# I. UPTAKE OF PHENYLACETIC ACID BY *PENICILLIUM CHRYSOGENUM* WIS 54-1255: A CRITICAL REGULATORY POINT IN BENZYLPENICILLIN BIOSYNTHESIS

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(Received for publication April 17, 1989)

The transport system of phenylacetic acid (PA) in *Penicillium chrysogenum* was studied. Kinetic measurements were carried out "*in vivo*" at 25°C in 0.06 M phosphate buffer at pH 6.5. Uptake was a linear function of time over 3 minutes and the *Km* was 5.2 $\mu$M. PA uptake was inhibited by 2,4-dinitrophenol, 4-nitrophenol, sodium azide, potassium cyanide, *N*-ethylmaleimide, amino acids, xylose and fatty acids whereas lactose and ribose stimulated it. Benzylpenicillin, phenoxymethylpenicillin, penicillins DF, K and 6-aminopenicillanic acid did not modify uptake whereas phenoxyacetic acid and many phenyl derivatives strongly inhibited the incorporation of PA.

PA transport is an inducible system that is strictly regulated by the carbon source used for *P. chrysogenum* growth. Uptake is not induced by phenoxyacetic acid and is repressed by L-lysine.

The absence of the PA transport system when *P. chrysogenum* is grown in the presence of readily metabolized sugars and its repression by L-lysine suggests that this is a critical regulatory point in the control of benzylpenicillin biosynthesis.

The last step in penicillin biosynthesis by *Penicillium chrysogenum* involves exchange of the $\alpha$-aminoadipic moiety, present in the isopenicillin N molecule, by other side chain precursors (phenylacetic acid (PA), phenoxyacetic acid (POA), or $C_6 \sim C_8$ fatty acids) to generate different penicillins (benzyl-, phenoxymethyl-, F, DF and K) (Fig. 1)[1~3]. Although the benzylpenicillin biosynthetic pathway has received considerable attention[4,5], very little is known about the mechanisms through which side chain precursors may be incorporated by *P. chrysogenum*[6]. Some of these precursors (hexanoic, heptanoic and octanoic acids) can be obtained from an endogenous origin (fatty acid biosynthesis or by fatty acid $\beta$-oxidation) whereas others, such as PA and POA, must be taken up from the fermentation broths. Thus, when industrial fermentation takes place in PA/POA-limiting conditions, the final titers of benzylpenicillin or phenoxymethylpenicillin are greatly decreased whereas 6-aminopenicillanic acid (6-APA) and natural penicillins are produced efficiently.

Knowledge of the mechanisms involved in the control of the uptake of these side chain precursors is important not only because it should help to clarify the biosynthetic pathway of penicillins but also for economic reasons.

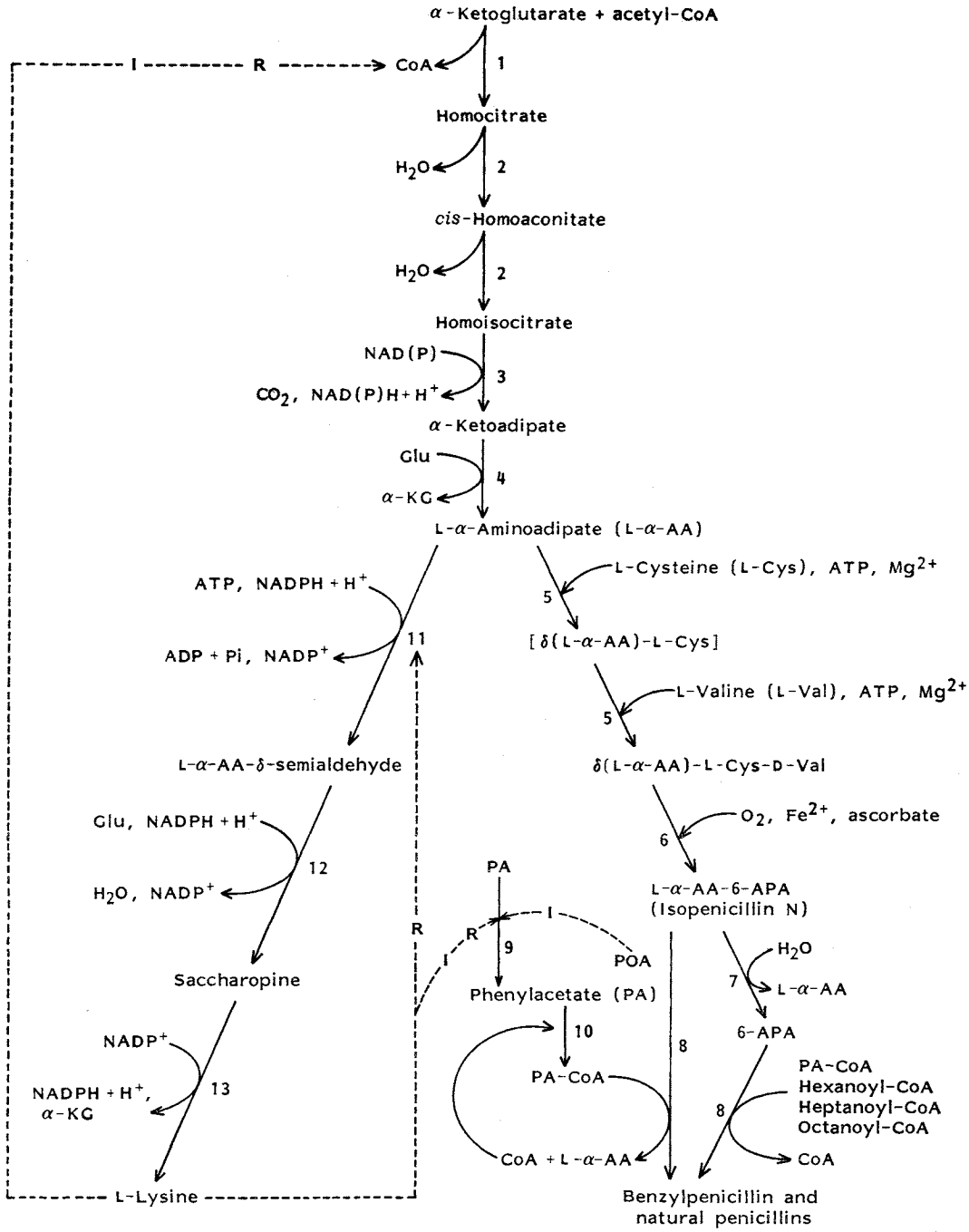
In this report we describe for the first time a biochemical study of the PA transport system in *P. chrysogenum* and evidence concerning its "*in vivo*" regulation is offered.

