CARBON CATABOLITE REGULATION OF THE CONVERSION OF PENICILLIN N INTO CEPHALOSPORIN C

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(Received for publication January 7, 1983)

Cephalosporin C biosynthesis by *Cephalosporium acremonium* was delayed until most glucose in the medium was used. Addition of increasing concentrations of glucose up to 55 g/liter decreased cephalosporin C biosynthesis but stimulated growth. Sequential formation of penicillin N (an intermediate in the cephalosporin C biosynthetic pathway) and cephalosporin C was found when the culture was developed synchronously. Little cephalosporin C formation was observed until most penicillin N had already been formed. The sequential formation of penicillin N and cephalosporin C was due to the sequential formation of the "penicillin N synthetase system" and the "cephalosporin C synthetase system". Cells grown in the presence of glucose showed an increased accumulation of penicillin N and clear reduction of the conversion of penicillin N to cephalosporin C. Resting cell studies indicated that the glucose effect was due to the repression of one or more of the enzymes converting penicillin N into cephalosporin C. Little inhibition by glucose of the activity of these enzymes, once formed, was observed. Glucose did not effect significantly the pool sizes of either precursor amino acids of cephalosporin (α-aminoadipic acid and valine) or methionine (an inducer of penicillin N and cephalosporin C biosynthesis). On the basis of these data it is suggested that glucose catabolism specifically represses the enzyme system converting penicillin N into cephalosporin C.

Carbon catabolite regulation of antibiotic biosynthesis is a general regulatory phenomenon affecting the production of many antibiotics belonging to different biosynthetic groups[1]. The biosynthesis of fungal and bacterial β-lactams is subject to negative feedback regulation by glucose[2~5].

Initial studies by DEMAIN[2] suggested that glucose was the best carbon source for growth of *Cephalosporium acremonium*. KENNEL and DEMAIN[3] used several sugars for cephalosporin production by *Cephalosporium acremonium* and found an inverse relationship between the growth rate achieved on different carbon sources and the antibiotic production obtained. Similar results were reported by MATSUMURA *et al.*[5] in batch and continuous cultures. The highest cephalosporin C yield was obtained in continuous culture at low dilution rates, using glucose-limited cultures. The molecular mechanism of carbon catabolite regulation is not known. KUENZI[6] has reported a combined effect exerted by glucose and phosphate. Similar results showing a requirement for phosphate in catabolite regulation of penicillin biosynthesis by *Penicillium chrysogenum* have been found by MARTÍN and ANTEQUERA (submitted manuscript).

Cephalosporin C (CPC) is synthesized either by *C. acremonium* or several Streptomyces species following a similar biosynthetic pathway which includes: 1) formation of the tripeptide δ (α-aminoadipyl)-cysteinylvaline by condensation of the three component amino acids, 2) cyclization to form penicillin N (Pen N) in a two-step process through the intermediate isopenicillin N and 3) conversion by ring expansion of Pen N into deacetoxycephalosporin C (DACPC), which is finally transformed to

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cephalosporin C (for a review see DEMAIN[7]). The time-sequence of formation of different intermediates in cephalosporin biosynthesis and its regulation is unknown. However, in several fungi the formation of secondary metabolites follows a clearcut sequential pattern[8,9]. It was therefore of great interest to study the formation of the intermediate Pen N and its conversion to CPC, and the possible role of glucose in controlling specific enzymes of cephalosporin biosynthesis. Our results indicate that there is a sequential formation of Pen N and CPC and that glucose specifically controls the conversion of Pen N into CPC.

Materials and Methods

Microorganisms and Culture Conditions

Cephalosporium acremonium (synonym: *Acremonium chrysogenum*) strain CW-19 is an improved cephalosporin producing strain derived from the initial *Cephalosporium acremonium* Corda ATCC 11550 by several mutational steps[10]. Cultures were grown on solid sporulation medium[11].

Escherichia coli ESS 22–31, supersensitive to β-lactam antibiotics, was a gift of Professor A. L. DEMAIN, MIT, Cambridge, Massachusetts. *Alcaligenes faecalis* ATCC 8750 and *Micrococcus luteus* ATCC 9341 were kept and grown in TSA medium (Difco).

