e., pH 5 to 8. To establish the presence of this PA transport system and to characterize it in detail, we studied PA uptake in mycelial suspensions. Furthermore, we used these mycelial suspensions, liposomes, and cytochrome *c* oxidase-containing liposomes to study the passive influx of PA. The data obtained from these studies suggest that the main PA flux across the plasma membrane during penicillin biosynthesis occurs through passive diffusion rather than via a specific transport system. At pH 6.5 and an extracellular PA concentration of 3 mM, the rate of PA influx was found to be at least 250 times higher than the overall rate of penicillin synthesis in *P. chrysogenum* P2. From these observations, it was concluded that the influx of PA is not the rate-limiting factor in the synthesis of this antibiotic.

MATERIALS AND METHODS

Abbreviations used in this paper. PA, phenylacetic acid; POA, phenoxyacetic acid; AT, acyltransferase; CoA, coenzyme A; COV, cytochrome *c* oxidase vesicle; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; MES, morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TPP⁺, tetraphenylphosphonium ion.

Organism and culture conditions. *P. chrysogenum* Wisconsin 54-1255 and Panlabs P2 (kindly supplied by Gist-Brocades NV) were grown on production medium (pH 6.3) supplemented with 10 mM glutamate and 10% (wt/vol) glucose as described by Lara et al. (18). Cultures were incubated for approximately 70 h in a rotary shaker at 200 rpm and 25°C. The Wisconsin 54-1255 strain was

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precultured for 24 h on production medium, with the omission of PA and lactose but containing 16% (wt/vol) glucose. The P2 strain was precultured on YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, and 2% [wt/vol] glucose) for 72 h at pH 7.

Isolation and reconstitution of cytochrome *c* oxidase. Bovine heart mitochondria were obtained according to the procedure described by King (17). Cytochrome *c* oxidase was isolated from these mitochondria as described by Yu et al. (32), suspended in 50 mM sodium phosphate (pH 7.5) containing 1.5% (wt/vol) cholic acid, and stored in liquid nitrogen. Cytochrome *c* oxidase was reconstituted in liposomes composed of 75% (wt/wt) acetone-ether-washed *E. coli* lipids and 25% (wt/wt) egg yolk L-phosphatidylcholine. A protein/lipid ratio of 0.16 nmol of heme *a* per mg of lipid was used (7). These proteoliposomes are termed COVs.

Determination of the electrical and pH gradients across the membrane. The transmembrane electrical potential ($\Delta\psi$, interior negative) was calculated from the distribution of TPP⁺, assuming concentration-dependent binding to the membranes as described previously (19). The external concentration of TPP⁺ was monitored with a TPP⁺-selective electrode. COVs (0.23 nmol of oxidase) were added to a buffer of the indicated pH, containing 50 mM potassium phosphate, 5 mM MgSO₄, and 2 μ M TPP⁺. A proton motive force (Δp) was generated by the addition of 10 mM ascorbate (adjusted to the desired pH), 200 μ M TMPD, and 20 μ M cytochrome *c*. Where indicated, the ionophores nigericin and valinomycin were used at concentrations of 10 and 100 nM, respectively. The pH gradient across the membrane (Δp H, interior alkaline) was determined from the fluorescence of pyranine (excitation, 450 nm; emission, 508 nm) as measured with a Perkin-Elmer LS50B luminescence spectrophotometer. Pyranine (100 μ M) was entrapped in proteoliposomes by freeze-thaw sonication (7). External pyranine was removed by chromatography of the proteoliposome suspension over a Sephadex G-25 column (coarse, 1 by 20 cm). Valinomycin was added to a final concentration of 50 nM. Energization of proteoliposomes was carried out as described for $\Delta\psi$ measurements. When indicated, nigericin was added to a final concentration of 1 μ M.

Stopped-flow measurements. Rates of influx of protonated acid were determined from the decrease in pyranine fluorescence by using a Milliflow stopped-flow reactor and an Aminco SLM 4800C time-resolved fluorescence spectrophotometer. Pyranine (100 μ M) was entrapped in liposomes composed of 75% (wt/wt) acetone-ether-washed *E. coli* lipids and 25% (wt/wt) egg yolk L-phosphatidylcholine as described for Δp H measurements; instead of sonication, liposomes were obtained by extrusion through polycarbonate filters (Avestin Inc., Ottawa, Canada) with pore sizes of 400 and 200 nm in a low-volume extrusion apparatus (Avestin) (13). Liposomes were diluted to a final concentration of 2 mg of lipid per ml, and valinomycin was added to the liposomal suspension to a final concentration of 50 nM. Stopped-flow measurements were performed at 20°C in 5 mM MOPS-KOH (pH 7) containing 1 mM MgSO₄.

Oxygen consumption. O₂ consumption by proteoliposomes was measured with a Clark-type oxygen electrode (YSI, Yellow Springs, Ohio) in a 1-ml vessel at 25°C under the same conditions as described for $\Delta\psi$ measurements. Endogenous O₂ consumption was determined after addition of 1 mM KCN.

Penicillin G and V concentrations. Penicillin G concentrations were determined by reversed-phase HPLC as described by Terada and Sakabe (30) with penicillin V as an internal standard. Penicillins were separated with a stainless steel Nucleosil C₁₈ column (5 μ M; 250 by 4.6 mm; Alltech Associates, Inc.). As a mobile phase, methanol-water-0.2 M potassium phosphate (pH 5.0) (5:13:1) containing 10 mM sodium alkylsulfonate was used. The flow rate was set at 1 ml/min, and a column temperature of 25°C was applied. For detection of penicillins by UV (210 nm), a Waters 481 LC spectrophotometer (Millipore) was used. Data were processed by the JCL6000 chromatography system (Jones Chromatography).

