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Glucose Represses Formation of δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine and Isopenicillin N Synthase but Not Penicillin Acyltransferase in *Penicillium chrysogenum*

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The content of α-aminoadipyl-cysteinyl-valine, the first intermediate of the penicillin biosynthetic pathway, decreased when *Penicillium chrysogenum* was grown in a high concentration of glucose. Glucose repressed the incorporation of [^{14}C]valine into α-aminoadipyl-cysteinyl-[^{14}C]valine in vivo. The pool of α-aminoadipic acid increased sevenfold in control (lactose-grown) penicillin-producing cultures, coinciding with the phase of rapid penicillin biosynthesis, but this increase was very small in glucose-grown cultures. Glucose stimulated homocitrate synthase and saccharopine dehydrogenase activities in vivo and increased the incorporation of lysine into proteins. These results suggest that glucose stimulates the flux through the lysine biosynthetic pathway, thus preventing α-aminoadipic acid accumulation. The repression of α-aminoadipyl-cysteinyl-valine synthesis by glucose was not reversed by the addition of α-aminoadipic acid, cysteine, or valine. Glucose also repressed isopenicillin N synthase, which converts α-aminoadipyl-cysteinyl-valine into isopenicillin N, but did not affect penicillin acyltransferase, the last enzyme of the penicillin biosynthetic pathway.

Glucose exerts a negative control on the biosynthesis of penicillin in *Penicillium chrysogenum* (17, 22) similar in many aspects to carbon catabolite regulation of the biosynthesis of cephalosporins in *Acremonium chrysogenum* (3, 24) and cephamycins in *Streptomyces clavuligerus* (1, 23) and *Streptomyces lactamdurans* (6). Glucose (28 to 140 mM) produces a concentration-dependent repression of the incorporation of [^{14}C]valine into penicillin but does not have an inhibitory effect on such incorporation by enzymes formed before glucose addition (22). The total activity of the penicillin-synthesizing enzymes in *P. chrysogenum* is repressed by glucose (22). Derepression of penicillin biosynthesis occurs after depletion of glucose. However, the overall biosynthetic activity of the pathway in vivo is determined by the availability of precursors of the L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) tripeptide and by the activities of the (at least) three enzymes involved in penicillin biosynthesis.

The first well-established intermediate in the penicillin biosynthetic pathway, ACV (2, 11), appears to be formed by a sequential condensation of the three precursor amino acids (8) (Fig. 1). ACV is then cyclized to form isopenicillin N by the action of isopenicillin N synthase, an enzyme that has been purified to near homogeneity from extracts of *P. chrysogenum* (19, 21). In the last step of penicillin biosynthesis, the α-aminoadipyl side chain of isopenicillin N is exchanged for phenylacetic acid, which is previously activated in the form of phenylacetyl coenzyme A (CoA) (20; B. Spencer and C. Maung, Biochem. J. **118**:29p–30p, 1970).

The recent development of reliable assay methods to quantify the tripeptide ACV (11) and to measure the activities of isopenicillin N synthase (21) and penicillin acyltransferase (E. Alvarez and J. F. Martín, unpublished data) prompted us to study the effect of glucose on the synthesis and activity of the different enzymes involved in penicillin biosynthesis, as well as on the availability of precursor amino acids for tripeptide biosynthesis.

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MATERIALS AND METHODS

Microorganisms and culture conditions. *P. chrysogenum* AS-P-78, a high-penicillin-producing strain (donated by Antibióticos, S.A., León, Spain), was used in this study. The growth characteristics, penicillin production kinetics, and lysine regulation of penicillin biosynthesis in this strain have been previously reported (13, 14). Conidia obtained from sporulation medium PM1 (11) were kept at −20°C in 20% glycerol.