## Experimental

### Chemicals

Phenoxymethylpenicillin (1,520 U/mg) was from Marsing and Co., Ltd. (Denmark). Penicillin K

Fig. 1. Postulated branched pathway to L-lysine and benzylpenicillin biosynthesis in *Penicillium chrysogenum*.




I: Inhibition, R: repression, 1: homocitrate synthase, 2: homoaconitase (homoaconitate hydratase), 3: isocitrate dehydrogenase, 4: keto-adipate-glutamate-transaminase, 5: L-α-aminoadipyl-L-cysteinyl-D-valine synthetase (ACV synthetase), 6: isopenicillin N synthase (ACV cyclase), 7: isopenicillin N amidolyase, 8: acyl-CoA: 6-APA acyltransferase (acyl-CoA: isopenicillin N acyl-transferase); enzymatic activities 7 and 8 may be associated "*in vivo*" in a single functional complex (acyl-CoA: isopenicillin N acyltransferase, so-called transacylase), 9: PA transport system, 10: phenylacetyl-CoA ligase, 11: α-aminoadipate semialdehyde dehydrogenase, 12: saccharopine dehydrogenase (glutamate-forming), 13: lysine-α-ketoglutarate reductase (lysine forming saccharopine dehydrogenase).

(2,543 U/mg) and penicillin DF (1,667 U/mg) were synthesized by us[3]. Isopenicillin N was a gift from Antibioticos, S.A. (Madrid, España). δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV) was purchased from Incell Corporation (U.S.A.). Carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) was obtained from DuPont (U.S.A.). [1-$^{14}$C]PA (24 mCi/mmol) was purchased from CEA (France). L-[*U*-$^{14}$C]-Lysine monohydrochloride (270 mCi/mmol) was supplied by The Radiochemical Centre, Amersham (UK). [1-$^{14}$C]POA (10 mCi/mmol) was obtained from ICN (U.S.A.).

All the other chemicals used were of analytical quality.

### Microorganisms

*P. chrysogenum* Wis 54-1255 (ATCC 28089), *Micrococcus luteus* ATCC 9341 and *Bacillus subtilis* ATCC 6633 were obtained from the American Type Culture Collection. The strains were kept lyophilized.

### Culture Media and Growth Conditions

*P. chrysogenum* Wis 54-1255 was maintained in sporulation medium with the following composition (g/liter); Malt extract 20, Bacto-peptone 1, glucose 20, Bacto-agar 20. Incubations were carried out on slants of 3×20 cm containing 25 ml of medium at 25°C for 7 days. Spores from the slants were collected by adding 10 ml of sterile saline solution and the suspension was filtered through a glass fiber filter. The final spore suspension containing $10^8$ spores/ml was used to inoculate liquid medium. Each 250-ml Erlenmeyer flask containing 30 ml of growth medium (see below) was seeded with 1.0 ml of the spore suspension and incubated on a rotary shaker at 250 rpm and 25°C for 50 hours or the time specified in each set of experiments. The liquid medium contained (g/liter): Corn-steep solids 30, lactose 30, PA 1, distilled water, to 1 liter. pH was adjusted to 6.5 with NaOH (30% w/v) before sterilization and later 10 g of $CaCO_3$ were added. When required, PA was replaced by POA keeping the final concentration constant.

