CARBON CATABOLITE REPRESSION OF PENICILLIN BIOSYNTHESIS BY *PENICILLIUM CHRYSOGENUM*

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The addition of glucose to batch cultures of *Penicillium chrysogenum* AS-P-78 reduced the biosynthesis of penicillin. This regulatory effect was also observed in penicillin biosynthesis by nitrogen-limited resting cells when cultures were previously grown in high concentrations of glucose. The effect of glucose was concentration-dependent in the range of 28~140 mM. Incorporation of L-[*U*-^{14}C]valine into penicillin in nitrogen-limited resting cultures was reduced by 70% when cells were grown on 140 mM glucose, as compared with that grown on lactose. It was not affected when the sugar was added to the resting cell system, in which penicillin biosynthesis took place without growth. Fructose, galactose and sucrose exerted the regulatory effect to the same extent as glucose (64 to 70%). Lactose did not exert suppression of penicillin biosynthesis. Penicillin-synthesizing activity in control cultures with lactose reached a peak at 24 hours of incubation and decreased slowly thereafter, as studied with resting cell cultures in which further protein synthesis was blocked with cycloheximide. Glucose repressed the formation of penicillin-synthesizing enzymes, but had no effect on the activity of these enzymes. These results suggest that glucose represses but does not inhibit penicillin biosynthesis.

Glucose is the best carbon and energy source for the growth of most antibiotic-producing microorganisms. However, rapid utilization of glucose decreases the biosynthesis of many antibiotics[1].

In a medium containing glucose plus a second carbon source glucose is generally used first, thereby suppressing antibiotic biosynthesis. When glucose is depleted (or its concentration falls below a repressing threshold) the second carbon source is used and antibiotic formation occurs.

This phenomenon, which is similar to the initially named "glucose effect"[2], is now understood in terms of carbon catabolite regulation[3,4].

Carbon catabolite regulation of antibiotic biosynthesis is a general mechanism controlling the biosynthesis of antibiotics belonging to different biosynthetic groups. These include actinomycin production by *Streptomyces antibioticus*[5], puromycin by *Streptomyces alboniger*[6], cephalosporin by *Cephalosporium acremonium*[7,8,9] and *Streptomyces clavuligerus*[10] and penicillin by *Penicillium chrysogenum*[11].

Early studies on media development for penicillin production indicated that di-, oligo- and polysaccharides were better carbon sources than glucose for penicillin production[12]. Industrial production is therefore carried out using lactose which is slowly utilized. Carbon catabolite regulation of penicillin biosynthesis is bypassed when glucose is slowly fed to the culture[13]. The same occurs in cephalosporin production[7]. These results suggest that carbon catabolite regulation is exerted by an effector formed during the transport or catabolism of glucose.

The precise molecular mechanism of carbon catabolite regulation of penicillin biosynthesis is unknown, although there appears to exist a close relationship with the energy metabolism of the cells[4].

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Previous studies have been carried out in long-term fermentations in complex media. In order to characterize the molecular mechanism, we have developed a resting cell system in which the incorporation of [^{14}C]valine into penicillin in short-term experiments was followed. The results indicate that glucose exerts repression of the formation of the penicillin-synthesizing enzymes but not inhibition of the enzyme activity.

Materials and Methods

Microbial Strains

A high penicillin-producing strain *P. chrysogenum* AS-P-78 was kindly provided by Antibióticos, S. A. León, Spain. Growth characteristics and penicillin-producing ability of this strain have been reported previously[14,15].

Culture Medium and Growth Conditions

The inoculum was grown in complex seed medium (CSM) (containing in g/liter: corn-steep liquor 20, distiller solubles 20, sucrose 20, $CaCO_3$ 5, pH 5.7 before sterilization) as described previously[14].

Seed media (50 ml) in 250-ml triple-baffled flasks were inoculated with a conidial suspension (final density of 10^7 conidia/ml). After growing for 40 hours at 25°C in a rotary shaker at 250 rpm mycelium was collected, washed by centrifugation and suspended in sterile water (10 ml). Portions (2.5 ml) of this mycelial suspension were used to inoculate complex production medium (50 ml) in 500-ml flasks. Penicillin production in long-term incubations was carried out in complex production medium (CPM) (corn-steep solids 35, lactose 25, potassium phenylacetate 2.5, $MgSO_4 \cdot 7H_2O$ 3, KH_2PO_4 7, corn oil 2.5, $CaCO_3$ 10)[14]. Production was carried out at 25°C for 120 hours (long-term fermentations). Glucose or other effectors were added at the final concentrations indicated. In experiments to test the effect of different carbon sources, the sugar existing in the CPM medium (lactose) was replaced by other carbon sources at the same final concentration (140 mM).