Purified Preparations of Conidia

Inoculation was carried out with conidia obtained from LE PAGE medium[11]. Conidia were suspended in tris-HCl buffer 0.1 M, pH 7.0, and filtered through sterile gauze and cotton to remove mycelial fragments. After centrifugation the conidia were suspended in tris-HCl buffer at a final concentration of 10^8 conidia/ml.

Antibiotic Production Medium

Studies on antibiotic biosynthesis were carried out in the defined medium described by DREW and DEMAIN[12] containing per liter: sucrose 36 g, glucose 27 g, ammonium sulfate 7.5 g, oleic acid 1.5 g, ammonium ferrous sulfate 7.5 g, and salts solutions 135 ml. Salts solution contained KH_2PO_4 62 g, K_2HPO_4 69.3 g, Na_2SO_4 3.33 g, $MgSO_4 \cdot 7H_2O$ 1.633 g, $MnSO_4 \cdot H_2O$ 0.133 g, $ZnSO_4 \cdot 7H_2O$ 0.133 g, $CuSO_4 \cdot 5H_2O$ 0.033 g, $CaCl_2$ 0.25 g in 600 ml of distilled water. The pH of the medium was adjusted to 7.3 prior to sterilization. When required the medium was buffered with 200 mM 3-[*N*-morpholino]-propanesulfonic acid (Sigma). Production of β-lactams (Pen N and CPC) in this medium reached about 400 μg/ml. Some experiments were carried out in the complex medium described by CALTRIDER and NISS[13] where production reached 1,000 μg/ml. Biosynthesis of cephalosporin in both media was induced with DL-methionine (3 g/liter).

Resting Cell Cultures

Cells grown for 84 hours in defined medium were collected, washed thoroughly and suspended in a nitrogen-free medium as described by MASUREKAR and DEMAIN[14] containing per liter: sucrose 36 g, salts solution ($\times$10) 160 ml in 0.05 sodium phosphate buffer pH 7.0. Salts solution ($\times$10) contained per liter: KH_2PO_4 30 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, $FeSO_4 \cdot 7H_2O$ 1 g, $CuSO_4 \cdot 5H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 0.2 g, Na_2SO_4 5 g, $MnSO_4 \cdot 1H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.5 g. The sucrose, salts solution and sodium phosphate buffer were sterilized separately. Growth in this medium was limited by the absence of nitrogen. Biosynthesis of antibiotics by resting cell cultures was linearly dependent on incubation time and cell-mass.

Assays of Pen N and CPC

Pen N and CPC were routinely determined microbiologically. CPC was also measured by HPLC. *E. coli* ESS 22–31 was used for the determination of both Pen N and CPC[15]. Bioassays were carried out in TSA medium. *Alcaligenes faecalis* was used for the determination of CPC, since it is resistant to Pen N[16]. *Micrococcus luteus*, which is resistant to CPC (up to 500 μg/ml) was used for the determination of Pen N. CPC in broths was also measured by a difference after treatment with *Bacillus cereus* UL-1 penicillinase (10 μg per 100 μl of broth; incubation at 35°C for 1 hour). Bioas-

says with *A. faecalis* and *M. luteus* were carried out in Bacto-Antibiotic Medium 5 (Difco). In all cases the assay medium (15 ml) was inoculated with 0.5 ml of an exponential culture of the test bacteria. Assays were carried out with 10 and 20 μl of the broths (or control solutions) impregnated in 6-mm glass-fiber discs (Whatman GF/C). After diffusion at 4°C for 4 hours, the plates were incubated for 24 hours at 37°C[17].

CPC, DACPC and deacetylcephalosporin C were also determined by HPLC after filtration through a 0.45 μm filter (Millipore) and deproteinization with acetone. Analysis was carried out in a Varian 5000 chromatograph with a Micro-Pak CH-10 column of 30 cm, using a 254 nm detector. Mobile phase was acetonitrile - acetate buffer (10 mM, pH 4.7). The elution mixture was 98% acetate buffer and 2% acetonitrile with a flow of 0.3 ml/minute.

Total protein was determined as described by LOWRY *et al.*[18] and glucose by the glucose oxidase method.

