Chapter 5

Penicillium chrysogenum takes up the penicillin G precursor phenylacetic acid by passive diffusion


SUMMARY

Penicillium chrysogenum is used in the industrial production of penicillin G. During the production stage, the side-chain precursor phenylacetic acid is fed in small amounts to the medium. P. chrysogenum takes up phenylacetic acid and couples it intracellularly to 6-aminopenicillanic acid rendering penicillin G. The process by which phenylacetic acid passes the plasma membrane was studied in mycelia and liposomes. Uptake of phenylacetic acid in mycelium was non-saturable and the initial velocity of uptake increased logarithmically with decreasing external pH. Studies with liposomes demonstrated a fast passive flux of the protonated species. This indicates that phenylacetic acid passes membranes via passive diffusion of the protonated species. At an external concentration of 3 mM, the rate of phenylacetic acid uptake is at least 250-fold higher than the penicillin production rate in the Panlabs P2 strain. In this strain, uptake of phenylacetic acid is not a rate limiting step in penicillin G production.
**INTRODUCTION**

*P. chrysogenum* is a filamentous fungus, used for commercial production of penicillins. It is capable of synthesizing penicillins with specific hydrophobic side-chains when the appropriate precursor is fed to the production medium [14, 65, 73, 125]. Phenylacetic acid (PA) appears to be the best precursor, but the phenyl group can be substituted by other ring systems [14]. Since benzoic acid derivatives cannot be utilized, and only mono-substituted acetic acids are incorporated, an α-methylene group seems to be essential. The effectiveness of the side-chain precursor appears to depend on its toxicity and its resistance to oxidation by *P. chrysogenum*. In commercial processes, only PA, phenoxyacetic acid (POA), and allylmercapto-acetic acid have been used to produce penicillin G, penicillin V and penicillin O, respectively [65]. In the absence of an exogenous side chain precursor, *P. chrysogenum* produces mainly 6-aminopenicillanic acid and some isopenicillin N [63, 186]. Under these conditions, mono-substituted acetic acids present at low intracellular concentrations, are used to form small amounts of penicillins like benzylpenicillin, 2-pentenylpenicillin, n-amylpenicillin, n-heptylpenicillin and *p*-[3,4-hydroxybenzylpenicillin [186].

Incorporation of the side-chain precursor, carried out by the acyl CoA: isopenicillin N acyltransferase (AT), is the last step in the penicillin biosynthetic pathway [186, 214]. AT converts isopenicillin N into penicillin G by exchange of the α-amino acid moiety for PA. Prior to this transacylation, PA has to be activated to phenylacyl-CoA. Recent studies suggest that this activation takes place by a general acetyl-CoA synthetase (ACS) which is involved in the primary metabolism of *P. chrysogenum* [191]. Electron microscopic studies with specific antibodies demonstrated that AT is present solely in specific microbodies [204, 205, 206]. Furthermore, AT contains a typical peroxisomal targeting sequence (alanine-arginine-leucine) at the C-terminus. AT lacking this targeting signal was found to be localized in the cytosol or small vacuoles, and transformants containing this mutant AT could not produce penicillin [Müller et al., 1992]. The acetyl-CoA synthetase seems to be an intracellular enzyme, yet its exact location is unknown. An intracellular localization of the acetyl-CoA synthetase and AT implies that PA is taken up by *P. chrysogenum* to synthesize penicillin G.

Uptake of PA by *P. chrysogenum* can either take place through simple diffusion or via a specific transport system. PA is a weak acid and the undissociated species might rapidly diffuse across lipid bilayers [125, 300]. Fernández-Cañón and coworkers [100, 101, 189, 276] 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 PA values, i.e. pH 5 to 8. To establish the presence of the PA
transport system and to characterize it in detail, the uptake of PA in mycelial suspensions was studied extensively. Mycelial suspensions, liposomes and cytochrome c-oxidase containing liposomes were used to examine the passive influx of PA. The data obtained from these studies suggest that the main PA flux across the plasma membrane results from passive diffusion rather than a specific transport process. 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 faster than the overall rate of penicillin production by \textit{P. chrysogenum} P2. From these observations, it was concluded that the influx of PA is not a rate-limiting factor in the production of penicillin.