### Analytical Determinations

Benzylpenicillin determinations in broth were carried out by bioassay against *B. subtilis* and *M. luteus*[2,7]. Benzylpenicillin was separated from 6-APA by $(NH_4)_2SO_4$ precipitation[8] and from the natural penicillins by HPLC[9] and by paper chromatography[1].

In the induction experiments the concentrations of natural β-lactam antibiotics were calculated by subtracting from the total quantity of β-lactam the titer of benzylpenicillin present in the broth. For this kind of experiments, 2 liters of culture broth were processed in order to obtain a sufficient quantity of antibiotic.

When fermentations of *P. chrysogenum* were carried out in the absence of exogenously added side chain precursors, the natural β-lactam antibiotics produced by this strain were: 6-APA (50%), penicillin DF (15%), penicillin K (30%) and unidentified molecules (probably penicillin F) (4%). When PA was supplied to the fermentation broth at zero time benzylpenicillin was mainly produced, penicillin K constituting only 10% of the total whereas the percentages of penicillin DF and 6-APA were lower than 3%.

L-Lysine consumption was checked by TLC using L-[*U*-$^{14}$C]lysine monohydrochloride (270 mCi/mmol)[7].

Sugar determinations were carried out by the phenolsulfuric acid method[10]. Glycerol was determined by the procedure indicated by BURTON[11]. Isopenicillin N synthetase and acyltransferase were obtained and assayed as previously described[2,3,12].

### Transport Experiments

Mycelia of *P. chrysogenum* grown in the above media and conditions were harvested at different times and washed four times with sterile distilled water. Aliquots of 35 mg (wet weight; about 10 mg dry weight) were suspended in 25-ml Erlenmeyer flasks containing 1.4 ml of 0.06 M phosphate buffer, pH 6.5 and preincubated at 25°C for 5 minutes in a thermostatically controlled bath at 160 strokes/minute before adding PA (6.4 μM containing 1.4 μM of labeled PA). Incubations were carried out for 30 seconds of the required time, halted by addition of 10 volumes of water, rapidly filtered through Millipore filters (0.45 μm pore size) and washed with 3×10 ml of sterile distilled water. The filters

were dissolved in 10 ml of scintillation fluid and counted as described[13].

When required, several effectors were added to the uptake mixture at 2.5 mM or the indicated final concentration. In these cases the effector tested was added 2 or 5 minutes before [$^{14}$C]PA.

[$^{14}$C]PA uptake is given as pmol/minute or as [$^{14}$C]PA uptake (pmol) when different uptake periods were employed.

Induction Experiments

To establish the effect of different molecules on the induction of the PA transport system, *P. chrysogenum* was grown in the same media described above under identical physical conditions but without PA. At 50 hours of growth, PA (0.1% w/v), POA (0.1% w/v) plus different sugars (2% w/v) or L-lysine (50 mM) were added to the flasks. The induction of the transport system was followed at different times.

## Results and Discussion

### Time Course of the Appearance of the PA Uptake System

During penicillin fermentation the PA transport system was followed in *P. chrysogenum* Wis 54-1255 grown in the above medium. Uptake of [$^{14}$C]PA was measured at different times. Fig. 2 indicates that the transport system appeared when *P. chrysogenum* reached the stationary phase of growth, just before benzylpenicillin started to be produced, and was maximal at 50 hours of growth. From thereafter up to 97 hours the rate of transport decreased continuously. These synthesis kinetics indicate that the transport system was induced when nutrient-limiting conditions existed in the broth.

### Characteristics of the PA Transport System

PA uptake was a linear function of time for at least 3 minutes although in order to carry out the assays under good conditions of linearity, 30 seconds periods of uptake were routinely employed (see Fig. 3).

The optimal temperature for PA uptake was found to be 25°C (Fig. 4), the uptake rates at lower (15°C) or higher temperatures (37°C) being much lower. The uptake of PA was maximal at pH 6.5 in both 0.06 M phosphate and Tris-maleate buffers (Fig. 5). At lower (5.5) or higher (7.5) pH values the uptake rates decreased. However, when PA transport was tested at pH values lower than 4.5 (the p*Ka* of PA is 4.3) a passive influx of PA was detected (this system was insensitive to FCCP, did not

Fig. 2. Time of appearance of the PA transport system (●) and benzylpenicillin production (▲), pH (○), growth (△) and lactose consumption (■) during *Penicillium chrysogenum* Wis 54-1255 fermentation.