Penicillin Biosynthesis by Resting Cell Cultures

Penicillin biosynthesis by nitrogen-limited resting cell cultures was studied in short-term experiments (1~3 hours). *P. chrysogenum* AS-P-78 was grown as described above for 24 hours and collected by filtration. After washing 3 times by centrifugation with NaCl solution (9 g/liter), cells were suspended at a final concentration of 12 mg cell dry weight per ml in nitrogen-free suspension medium, containing g/liter: lactose 48, sodium phenylacetate 1.6, KH_2PO_4 4.8, $MgSO_4 \cdot 7H_2O$ 0.4, $FeSO_4 \cdot 7H_2O$ 0.16, $CuSO_4 \cdot 5H_2O$ 0.008, $ZnSO_4 \cdot 7H_2O$ 0.032, Na_2SO_4 0.8, $MnSO_4 \cdot H_2O$ 0.0032, $CaCl_2 \cdot 2H_2O$ 0.08 and 320 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4), pH 7.0.

Resting cells were incubated at 25°C in a rotary shaker at 250 rpm in 50-ml baffled flasks containing 10 ml of suspension medium.

Determination of Penicillin

Culture broths were centrifuged at 1,800×*g* for 10 minutes. The supernatant fluid was centrifuged again for 5 minutes at 15,000×*g* and the cleared supernatants were used for the assay of penicillin with *Bacillus subtilis* ATCC 6633 as test organisms[16]. Penicillin was also determined colorimetrically with the hydroxylamine reagent[17] to confirm the results of the bioassay.

Incorporation of L-[*U*-^{14}C]Valine into Penicillin

P. chrysogenum AS-P-78 was grown in the CPM medium with and without glucose for 24 hours. Cells were harvested by filtration, washed with sterile saline, and transferred to suspension medium.

After incubation for 90 minutes at 25°C, L-[*U*-^{14}C]valine (285 mCi/mmol) was added to the medium (0.25 μCi/ml). Samples were taken at 15-minute intervals over a 60-minute period. Mycelium was removed by centrifugation (10 minutes at 2,000×*g*), and the penicillin in 1 ml of supernatant was extracted with 0.5 ml of amyl acetate previously acidified to pH 2.0 with phosphoric acid. The organic phase was chromatographed on Silica Gel G thin-layer plates using acetone - acetic acid (95: 5) as the developing solvent. Rf values in this solvent system were 0.05 for valine and 0.87 for penicillin. Penicillin was also identified by previous treatment of the sample with penicillinase (Difco) (100 μg/ml at

35°C for 1 hour) that destroyed the radioactive product migrating with an Rf of 0.87. Radioactivity was measured in Packard Tri-Carb 3320 or a Phillips PW 4700 scintillation counter with automatic quenching correction.

Determination of Protein Synthesis

Protein synthesis was followed by measuring the incorporation of [^{14}C]leucine (351 mCi/mmol) at final radioactivity of 0.5 μCi/ml into trichloroacetic acid (TCA)-insoluble materials. Samples (250 μl) were precipitated in ice-cold 5% TCA. After 30 minutes in ice, samples were heated at 75°C to solubilize RNAs and then filtered through Whatman GF/C glass fiber filters. Filters were dried and the radioactivity counted as before. The effect of cycloheximide on protein synthesis was studied by adding cycloheximide to the flasks (150 μg/ml) at the time indicated. Determination of total protein was carried out by the method of LOWRY *et al.*[18]. Glucose was determined by the glucose oxidase method.