Rate of penicillin production. Penicillin production rates of the Panlabs P2 strain were measured by a fast filtration assay combined with determination of penicillin G by HPLC. Mycelium was harvested, washed once with 0.9% (wt/vol) NaCl, and resuspended to a density of 15 to 20 mg (dry weight)/ml in production medium adjusted to the desired pH with KOH. This medium contained no glucose or PA, while potassium phosphate was replaced by 50 mM MES-PIPES-HEPES. Measurements were performed at 25°C in 250-ml Erlenmeyer flasks containing 25 ml of cell suspension under continuous stirring (300 rpm). Penicillin production was started by addition of the desired amount of PA. At different time intervals, 1-ml samples were withdrawn and filtered on paper filters (296 PE, type 0860; Schleicher & Schuell) to remove whole cells and debris. The filtrates were collected and stored at -20°C prior to HPLC analysis. Under these conditions, the penicillin production rate was constant for at least 1 h.

Transport assays. Uptake of phenylacetic acid and valine was studied at 25°C and pH 6.5, unless stated otherwise. Mycelia, harvested by suction filtration, were suspended in 50 mM potassium phosphate at final densities of 10 mg (dry weight)/ml (P2 strain) or 6 mg (dry weight)/ml (Wisconsin 54-1255 strain) and stored on ice until further use. Initial rates of uptake were determined in triplicate 5, 10, and 20 s after addition of the radiolabelled PA or valine to the mycelial suspension. To lower nonspecific binding and reduce the effect of metabolism, high concentrations of PA (>60 μ M) with a relatively low specific activity were used. Nonspecific binding was measured for cells permeabilized by treatment with 2% toluene for 2 h at 25°C. L-[U-¹⁴C]phenylacetic acid (3.4

mCi/mmol; Sigma) and L-[U-¹⁴C]valine (280 mCi/mmol; Amersham), previously diluted 10-fold with nonradiolabelled substrate, were added to the mycelial suspension to final concentrations of 63 and 30 μ M, respectively. Samples of 0.5 ml were taken at given time intervals, added to 2 ml of ice-cold 0.1 M LiCl, and filtered immediately on paper filters (296 PE, type 0860). The filters were washed once with 2 ml of ice-cold 0.1 M LiCl, and the radioactivity was determined with a liquid scintillation counter (Tri-Carb 460 CD; Packard Instruments Corp.). Nonenergized uptake was measured by using mycelial suspension preincubated with the protonophore 10 μ M CCCP for 5 min at 25°C.

HPLC analysis of intracellular pools. Cell pellets obtained as described for transport assays were immediately frozen in liquid N₂. A 250- μ l volume of ice-cold chloroform-methanol (1:2 [vol/vol]) was added, and the pellets were stored overnight at -20°C. By this procedure, more than 95% of the labelled material was extracted. After centrifugation in an Eppendorf-type centrifuge, supernatants were fractionated by HPLC as described above. Intracellular pools of radiolabelled PA, penicillins, and derivatives were determined with a liquid scintillation counter (Tri-Carb 460 CD).

Other methods. Protein concentrations were determined in the presence of 0.5% (wt/vol) sodium dodecyl sulfate by a modified Lowry assay (20). Bovine serum albumin was used as a standard.

RESULTS

Uptake of PA in *P. chrysogenum*. When a specific protein is involved in the transport of PA, uptake of this solute should show saturation kinetics. Therefore, the initial rates of PA uptake in *P. chrysogenum* P2 and Wisconsin were determined at different external PA concentrations (Fig. 1A). The Wisconsin and P2 strains strongly differ in morphology and penicillin production capacity. However, the uptake of PA showed similar kinetics (Fig. 1). The initial rate of PA uptake increased linearly up to an external PA concentration of 30 mM. This suggests that uptake occurs via a nonsaturable process or a process with an extremely low substrate affinity.

Since the extent of deprotonation of PA (pK_a 4.3) depends on the pH, the effect of the external pH on the initial rate of PA uptake was studied. At an external PA concentration of 63 μ M, the initial rate of PA uptake increased logarithmically with decreasing external pH (Fig. 1B). The increase was about 10-fold per pH unit, and no pH optimum was found within the pH range studied. Since this increase is proportional to the increase in concentration of protonated PA, these results suggest that PA is taken up via passive diffusion of the protonated species.

Since under normal physiological conditions the cytosolic pH is higher than the extracellular pH (28), PA dissociates intracellularly to a higher extent and will accumulate in the cell. If metabolic conversion is slow compared with the rate of influx, the pH difference between cytosol and medium will be the main factor that determines PA accumulation. Steady-state levels of PA accumulation were reached within 10 min after addition of radiolabelled PA to mycelial suspensions (see Fig. 3). The steady-state level of PA accumulation was highest at pH 5.5 and decreased dramatically above an external pH of 7 (Fig. 1C). These results point to an important role of the Δp H in PA accumulation. However, the observed relationship cannot be interpreted unequivocally, since both the Δp H (27) and the concentration of the protonated species decrease with increasing external pH.

