ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1976, p. 824-830

Vol. 9, No. 5
Printed in U.S.A.

Enzymatic Hydrolysis of Cephalosporin C by an Extracellular Acetylhydrolase of *Cephalosporium acremonium*

A. HINNEN AND J. NÜESCH*

Research Laboratories, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

Received for publication 31 October 1975

Extracellular hydrolases from *Cephalosporium acremonium* were analyzed according to their ability to deacetylate the β-lactam antibiotic cephalosporin C. One out of at least six hydrolases exhibits appreciable cephalosporin C acetylhydrolase (CAH) activity. This enzyme was separated from other hydrolases and purified 220-fold. The purified CAH has a relatively low affinity for cephalosporin C (K_m, 20 mM) and is strongly inhibited by diisopropylfluorophosphate and less markedly affected by fluoride. Addition of glucose, maltose, and sucrose to the culture broth suppresses CAH production, whereas glycerol and succinate have no effect. Verrucarin A prevented the enzyme from appearing in the medium, which indicates the necessity of protein synthesis for CAH formation. When 1-thio-D-glucose was added to the culture medium, the results suggested that this glucose analogue is able to inhibit CAH synthesis. Our data provide evidence for a regulation of CAH synthesis similar to the catabolite repression system in bacteria.

Cephalosporin C, a β-lactam antibiotic produced by different mutant strains of *Cephalosporium acremonium* (4, 7, 9, 26), was first isolated by Newton and Abraham (24).

The biosynthesis of this cyclic tripeptide is not yet understood in detail, but it is now well established that the final steps proceed via deacetoxycephalosporin C and deacetylcephalosporin C to cephalosporin C (Fig. 1; 19). Deacetylcephalosporin C has been detected in both intracellular and extracellular fluids of *C. acremonium* (31). The presence of this compound in the culture broth raises questions about its origin. Deacetylcephalosporin C could be an excreted intermediate product of biosynthesis or the result of enzymatic or non-enzymatic hydrolysis of cephalosporin C.

Huber et al. (15) have reported that their cell-free extracts of *C. acremonium* exhibit hydrolase activity, which fails to deacetylate cephalosporin C. The same authors came to the conclusion that extracellular deacetylcephalosporin C is the result of non-enzymatic hydrolysis. Recently Fujisawa et al. (10) described a mutant of *C. acremonium* with extracellular cephalosporin C acetylhydrolase (CAH; EC 3.1.1.?). We have shown independently that under certain conditions *C. acremonium* is capable of producing CAH (25). We were able to purify and characterize this enzyme. In addition, evidence is presented that the synthesis of CAH is regulated by the nature of the carbon source.

(This work was carried out by A. H. in partial fulfillment of the Ph.D. requirement at the University of Basel, Basel, Switzerland.)

MATERIALS AND METHODS

Strain and culture conditions. A superior cephalosporin C-producing strain, C462, derived from *C. acremonium* C.M.I. 49137/8650 was used for the experiments. Shake flasks (500 ml) with 100 ml of seed medium were inoculated with 5% of a lyophilized spore-mycelium suspension. The flasks were incubated on a rotary shaker at 23 C and 250 rpm for 72 h. The seed cultures were used to inoculate 200-ml shake flasks with 40 ml of production medium, inoculated under the same conditions. The seed medium has been described (3). The production medium contained: 10% peanut meal, 1.2% DL-methionine, 0.2% ammonium acetate, 0.5% methyl oleate, 2.5% D-glucose, 1.3% calcium carbonate, and deionized water. The medium was sterilized for 45 min at 120 C; the pH after sterilization was 7.2 to 7.4.

Measurement of enzyme activities. Acetylhydrolase activity was measured according to Pocker and Stone (29) with *p*-nitrophenylacetate as substrate. CAH activity was followed by a modified acidimetric method (30). The reaction mixture contained cephalosporin C, 100 mM, tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, 5 mM, and phenol red, 0.0006% (wt/vol), at pH 7.5 in a total volume of 1.1 ml. The enzyme extracts were passed through a column of Sephadex G-25, equilibrated with 5 mM Tris-hydrochloride buffer, pH 7.5, prior to measuring the CAH activity. Both enzyme activities are expressed as micromoles of product formed per minute at 25 C under the respective assay conditions (1 unit = 1 μmol/min).

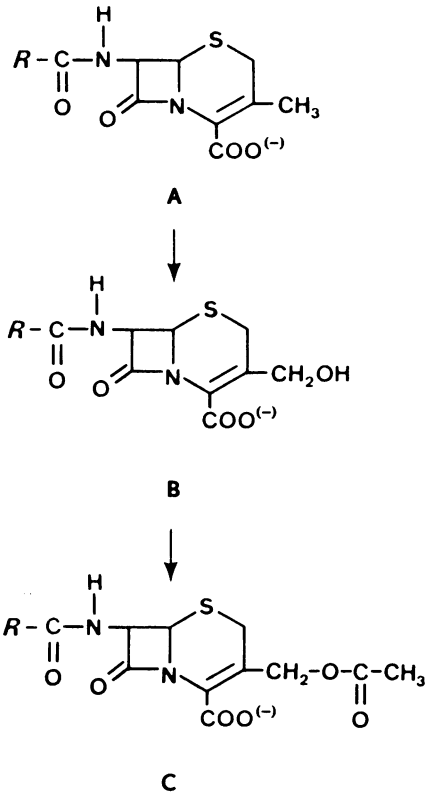

FIG. 1. *Final steps in the biosynthesis of cephalosporin C. (A) Deacetoxycephalosporin C; (B) deacetylcephalosporin C; (C) cephalosporin C. R = HOOC—CH(NH$_2$)—(CH$_2$)$_3$—.*

Assays for cephalosporin C. Cephalosporin C was determined either by a microbiological assay (26) or by high-pressure liquid chromatography (18).