"Pen N Synthetase" and "CPC Synthetase" Systems

The "Pen N synthetase system" refers to the enzyme complex involved in the *in vivo* formation of Pen N. Activity of the "Pen N synthetase system" was determined in cells washed three times with 0.05 M phosphate buffer pH 7.3 and suspended in resting-cell medium supplemented with cycloheximide (100 μg/ml). Cycloheximide at this concentration fully inhibits protein synthesis in *C. acremonium.* Pen N biosynthesis by cells in which *de novo* protein synthesis has been blocked by cycloheximide, is due to preexisting Pen N-synthesizing enzyme. One unit of the "Pen N synthetase system" is defined as the activity which forms 1 μg of Pen N in 1 hour at 25°C. Similarly, the "CPC synthetase system" is defined as the CPC-forming activity in cells in which protein synthesis has been blocked with cycloheximide[20].

Analysis of Amino Acids

The mycelium was collected by filtration, washed with 10 mM HCl and distilled water, and resuspended in 75 g/liter trichloroacetic acid. After extraction the mycelium was filtered again and the filtrate adjusted to pH 2.5. Amino acid analysis was carried in a Beckman 119 BL/CL amino acid analyzer.

Results

Kinetics of β-Lactam (Pen N and CPC) Biosynthesis

Synchronous swelling of conidia, emission of germ-tubes, growth of thin hyphae and differentiation into arthrospores were obtained in methionine-supplemented cultures by inoculating directly the culture with a purified preparation of conidia. Results shown in Fig. 1 indicate that under these conditions glucose in the control cultures (see Materials and Methods) was rapidly utilized and only low levels remained after 72 hours. Growth took place until 96 hours of incubation. A 24-hour lag phase was clearly seen as determined by dry weight when the medium was inoculated with conidia. The phase of high rate of antibiotic biosynthesis was delayed until most glucose had been consumed.

Sequential Formation of Pen N and CPC: "Pen N Synthetase System" and "CPC Synthetase System"

Sequential formation of Pen N and CPC by strain CW-19 was observed during cephalosporin fermentation under the conditions of synchronous growth and differentiation (Fig. 2). Until 96 hours of incubation, when most Pen N had already been formed, little conversion of Pen N into CPC was observed. These results suggest that the ring expansion system that converts Pen N into CPC is not active until it is derepressed later in the fermentation. This effect is strain-specific and also depends on biosynthetic enzymes induction by methionine[10].

The sequential synthesis of Pen N and CPC appears to be the result of the earlier formation of the

Fig. 1. Kinetics of growth, specific β-lactam (Pen N and CPC) production and glucose utilization.

A synchronous culture was developed by inoculating production medium with a purified preparation of conidia (see Materials and Methods).

Symbols: ●, cell dry weight; ○, specific production of β-lactam antibiotic; △ glucose; ■ pH.

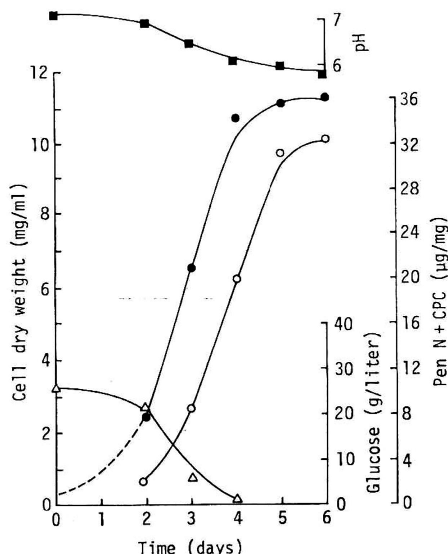


Fig. 2. Sequential formation of Pen N and CPC in methionine-induced (3 g/liter) cultures under conditions of synchronous growth.

Symbols: ●, volumetric β-lactam (Pen N+CPC) production; ▲, Pen N; ○, CPC.