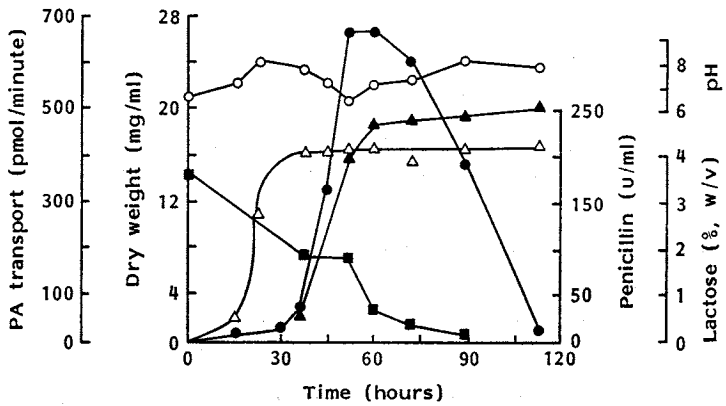


Fig. 3. Effect of incubation time on the uptake of PA.

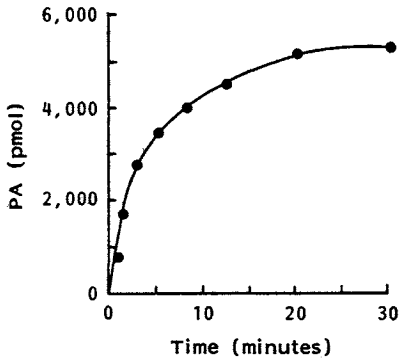



Fig. 4. Effect of temperature on the uptake of PA.

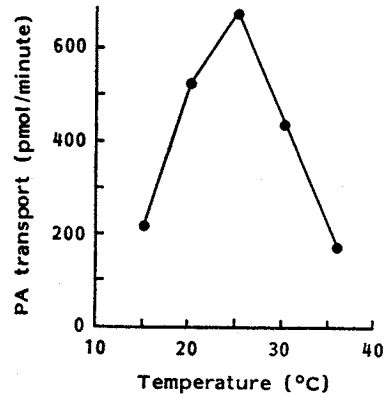



Fig. 5. Effect of pH on the uptake of PA.

$Na_2HPO_4$ - $KH_2PO_4$ buffer 0.06 M (●), Tris-maleate buffer 0.06 M (○) and passive influx (■).

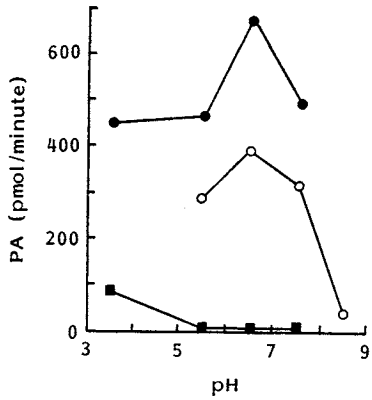
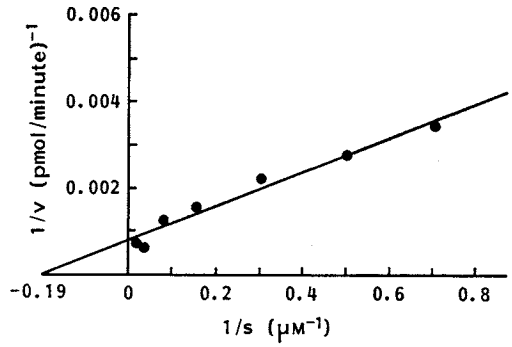



Fig. 6. Kinetics of PA transport (1/v *versus* 1/s). $Km$=5.2 μM.




show saturation kinetics and was not inhibited by POA). This effect could be due to the existence (at pH below 5) of a principal non-ionic form of this aromatic acid without charge ($C_6H_5$-COOH) that can be easily transported across cell membranes. Similar results have been reported by HUNTER and SEGEL testing different weak acids[14]. When the passive transport was measured in non-induced mycelia (grown in the absence of PA) the incorporation of labeled PA was maximal at pH 3.5, decreasing at higher pH values and becoming null at the optimal pH of the active transport (pH 6.5). At pH ranges lower than 4.5 both the active and passive transports function, and this might explain why at pH 5.5 and 3.5 the same rate of PA transport was observed (see Fig. 5). Under the above conditions the *Km* for the PA uptake system was 5.2 μM (Fig. 6).

The specificity of the PA transport system was examined by adding different molecules to the uptake mixture. When carbohydrates (D-glucose, D-galactose, D-ribose, D-xylose, L-arabinose, lactose, sucrose and trehalose) were tested, only D-xylose inhibited PA uptake (35%) whereas D-ribose and D-lactose stimulated the transport of this compound (118.7 and 125.9% respectively). Other carbohydrates (glucosamine and galactosamine), glycerol and Krebs-cycle intermediates did not cause any significant effect.