Determination of Penicillin-synthesizing Activity

Mycelium grown in CPM medium containing 140 mM glucose was collected at 0, 24, 48, 72, 96 and 120 hours, washed three times with 0.9% NaCl and suspended in nitrogen-free suspension medium supplemented with cycloheximide (150 μg/ml) to inhibit further protein synthesis. Cycloheximide-supplemented cultures were incubated in a rotary shaker as above for 3 hours. During this time biosynthesis of penicillin by the pre-existing penicillin-synthesizing enzymes was linear. Specific activity of penicillin "synthetase" is given as units/mg dry weight. One unit is defined as the activity synthesizing 1 nmol of benzylpenicillin/minute at 25°C.

Uptake of [^{14}C]Valine

L-[*U*-^{14}C]Valine (0.25 μCi/ml) was added to the penicillin-synthesizing resting cell system. Samples (250 μl) were taken in 5 ml of an ice-cold solution containing 1 mg/ml of unlabelled valine, filtered immediately through Whatman GF/C glass fiber filters, and washed with 20 ml of ice-cold valine solution. After drying the filters, radioactivity was counted as before.

Results

Effect of Growth in Glucose on Penicillin Biosynthesis in Batch and Nitrogen-limited Resting Cell Cultures

Addition of 140 mM (2.5%) glucose drastically reduced the biosynthesis of penicillin (Fig. 1a).

Fig. 1a. Effect of glucose (140 mM) at inoculation time on penicillin production in long-term fermentations.
A: Control cultures without glucose.
B: Cultures supplemented with glucose.
Cell dry weight (●), penicillin (○), residual glucose (▲), pH (△).

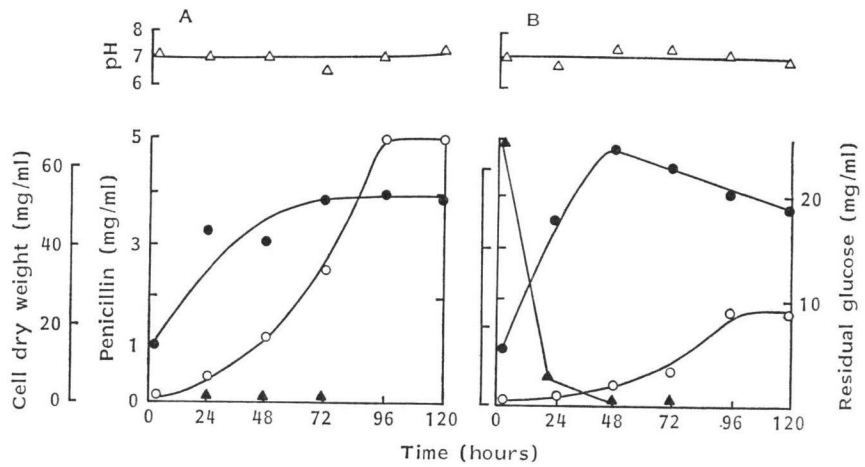


Fig. 1b. Effect of glucose on penicillin production in short-term resting-cell cultures.

Cells were grown without glucose (●) or with 140 mM (○) glucose, collected and suspended in nitrogen-free suspension medium without glucose.

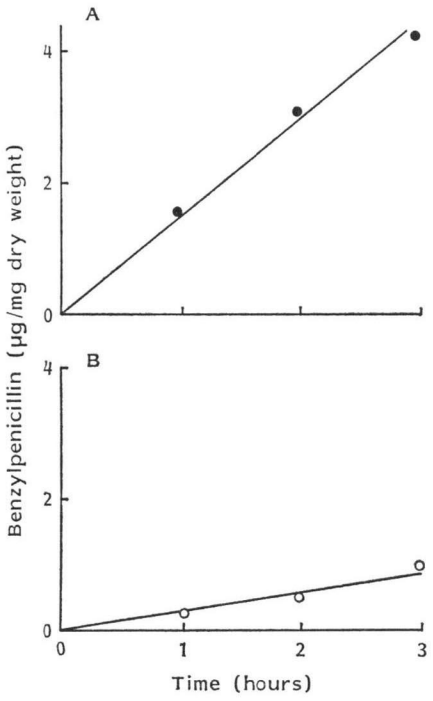
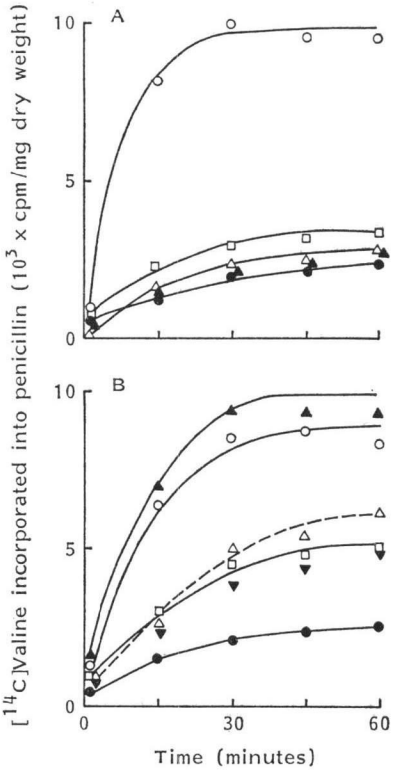


