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Cycloheximide Production by *Streptomyces griseus*: Control Mechanisms of Cycloheximide Biosynthesis

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Cycloheximide accumulation in a fermentation medium has been shown to be the product of the balance between synthesis and degradation of this antibiotic. Glucose has been shown to prevent cycloheximide degradation. Cycloheximide has been shown to interfere with its own synthesis probably due to feedback inhibition. Approaches for increasing cycloheximide titers in the light of these findings are discussed.

Cycloheximide is a glutarimide antibiotic noted particularly for its antifungal properties. It is composed of a dimethyl cyclohexanone nucleus attached to the glutarimide ring. Vanek et al. (8, 9) have shown, using *Streptomyces noursei*, that cycloheximide is formed by the condensation of six malonate units, with one of the malonate units in the glutarimide ring remaining in the carboxylated form. Both methyl groups of the cyclohexanone rings are formed by transmethylation with methionine. Studies by Spizek et al. (6) with *S. noursei* have shown that cycloheximide added to the culture medium interfered with the further accumulation of this antibiotic, indicating that a control mechanism of repression or feedback inhibition was in operation. This paper deals with the production of cycloheximide by *Streptomyces griseus* and the control mechanisms which govern the accumulation of this antibiotic.

MATERIALS AND METHODS

Microorganisms and cultivation. *S. griseus* was used throughout these studies. The organism was maintained on agar slants consisting of the following: glucose, 10 g; Torula yeast, 10 g; distiller solubles, 5 g; KCl, 4 g; $CaCO_3$, 1 g; agar (Difco) 15 g; and tap water to 1,000 ml. Spores from these slants were suspended in distilled water and used as the inoculum for a seed medium of the following composition: glucose, 10 g; beef extract, 5 g; peptone, 5 g; NaCl, 5 g; and tap water to 1,000 ml. The inoculated medium was incubated at 28 C on reciprocating shaker for 2 days. Erlenmeyer flasks (500 ml) containing 100 ml of medium were used for the seed.

The fermentation medium (1) was composed of the following: glucose, 60 g; defatted soybean flour, 15 g; yeast, 2.5 g; $(NH_4)_2SO_4$, 5 g; $CaCO_3$, 8 g; NaCl, 4 g; KH_2PO_4, 0.2 g; and tap water to 1,000 ml. This medium was inoculated with 5% of the seed medium and incubated in stippled 500-ml Erlenmeyer flasks containing 100 ml of fermentation medium at 25 C on a rotary shaker (240 rpm). The inoculum for the fermentation medium was blended under aseptic conditions for 3 min, in a Waring blender prior to addition, to obtain a uniform seed. All media and media components were sterilized by autoclaving at 121 C at 15 lbs/in^2 pressure for 25 min.

Preparation of washed cells. Mycelia were harvested from the fermentation medium by centrifugation in sterile polycarbonate bottles and washed twice with distilled water. The cells were resuspended in water to the concentrations specified in these experiments utilizing the washed cells. All manipulations of harvesting, washing, and resuspension were made at 4 C under aseptic conditions. Washed cells upon resuspension were incubated at 25 C on a rotary shaker in 500-ml stippled Erlenmeyer flasks containing 100 ml total volume.

Dry weights of the washed cell preparations were determined by centrifuging a portion to concentrate the cells. These were washed twice in cold distilled water and dried to a constant weight in tared aluminum dishes at 80 C in a vacuum oven. The dry weights of cells found by this procedure are slightly inflated due to the fact that the fermentation medium contained insoluble components (soybean flour, yeast, $CaCO_3$).

Analytical methods. Cycloheximide concentration in fermentation beer was determined by microbiological assay or a colorimetric assay. For the colorimetric assay 10 ml of whole beer was extracted with 25 ml of butyl acetate by shaking for 10 min and then centrifuged. A suitable portion of the solvent layer was removed, taken to dryness, and used in the colorimetric assay described by Takeshita et al. (7). This method is based on the reaction of cycloheximide with resorcinol. Related compounds such as isocycloheximide and anhydrocycloheximide give full response in this assay but neither compound is present in significant amounts in the fermentation beer. Microbiological assays were performed using the plate-disk method with *Saccharomyces cerevisiae* as the test organism. A comparison between the chemical and microbiological assays on fermentation beers of increasing age resulted in equivalent cycloheximide titers.