To investigate if metabolism of PA plays a significant role in these uptake experiments, the composition of intracellular pools of radioactively labelled compounds was analyzed after extraction and fractionation by HPLC. After a 10-min incubation with 63 μ M PA, PA made up at least 90% of the internalized label (Fig. 2). Preincubation of the mycelium with the protonophore CCCP to collapse the proton motive force (Δp) resulted in a fourfold-lower accumulation of PA. These results indicate that under the conditions applied, the metabolic conversion of PA is much slower than the rate of PA uptake. The intracellular pool of PA could not be chased by a 30-fold excess

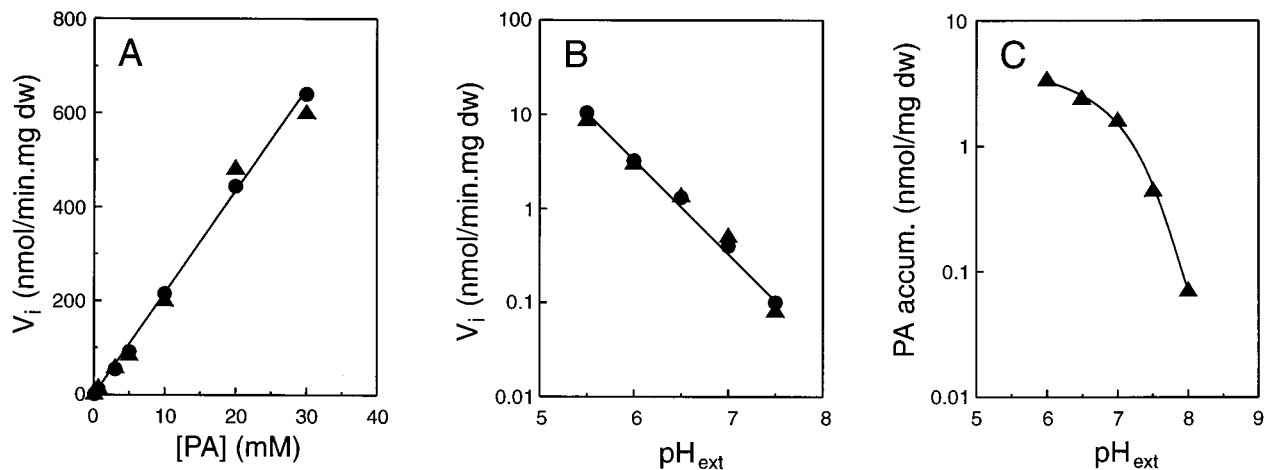


FIG. 1. Uptake of PA by *P. chrysogenum* Wisconsin 54-1255 (●) and Panlabs P2 (▲). (A) Initial rate of PA uptake (V_i) as a function of the external PA concentration. (B) Effect of the external pH on the initial rate of PA uptake. (C) PA accumulation as a function of the external pH. Unless indicated otherwise, PA was added to an external concentration of 63 μ M. Steady-state levels of accumulation were determined 10 min after addition of radiolabelled PA to the mycelial suspensions. dw, dry weight.

of unlabelled substrate (Fig. 3), whereas addition of CCCP resulted in a rapid efflux of PA. Furthermore, addition of PA analogs like POA, 2-OH-PA, 3-OH-PA, and phenylalanine at 50-fold-higher concentrations than PA did not affect the initial rate of PA uptake (data not shown). These results indicate that PA is taken up through a nonspecific diffusion process and that the accumulation of PA is determined mainly by the ΔpH across the plasma membrane.

Effect of PA on the ΔpH . To establish that PA is able to dissipate a ΔpH by passive diffusion, beef heart COVs were used (Fig. 4). The ionophore valinomycin was added to ensure that only a ΔpH was present in this model system. The internal pH was measured with the pH-sensitive fluorophore pyranine.

Upon addition of the electron donors ascorbate, TMPD, and cytochrome *c*, a ΔpH is rapidly generated in these COVs. Subsequent addition of increasing concentrations of PA to the COVs at pH 7 resulted in an initially fast decrease of pyranine fluorescence followed by a slower partial restoration of the fluorescence level (Fig. 5A). Influx of protonated PA therefore appears to be a fast process that results in acidification of the interior of the COVs. Equilibration of the protonated species across the membrane occurs rapidly, and the initial acidification is counteracted by the activity of cytochrome *c* oxidase, resulting in a new equilibrium at a lower internal pH. The decrease of the internal pH after addition of these weak acids is not caused by inhibition of the cytochrome *c* oxidase activity,

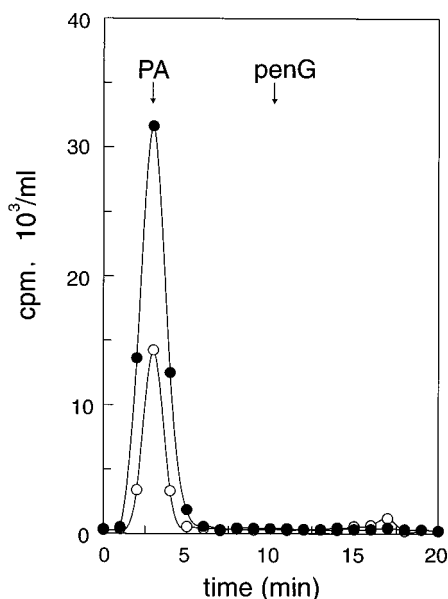


FIG. 2. Radioactivity in HPLC fractions from extracts of mycelial pellets. Pellets were obtained from mycelia incubated for 10 min with 63 μ M PA in the presence (●) or absence (○) of CCCP. Retention times of PA and penicillin G (pen G) are indicated. Approximately 90% of the radioactivity taken up was found in the PA peak. Radiolabelled penicillin was not detected in the extracts.