Fig. 2. Effect of growth in glucose and other sugars (140 mM) on incorporation of [^{14}C]valine into penicillin.

A: Control cells grown without glucose (corn steep solids 35 g/liter, lactose 140 mM) (○), cells grown with added glucose (●), cells grown with galactose (△), with fructose (▲), with sucrose (□).

B: Cells grown without glucose (control) (○), with glucose (●), with maltose (□), with starch (△), with dextrins (▼), with lactose (280 mM) (▲).



Small amounts of penicillin were formed in the first 72 hours of fermentation in cultures supplemented with 140 mM glucose. Lower concentrations of glucose (55 mM) had smaller effects. Free glucose existing in unsupplemented cultures (coming from corn steep or lactose) was rapidly exhausted but remained for up to 48 hours in cultures supplemented with 140 mM glucose. In glucose-supplemented cultures penicillin biosynthesis started after glucose was almost depleted from the broth although at a lower rate. Final titer of penicillin in glucose-supplemented cultures (140 mM) was 37% of that in control unsupplemented flasks. At this concentration glucose produced a small increase in dry weight.

Glucose addition up to 140 mM did not change the pH of the culture that remained in the optimal range for penicillin biosynthesis. Glucose concentrations of 280 mM and above lowered the pH of the culture and therefore were not used.

A similar effect of growth in glucose on penicillin biosynthesis by resting cells was observed in short-term experiments (Fig. 1b). In nitrogen-limited resting cell cultures glucose added to the suspension medium did not stimulate growth of the cells.

Effect of Growth in Glucose and Other Carbon Sources on the Incorporation of [^{14}C]Valine into Penicillin by Nitrogen-limited Resting Cells of *P. chrysogenum*

Short-term incorporation of L-[*U*-^{14}C]valine into penicillin by nitrogen-limited resting cell cultures was initially linear but ceased after 30 to 40 minutes. Inorganic phosphate at the concentration in the buffer (320 mM) used for incubation of the resting cell cultures did not affect cellular incorporation of [^{14}C]valine. The incorporation of [^{14}C]valine into penicillin was demonstrated by treatment penicillinase (see Methods). The radioactive penicillin spot (Rf 0.88) was transformed after penicillinase treatment in a new spot (Rf 0.50) corresponding to penicilloic acid. Glucose added at the beginning of the fermentation greatly reduced the incorporation of valine into penicillin (Fig. 2A).

Different carbon sources exerted a distinct inhibitory effect on the incorporation of [^{14}C]valine into penicillin. Control cells were grown in CPM as indicated in methods (corn steep solids 35 g/liter; lactose 25 g/liter). Incorporation was highest when cells were grown in cultures supplemented with additional lactose (Fig. 2B). Addition of sucrose, fructose or galactose to the growth medium resulted in a 64% to 70% decrease in the total incorporation of [^{14}C]valine into penicillin (Fig. 2A). Maltose and polymeric carbon sources as dextrin or starch exerted a smaller inhibitory effect (50% at the maximum) (Fig. 2B). There were no significant differences in the final dry weight obtained in the different carbon sources. The small differences in dry weight were taken into account during preparation of the resting cell systems, all of which were adjusted to 12 mg cell dry weight/ml (see Methods).

Fig. 3. Effect of increasing concentrations of glucose on incorporation of [^{14}C]valine into penicillin.

Cells were grown in 0 mM (○), 28 mM (●), 56 mM (△), 140 mM (▲) glucose.

Upper: Uptake of [^{14}C]valine by cells grown in absence of (○) and presence of 140 mM (●) glucose.