Penicillin biosynthetic enzymes and intermediates were studied in a complex production medium (corn-steep solids, 35 g/liter; lactose, 25 g/liter; potassium phenylacetate, 2.5 g/liter; $MgSO_4 \cdot 7H_2O$, 3 g/liter; KH_2PO_4, 7 g/liter; corn oil, 2.5 g/liter; calcium carbonate, 10 g/liter) which supports high penicillin production. The inoculum was developed from conidia in a complex seed medium as previously reported (22). Baffled flasks (500 ml) containing 50 ml of complex production medium were inoculated with 2.5 ml of the seed culture and incubated at 25°C, and penicillin was determined by bioassay (22). Control and β-lactamase-treated samples of the filtered broth were assayed by high-pressure liquid chromatography to determine the formation of penicillin G or isopenicillin N, using a Varian 5000 high-pressure liquid chromatograph equipped with a micropack mCH-10 column as described elsewhere (12, 23).

Glucose was added at the time of inoculation to complex production medium at a final concentration of 140 mM (previously determined to be the optimal conditions to observe glucose repression).

Uptake of L-[U-^{14}C]valine and [α-6-^{14}C]aminoadipic acid were as reported before (13, 14). In all cases the cells were preincubated for 1 h with a 1 mM unlabeled amino acid at

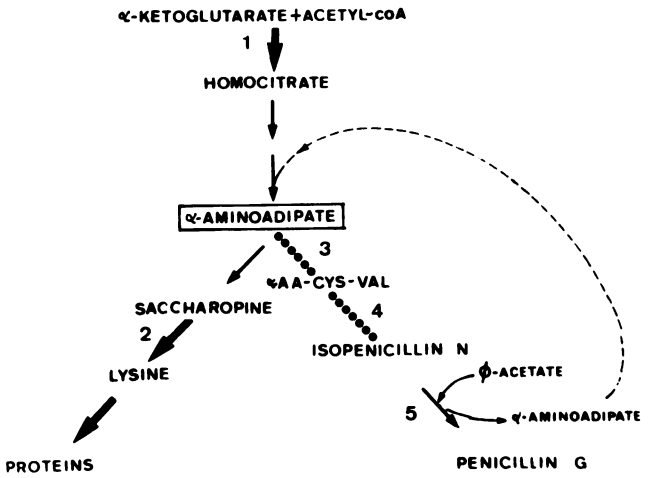

FIG. 1. Biosynthetic pathways of penicillin and lysine. 1, Homocitrate synthase; 2, saccharopine dehydrogenase; 3, ACV synthase; 4, isopenicillin N synthase; and 5, penicillin acyltransferase. The steps indicated by dots (3 and 4) are negatively regulated by glucose. The thick arrows correspond to steps in the pathway which are stimulated by glucose.

25°C to induce the transport system before addition of the labeled amino acid.

Incorporation of L-[U-^{14}C]valine into penicillin by resting cells. Growth-independent penicillin biosynthesis was studied in short-term experiments (60 min) with resting cell cultures prepared as described before (22). After the culture was incubated for 90 min at 25°C, L-[U-^{14}C]valine was added to the medium (9.25×10^3 Bq/ml). Samples were taken at intervals, and the penicillin was extracted, purified by thin-layer chromatography, and quantified as described elsewhere (22). The radioactivity incorporated was counted in a Phillips PW4700 scintillation counter.

Purification of sulfur-containing peptides and incorporation of valine into ACV. Peptides from mycelium of *P. chrysogenum* were extracted, oxidized to their sulfonic derivatives with performic acid, purified by chromatography on Dowex 50W × 4 (H^+ form), and separated by electrophoresis at pH 1.8 by a modification of the Arnstein and Morris method (2) as previously reported (11). Incorporation of L-[U-^{14}C]valine into ACV was carried out as described by López-Nieto et al. (11).

Analysis of intracellular concentration of amino acids. Mycelium collected by filtration was washed three times with distilled water and dried by pressing it between filter papers. The amino acids were extracted with 7.5% trichloroacetic acid (5 ml/g [wet weight]) at 25°C for 30 min. The extracts were filtered, and the filtrates, adjusted to pH 2.2 with 5 M NaOH, were chromatographed through Dowex 50W × 4 (H^+ form). The amino acids were eluted with 1 mM pyridine, concentrated to near dryness, and dissolved in 5 ml of lithium hydroxide buffer (pH 2.2) containing (per liter): lithium hydroxide, 3.6 g; citric acid monohydrate, 10.5 g; HCl, 13 ml; thiodiglycol (25%), 20 ml; and caprylic acid, 0.1 ml. The concentration of amino acids was determined in a Beckman 119 BL/CL amino acid analyzer.