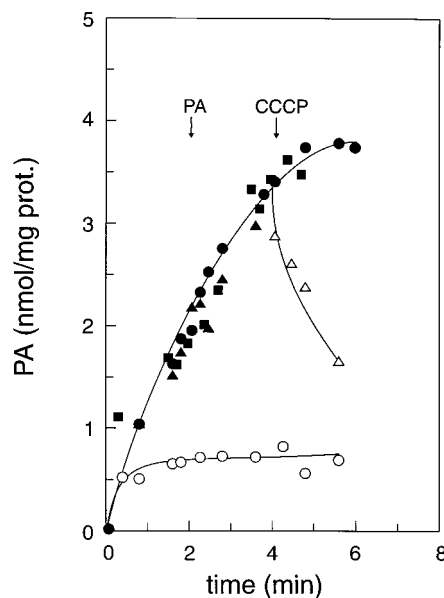


FIG. 3. Effect of the addition of the protonophore CCCP or nonradiolabelled PA on accumulation of radiolabelled PA. Nonradiolabelled PA was added at a 50-fold-higher concentration than radiolabelled PA at $t = 0$ (■) or $t = 5$ min (▲). CCCP (1 μ M) was added at $t = -5$ min (○) or $t = 10$ min (△). The [14 C]PA concentration used was 63 μ M.

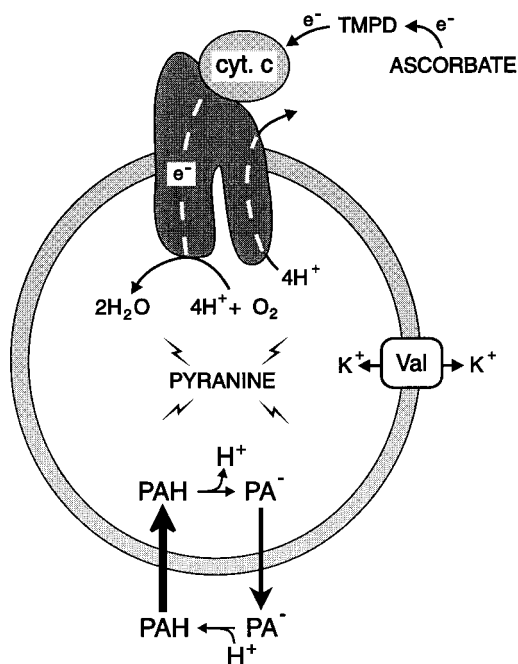


FIG. 4. Model system used to study dissipation of ΔpH by PA and acetic acid. Liposomes made of *E. coli* phospholipids and phosphatidylethanolamine from egg yolk (3:1, wt/wt) were reconstituted with beef heart cytochrome *c* (cyt. *c*) oxidase. Pyranine, a fluorescent pH indicator, was enclosed in the liposomes by freeze-thaw sonication. The ΔpH uncoupling PA flow is indicated. Valinomycin (Val) was added to prevent the formation of a $\Delta\psi$.

since up to 50 mM PA or acetic acid did not affect the rate of oxygen consumption by cytochrome *c* oxidase (data not shown).

The decrease of the steady-state internal pH after addition of PA or acetic acid to the COVs suggests that a constant net

influx of protonated acid takes place. Since the protonated acid equilibrates quickly across the membrane, a constant influx of this species can be sustained only when the deprotonated form fluxes out of the COVs at a similar rate. Under the conditions applied, this outward flow of deprotonated acid determines the extent to which the internal pH and thus the ΔpH are decreased by addition of these weak acids. As shown in Fig. 5B, PA has a stronger effect on the ΔpH than does acetic acid at the same external concentration. Addition of PA or acetic acid to pyranine-containing liposomes ($\text{pH}_{\text{in}} = \text{pH}_{\text{ext}} = 7$) results in a fast decrease of pyranine fluorescence and thus of the internal pH (Fig. 6) as a result of the influx of protonated PA or acetic acid. This process was studied by stopped-flow analysis. Under the conditions applied, equilibration of PA and acetic acid occurred very fast. Single exponential fits of the initial decay of pyranine fluorescence indicated that protonated PA diffuses across the liposomal membrane about 10 times faster than protonated acetic acid does (Fig. 6, inset). These studies clearly show that PA in its protonated form is able to pass a lipid bilayer rapidly by simple diffusion.

Effect of PA on growth and valine uptake. The toxicity of PA toward the fungal cell is suspected to result from dissipation of transmembrane pH gradients and lowering of the internal pH (12). It is therefore likely that lowering of the external pH increases the PA toxicity. This aspect was studied by growth experiments with the Wisconsin strain. Production media containing different PA concentrations were set at the appropriate pH value and inoculated from 45-h-old cultures growing logarithmically in production media of the same pH containing 3 mM PA. Growth was monitored for 10 h by measuring the increase in the amount of cellular protein. Growth rates of cultures without PA were arbitrarily set at 1. At decreasing external pH, the growth inhibition exerted by PA clearly increased (Fig. 7A).

Dissipation of the ΔpH across the plasma membrane by high external PA concentrations will result in inhibition of ΔpH -driven transport systems (16). Uptake of valine in *P. chrysog-*