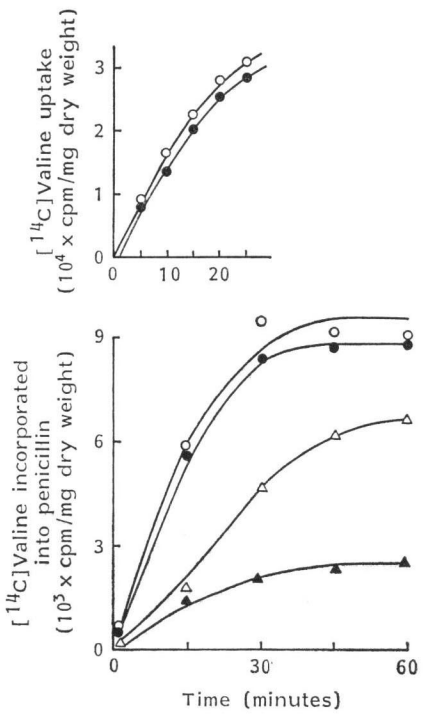


Fig. 4. Repression and lack of inhibition of penicillin biosynthesis by glucose.

Cells were grown in the presence or absence of glucose (140 mM), and then were suspended in nitrogen-free suspension medium with or without glucose (140 mM).

A: Cells grown without glucose, suspended without glucose (○), cells grown with glucose, suspended with glucose (△), cells grown with glucose, suspended without glucose (●).

B: Cells grown without glucose, suspended without glucose (○), cells grown without glucose, suspended with glucose (▲).

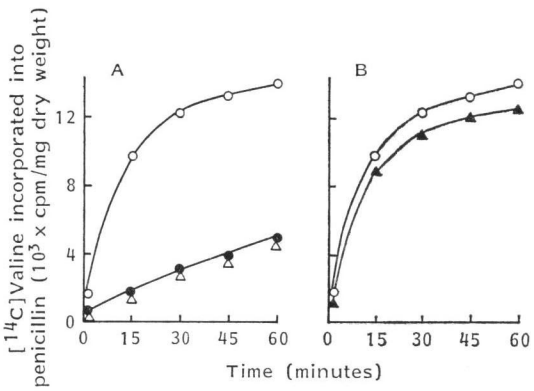
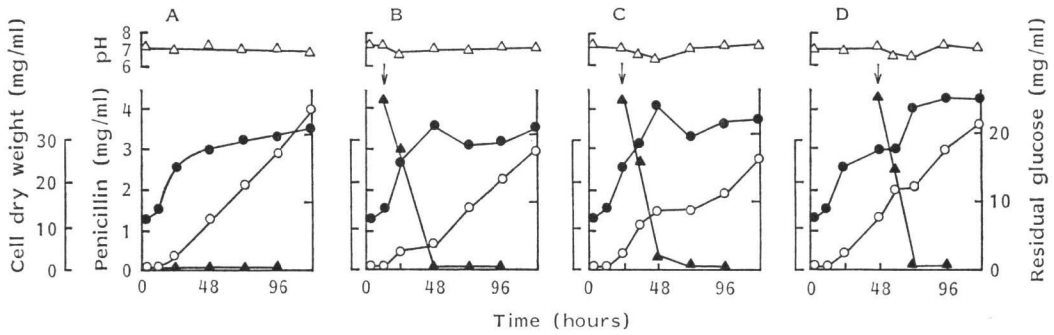


Fig. 5. Effect of the time of addition of glucose on growth of *P. chrysogenum* AS-P-78 and penicillin biosynthesis.

Glucose (140 mM) was added at 12 hours (B), 24 hours (C), 48 hours (D) (arrows). Control without addition (A). Cell dry weight (●), penicillin (○), residual glucose (▲), pH (△).



Effect of Increasing Concentration of Glucose on Penicillin Biosynthesis

Biosynthesis of penicillin (determined as [^{14}C]valine incorporated into penicillin) by nitrogen-limited resting cell cultures of *P. chrysogenum* previously grown on various concentrations of glucose was not affected by growth in 28 mM glucose and was reduced about 30% by 56 mM, and 70% by 140 mM glucose (Fig. 3).