Qualitative estimation of cycloheximide was performed by thin-layer chromatography. After extraction of the whole beer as described above, a portion of the solvent was taken to dryness and redissolved in acetone. The acetone solution was spotted on a silica gel GF plate (Analtech, Inc.) to contain 50 to 200 μg of cycloheximide per spot. The solvent system used for development was isopropanol:ethyl acetate (2:98) and detection was accomplished by spraying with the following solution: molybdic acid (2.5 g); ceric sulfate (1.0 g); H_2SO_4 (11.8 ml); and H_2O (88.0 ml). After spraying the plates were heated and cycloheximide appeared as a blue spot on a white background with an R_f of $\simeq$0.7.

Glucose was determined enzymatically by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.).

Radiochemical determinations. The products of [^{14}C]cycloheximide degradation by washed cells were determined using a 1-liter fermentor (Fermentation Design, Inc.). The resuspension medium consisted of: [^{14}C]cycloheximide, 1 mg/ml (3,927 dpm/ml); tris(hydroxymethyl)aminomethane-hydrochloride buffer, 0.05 M (pH 7.0); and cells, 18.8 mg/ml. Total volume of the medium in the fermentor was 500 ml and incubation was at 25 C for 4 days with an aeration rate of 1.0 standard liter per min. The effluent gas was passed through an ethanolamine solution to trap CO_2. ^{14}C-labeled carbon dioxide was determined by the method of Jaffery and Alverez (3). A portion of the beer was separated into solid and aqueous fractions by centrifugation. The solids were washed two times with cold distilled water and the aqueous and washes were combined. The aqueous and solid phases were counted after dispersion in silica (Cab-O-Sil M-5) and Diotol counting solution with a Beckman scintillation counter (model LS-133). Samples of beer were also extracted with butyl acetate by the procedure normally used for the colorimetric assay of cycloheximide. In one case the beer was acidified to pH 2 with HCl prior to the usual extraction procedure. In both cases portions of the solvent layer were taken to dryness and the material was redissolved in chloroform. A suitable portion of the chloroform solution was measured for radioactivity in Diotol counting solution with a Beckman scintillation counter.

Chemicals. Radiochemicals were purchased from the New England Nuclear Corp., Boston, Mass. [^{14}C]cycloheximide was prepared by fermentation in the presence of [1,2-^{14}C]acetate. Anhydrocycloheximide was obtained from L. W. Brown and isocycloheximide from W. J. Haak, both of our laboratories. Diotol scintillation solution was purchased from Burdick & Jackson Lab., Inc., Muskegon, Mich., and was composed of the following: dioxane, 340 ml; methanol, 205 ml; toluene, 385 ml; naphthalene, 70 g; 2,5-diphenyloxazole, 4.8 g; and *p*-bis[2-(5-phenyloxazoyl)]benzene, 0.059 g.

RESULTS

Fermentation studies. The production of cycloheximide in relation to glucose utilization during a standard and glucose fed fermentation is illustrated in Fig. 1. Rapid glucose utilization began 1 day after inoculation and corresponds to the start of cycloheximide accumulation in the medium. Glucose exhaustion corresponds to the cessation of antibiotic accumulation which is followed by rapid degradation of cycloheximide. When a glucose feed is used starting on day 2 of the fermentation, degradation of cycloheximide is not apparent and titers are increased by $\simeq$20%. However, in the presence of excess glucose, the titers still leveled off even with extended fermentation time.

Figure 2 compares the rate of cycloheximide production by *S. griseus* in the presence of various levels of cycloheximide added to a standard fermentation in shake flasks. The net synthetic rate was found to be inversely proportional to the concentration of cycloheximide in the medium. Isocycloheximide and anhydrocycloheximide, both antibiotically inactive, were found to produce the same inhibitory effect when added to a standard fermentation as did cycloheximide (Table 1).