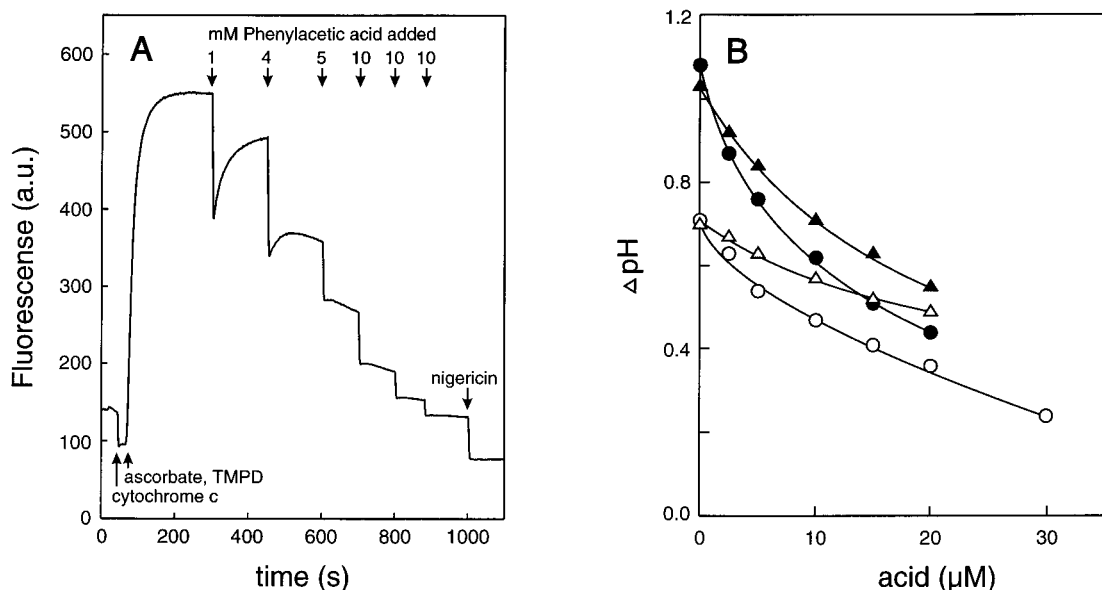


FIG. 5. Decrease of the ΔpH in COVs by the addition of PA or acetic acid. (A) Fluorescent trace of COVs loaded with pyranine, showing the effect of subsequent PA additions on the internal pH at an external pH of 7. (B) Effect of addition of PA (○, ●) or acetic acid (▽, ▼) on the ΔpH at an external pH of 7 (open symbols) or 6.4 (solid symbols).

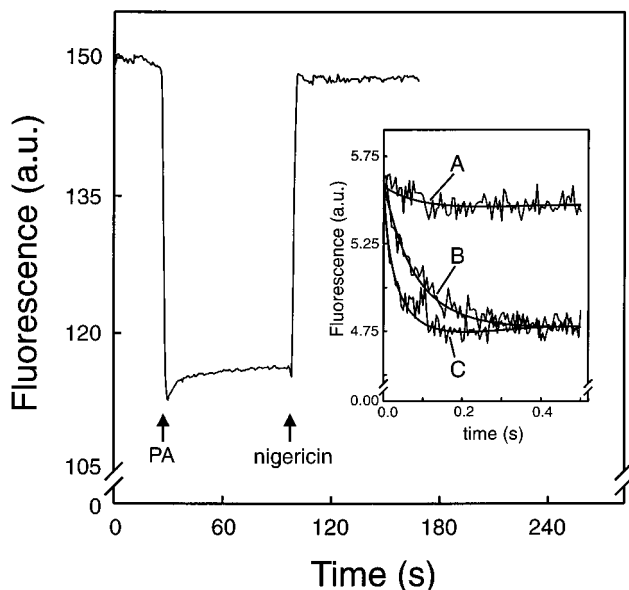


FIG. 6. Fluorescent trace of liposomes loaded with pyranine, showing the effect of subsequent addition of PA (5 mM) and nigericin (1 μ M) on the internal pH at an external pH of 7. Valinomycin (50 nM) was added to the liposomal suspension (2 mg of lipid per ml) 10 min before the start of the experiment. (Inset) Pyranine fluorescence traces of liposomes loaded with pyranine obtained by averaging 10 separate stopped-flow measurements. The different traces were obtained by mixing a liposomal suspension with buffer (trace A) and with buffer containing 5 mM acetic acid (trace B) or 5 mM PA (trace C).

num occurs in symport with one proton and is driven by both the Δ pH and the $\Delta\psi$ (14). Addition of 50 mM PA to a mycelial suspension resulted in a strong reduction in the steady-state level of valine uptake (Fig. 7B). Addition of acetic acid had a smaller effect on valine uptake. Initial rate studies at different

concentrations revealed that PA and acetic acid affected only the V_{max} , and not the K_m , of valine uptake, indicating that these compounds did not compete with valine for uptake (data not shown). Concentrations of up to 100 mM PA or acetic acid also had no effect on the activity of the plasma membrane ATPase in isolated plasma membranes (data not shown) (11). The observed effects of PA and acetic acid are thus not due to an effect on the activity of the primary proton pump. Therefore, dissipation of the transmembrane pH gradient by passive diffusion of PA and acetic acid is the most plausible explanation for the observed decrease in valine uptake.

Effect of the external PA concentration on penicillin production. An important question is whether the rate of PA influx can be a limiting factor in penicillin production. The relation between the external PA concentration and the rate of penicillin production by the P2 strain was determined by a simple filtration assay followed by analysis of the obtained filtrate by HPLC. At 25°C and pH 6.5, the production of penicillin was constant for at least 1 h (Fig. 8A). Under these conditions and at an external concentration of 3 mM, PA is taken up at an initial rate of at least 50 nmol/min/mg (dry weight) while the initial rate of penicillin production is about 0.20 nmol/min/mg (dry weight). The initial rate of penicillin production by the P2 strain depends on the external PA concentration in a Michaelis-Menten-like manner (Fig. 8B). The apparent K_m was approximately 200 μ M, and the V_{max} was 0.25 nmol/min/mg (dry weight). The observed dependency of penicillin production on the external PA concentration is probably due to a step in the internal conversion of PA, since influx of PA occurs much faster than the formation of penicillin. Thus influx of PA does not seem to determine the overall rate of penicillin biosynthesis in this strain.