The uptake of [^{14}C]valine by resting cells of *P. chrysogenum* was not significantly affected by previously growing the cells in the presence or absence of glucose (Fig. 3 upper).

Repression or Inhibition by Glucose of Penicillin-synthesizing Enzymes

In order to elucidate whether the glucose effect is exerted by repression or inhibition of penicillin-synthesizing enzymes we carried out experiments in which cells were grown in the presence of glucose and then the sugar was washed out before incorporation of [^{14}C]valine, or cells were grown in the absence of glucose and then supplemented with the sugar prior to addition of [^{14}C]valine. The glucose effect was only exerted when cells were grown in the presence of the sugar (Fig. 4A), but not when grown in the absence of the sugar and later supplemented with glucose (Fig. 4B). These results suggest that the mechanism of carbon catabolite regulation of penicillin biosynthesis involves repression of penicillin-synthesizing enzymes but not inhibition of these enzymes after they are formed.

Time-course of Penicillin Biosynthesis: Repression by Glucose of Penicillin-synthesizing Enzymes

If the glucose effect is due to repression of the penicillin-synthesizing enzymes, reduction should occur when glucose is added before penicillin-synthesizing enzymes have been formed.

The glucose effect was more drastic when glucose was added at the beginning of the growth phase than when added at 12 or 24 hours of incubation. The sugar had almost no effect when added later (Fig. 1 compared with Fig. 5).

To measure the total penicillin-synthesizing activity (the so-called penicillin "synthetase") at different times during the fermentation, protein synthesis was inhibited with cycloheximide (Fig. 6A, B). Cycloheximide (150 μg/ml) completely blocked protein synthesis (as measured by the incorporation of [^{14}C]leucine into TCA-insoluble material) for at least 3 hours. In such translation-inhibited resting cell cultures the biosynthesis of penicillin was followed for 3 hours at different times during the fermentation (Fig. 6B).

Fig. 6. Time-course of penicillin-synthesizing activity.

Penicillin synthesizing activity was determined after blocking protein synthesis with cycloheximide.

A: Effect of cycloheximide (150 μg/ml) on [^{14}C]-leucine incorporation into TCA-insoluble material. Control (●), with cycloheximide (○).

B: Time-course of growth (△), penicillin synthetase activity (●), and penicillin (○).

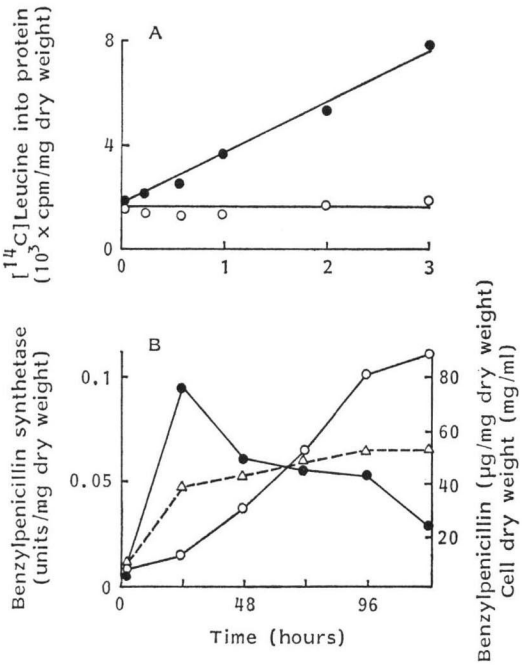


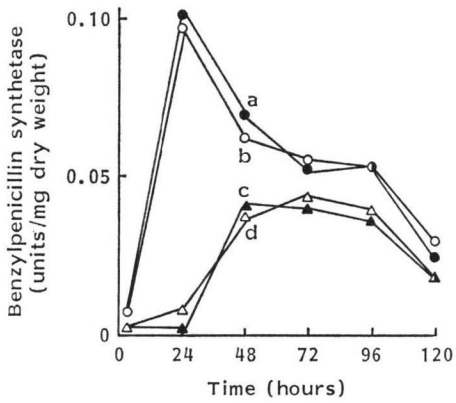
Fig. 7. Repression of penicillin-synthesizing activity by glucose.

a: Penicillin synthetase activity at different times during the fermentation in control cells without addition of sugar.

b: Penicillin synthetase activity of cells grown in the absence of glucose, collected at different times during the fermentation and supplemented with glucose (140 mM) during the resting cell incubation.

c: Penicillin synthetase activity of cells grown in presence of 140 mM glucose.

d: Penicillin synthetase activity of cells grown in presence of glucose and supplemented with additional glucose (140 mM) during the resting cell incubation.