**EXPERIMENTAL PROCEDURES**

**Organisms and culture conditions**

\textit{P. chrysogenum} strains Wisconsin 54-1255 and Panlabs P2 (kindly supplied by Gist-brocades NV) were grown on production medium (pH 6.3) as described by Lara \textit{et al.} (1982) supplemented with 10 mM glutamate and 10 \% (mass/vol.) glucose. Cultures were incubated for approximately 70 h in a rotary shaker at 200 rpm and 25 °C. The Wisconsin 54-1255 strain was previously cultured for 24 h on production medium, with the omission of phenylacetic acid and lactose, containing 16\% (mass/vol.) glucose. The P2 strain was previously cultured on YPG medium [1 \% (mass/vol.) Yeast extract, 2 \% (mass/vol.) Peptone and 2 \% (mass/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 (1967). Cytochrome-c oxidase was isolated from these mitochondria as described by Yu \textit{et al.} (1975), suspended in 50 mM sodium phosphate (pH 7.5) containing 1.5 \% (mass/vol.) cholic acid and stored in liquid nitrogen. Cytochrome-c oxidase was reconstituted in liposomes, composed of 75 \% (by mass) acetone/ether washed \textit{Escherichia coli} lipids and 25 \% (by mass) Egg Yolk L-phosphatidylcholine, at a protein/lipid ratio of 0.16 nmol heme a/mg lipid [82].

**Electrical and pH gradients across the membrane**

The transmembrane electrical potential (\(\Delta \psi\), interior negative) was calculated from the distribution of the tetraphenylphosphonium ion (TPP\(^+\)), assuming concentration dependent binding to the membranes as described [178]. The external
concentration of TPP⁺ was determined with a TPP⁺-selective electrode. Cytochrome-c oxidase vesicles (corresponding to 0.23 nmol cytochrome-c oxidase) were added to 50 mM potassium phosphate of the indicated pH, containing 5 mM MgSO₄ and 2 µM TPP⁺. A proton-motive force (Δp) was generated by the addition of ascorbate (10 mM, adjusted to the desired pH), N,N,N',N'-tetramethyl-p-phenylenediamine (Ph(NMe₂)₂; 200 µM) and horse heart cytochrome c (20 µM). When indicated, the ionophores nigericin and valinomycin were used at concentrations of 10 nM and 100 nM, respectively. The pH gradient across the membrane (ΔpH, interior alkaline) was determined from the fluorescence of pyranine (excitation, 450 nm; emission, 508 nm), measured with a Perkin Elmer LS50B luminescence spectrophotometer. Pyranine (100 µM) was entrapped in proteoliposomes by freeze-thaw-extrusion [82]. External pyranine was removed with a Sephadex G-25 column (coarse, 1/20 cm). Valinomycin was added to a final concentration of 50 nM. A Δp was generated by addition of the electrondonor system as described for Δψ-measurements. When indicated, nigericin was added to a final concentration of 1 µM.

**Stopped-flow measurements**

Rates of influx of protonated acid were determined from the decrease in pyranine fluorescence using a Milliflow stopped-flow reactor and an Aminco SLM 4800C time-resolved fluorescence spectrophotometer. Pyranine (100µM) was entrapped in liposomes composed of 75 % (by mass) aceton/ether washed E. coli lipids and 25 % (by mass) Egg Yolk L-phosphatidylcholine that were prepared by extrusion through polycarbonate filters (Avestin) with pore sizes of 400 and 200 nm using a small-volume extrusion apparatus (Avestin Inc., Ottawa, Canada) [184]. Liposomes were diluted to a final concentration of 2 mg lipid per ml., 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**

Oxygen 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 Δψ-measurements. Endogenous oxygen consumption was determined after addition of KCN (1 mM).