DISCUSSION

Although PA uptake in *P. chrysogenum* has never been studied in detail, it was assumed to take place through passive

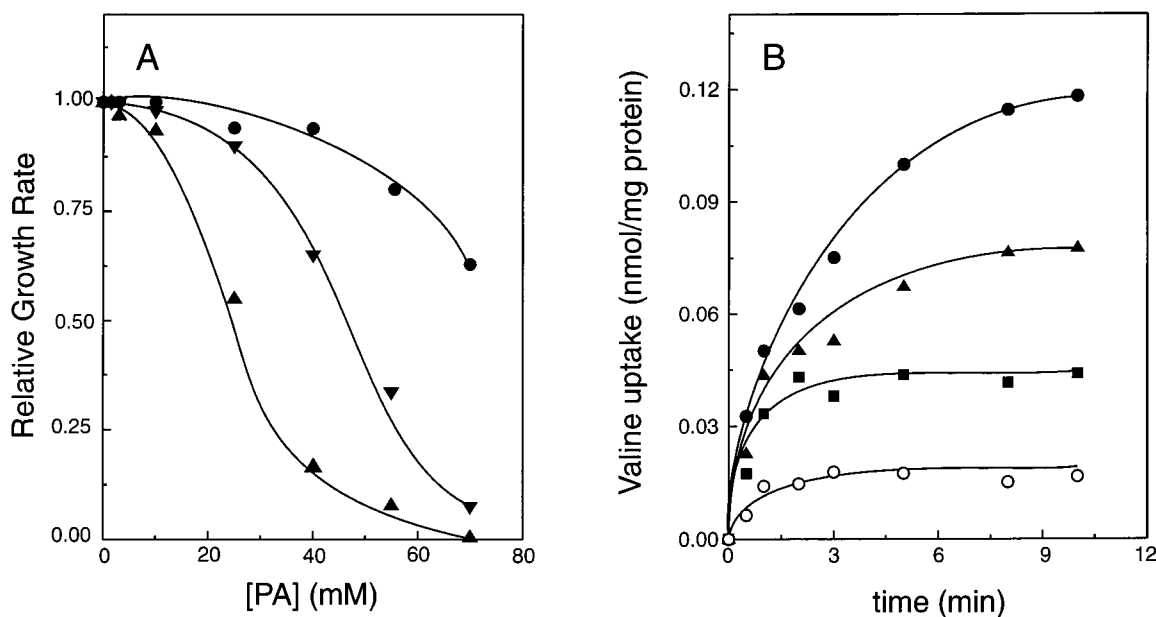


FIG. 7. Effect of PA on growth rate and valine uptake. (A) Inhibition of growth rate of *P. chrysogenum* Wisconsin 54-1255 by PA at different pH values of the culture medium: \bullet , pH 7; \blacktriangledown , pH 6.5; \blacktriangle , pH 6. Growth rates of cultures containing no PA were set at 1. (B) Effect of 50 mM PA or acetic acid on valine uptake at pH 6.5 in the Wisconsin 54-1255 strain: \bullet , no additions; \blacktriangle , 50 mM acetic acid; \blacksquare , 50 mM PA; \circ , 1 μ M CCCP. Mycelial suspensions were incubated for 5 min with the effector prior to the addition of valine.

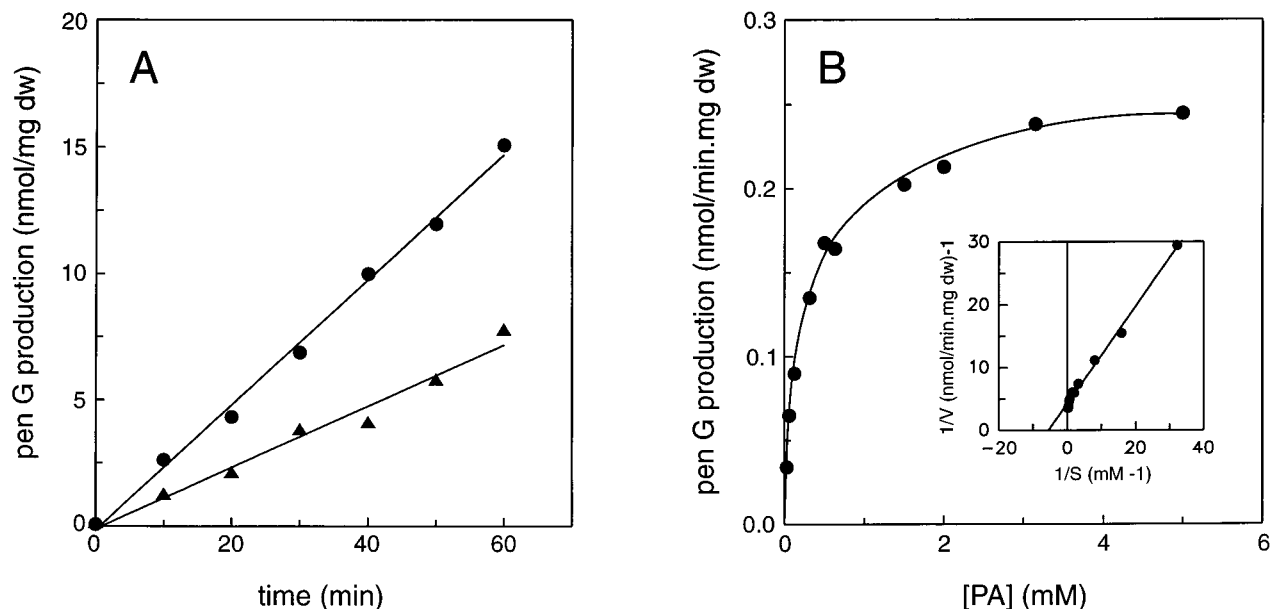


FIG. 8. Penicillin production by *P. chrysogenum* Panlabs P2. (A) Production of penicillin by mycelium grown in production medium containing 3 mM PA and harvested after 52 h (▲) or 72 h (●). (B) Dependency of penicillin production on the external PA concentration, with suspensions of mycelium harvested after 72 h. (Inset) Lineweaver-Burk plot showing an apparent K_m of 200 μ M and V_{max} of 0.25 nmol/min/mg (dry weight). pen G, penicillin G.

diffusion (12). However, recently it was suggested that a specific transport system is involved in PA uptake in penicillin-producing mycelium (9, 10). The presence of transport systems for monocarboxylic acids has been demonstrated for several cell types and organisms (2, 6). Therefore, the existence of a specific PA permease is not unlikely, and such a transport system could play an important role in the biosynthesis of penicillins by *P. chrysogenum*.