Washed cell studies. A washed cell system was developed to study the biosynthesis and degradation of cycloheximide. The rate of cycloheximide synthesis in this system is linear with respect to cell concentration as shown in Fig. 3. Figure 4 illustrates the synthesis of cycloheximide under various conditions in

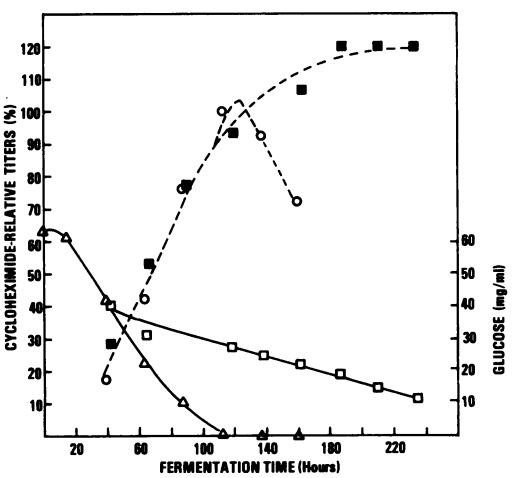

FIG. 1. *Cycloheximide production and glucose utilization during a standard and a glucose-fed fermentation. The fermentation was run in a tank containing 250 liters of medium at 25 C with aeration and agitation of 250 standard liters per min and 300 rpm, respectively. Cycloheximide titer was determined by the colorimetric assay. The glucose feed was initiated at 42 h at a rate of 0.24 g/h per liter. Standard fermentation: cycloheximide, O; glucose, Δ. Glucose-fed fermentation: cycloheximide ■; glucose, □.*

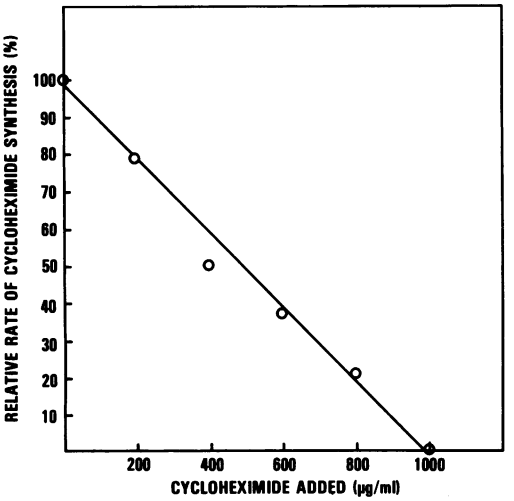

FIG. 2. *The rate of cycloheximide biosynthesis in the presence of added cycloheximide of increasing concentration.*

TABLE 1. *The effect of cycloheximide, isocycloheximide, and anhydrocycloheximide on cycloheximide production in a standard fermentation*

Compound added[a]	Cycloheximide synthesized at 5 days relative to control (%)[b]
None (control)	100
Cycloheximide	0
Isocycloheximide	0
Anhydrocycloheximide	0

[a] A sterile solution of the indicated compound was aseptically added to a standard fermentation at 48 h. The final concentration of the added compound in the fermentation was 1 mg/ml.

[b] Cycloheximide production was determined by bioassay.

a washed cell system as determined by chemical assay (Fig. 4A) and by the incorporation of [^{14}C]glucose into cycloheximide (Fig. 4B). Addition of cycloheximide to this system results in greatly reduced production of cycloheximide when determined by chemical assay (ca. 14% of control) but [^{14}C]glucose incorporation (ca. 30% incorporation of control) indicates that the synthetic process is functioning at a faster rate than indicated by chemical assay. This observation can be explained by the fact that feedback inhibition reduces the effectiveness of the synthetic enzyme(s) sufficiently so that the rate of cycloheximide biosynthesis approximates the rate of cycloheximide degradation. Chloramphenicol was found to severely inhibit cycloheximide synthesis and after an initial lag period this inhibition was complete. Chloramphenicol did not have an effect on the rate of glucose utilization so it appears this inhibition is specific for cycloheximide biosynthesis and unrelated to protein synthesis.

The effect of glucose on cycloheximide degradation is illustrated in Fig. 5. The presence of glucose prevents cycloheximide degradation. This is not due solely to synthesis in its presence since the addition of chloramphenicol which prevents biosynthesis does not increase the rate of degradation. The addition of chloramphenicol in the absence of glucose has no effect on cycloheximide degradation.