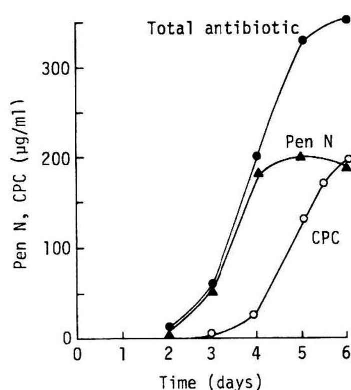
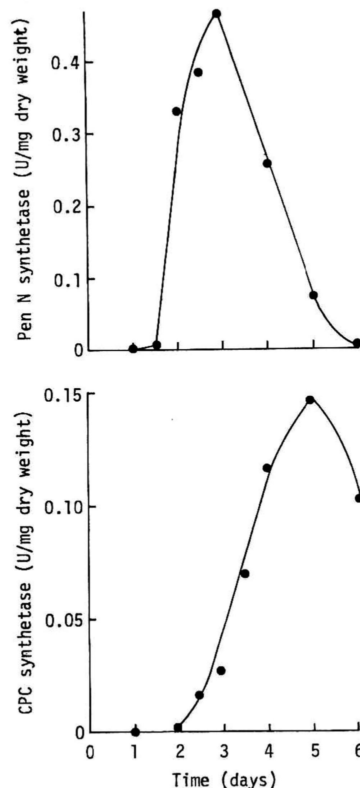


Fig. 3. Sequential formation of the "pen N synthetase system" (upper) and "CPC synthetase system" (lower) in methionine induced (3 g/liter) cultures under conditions of synchronous growth.



"Pen N synthetase system" (Fig. 3). When the activity of each of these enzyme systems was determined, "Pen N synthetase" reached a peak in methionine-induced fermentations at 60 to 72 hours and decreased rapidly thereafter. By contrast, "CPC synthetase" in methionine-induced fermentations reached a maximum at 96 to 120 hours and remained at a high activity level even at 144 hours (Fig. 3). The sequential formation of the "Pen N synthetase system" and the "CPC synthetase system" may be the result of a differential derepression of these enzymes by different threshold levels of glucose; alternatively the "CPC synthetase" might be induced by high levels of Pen N.

Effect of Glucose on Total β-Lactam (Pen N+CPC) Biosynthesis

Addition of increasing concentrations of glucose at the beginning of incubation produced a glucose-dependent decrease of antibiotic biosynthesis (Fig. 4). The inhibitory effect is not due to changes in the

Fig. 4. Effect of increasing concentrations of glucose added at inoculation time on specific β-lactam (Pen N+CPC) biosynthesis.

Note that there is a significant reduction of β-lactam biosynthesis, but no change in pH.

Symbols: specific production of β-lactam antibiotics in control (C) cultures (27 g/liter) (●), and after addition of 35 g/liter (○), 45 g/liter (△) and 55 g/liter (▲) of glucose. pH in control cultures (□) and in cultures supplemented with 55 g/liter of glucose (■).

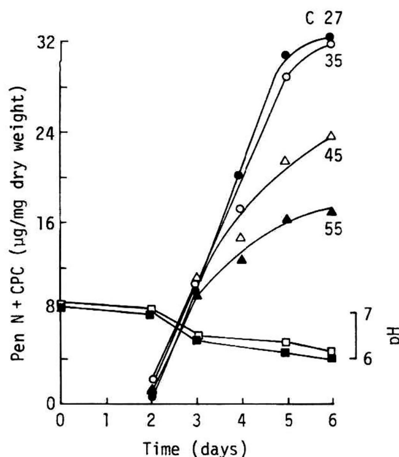
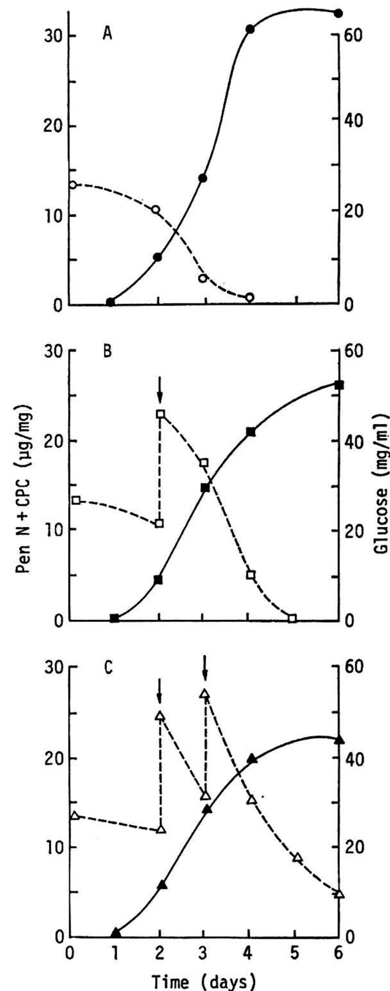


Fig. 5. Effect of glucose added at different incubation times on specific β-lactam production.