On the other hand, the addition of amino acids (Table 1) to the uptake system inhibited

Table 1. Effect of different amino acids on the uptake of [$^{14}$C]PA by *Penicillium chrysogenum*.

| Effectors | Percentage of the control | Effectors | Percentage of the control |
|---|---|---|---|
| Control | 100 | +L-Thr | 62.5 |
| +Gly | 65.1 | +L-Cys | 63.8 |
| +L-Ala | 70.3 | +L-Tyr | 82.5 |
| +L-Val | 58.5 | +L-Asn | 61.3 |
| +D-Val | 76.9 | +L-Gln | 72.1 |
| +L-Leu | 76.2 | +L-Asp | 65.7 |
| +L-Ile | 79.3 | +L-Glu | 68.6 |
| +L-Phe | 68.3 | +L-α-Aad | 77.3 |
| +L-Trp | 80.2 | +L-Lys | 48.7 |
| +L-Met | 65.4 | +L-His | 51.0 |
| +L-Ser | 66.7 | +L-Arg | 48.2 |

All effectors (2.5 mM) were added 2 minutes before PA. The results are means of three different experiments.

between 18 and 52%. It is interesting to note that L-lysine, an amino acid related to penicillin biosynthesis, caused a great inhibition (Table 1).

Benzylpenicillin, phenoxymethylpenicillin and different aliphatic penicillins (K, DF) and the related molecule 6-APA did not inhibit the "*in vivo*" uptake of PA (data not shown), whereas fatty acids notably affected the uptake rates (Table 2). Fatty acid inhibition was directly related to the length of the carbon chain of the molecules and was maximal with nonanoic acid (99.6%). This effect, which could be explained by the detergent properties of some of these compounds, strongly suggests that intact membranes are required for maintaining a functional transport system. The fact that many enzymes located at the cellular surface of biological membranes are strongly inhibited by fatty acids[15,16] agrees with these findings.

Table 2. Effect of acetic acid and different fatty acids on the uptake of [$^{14}$C]PA by *Penicillium chrysogenum*.

| Effectors | Percentage of the control |
|---|---|
| Control | 100 |
| +Acetic acid | 68.5 |
| +Propionic acid | 91.2 |
| +Butyric acid | 87.5 |
| +Pentanoic acid | 62.1 |
| +Hexanoic acid | 55.0 |
| +Heptanoic acid | 26.1 |
| +Octanoic acid | 2.5 |
| +Nonanoic acid | 0.4 |

Effectors were added as in the preceding table at 2.5 mM final concentration. All them were adjusted to pH 6.5 before addition.

Phenyl derivatives also inhibited the uptake of PA (Table 3) although it is still not clear whether these molecules affect the transport of labeled PA due to their structural similarity to the substrate or owing to the irreversible damage caused to the membrane or on the energized state of the cells[14]. It is interesting to note that the inhibition caused by POA, a direct precursor of phenoxymethylpenicillin, on the uptake of PA decreased when the PA/POA ratio increased (Table 3). This effect will be discussed further below.

## Effect of Thiol-containing and Thiol-modifying Reagents

Mercaptoethanol (MSH), glutathione (GSH) and dithiothreitol (DTT) slightly stimulated the uptake of PA (30, 20 and 20%, respectively). These results suggest that an extracellular environ-

Table 3. Effect of different phenyl derivatives on the uptake of [$^{14}$C]PA by *Penicillium chrysogenum*.

| Effectors | Percentage of the control | Effectors | Percentage of the control |
|---|---|---|---|
| Control | 100 | +2-OH-Phenylacetic acid | 0 |
| +Phenylphosphate | 42.5 | +3-OH-Phenylacetic acid | 0 |
| +Benzoic acid | 32.1 | +4-OH-Phenylacetic acid | 0 |
| +2-OH-Benzoic acid | 41.8 | +Phenoxyacetic acid (2.5 mM) | 0 |
| +2-Acetoxy benzoic acid | 50.1 | +Phenoxyacetic acid (0.25 mM) | 5.7 |
| +3-OH-Benzoic acid | 47.8 | +Phenoxyacetic acid (0.025 mM) | 23.4 |
| +4-OH-Benzoic acid | 64.8 | +Phenoxyacetic acid (0.006 mM) | 50.1 |
| +1,2-Benzenediol | 90.1 | +Phenylpropionic acid | 19.3 |
| +1,3-Benzenediol | 96.4 | +2-OH-Phenylpropionic acid | 65.3 |
| +1,4-Benzenediol | 96.2 | +3-OH-Phenylpropionic acid | 30.0 |
| +2-$NH_2$-Benzoic acid | 14.6 | +4-OH-Phenylpropionic acid | 9.8 |
| +3-$NH_2$-Benzoic acid | 9.3 | +$\alpha$-Phenylpyruvic acid | 6.1 |
| +4-$NH_2$-Benzoic acid | 8.1 | +$\gamma$-Phenylpyruvic acid | 26.1 |
| +Phenol | 15.8 | | |

All effectors (2.5 mM except in the cases indicated) were added as in the preceding tables. Results are also means of three different experiments.

ment containing reduced thiols might be necessary for the transport of this weak acid, in turn pointing to the notion that maintenance of membrane-associated disulfide groups (which must be present in a reduced state) are necessary for the bioactivity of this system[17]. Moreover, the thiol-modifying reagent *N*-ethylmaleimide (NEM)[18] inhibited the uptake by more than 60%. Similar results have been described for other transport systems in *P. chrysogenum*[19] and the activity of proteins containing reactive thiolgroups[2,20,21].