**Penicillin G and V concentrations**

Penicillin G concentrations were determined by reversed phase high-performance liquid chromatography (HPLC) as described by Terada and Sakabe (1985)
using penicillin V as an internal standard. Penicillins were separated with a stainless steel Nucleosil C18 column (5 µM; 250 x 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 the column temperature was maintained at 25 °C. 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 % (w/v) NaCl, and resuspended to a density of 15-20 mg dw/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 the addition of the desired amount of PA. At different time intervals, samples of 1 ml were withdrawn and filtrated (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 the applied conditions the penicillin production rate was constant for at least one hour.

**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/ml (P2) or 6 mg/ml (Wisconsin-54-1255) (dry masses), and stored on ice until further use. Initial rates of uptake were determined in triplicate, 5, 10 and 20 s after addition of the radiolabeled PA or valine to the mycelial suspension. To lower aspecific binding and reduce the effect of metabolism high concentrations of PA (>60 µM) with a relatively low specific activity were used. Aspecific binding was measured using mycelia permeabilized by treatment with 2 % (vol./vol.) toluene for 2 h at 25 °C. L-[U-14C]-phenylacetic acid (Sigma, 3.4 Ci/mol), or L-[U-14C]-valine (Amersham, 28 Ci/mol), were added to mycelial suspensions to a final concentration of 63 and 30 µM, respectively. Samples of 0.5 ml, were taken at given time intervals, added to 2 ml ice cold 0.1 M LiCl, and filtered immediately (paper filters, grade 520b; Schleicher & Schüll). Filters were washed once with 2 ml ice cold 0.1 M LiCl, and the radioactivity was determined with a liquid scintillation counter (Packard Tri-Carb 460 CD; Packard Instruments Corp.). Non-
energized uptake was measured using mycelial suspension pre-incubated with the protonophore carbamoyl-cyanide-\(m\)-chloro-phenylhydrazone (10 µM) for 5 min at 25 °C. Kinetic data were analyzed with the GraFit program (Erithacus Software Ltd.).

**Intracellular pools by HPLC**

Cell pellets obtained as described for transport assays were immediately frozen in liquid N\(_2\). 250 µl ice-cold chloroform/methanol (1:2; v/v) was added and the pellets were stored overnight at -20 °C. By this procedure more than 95 % of the labeled material was extracted. After centrifugation in an Eppendorf type centrifuge, supernatants were fractionated by HPLC as described above. Intracellular pools of radiolabeled PA, penicillins and derivatives were determined with a liquid scintillation counter (Packard Tri-Carb 460 CD; Packard Instruments Corp.).

**Other methods**

Protein concentrations were determined in the presence of 0.5 % (wt/vol) SDS using a modified Lowry assay [289]. Bovine serum albumin was used as a standard.

**RESULTS**

**Uptake of phenylacetic acid in *P.chrysogenum***

When a specific enzyme is involved in the transport of phenylacetic acid (PA), uptake of this solute should show saturation kinetics. Therefore, the initial rates of PA uptake in the *P.chrysogenum* strains P2 and Wisconsin were determined at different external concentrations (Fig. 5.1A). Although the Wisconsin and P2 strains significantly differ in morphology and penicillin production capacity, the uptake of PA showed similar kinetics (Fig. 5.1). The initial rate of PA uptake increased linearly up to an external PA concentration of 30 mM, indicating that uptake occurs via a non-saturable 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 examined. At an external PA concentration of 63 µM, the initial rate of PA uptake increased logarithmically with decreasing external pH (Fig. 5.1B). The increase was about 10-fold per pH unit and no pH optimum was found within the pH range studied. This increase is proportional to the increase in concentration of protonated PA, which suggest that PA is taken up via passive diffusion of the protonated species.
Under normal physiological conditions the cytosolic pH is higher than the extracellular pH [245], consequently PA dissociates intracellularly to a higher extent and will accumulate in the cell. If metabolic conversion is slow compared to the rate of influx, the pH difference between cytosol and medium will be the main factor determining PA accumulation. Steady state levels of PA accumulation were reached within 10 min after addition of radiolabeled PA to mycelial suspensions (Fig. 5.2B). The steady state level of PA accumulation showed to be highest at pH 5.5, and decreased severely above an external pH of 7 (Fig. 5.1C). These results point to a major role of the ΔpH in PA accumulation. A straightforward interpretation of observed relationship is not feasible since both the ΔpH [245] and the concentration of the protonated species decrease with increasing external pH.