Washed cells were used to determine the

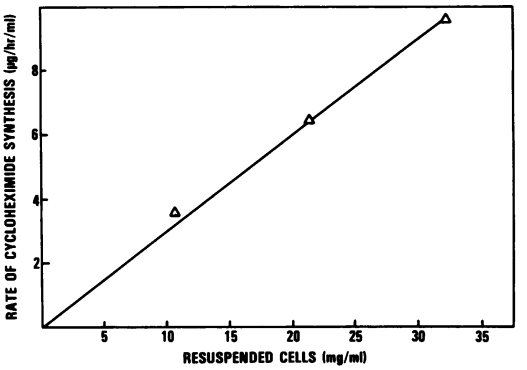

FIG. 3. *Cycloheximide biosynthesis in a washed cell system with respect to cell concentration. Resuspension medium consists of glucose, 60 mg/ml; tris(hydroxymethyl)aminomethane buffer, 0.05 M (pH 7.0); and washed cells as indicated.*

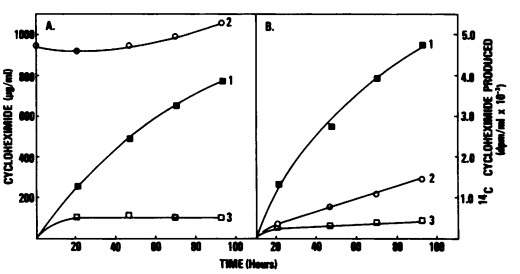

FIG. 4. *The effect of cycloheximide and chloramphenicol on cycloheximide biosynthesis in a washed cell system. (A) Cycloheximide titer as determined by the colorimetric assay. (B) Incorporation of [^{14}C]glucose (UL) into cycloheximide. Resuspension medium consists of: (1) [^{14}C]glucose (UL) (2.22×10^6 dpm/ml), 60 mg/ml; tris(hydroxymethyl)aminomethane buffer, 0.05 M (pH 7.0); and washed cells, 21.5 mg/ml. (2) Same as (1) plus cycloheximide (1 mg/ml). (3) Same as (1) plus chloramphenicol (0.1 mg/ml). Incorporation of [^{14}C]glucose into cycloheximide was determined by extracting a portion of washed cell resuspension with equal volume of chloroform and placing a suitable portion of the solvent in Diotol counting solution, and the radioactivity was determined in a Beckman scintillation counter.*

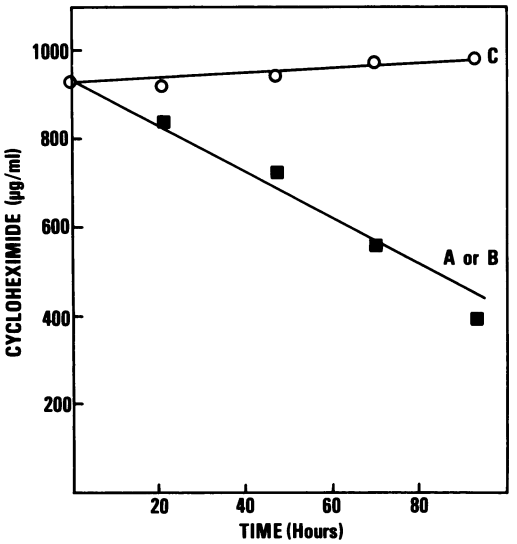

FIG. 5. *Degradation of cycloheximide in a washed cell system. Cycloheximide concentration determined by the colorimetric assay. Resuspension medium consists of: (A) cycloheximide, 1 mg/ml; tris(hydroxymethyl)aminomethane buffer, 0.05 M (pH 7.0) and washed cells, 21.5 mg/ml; (B) same as (A) plus chloramphenicol, 0.1 mg/ml; (C) Same as (A) plus chloramphenicol, 0.1 mg/ml plus glucose, 60 mg/ml.*

ability of cells of increasing age to synthesize or degrade cycloheximide. The results are shown in Fig. 6. The rates of cycloheximide synthesis and degradation were based on equal volumes of beer harvested from a standard fermentation rather than dry weight of the cells because of the insolubles present in the fermentation medium. Differential sedimentation of insoluble medium components and mycelia was possible by centrifugation. Determination of packed cell volume on a daily basis in a standard fermentation indicated that the bulk of growth had taken place in the first 24 h and little change in cell concentration occurs after day 2. Therefore, comparisons based on the volume of beer harvested presents a fair estimate of the relative activity of cells with regard to age.