Table 4. Effect of uncouplers and metabolic inhibitors on the uptake of [$^{14}$C]PA by *Penicillium chrysogenum*.

| Effectors | Percentage of the control |
|---|---|
| Control | 100 |
| 2,4-DNP | 6.7 |
| 2-NP | 12.7 |
| 4-NP | 4.6 |
| FCCP | 6.2 |
| Arsenate | 96.7 |
| KCN | 6.5 |
| $NaN_3$ | 2.3 |

All effectors were added 2 minutes before [$^{14}$C]-PA. The final concentrations were: 2,4-DNP, 2-NP, 4-NP, 1 mM; FCCP, 0.1 mM and arsenate, 5 mM. When mycelia were preincubated in the presence of 5 mM arsenate for 5 minutes, similar results were obtained.

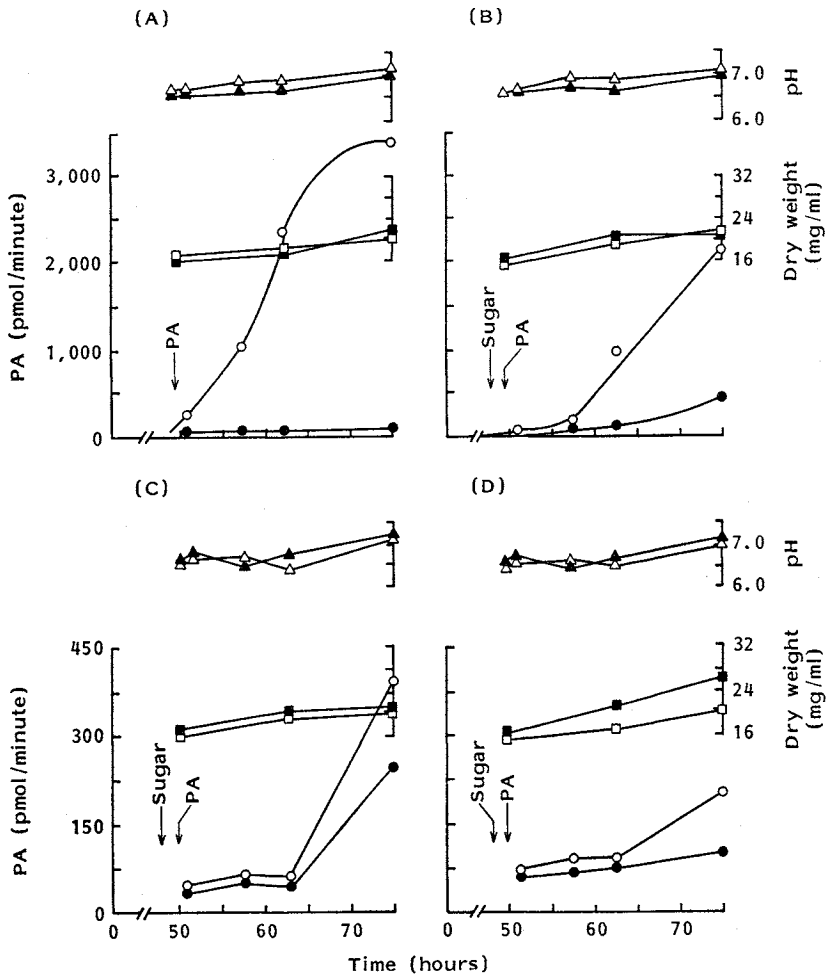
### Effect of Metabolic Inhibitors

$NaN_3$ and KCN inhibited PA uptake in *P. chrysogenum* by more than 90% whereas arsenate did not cause significant effect (Table 4). The energy poisons 2,4-dinitrophenol (DNP) and 4-nitrophenol (NP) also inhibited uptake (90%). These results, like those described by HUNTER and SEGEL for the basic amino acid transport systems of *P. chrysogenum*[19], suggest that PA transport is energy-dependent and that energy production may be intimately coupled with transport within the cell membrane. The ineffectiveness of arsenate (even when preincubated with mycelia for 5 minutes) further suggests that endogenous ATP is not the energy source for this active transport[19].