Homocitrate synthase and saccharopine dehydrogenase assays. Homocitrate synthase (3-hydroxy-3-carboxyadipate 2-oxoglutarate lyase) was assayed in dialyzed extracts of *P. chrysogenum* by following the incorporation of [1-^{14}C]acetyl-CoA into homocitric acid as described by Luengo et al. (13).

Saccharopine dehydrogenase (saccharopine:NAD oxidoreductase) was assayed spectrophotometrically by measuring the lysine- and α-ketoglutarate-dependent oxidation of NADH (reverse reaction) as described by Broquist (4).

Isopenicillin N synthase. Isopenicillin N synthase (cyclase) was determined in cell extracts of *P. chrysogenum* as described by Ramos et al. (21) after filtration through a Sephadex G-25 column to remove residual penicillin G and other low-molecular-weight molecules in the extract.

Penicillin acyltransferase assay. Penicillin acyltransferase (isopenicillin N:phenylacetyl-CoA acyltransferase) was determined by the Spencer and Maung method (Biochem. J. **118**:29–30, 1970).

Reagents. Pure ACV (dimer form) and isopenicillin N were kindly provided by P. van Dijck (Gist-Brocades, Delft, Holland). L-[U-^{14}C]leucine (370 MBq/mmol), L-[U-^{14}C]valine (10,540 MBq/mmol), L-[U-^{14}C]lysine (370 MBq/mmol), and [1-^{14}C]acetyl-CoA (22.01×10^2 MBq/mmol) were obtained from the Radiochemical Centre (Amersham, England), [α-6-^{14}C]aminoadipic acid (1,735.3 MBq/mmol) was from CEA (Gif Sur Yvette, France), and acetyl-CoA and phenylacetyl-CoA were from Sigma Chemical Co. (St. Louis, Mo.) All other chemicals were of reagent quality.

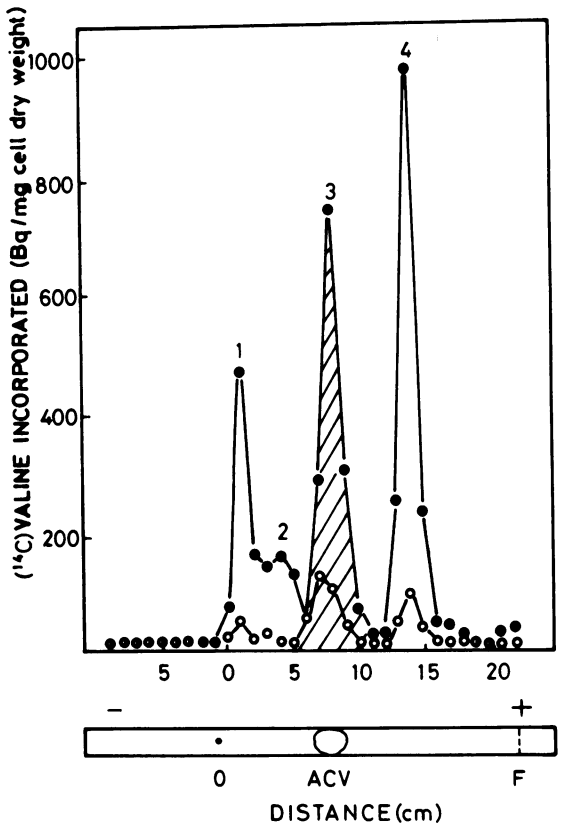

FIG. 2. Thin-layer electrophoretic separation of sulfonated peptides in 48-h-old cells labeled by incorporation of [^{14}C]valine. The sulfonated derivative of ACV is peak 3 migrating at 6 to 9 cm. Peaks 1 and 2 are unknown sulfonated peptides. Peak 4 is penicillaminic acid. A total of 1 μCi (37,000 Bq) of [^{14}C]valine was added to 1 ml of culture (10 mg of cell dry weight). After extraction of the peptides, the total extract was dried, redissolved in 20 μl of distilled water, and electrophoresed. The same amount of sample was applied in all cases. Closed symbols show lactose-grown cultures and open symbols show glucose-grown cultures.