The ability to synthesize cycloheximide appears to be inducible and reaches a maximum between 3 and 5 days of the fermentation. After 5 days the ability to produce cycloheximide drops off significantly at a point that corresponds to glucose exhaustion in the fermentation medium from which these cells were harvested. The ability to degrade cycloheximide decreases from the beginning of the fermentation and reaches a minimal value between 4 and 5 days. Degradative ability increases from this time which again corresponds with exhaustion of glucose from the fermentation medium.

The product of cycloheximide degradation was studied using ^{14}C-labeled cycloheximide and washed cells as described in Table 2. Cycloheximide was not completely degraded to carbon dioxide but rather to a material which was extractable into butyl acetate only upon acidification of the aqueous phase. This suggests that the degradation product is an acid. Comparison of cycloheximide and the degradation product by thin-layer chromatography shows it to be more polar than the antibiotic, moving just above the origin.

DISCUSSION

The accumulation of cycloheximide in a fer-

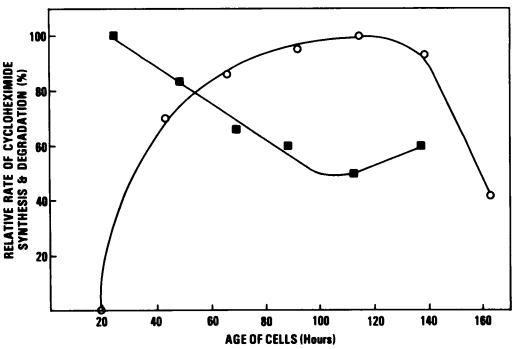

FIG. 6. *Rate of cycloheximide biosynthesis and degradation by cells of increasing age. Rate of synthesis (○): 200 ml of whole beer was harvested at the indicated time interval, washed, and resuspended in 100 ml of a medium containing glucose (60 mg/ml) and tris(hydroxymethyl)aminomethane-hydrochloride buffer, 0.05 M (pH 7.0). Rate of degradation (■): 200 ml of whole beer was harvested at the indicated time interval, washed, and resuspended in 100 ml of a medium containing cycloheximide (1 mg/ml) and tris(hydroxymethyl)aminomethane-hydrochloride buffer, 0.05 M (pH 7.0). In both cases cycloheximide was determined by chemical assay.*

TABLE 2. *Products of [^{14}C]cycloheximide degradation by washed cells of S. griseus*

Determinants	dpm/ml	% of total
[^{14}C]cycloheximide added	3927	100
Total ^{14}C-labeled material recovered[a] at 4 days	3714	94.6
Beer supernate and washes (aqueous phase)	3623	92.3
Cells from beer (solid phase)	49	1.2
$^{14}CO_2$ evolved (gas phase)	42	1.1
^{14}C-labeled material in beer extracts		
No pH adjustment (pH 7.0)	405	10.3
pH adjusted to 2.0	2311	58.8

[a] Chemical assay and bioassay indicated 91.5 and 94% degradation of cycloheximide, respectively.

mentation medium by *S. griseus* has been found to be a dynamic process involving both synthesis and degradation of this antibiotic. The biosynthetic system responsible for cycloheximide formation appears to be inducible and retains a high level of activity throughout the fermentation provided glucose is present. The data suggests that the cycloheximide degrading system is repressible and subject to catabolite inhibition in the presence of glucose. Cycloheximide is not completely degraded to CO_2 but to a bio-inactive acidic compound which has not yet been identified. Cycloheximide has been found to interfere with its own production probably due to feedback inhibition. Therefore, the accumulation of cycloheximide is a product of the balance between synthesis and degradation. When product inhibition becomes great enough to balance the rate of synthesis to that of degradation, accumulation of this antibiotic ceases.

In many respects the factors involved in cycloheximide production appear similar to those affecting chloramphenicol accumulation. Chloramphenicol titer reflects the sum of both catabolic and biosynthetic reactions with biosynthesis subject to product inhibition (2, 5, 10).

Efforts aimed at increasing cycloheximide titer should be directed toward alleviating end-product inhibition of this antibiotic. Mutants with increased resistance to product inhibition would be one approach. Another approach would be the physical removal of cycloheximide during its formation in the fermentation. This latter approach is the subject of the companion paper (4).

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