Effect of Carbohydrates and Other Molecules on the Regulation of PA Uptake

The PA transport system was not present until PA was added to the broth (Fig. 7). However, in these conditions *P. chrysogenum* produced a small amount of penicillins K, DF and 6-APA but not benzylpenicillin. These results are fairly consistent with the identical levels of isopenicillin N synthase and acyl-CoA: 6-APA acyltransferase found in both induced and non-induced mycelia of *P. chrysogenum* (data not shown) suggesting that there is no coordinated induction of the PA transport system and of these enzymes. When glucose, xylose, sucrose, lactose, galactose or glycerol were added to the medium (1 hour before adding PA) the induction was greatly delayed (Fig. 7) indicating that PA uptake is under a carbon catabolic mode of regulation. Therefore, benzylpenicillin did not

Fig. 7. Effect of different sugars and glycerol on the induction of the PA transport system.




Uptake of PA (○, ●), pH (△, ▲) and dry weight (mg/ml) (□, ■) when *Penicillium chrysogenum* was grown for 50 hours in absence of side chain precursors and at this time: PA or PA and different molecules were added: (A) Control without additions (●, ▲, ■), PA (○, △, □), (B) PA+lactose (○, △, □), PA+galactose (●, ▲, ■), (C) PA+glucose (○, △, □), PA+xylose (●, ▲, ■), (D) PA+sucrose (○, △, □), PA+glycerol (●, ▲, ■).

Arrow indicate the time of addition. Sugars were added 1 hour before PA.

Table 5. Benzylpenicillin and natural β-lactam-containing molecules produced by mycelia of *Penicillium chrysogenum*.

| | Benzylpenicillin (u/ml) | | | Natural β-lactams[a] | | |
|---|---|---|---|---|---|---|
| | 0 hour | 12 hours | 24 hours | 0 hour | 12 hours | 24 hours |
| Control | ND | ND | ND | 65 | 70 | 129 |
| +PA | ND | 50 | 100 | 55 | 72 | 78 |
| +PA+glucose | ND | 2 | 12 | 63 | 131 | 190 |
| +PA+xylose | ND | 1 | 8 | 66 | 146 | 187 |
| +PA+sucrose | ND | 2 | 6 | 67 | 137 | 180 |
| +PA+galactose | ND | 2 | 13 | 61 | 160 | 210 |
| +PA+lactose | ND | 22 | 65 | 57 | 69 | 94 |
| +PA+glycerol | ND | 1 | 3 | 69 | 71 | 90 |
| +PA+lysine | ND | 9 | 27 | 69 | 101 | 121 |

Mycelia were grown in the absence of PA for 50 hours and at this time PA (0.1% w/v), PA plus different sugars or PA plus L-lysine (50 mM) were added to the broth.

[a] Results are given as units of benzylpenicillin per ml.

ND: Not detected.

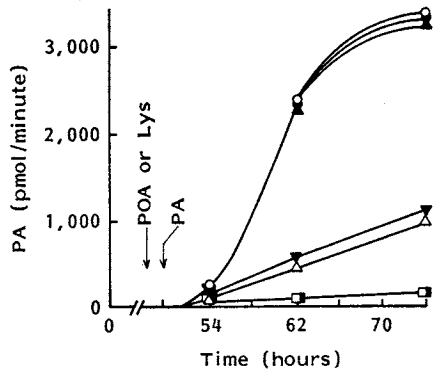
start to be produced until the transport of PA was induced. It had been previously reported[22,23] that some enzymes involved in benzylpenicillin biosynthesis seem to be under catabolic regulation. However, this is the first description of a clear effect of the carbon source on the incorporation of a direct precursor of benzylpenicillin. The fact that the quantity of natural β-lactam antibiotics increases even in the presence of readily metabolizable sugars (Table 5) is due to the presence in non-induced mycelia of high levels of cyclase and acyltransferase (before the carbon source is added). In the presence of glycerol, which caused the greatest repression on PA uptake, the final level of natural β-lactam-containing molecules was minimal, as was the final titer of benzylpenicillin (Table 5).

Moreover, L-lysine, which inhibits the biosynthesis of penicillin by blocking homocitrate synthase as has been well established by DEMAIN and co-workers[24~26], also repressed the biosynthesis of the PA uptake system (Fig. 8). Thus, L-lysine also decreased the synthesis of 6-APA and natural penicillins when added (50 mM) at zero fermentation time (Fig. 9). Moreover, the total levels of β-lactam-containing molecules continued to increase when L-lysine was added together with PA at induction time (Table 5). This fact suggests that although L-lysine blocked homocitrate synthase the levels of enzymes and penicillin biosynthetic intermediates in non-induced mycelia at 50 hours are high enough to efficiently continue the synthesis of these β-lactam antibiotics. However, the final titer of benzylpenicillin reached in the broth by the mycelia induced with PA in the presence of L-lysine was considerably lower than that reached in the controls (induced only with PA) (Table 5). These results suggest that L-lysine affects benzylpenicillin biosynthesis at least at two levels; at homocitrate synthase level[27] and at PA transport level.