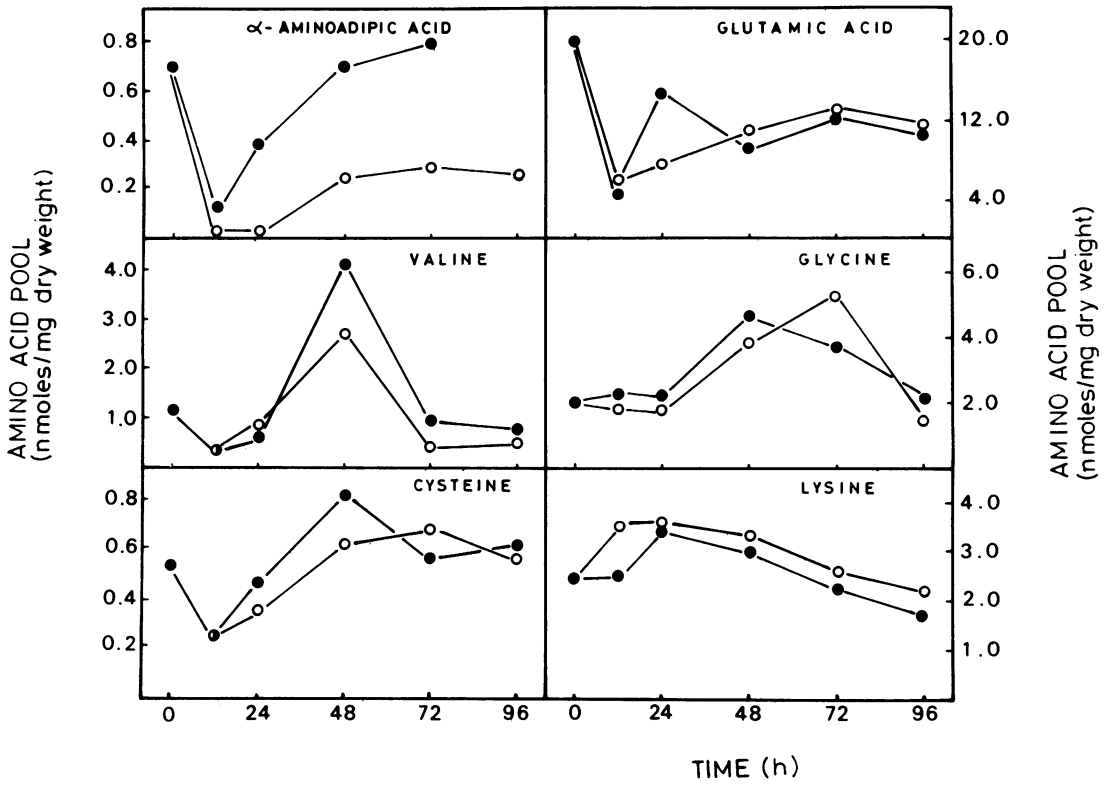

FIG. 3. Time course of the pools of different amino acids during growth. Cells were grown in lactose (●) or in glucose (○), and the amino acids were extracted and quantified as indicated in the text.

RESULTS

Glucose repression of ACV formation. The biosynthesis of ACV in vivo, as measured by the incorporation of [^{14}C]valine into ACV, was much lower in cultures grown in 140 mM glucose than in control cultures grown in lactose (Fig. 2). This was observed very clearly in cultures at 24 or 48 h of incubation. Glucose repressed not only the formation of ACV, but also the incorporation of [^{14}C]valine into other sulfonated peptides (Fig. 2, peaks 1 and 2) and the accumulation of penicillaminic acid (peak 4), previously identified as a degradation product of isopenicillin N and penicillin G (11).