Since metabolism of PA might interfere with uptake, the composition of intracellular pools of radioactively labeled compounds was analyzed after extraction and fractionation by HPLC. After a 10-min incubation with 63 µM PA, PA made up at least 90% of the internalized label (Fig. 5.2A). Pre-incubation of mycelium with the protonophore CF$_3$OPh$_2$(CN)$_2$ to collapse the proton-motive force (Δ$p$) resulted in a four-fold lower accumulation PA. Thus 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 50-fold excess of unlabeled PA (Fig. 5.2B), whereas addition of CF$_3$OPh$_2$(CN)$_2$ resulted in a rapid efflux. Addition of a 50-fold excess of PA analogues like phenoxyacetic acid (POA), 2-OH-phenylacetic acid,
Fig. 5.2 Phenylacetic acid uptake and efflux. A. Radioactivity in HPLC fractions from extracts of mycelial pellets. Pellets were obtained from mycelia incubated 10 min with 63 µM PA in the presence (○) or absence (●) of CF$_3$OPh$_3$(CN)$_2$. Retention times of PA and penicillin G are indicated. Approximately 90% of the radioactivity taken up was found in the PA peak. Radiolabeled penicillin was not detected in the extracts. B. Effect of the addition of the protonophore CF$_3$OPh$_3$(CN)$_2$ or non-radiolabeled PA on accumulation of radiolabeled PA. Non-radiolabeled PA was added at an 50-fold higher concentration than radiolabeled PA at t=0 ( ) or t=5 min (▲). CF$_3$OPh$_3$(CN)$_2$ (1µM) was added at t=-5 min (○) or t=10 min (△). The $[^{14}$C]PA concentration used was 63 µM.

3-OH-phenylacetic acid and phenylalanine did not affect the initial rate of PA uptake (data not shown). These results support the view that PA is taken up through a non-specific diffusion process and accumulation of PA is determined mainly by the ΔpH across the plasma membrane.

Effect of PA on the ΔpH

To establish that PA can dissipate a ΔpH by passive diffusion, beef heart cytochrome-c oxidase vesicles were used (Fig. 5.3). The ionophore valinomycin was added to ensure that only a ΔpH was present. The internal pH was measured with the pH-sensitive fluorophore pyranine. Upon addition of the electron donor system ascorbate-Ph(NMe$_2$)$_2$-cytochrome c, a ΔpH is generated rapidly in these cytochrome-c oxidase vesicles. Subsequent addition of PA to the Cytochrome-c oxidase vesicles at pH 7 resulted in a fast initial decrease of pyranine fluorescence, followed by a partial restoration of the fluorescence level (Fig. 5.4A). This implies that influx of protonated
PA is a fast process which results in acidification of the interior of the cytochrome-c oxidase vesicles. 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 PA or acetic acid did not result from inhibition of the cytochrome-c oxidase activity. Up to 50 mM PA or acetic acid did not effect 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 cytochrome-c oxidase vesicles suggest a constant net influx of protonated acid. As the protonated acid equilibrates fast across the membrane a constant influx of this species can only be sustained by the efflux of an equivalent amount of deprotonated acid. This outward flux of deprotonated acid determines the extent to which the internal pH and thus the ΔpH are reduced. As shown in Fig. 4B, at the same external concentration PA has a stronger effect on the ΔpH than does acetic acid. Addition of PA or acetic acid to pyranine containing liposomes (pH$_{in}$ = pH$_{ext}$ = 7) results in a fast decrease of pyranine fluorescence and thus of the internal pH (Fig. 5.5) due to the influx of protonated PA or acetic acid. This process was studied by stopped-flow analysis. Equilibration of PA and acetic acid occurred very fast, and single exponential fits of the initial decay of pyranine fluorescence indicated that protonated PA diffuses about 10 times faster across the liposomal membrane than protonated acetic acid does (Fig. 5.5, inset).
Fig. 5.4 Decrease of the ΔpH in cytochrome-c oxidase vesicles by the addition of PA or acetic acid. Panel A: Fluorescent trace of cytochrome-c oxidase vesicles loaded with pyranine showing the effect of subsequent PA additions on the internal pH at an external pH of 7. Panel B: effect of addition of PA (○, ▲) or acetic acid (∆, △) on the ΔpH at an external pH of 7 (open symbols) or 6.4 (closed symbols).