A, control without glucose addition; B, addition of glucose (25 g/liter) at 48 hours; C, addition of glucose (25 g/liter) both at 48 and 72 hours. Continuous line: specific production of β-lactam antibiotics; dashed line: glucose.

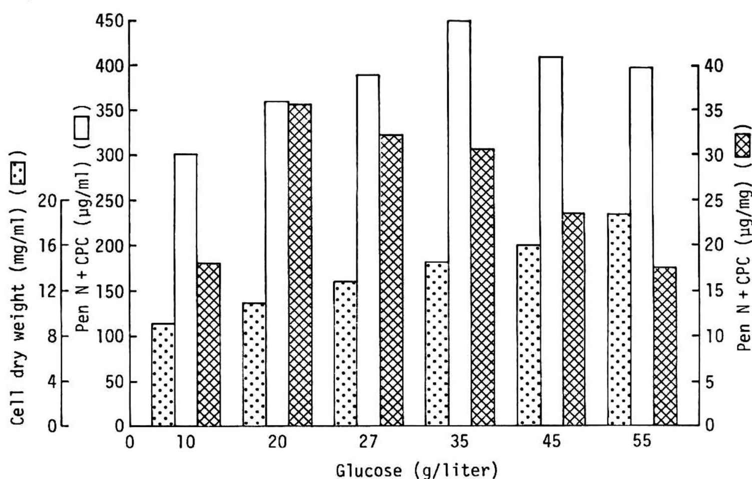


pH of the culture which is not significantly effected. Moreover, the same effect was observed when experiments were carried out in buffered systems (not shown).

When glucose (140 mM) (25 g/liter) was added to the fermentation medium at 48 hours of incubation, coinciding with the phase of hyphal growth, the production of total β-lactam (Pen N plus CPC) was reduced (Fig. 5). An additional reduction was observed when glucose was added both at 48 hours and 72 hours (Fig. 5). Later additions had a smaller inhibitory effect, suggesting that after the biosynthetic enzymes are formed, addition of glucose has little or no effect. The highest effect was observed when glucose was added at the beginning of fermentation (compare with Fig. 4 in which additions were made at the beginning of fermentation).

A summary of the results of several experiments shown in Fig. 6 indicates that growth of *C. acremonium* was linearly stimulated by up to 55 g/liter of glucose, but specific β-lactam biosynthesis (that is antibiotic produced per unit of dry weight) was highest when glucose concentration was about 20 g/liter. Concentrations of glucose below 20 g/liter were limiting both for growth and antibiotic biosynthesis. These results clearly indicate that specific β-lactam production is higher at glucose concentrations limiting for growth, but is lower at concentrations supporting high growth.

Fig. 6. Comparative effect of increasing concentrations of glucose added at inoculation time on cell dry weight (dotted bars), volumetric β-lactam biosynthesis (open bars) and specific β-lactam production (dashed bars).



Increase of Pen N Accumulation and Decrease of CPC Formation after Glucose Addition

The cephalosporin biosynthetic pathway can be divided into two parts; the reactions starting from the component amino acids leading to Pen N, and the ring expansion and late modification reactions that convert Pen N into CPC.

To elucidate whether the glucose effect was exerted on Pen N-synthesizing enzymes, or enzymes converting Pen N into CPC, resting-cell experiments were carried out in which cells from cultures supplemented or non-supplemented with glucose were suspended in a nitrogen-free growth-limiting medium. As observed in Fig. 7, cells grown in the presence of glucose showed a slightly lower capability than the control cells to synthesize total β-lactams (Pen N plus CPC) when suspended in a resting-cell medium without supplementation with glucose. When Pen N and CPC were determined separately it was observed that cells grown in glucose-supplemented medium produced somewhat higher levels of Pen N, but clearly reduced levels of CPC, indicating that glucose affected the conversion of Pen N into CPC. In all cases, CPC biosynthesis was delayed as compared to Pen N production, suggesting again a sequential formation of Pen N and CPC. Cultures grown in presence of glucose showed not only a decrease of CPC formation but also a delayed conversion of Pen N into CPC.