When the sulfonated peptides were quantified by densitometry after electrophoresis, there were only traces of ACV in glucose-grown (140 mM) cells at 24 h and less than 10% of the ACV content of control cells at 48 h. However, there was no large difference in the ACV pool of control and glucose-grown cells after 72 h. Readdition of glucose before 48 h prevented formation of ACV and penicillin, but the inhibitory effect was smaller when glucose was added after 48 h of incubation. These results suggest that peptide formation is derepressed in glucose-supplemented cultures after glucose depletion, which occurs at about 48 h (22). The content of total glutathione (a structurally related peptide) in the same samples was very low at 24 h and increased at 48 h, but in both cases it was not significantly reduced in glucose-grown cells.

Reduction of α-aminoadipic acid pool in glucose-grown cells. The pools of three amino acids (α-aminoadipic acid, proline, and alanine) in control cells were different from those in cells grown in the presence of glucose. The pool of α-aminoadipic acid in lactose-grown cells increased about sevenfold from 12 to 72 h, coinciding with the active phase of penicillin biosynthesis, but the increase was three to four times less in glucose-grown cells (Fig. 3).

The pools of valine and cysteine (another two precursors of ACV), glutamic acid and glycine (components of glutathione), and lysine (the end product of the penicillin-lysine branched pathway) were not significantly affected in cells grown in glucose. The pools of proline and alanine were reduced by between 20 and 30% (data not shown) by incubation in the presence of glucose, whereas the pools of isoleucine, leucine, phenylalanine, and tyrosine were not affected.

Lack of reversal of the glucose effect by α-aminoadipic acid, cysteine, and valine. Since the decrease of the α-aminoadipic acid pool could impair penicillin production, attempts were made to reverse the glucose repression of penicillin biosynthesis by the addition of α-aminoadipic acid, cysteine, and valine individually (2 mM) or in mixtures (2 mM each).

Penicillin biosynthesis (measured by the incorporation of [^{14}C]valine into penicillin G) was stimulated about 30 to 40% when control cells were supplemented with α-aminoadipic acid (2 mM) for 2 h (Fig. 4a). However, α-aminoadipic acid did not reverse the repression of penicillin biosynthesis exerted by glucose, although it was able to reverse the inhibition exerted by lysine on penicillin biosynthesis (Fig. 4b).

In this type of experiment the addition of α-aminoadipic acid could affect the rate of uptake of the [^{14}C]valine used as a precursor for penicillin biosynthesis. To avoid any interference, we carried out similar experiments to determine the effect of α-aminoadipic acid, cysteine, and valine on total de novo penicillin G synthesis in lactose (control)- and glucose-