In contrast to the conclusions of Fernández-Cañón and coworkers (9, 10, 23), our results demonstrate that uptake of PA occurs through passive diffusion. This conclusion is based on the following observations. (i) The rate of PA uptake by mycelial suspensions does not saturate up to a concentration of 30 mM and does not show a clear saturable component, as would be expected if transport of PA were carrier mediated. (ii) The initial rates of PA uptake increased 10-fold when the external pH was lowered by 1 unit. (iii) Accumulation of PA depends strongly on the Δ pH across the plasma membrane. Dissipation of this Δ pH by CCCP results in a strongly reduced uptake and a fast efflux of accumulated PA. (iv) Addition of an excess of cold PA does not result in efflux of intracellular PA. However, since unidirectional transport systems in fungi have been reported (15), this observation in itself does not exclude the presence of a specific uptake system. Both strains used in these studies showed the same uptake characteristics for PA, although the P2 strain and the Wisconsin strain differ strongly in morphology and penicillin production capacity.

The differences between our observations and those made by Fernández-Cañón and coworkers (9, 10, 23) may have two reasons. First, the washing step in our transport assay is much faster and therefore reduces drastically the efflux of free PA during the assay. Second, we used radiolabelled PA with a low specific activity at a relatively high concentration to minimize effects of binding and metabolism. Metabolism and compartmentation of PA were avoided by determining initial uptake rates. The results reported by Fernández-Cañón and coworkers (9, 10, 23) might be affected by metabolism as a conse-

quence of the use of low concentrations of PA with a high specific activity. This would explain why regulation and induction of the "putative" PA transport system coincides with the regulation and induction of penicillin biosynthesis (10) and why strong inhibition of PA uptake by several analogs was observed (10, 30). The same compounds had no effect on PA uptake in our studies. When PA and POA are added in equimolar amounts to the medium, PA is preferentially utilized (8). That this is due to the existence of a specific POA permease which is repressed by PA seems unlikely. A more likely explanation is that the acetyl-CoA synthetase that activates PA and POA has a much higher affinity for PA or that the acyltransferase preferentially couples phenylacetyl-CoA to 6-aminopenicillanic acid. Studies with COVs and liposomes showed that PA in its protonated form can rapidly pass through bilayers. Although these studies did not allow calculations of the exact flux rate, they clearly indicate that the half time of PA equilibration across the membrane is very short. Furthermore, the rate at which PA fluxes across the liposomal membrane is about 10 times higher than for acetic acid. Acetic acid has a permeability coefficient of 6.9×10^{-3} cm s⁻¹ for egg phosphatidylcholine-decane bilayers (29). The composition of these artificial membranes is significantly different from that of the plasma membrane of *P. chrysogenum* (30). It is, however, unlikely that the permeability of the native plasma membrane toward PA is drastically different, since the effects of PA on growth and valine uptake are consistent with a fast passive-diffusion process. Our results indicate that the toxicity of PA results from lowering the internal pH and from dissipation of transmembrane pH gradients through passive diffusion. This leads to inhibition of processes like hyphal tip growth (11) and uptake of solutes.

At an external PA concentration of 3 mM, the rate of PA uptake is at least 200-fold higher than the penicillin production rate of the Panlabs P2 strain. This strain can produce up to 25 mM penicillin, while strains used in industry yield a penicillin titer of approximately 125 mM (4). The difference between the

rates of PA influx and penicillin production is large enough to ensure that PA uptake is not a rate-limiting step in the biosynthesis of penicillin by industrial strains used at present.