Fig. 5.5 Fluorescent trace of liposomes loaded with pyranine, showing the effect of subsequent addition of PA (5 mM) and nigericin (1 µM) on the internal pH at an external pH of 7. Valinomycin (50 nM) was added to the liposomal suspension (2 mg lipid/ml) 10 min before the start of the experiment. Inset: pyranine fluorescence traces of liposomes obtained by averaging ten separate stopped-flow measurements. The different traces were obtained by mixing a liposomal suspension with buffer (trace A) and with buffer containing acetic acid (5 mM, trace B) or PA (5 mM, trace C).
studies 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 towards the fungal cell is suspected to result from dissipation of transmembrane pH gradients and lowering of the internal pH [125]. It is therefore likely that lowering of the external pH increases the toxicity of PA. This was examined by growth experiments with the Wisconsin 54-1255 strain. Production media containing different PA concentrations were set at the appropriate pH value and inoculated with mycelium obtained 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 one. At decreasing external pH inhibition of growth exerted by PA increased significantly (Fig. 5.6A).

Dissipation of the ΔpH across the plasma membrane by high external PA concentrations will result in an inhibition of ΔpH driven transport systems [137]. Uptake of valine in *P. chrysogenum* occurs in symport with one proton and is driven by both the ΔpH and the ΔΨ [130]. Addition of 50 mM PA to a mycelial suspension resulted in a significant reduction of the steady-state level of valine uptake (Fig. 5.6B). Addition of acetic acid had a smaller effect on valine uptake. Initial rate studies at different concentrations revealed that PA and acetic acid only affected the $V_{\text{max}}$, and not the $K_m$ of valine uptake indicating that these compounds did not compete with valine for uptake (data not shown). Concentrations 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). The observed effects of PA and acetic acid are not due 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 issue in penicillin production is whether the rate of PA influx can be a limiting factor. 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 one h (Fig. 5.7A). 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 mass) while the initial rate of penicillin production is about 0.20 nmol/min.mg (dry mass). The initial rate of penicillin
Fig. 5.6 Effect of PA on growth rate and valine uptake. Panel A: Inhibition of growth rate of the *P.chrysogenum* strain Wisconsin 54-1255 by PA at different pH values of the culture medium: ●, pH 7; ▲, pH 6.5; and ■, pH 6. Growth rates of cultures containing no PA were set at 1. Panel B: Effect of 50 mM PA or acetic acid on valine uptake at pH 6.5 in the Wisconsin 54-1255 strain: ●, no additions; ▲, 50 mM acetic acid; ■, 50 mM PA; and ○, 1 µM CF$_3$OPh$_2$(CN)$_2$. Mycelial suspensions were incubated 5 min with the effector prior to the addition of valine.