Repression *versus* Inhibition by Glucose of the Enzymes Converting Pen N into CPC

Experiments were carried out to study if the glucose effect was due to repression or to inhibition of the activity of the enzymes converting Pen N into CPC. Cells grown without added glucose were collected and suspended in nitrogen-limited medium in which protein synthesis was inhibited with cycloheximide (100 µg/ml) and the resting cell cultures were incubated with and without glucose. When glucose was added to the resting cell cultures in the absence of cycloheximide, a stimulatory effect on Pen N biosynthesis and an inhibitory effect on CPC formation were observed, whereas when glucose was added to cells in which protein synthesis has been arrested with cycloheximide, only little effect was observed (results not shown). Cycloheximide by itself drastically reduced the conversion of Pen N into CPC. Similar results have been described by KENNEL *et al.*[20] Glucose does not exert a significant inhibitory effect on cephalosporin biosynthesis in cycloheximide-treated cells. However, due to the strong inhibitory effect of cycloheximide the action of glucose may be canceled.

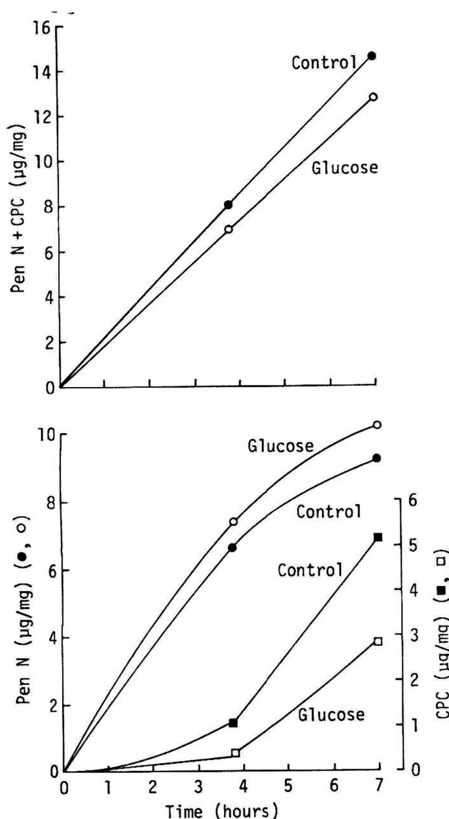


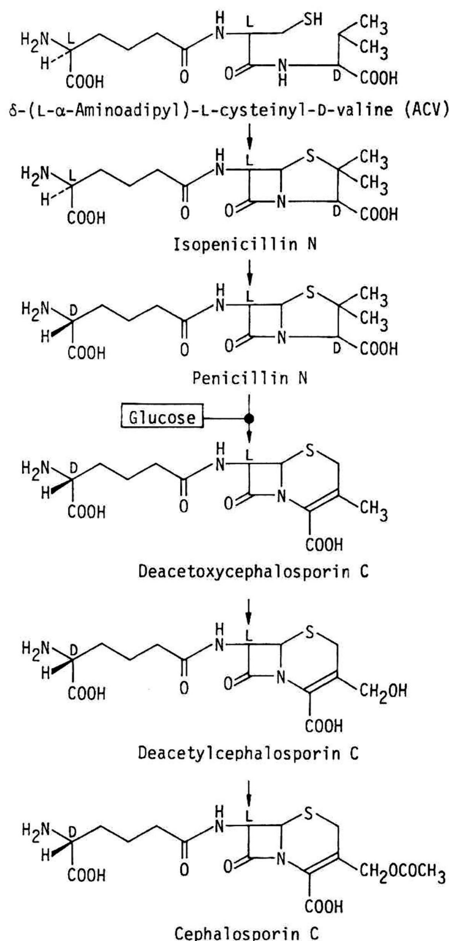
Fig. 7. Accumulation of Pen N and repression of conversion of Pen N into CPC by glucose in resting cell cultures.

Upper: Specific production of β-lactam antibiotics by control cells (●) and cells grown for 84 hours in the presence of glucose (55 g/liter) (○).

Lower: Pen N (●, ○) and CPC (■, □) biosynthesis by control cultures (closed symbols) and cells grown in the presence of 55 g/liter of glucose (open symbols).

Fig. 8. Biosynthetic pathway of CPC converting the ACV tripeptide precursor into CPC.

Glucose appears to act as an effector by repressing the ring expansion enzyme system that converts Pen N into CPC, most likely at the level of conversion of Pen N into DACPC (arrow).