When POA was added to the fermentation broths to replace PA, no induction of PA uptake was observed. Furthermore, when PA was supplied (0.1% w/v) at 50 hours to a culture of *P. chrysogenum* grown in the presence of POA, the rates of induction were similar to those observed in cultures without POA (Fig. 8).

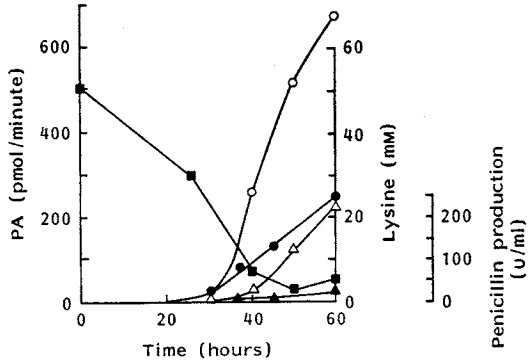
These data indicate that: a) POA is unable to induce the PA transport system and b) POA does not affect the rate of induction, suggesting that both molecules are transported by two different uptake systems. However, when tested as an effector, it did inhibit the incorporation of labeled PA

Fig. 8. Effect of POA and L-lysine on the induction of the PA transport system.




Control grown in absence of side chain precursor, (non-induced mycelia) (□). Mycelia grown in presence of POA (■). Mycelia grown with POA and PA added at induction time (▲). Mycelia grown in absence of side chain precursor and at 50 hours POA and PA were added together at the same concentration (0.1% w/v) (●). Control supplied with Lys (50 mM) and PA (0.1% w/v) at induction time (△). *Idem* with PA (0.1% w/v) at induction time (○). Transport of [$^{14}$C]POA by mycelia of *Penicillium chrysogenum* grown in presence of PA (▼).

Fig. 9. Effect of L-lysine (50 mM) on the uptake of PA in *Penicillium chrysogenum*.




Uptake of PA (△, ○) and penicillin production (benzylpenicillin, 6-APA and natural penicillins) (▲, ●) in presence (△, ▲) or absence (○, ●) of L-lysine and L-lysine consumption (■).

inside the cells (Table 3). The fact that no modification in the induction rates was observed could be explained by assumming that the quantity of PA needed for induction is much lower than that added at 50 hours of growth (0.1% w/v). It is also possible that when PA supplied to the non-induced mycelia, the quantity of POA decreased notably since *P. chrysogenum* produced phenoxymethylpenicillin during fermentation in the presence of this side chain precursor (data not shown). Under these conditions, the PA/POA ratio at 50 hours (the moment of induction) would be very high and therefore the inhibition caused by POA would be very slight. Attempting to check the real effect of POA, it was added together with PA at the same final concentration (0.1% w/v) to a non-induced culture of *P. chrysogenum*. Fig. 9 shows that the uptake rates were not modified with respect to controls suggesting that although POA inhibited the uptake of PA (Table 3), the quantity of PA taken up by the mycelia is able to efficiently induce the PA transport system. In order to clarify whether the PA uptake system might also be able to transport some POA, the uptake of [$^{14}$C]POA was measured in mycelia in which the PA transport system had been previously induced (see conditions described above). Fig. 8 shows that POA is transported with a lower efficiency (about 10% respect of PA) suggesting that this PA transport system is also able to take up POA. Accordingly, we think that the selection of high benzylpenicillin-producing strains based on their phenoxymethylpenicillin titer, a technique used by many important firms, could not be appropiate since it is possible to select mutants with a very efficient transport system for POA but not for PA. Moreover, we suggest that in industrial penicillin fermentations, which are usually carried out in two or more stages, PA should be added in the first stage (before the fermenters are seeded) in order to induce the PA transport system thus avoiding the massive synthesis of natural penicillins in the first hours of fermentations.

It is clear that the study of the POA transport system in *P. chrysogenum*, the establishment of

its biochemical properties and the knowledge of the molecules involved in the regulation of the PA uptake could provide greater insight into the biosynthesis of benzylpenicillin and phenoxymethylpenicillin, opening new aspects in the research into these $\beta$-lactam antibiotics. Further experiments about this exciting field are currently in progress and some of them are described in the following papers.

Acknowledgments

We are gratefully indebted to Mr. N. S. D. SKINNER for revising the English version of this manuscript and to R. REGLERO for typing it. We also thank M. J. ALONSO for her participation in the elaboration of this manuscript. This investigation was supported by a grant (No. 88/984) from the (Fondo de Investigaciones Sanitarias de la Seguridad Social, Madrid, España (FISSS). J.M.F.C. is a recipient of a FISSS fellowship. Thanks are also due to G. GONZALEZ DE BUITRAGO and A. PEREZ-ARANDA (Antibióticos, S.A., Madrid) for the gift of an authentic sample of isopenicillin N.

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