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REFERENCES

1. Arnstein, H. R. V., and P. T. Grant. 1956. The metabolism of the penicillia in relation to penicillin biosynthesis. *Bacteriol. Rev.* **20**:133–147.
2. Cássio, F., C. Leão, and N. van Uden. 1987. Transport of lactate and other short-chain monocarboxylates in the yeast *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **53**:509–513.
3. Cole, M. 1966. Microbial synthesis of penicillins. *Process Biochem.* **1**:334–338.
4. Crueger, W., and A. Crueger. 1992. *Biotechnology, a textbook of industrial microbiology*, p. 229–270. Sinauer Associates, Inc., Sunderland, Mass.
5. Demain, A. L. 1959. The mechanism of penicillin biosynthesis. *Adv. Appl. Microbiol.* **1**:23–47.
6. Deuticke, B. 1989. Monocarboxylate transport in red blood cells: kinetics and chemical modification. *Methods Enzymol.* **173**:300–329.
7. Driessen, A. J. M., W. de Vrij, and W. N. Konings. 1985. Incorporation of beef heart cytochrome c oxidase as a proton-motive force-generating mechanism in bacterial membrane vesicles. *Proc. Natl. Acad. Sci. USA* **82**:7555–7559.
8. Eriksen, S. H., B. Jensen, I. Schneider, S. Kaasgaard, and J. Olsen. 1994. Utilization of side-chain precursors for penicillin biosynthesis in a high-producing strain of *Penicillium chrysogenum*. *Appl. Microbiol. Biotechnol.* **40**:883–887.
9. Fernández-Cañón, J. M., A. Reglero, H. Martínez-Blanco, M. A. Ferrero, and J. M. Luengo. 1989. Phenylacetic acid transport system in *Penicillium chrysogenum* WIS 54-1255: molecular specificity of its induction. *J. Antibiot.* **42**:1410–1415.
10. Fernández-Cañón, J. M., A. Reglero, H. Martínez-Blanco, and J. M. Luengo. 1989. Uptake of phenylacetic acid by *Penicillium chrysogenum* WIS 54-1255: a critical regulatory point in benzylpenicillin biosynthesis. *J. Antibiot.* **42**:1398–1409.
11. Gibbon, B. C., and D. L. Kropf. 1994. Cytosolic pH gradients associated with tip growth. *Science* **263**:1419–1421.
12. Hersbach, G. J. M., C. P. van der Beek, and P. W. M. van Dijck. 1984. The penicillins: properties, biosynthesis, and fermentation. *Biotechnol. Ind. Antibiot.* **22**:45–140.
13. Hillenga, D. J., J. M. Versantvoort, A. J. M. Driessen, and W. N. Konings. 1994. Structural and functional properties of plasma membranes from the filamentous fungus *Penicillium chrysogenum*. *Eur. J. Biochem.* **224**:581–587.
14. Hillenga, D. J., J. M. Versantvoort, A. J. M. Driessen, and W. N. Konings. Unpublished data.
15. Horák, J. 1986. Amino acid transport in eucaryotic microorganisms. *Biochim. Biophys. Acta* **864**:223–256.
16. Hunter, D. R., and I. H. Segel. 1973. Effect of weak acids on amino acid transport by *Penicillium chrysogenum*: evidence for a proton or charge gradient as the driving force. *J. Bacteriol.* **113**:1184–1192.
17. King, T. E. 1967. The Keilin-Hartree heart muscle preparation. *Methods Enzymol.* **10**:202–208.
18. Lara, F., R. C. Mateos, C. Vázquez, and S. Sánchez. 1982. Induction of penicillin biosynthesis by L-glutamate in *Penicillium chrysogenum*. *Biochem. Biophys. Res. Commun.* **105**:172–178.
19. Lolkema, J. S., K. J. Hellingwerf, and W. N. Konings. 1982. The effect of 'probe binding' on the quantitative determination of the proton-motive force. *Biochim. Biophys. Acta* **681**:85–94.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
21. Martin, J. F., and P. Liras. 1989. Enzymes involved in penicillin, cephalosporin and sephamycin biosynthesis. *Adv. Biochem. Eng.-Biotechnol.* **39**:153–187.
22. Martínez-Blanco, H., A. Reglero, M. Fernández-Valverde, M. A. Ferrero, M. A. Moreno, M. A. Peñalva, and J. M. Luengo. 1992. Isolation and characterization of the acetyl-CoA synthetase from *Penicillium chrysogenum*. *J. Biol. Chem.* **267**:5474–5481.
23. Martínez-Blanco, H., A. Reglero, M. A. Ferrero, J. M. Fernández-Cañón, M. A. Luengo. 1989. Repression of phenylacetic acid transport system in *Penicillium chrysogenum* WIS 54-1255 by free amino acids and ammonium salts. *J. Antibiot.* **42**:1416–1423.
24. Müller, W. H., R. A. L. Bovenberg, M. H. Groothuis, F. Kattavilder, E. B. Smaal, L. H. M. van der Voort, and A. J. Verkleij. 1992. Involvement of microbodies in penicillin biosynthesis. *Biochim. Biophys. Acta* **1116**:210–213.
25. Müller, W. H., T. P. van der Krift, A. J. J. Krouwer, H. A. B. Wösten, L. H. M. van der Voort, E. B. Smaal, and A. J. Verkleij. 1991. Localization of the pathway of penicillin biosynthesis in *Penicillium chrysogenum*. *EMBO J.* **10**:489–495.
26. Nüesch, J., J. Heim, and H.-J. Treichler. 1987. The biosynthesis of sulfur-containing β -lactam antibiotics. *Annu. Rev. Microbiol.* **41**:51–75.
27. Queener, S., and N. Neuss. 1982. The biosynthesis of β -lactam antibiotics, p. 1–810. In R. B. Morrin and M. Morgan (ed.), *The chemistry and biology of β -lactam antibiotics*, vol. 3. Academic Press, Inc., New York.
28. Sanders, D., and C. L. Slayman. 1982. Control of intracellular pH. Predominant role of oxidative metabolism on proton transport in the eucaryotic microorganism *Neurospora*. *J. Gen. Physiol.* **80**:377–402.
29. Tan, I. K. P., J. M. Fernández-Cañón, A. Reglero, and J. M. Luengo. 1993. Effect of analogues of phenylacetic acid (PA) on the PA transport system in *Penicillium chrysogenum* strains H1107 and M223. *Appl. Microbiol. Biotechnol.* **40**:113–116.
30. Terada, H., and Y. Sakabe. 1985. Studies on residual antibacterials in foods. Simultaneous determination of penicillin G, penicillin V and ampicillin in milk by high-performance liquid chromatography. *J. Chromatogr.* **348**:379–381.
31. Walter, A., and J. Gutknecht. 1986. Permeability of small nonelectrolytes through bilayer membranes. *J. Membr. Biol.* **90**:207–217.
32. Yu, C. A., L. Yu, and T. E. King. 1975. Studies on cytochrome c oxidase. *J. Biol. Chem.* **250**:1383–1392.