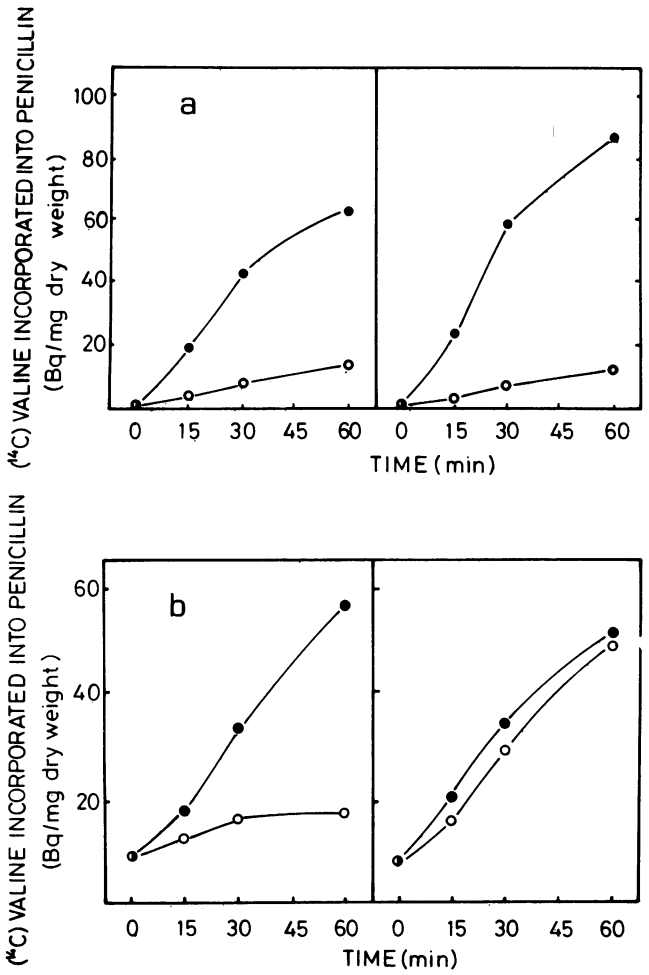

FIG. 4. (a) Effect of glucose on incorporation of [^{14}C]valine into penicillin and lack of reversal by α-aminoadipic acid of the glucose effect. Left panel, Cells grown in lactose (●) or glucose (○). Right panel, Cells grown in lactose (●) or glucose (○) were supplemented in both cases with 2 mM α-aminoadipic acid for 2 h, before carrying out the incorporation of [^{14}C]valine into penicillin, as indicated in the text. (b) Effect of lysine on incorporation of [^{14}C]valine into penicillin and reversal by α-aminoadipic acid of the lysine effect. Left panel, Cells grown in lactose with (○) or without (●) 10 mM lysine. Right panel, Cells grown in lactose with 10 mM lysine were supplemented with 1 (○) or 10 (●) mM α-aminoadipic acid for 2 h, before carrying out the incorporation of [^{14}C]valine into penicillin.

grown cells. A net increase of penicillin G was already detectable after 3 h of incubation of *P. chrysogenum* resting cells. Lactose-grown cells supplemented with α-aminoadipic acid, cysteine, and valine (2 mM each) showed up to 30 to 40% higher penicillin biosynthetic activity, but again neither α-aminoadipic acid nor a mixture of the three amino acids was able to reverse the repression exerted by glucose.

Effect of glucose on cellular incorporation of α-aminoadipic acid. The uptake of [^{14}C]valine and [α-^{14}C]aminoadipic acid by *P. chrysogenum* cells was studied in control and glucose-grown cells (Fig. 5). α-Aminoadipic acid appeared to require an inducible system to enter the cell since it needed 2 h to get to a significant intracellular level followed later by a rapid incorporation, whereas high incorporation levels of valine were obtained in 15 to 30 min. Supplementing the growth medium with glucose did not affect the uptake of valine, but glucose-grown cells incorporated only about 50% of the α-aminoadipic acid taken up by control cells (see Discussion).

Effect of glucose on α-aminoadipic acid-forming and -converting enzymes. α-Aminoadipic acid in *P. chrysogenum* is an intermediate in the lysine biosynthetic pathway. To establish whether glucose affects the rate of biosynthesis of α-aminoadipic acid or the conversion of α-aminoadipic acid into lysine, we studied the activities of homocitrate synthase (the first enzyme of the lysine pathway) and saccharopine dehydrogenase (a late enzyme that converts saccharopine into lysine). The activity of homocitrate synthase was clearly higher at 12 and 24 h of incubation in glucose-grown cells than in control cultures in three different experiments (Fig. 6). Saccharopine dehydrogenase behaved similarly (Fig. 6). Cultures grown in glucose (140 mM) showed a higher activity of saccharopine dehydrogenase at 12 h of incubation, coinciding with the rapid phase of growth in glucose. After 36 h the levels of both enzymes were similar in control and glucose-grown cells. Cells grown in glucose for 24 h showed a greatly stimulated protein synthesis as measured by the incorporation of either [^{14}C]leucine or [^{14}C]lysine into trichloroacetic acid-insoluble material (data not shown), in agreement with the stimulation of growth previously reported (22).

Glucose repression of isopenicillin N synthase. Isopenicillin N synthase converts the tripeptide ACV to isopenicillin N. The specific activity of this enzyme was severely reduced in glucose-grown cells during the first 48 h of incubation (Fig. 7). The cyclase activity was barely detectable at 24 h and increased at 36 and 48 h after glucose depletion. In vitro, glucose did not inhibit the activity of isopenicillin N synthase.

Lack of effect of glucose on penicillin acyltransferase. Unexpectedly, acyltransferase (the last enzyme of the penicillin biosynthesis pathway which converts isopenicillin N into penicillin G) was not repressed in glucose-grown cells under conditions in which penicillin biosynthesis was clearly reduced (Fig. 8).