These results suggest that the enzymes converting Pen N into CPC have a very high turnover (ZANCA and MARTÍN, unpublished results) and that glucose does repress, rather than inhibit, the *de novo* formation of such enzymes (Fig. 8).

Intracellular Concentration of Amino Acids Related to CPC Biosynthesis

To test if glucose affected the pool sizes of precursor amino acids of Pen N and CPC, which could explain the accumulation of Pen N, the pool of α-aminoadipic acid (α-AAA), valine, lysine and methionine at 84 hours of fermentation was determined (Table 1).

Table 1. Intracellular concentration of amino acids related to tripeptide ACV and CPC biosynthesis.*

Supplementations of the medium (g/liter)	Amino acids (nmoles/mg dry weight)			
	α-AAA	Val	Lys	Met
None (Control)	1.7	47.2	0.6	3.6
DL-Methionine (3)	1.9	64.0	7.4	5.1
DL-Methionine (3) and glucose (55)	2.0	66.0	7.3	5.1

* Samples were taken at 84 hours of incubation.

Cultures induced with methionine showed a higher pool of α-aminoadipic acid, valine, methionine and a much higher pool of lysine than non-induced cultures. However, cultures induced with methionine and grown in presence of glucose (55 g/liter) did not show any difference with respect to cultures without glucose (Table 1).

Discussion

The sequential biosynthesis of Pen N and CPC (Fig. 2) seems to be the result of the earlier formation of the "Pen N synthetase system" as compared to the "CPC synthetase system" (Fig. 3). These results suggest that the ring-expansion system that converts Pen N into CPC was repressed (or inhibited). Derepression was probably determined by some change in the pool level of an intracellular effector in response to a nutritional shift-down. Such change seemed to be the depletion (or reduction below a threshold level) of the concentration of glucose in the culture broth. When glucose levels were kept high by repeated additions of glucose little production of antibiotic occurred (Fig. 5). The antibiotic activity remaining in the presence of glucose was mostly due to Pen N (Fig. 7).

The sequential formation of "Pen N synthetase and CPC synthetase systems" might be the result of a differential derepression of enzyme formation from the effects of different threshold levels of glucose. Similar derepression mechanisms have been proposed for the phosphate and glucose control of the biosynthesis of macrolide and β-lactam antibiotics[1,4]. Sequential formation of patulin biosynthetic enzymes and intermediates was well established[9]. Mechanisms of control of sequential formation of enzymes involved in the biosynthesis of secondary metabolites by different threshold levels of intracellular effectors appear to be common in fungal systems[8].

CPC biosynthesis was strongly depressed by glucose while Pen N accumulated (Fig. 7). The increase of Pen N in presence of glucose was more likely due to the decreased conversion of Pen N into CPC than the result of stimulation of Pen N formation from its precursors. The pool of valine, α-aminoadipic acid (precursors of Pen N) and methionine (an inducer of cephalosporin biosynthesis) was not affected by growing the cells in high concentrations of glucose.

MATSUMURA *et al.*[21] suggested that the negative regulation by glucose of CPC biosynthesis might be exerted by reduction of the intracellular level of methionine. Our results (Table 1) did not show any effect of glucose on the methionine pools at 84 hours, but we cannot exclude an effect on the methionine pool during the growth phase. An effect of glucose on methionine uptake and/or endogenous biosynthesis (or degradation) under specific circumstances cannot be excluded but this effect would not explain the regulatory effect of glucose on conversion of Pen N to CPC observed in our studies. Methionine activates Pen N as well as CPC formation[10], while glucose reduces CPC biosynthesis, but does not decrease Pen N. Moreover, ZANCA[10] and SAWADA *et al.*[22] have observed that methionine increases several cephalosporin biosynthetic enzymes while our results show that glucose specifically affects the ring expanding system.

Carbon catabolite regulation of cephalosporin biosynthesis in *C. acremonium* seems to be exerted mainly by repression, rather than by inhibition, of enzymes converting Pen N into CPC. However, further experimental research is being carried out to establish unequivocally if there is any role of glucose on inhibition *in vivo* of enzymes converting Pen N into CPC.

Acknowledgments

This work was supported in part by grants of the "Comision Asesora de Investigacion Cientifica y Tecnica", Madrid, and from Antibioticos, S.A., León, Spain. Thanks are due to Ms. LOURDES VARA for typing the manuscript.

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