Fig. 5.7 Penicillin production by the *P.chrysogenum* Panlabs P2 strain. Panel A: Production of penicillin by mycelium grown in production medium containing 3 mM PA and harvested after 52 h (▲) or 72 h (●). Panel B: Dependency of penicillin production on the external PA concentration using suspensions of mycelium harvested after 72 h. Inset: Lineweaver-Burk plot showing an apparent $K_m$ of 200 µM and $V_{max}$ of 0.25 nmol/min.mg d.w.
production by the P2 strain depends on the external PA concentration in a Michaelis-Menten-like manner (Fig.5.7B). The apparent $K_m$ was approximately 200 µM and the $V_{\text{max}}$ was 0.25 nmol/min.mg (dry mass). Since influx of PA occurs much faster than the formation of penicillin, the observed dependency of penicillin production on the external PA concentration is probably due to a step in the conversion of PA. Clearly the influx of PA does not determine the overall rate of penicillin biosynthesis in this strain.

**DISCUSSION**

Although the uptake of PA in *P. chrysogenum* had never been studied in detail, it was commonly assumed to take place through passive diffusion [125]. Recently it was suggested that a specific transport system is involved in PA uptake in penicillin-producing mycelium [100, 101]. Since transport systems for monocarboxilic acids are found in several cell types and organisms [54, 77], 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 [100, 101, 189, 276], 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 in mycelial suspensions does not saturate up to a concentration of 30 mM and does not show a clear saturable component, as would be expected when transport of PA were carrier mediated; (ii) the initial rates of PA uptake increased 10-fold upon lowering the external pH by one unit; (iii) accumulation of PA depends clearly on the $\Delta$pH across the plasma membrane. Dissipation of this $\Delta$pH by CF$_3$OPh$_2$(CN)$_2$ results in a significantly reduced uptake and a fast efflux of accumulated PA; (iv) addition of an excess of unlabeled PA does not result in efflux of intracellular PA. Since uni-directional transport systems in fungi have been reported [132], the latter observation in itself does not exclude the presence of a specific PA uptake system. Both strains used in these studies showed the same uptake characteristics for PA, although the P2 strain and the Wisconsin 54-1255 strain differ strongly in morphology and penicillin production capacity.

The differences between our observations and those made by Fernández-Cañón and coworkers [100, 101, 189, 276] may have two main reasons. First, the washing step in our transport assay is considerable faster which reduces drastically the release of free PA through efflux. Second, we used radiolabeled PA with a low specific activity at a relatively high concentration to minimize effects of binding and metabolism. Interference of metabolism and compartmentalization with PA uptake were avoided by
determining initial uptake rates. As a consequence of using low concentrations of PA with a high specific activity, results reported by Fernández-Cañón and coworkers [100, 101, 189, 276] might be affected by metabolism. This would explain why regulation and induction of the “putative” PA transport system coincides with the regulation and induction of penicillin biosynthesis [100] and why inhibition of PA uptake by several analogues was observed [101, 276], whereas 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 [92]. That this is due to the existence of a specific POA permease which is repressed by PA seems unlikely. A more plausible 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-aminopenicilllic acid.

Studies in cytochrome-c oxidase vesicles and liposomes showed that PA in its protonated form rapidly passes lipid 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 ten times faster than acetic acid, which has a permeability coefficient of $6.9 \times 10^{-3}$ cm sec$^{-1}$ for egg PC-decane bilayers [300]. The composition of these artificial membranes is significantly different from that of P. chrysogenum plasma membranes [127]. It is, however, unlikely that the permeability of the native plasma membrane towards 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 dissipation of transmembrane pH gradients through passive diffusion. This leads to inhibition of processes like hyphal tipgrowth [113] and uptake of solutes. At an external PA concentration of 3 mM the rate of PA uptake is at least 250-fold faster than the penicillin production rate of the Panlabs P2 strain. This strain can produce up to 25 mM penicillin while strains presently used in industry are supposed to produce final penicillin yields of at least 125 mM [65]. 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 production of penicillin G by industrial strains used at present.

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