DISCUSSION

Glucose decreases the biosynthesis of penicillin in *P. chrysogenum* (17, 22), cephalosporin in *A. chrysogenum* (3, 18, 24), and cephamycin in *S. lactamdurans* (6, 7). The mechanism underlying carbon catabolite regulation may be similar in the different β-lactam-producing organisms although the enzymes repressed or inhibited by glucose are not necessarily the same (15, 16).

As observed in Fig. 2 almost no ACV was formed until 48 h under conditions of total repression of penicillin biosynthesis by glucose, but formation of this tripeptide was derepressed after this time. At this stage glucose had decreased below nonrepressive levels (22). Similarly, glucose strongly represses the formation of ACV in *S. lactamdurans* (6, 7), suggesting that carbon catabolite repression of ACV synthesis is probably a general mechanism in β-lactam-producing organisms.

Glucose also decreased the pool of α-aminoadipic acid in *P. chrysogenum*. The reduction by glucose of the α-aminoadipic acid pool appears to be the result of two different effects produced by this sugar. On one hand, glucose increased the levels of homocitrate synthase and saccharopine dehydrogenase (two enzymes of the lysine biosynthetic pathway involved in α-aminoadipic acid formation and its conversion to lysine) and also stimulated the

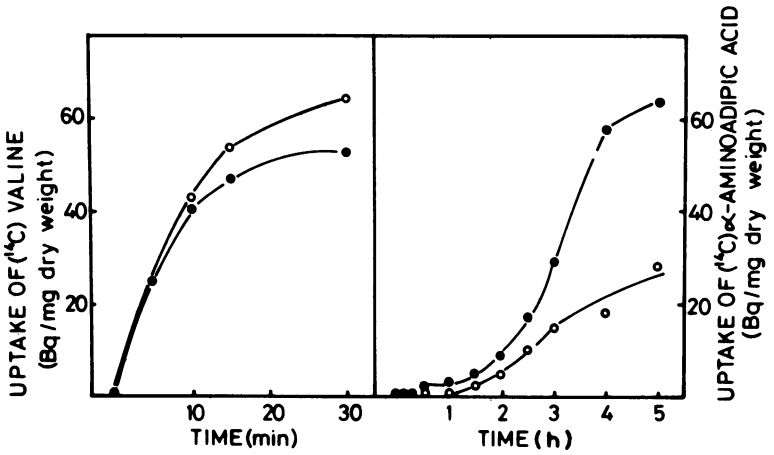

FIG. 5. Uptake of [^{14}C]valine and [α-^{14}C]aminoadipic acid by cells grown in lactose (●) or glucose (○). Uptake was induced by previous incubation with 1 mM unlabeled amino acid for 1 h (see the text).

incorporation of lysine into proteins. Therefore, the flux of intermediates through the lysine pathway is increased, resulting in a reduction of the α-aminoadipic acid pool. On the other hand, α-aminoadipic acid appears to be recycled during penicillin biosynthesis (9), although part of it accumulates in the broth (5), and glucose-grown cells showed a reduction in the uptake of extracellular α-aminoadipic acid (Fig. 5).

These results prompted us to study whether the glucose effect on penicillin biosynthesis was due simply to a deprivation of α-aminoadipic acid for ACV synthesis. This was not the case since the glucose effect on penicillin biosynthesis was not reversed by supplementing the culture with α-aminoadipic acid, cysteine, valine, or a mixture of the three amino acids. It seems that glucose represses the ACV-forming enzyme complex (ACV synthase) in addition to reducing the pool of α-aminoadipic acid.

Glucose also repressed isopenicillin N synthase, a well-characterized enzyme which converts ACV into isopenicillin N (21). The isopenicillin N synthase of *P. chrysogenum* is more sensitive to carbon catabolite regulation than is the isopenicillin N synthase of *A. chrysogenum* (3, 10) and *S. lactamdurans* (6).