Cultures of *P. chrysogenum* AS-P-78 showed a maximal penicillin-synthesizing activity in complex medium at 24 hours of fermentation, coinciding with the onset of penicillin biosynthesis. After 48 hours the penicillin-synthesizing activity was smaller, although it kept an intermediate level until 96 hours of incubation, decreasing rapidly thereafter. During this period production of most of the penicillin occurred. After 96 hours of fermentation no more accumulation of the antibiotic took place under the experimental conditions used.

Repression of Penicillin-synthesizing Enzymes by Glucose

As shown in Fig. 7 glucose added to the nitrogen-limited resting cell system after cells had been grown in the absence of glucose had no effect on penicillin synthetase activity (Fig. 7 line b). However, when cells were grown in the presence of glucose there was a strong repression of the synthetase activity (line c). Almost no penicillin-synthesizing activity was detected in the first 24 hours of glucose-supplemented fermentations, and only low levels were found after 48 hours, apparently due to a late derepression of the penicillin-synthesizing enzymes. No additional inhibitory effect was observed when cells were grown in the presence of glucose and supplemented with additional glucose in the nitrogen-limited resting cell system (line d).

Discussion

The molecular mechanism of carbon catabolite regulation of penicillin biosynthesis has received little attention so far[4]. The results shown in this paper (using short-term *in vivo* experiments to measure either total penicillin or the incorporation of [^{14}C]valine into penicillin) clearly suggest that the glucose effect is due to repression of penicillin-synthesizing enzymes. Glucose had no effect when added to the nitrogen-limited resting cell system (in which no growth takes place) prior to the addition of the labelled precursor [^{14}C]valine (Fig. 4). Similarly, no effect of glucose on penicillin-synthesizing activity was observed in cells in which further protein synthesis was blocked with cycloheximide (Fig. 7). The penicillin-synthesizing enzymes were already formed at the time (24 hours) cells were collected to prepare the resting cell system (Fig. 6B). This pattern of penicillin-synthesizing enzymes explains why the more severe suppressing effect of glucose on benzylpenicillin formation occurred when glucose was added at the time of inoculation (Figs. 1 and 5). The lack of effect of glucose in nitrogen-limited resting cell cultures in which some protein synthesis still occurs (Fig. 6A) suggest that the turnover of penicillin-synthesizing enzymes in the resting cell system is very small.

There are not many reports in the literature studying whether carbon catabolite regulation of other antibiotics is exerted at the repression or inhibition level or both. The reduction of actinomycin biosynthesis by glucose is due to repression of phenoxazinone synthetase, which is involved in the formation of phenoxazinone ring of actinomycin[5]. Glucose markedly repressed the ring expansion system (deacetoxycephalosporin C synthetase) involved in cephalosporin C biosynthesis[8,9]. *O*-Demethylpuromycin methyltransferase, the last enzyme of the puromycin biosynthetic pathway, is repressed by glucose[6]. In the cases of other antibiotics carbon catabolite regulation may involve inhibition of the activity of antibiotic synthetases in addition to repression of the formation of these enzymes[3]. Glucose interference in bacitracin production has been reported to be due to a decreased pH[19,20]. This is not the case in our studies on penicillin production since experiments were always carried out at low glucose concentrations which did not decrease the pH (Fig. 1). Moreover, experiments in systems buffered with MOPS indicated that glucose exerted the same repressive effect.

The results reported in this paper indicate that there is a clear correlation between depletion of glucose and derepression of antibiotic biosynthesis (Fig. 1). Lactose does not exert the carbon catabolite regulatory effect, even though it is well utilized as carbon source for growth. This suggests that lactose is transported and phosphorylated by a different transport system that does not form the repressing effector. Alternatively, lactose may be catabolized slowly without accumulation of enough glucose to reach the repressing threshold. Other carbon sources exert the carbon catabolite regulation to different extents. Similar results have been reported in the regulation of cephalosporin biosynthesis by different carbon sources[21].

Acknowledgments

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