A somewhat unexpected result was the finding that penicillin acyltransferase (the third enzyme involved in penicillin biosynthesis) was not reduced in glucose-grown cultures under experimental conditions in which glucose repression of penicillin biosynthesis was very clear. The lack of ACV and isopenicillin N formation in glucose-supplemented cul-

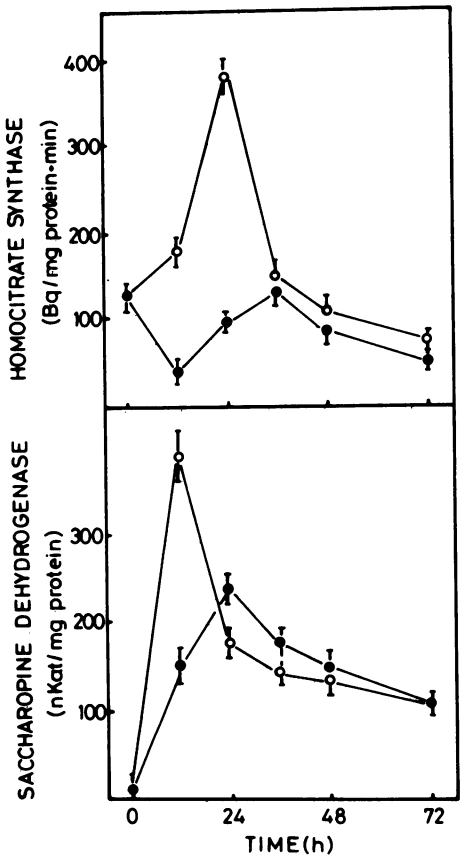

FIG. 6. Homocitrate synthase and saccharopine dehydrogenase activities in cultures of *P. chrysogenum* AS-P-78 grown in lactose (●) or glucose (○) as the carbon source. Vertical bars indicate the standard deviations of the values of the enzyme activities obtained.

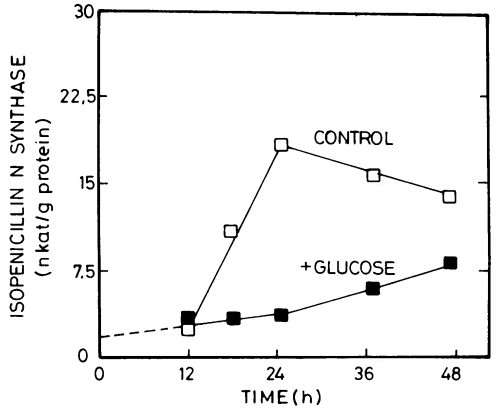

FIG. 7. Isopenicillin N synthase activity in cultures of *P. chrysogenum* AS-P-78 grown in lactose (control) or glucose during the initial 48 h of incubation.

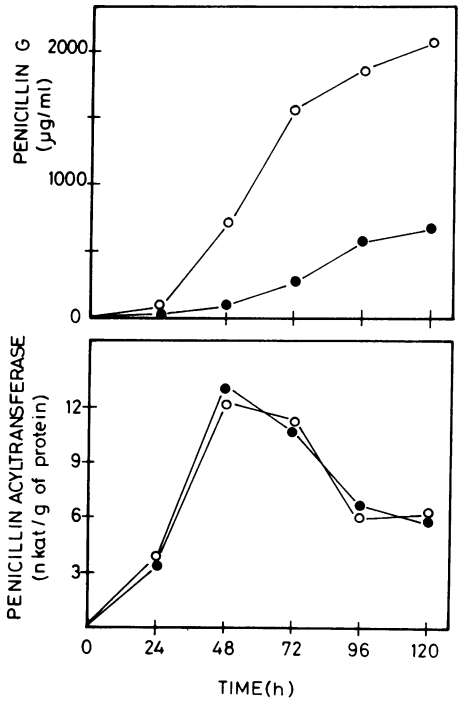

FIG. 8. Penicillin production (upper) and penicillin acyltransferase activity (lower) in cultures of *P. chrysogenum* AS-P-78 grown in lactose (○) or glucose (●) as the carbon source.

tures explains the reduction of penicillin biosynthesis even though penicillin acyltransferase levels are normal.

Penicillin acyltransferase is therefore not subject to the same type of control as the two preceding enzymes of the penicillin biosynthetic pathway, which suggests that acyltransferase is located in a separate chromosomal location or at least expressed in a different transcriptional unit.

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