Determination of protein, sedimentation coefficient, and isoelectric point. Protein was determined by the method of Lowry et al. (20), using bovine serum albumin as a standard. Sedimentation coefficients were determined by ultracentrifugation in a sucrose gradient as described by Martin and Ames (22). The centrifugation was carried out in a Heraeus Christ Omega II centrifuge at 4 C for 20 h at 100,000 × *g*, using myoglobin and hemoglobin as standards. The isoelectric point was determined by electrofocusing in polyacrylamide gel (12) with Ampholine, pH 3 to 5, as carrier ampholyte. After electrofocusing, the gel was cut into 1-mm slices, the slices were immersed in 0.2 ml of water, and, after 60 min, the pH was measured. The pH was adjusted to 7.5 by adding 0.05 ml of 0.5 M Tris-hydrochloride buffer, pH 7.5 (22 C), and hydrolase activity was determined.

Electrophoresis and activity staining. Polyacrylamide gel electrophoresis was carried out in the Tris-diethylbarbiturate system as described by Maurer (23). Sephadex G-25 (medium grade) was used instead of the spacer gel. The acrylamide concentration was 10.5%, and a current was applied at 4 mA/tube at room temperature. Acetylhydrolase activity was detected by activity staining using β-naphthyl acetate as substrate (11). Samples (20-μl) of the culture media were applied on each gel, and the staining period was 30 min.

Purification of CAH. All operations were carried out at 0 to 4 C, and all buffer solutions contained dithiothreitol, 10^{-3} M. Mycelium and other solid particles were removed by centrifugation at 40,000 × *g* for 30 min.

(i) Ammonium sulfate precipitation. A 500-ml amount of the supernatant was precipitated with solid ammonium sulfate added to 85% saturation. The suspension was centrifuged at 40,000 × *g* for 30 min, and the supernatant fluid was discarded.

(ii) Ultrafiltration and diethylaminoethyl-Sephadex A-50 chromatography. The ammonium sulfate precipitate was dissolved in 100 ml of 0.1 M Tris buffer, pH 7.9 (4 C), and centrifuged at 40,000 × *g* for 20 min. The supernatant was desalted by ultrafiltration in an Amicon ultrafiltration cell with an XM-50 DIAFLO membrane. No acetylhydrolase activity could be detected in the eluant as checked by polyacrylamide gel electrophoresis and activity staining. The salt-free solution was adsorbed on diethylaminoethyl-Sephadex A-50 in a batch procedure by adding ion exchanger (3-g dry gel), preequilibrated in 0.1 M Tris buffer, pH 7.9 (4 C), to give a final volume of 200 ml. After the mixture had been stirred for 3 h, solid NaCl was added to a concentration of 0.5 M. The ion-exchange material was subsequently removed by filtration through a Buchner funnel. Desalting of the eluate was performed using a UM-2 DIAFLO membrane. The salt-free solution was placed on a column (2.5 by 20 cm) of diethylaminoethyl-Sephadex A-50, equilibrated with the above-mentioned buffer, and eluted by a linear gradient (800 ml) from 0 → 0.5 M NaCl. The flow rate was 4 ml/cm^{-2} per h; 10-ml fractions were collected. By this procedure more than 95% of the unidentified dark pigment material, probably derived from the complex culture medium, was removed.

(iii) Sephadex G-75 chromatography. Fractions 47 to 53 (Fig. 2) were pooled, concentrated to 5 ml by ultrafiltration (PM-2 membrane), and passed through a Sephadex G-75 column (2.5 by 40 cm), preequilibrated with 1 mM phosphate buffer (pH 6.8). The flow rate was 4 ml/cm^{-2} per h; 3-ml fractions were collected.

(iv) Hydroxyapatite chromatography. The active fractions of the above eluate were placed on a column (2.5 by 15 cm) of hydroxyapatite, preequilibrated with 1 mM phosphate buffer, pH 6.8. The enzyme was eluted by increasing the phosphate concentration to 10 mM (linear gradient of 500 ml). The flow rate was 4 ml/cm^{-2} per h; 5-ml fractions were collected. The CAH activity was free from other hydrolase activities, as could be shown by gel electrophoresis and activity staining.

Preparation of cell-free extracts. The mycelium and other solid particles were separated from the culture broth by centrifugation at 40,000 × *g* and washed three times with 0.06 M phosphate buffer,

pH 7.0. A 30-g portion of the washed precipitate was disrupted in an X-press (AB BIOX, Nacka, Sweden) at about −30 C at 2,000 kg/cm² (28,000 lb/in²). The disintegrated cells were suspended in 300 ml of the same buffer, homogenized in a Potter Elvejhem homogenizer, and then centrifuged at 40,000 × *g* for 20 min. The supernatant fluid was used for studying the intracellular hydrolase pattern by electrophoresis. All buffer solutions were held at 0 to 4 C.

Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine, and ammonium persulfate were purchased from Serva (Heidelberg, Germany); Sephadex G-25, diethylaminoethyl-Sephadex A-50, and Sephadex G-75 from Pharmacia (Uppsala, Sweden); Bio-Gel HTP hydroxyapatite from Bio-Rad Laboratories (Richmond, Calif.); and Ampholine from LKB-Produkter (Bromma, Sweden). Dithiothreitol, hemoglobin, and myoglobin were obtained from Calbiochem (San Diego, Calif.); bovine serum albumin, β-naphthyl acetate, phenylmethylsulfonylfluoride, and diisopropylfluorophosphate from Sigma (St. Louis, Mo.); eserine sulfate from Nutritional Biochemicals (Cleveland, Ohio); and 1-thio-D-glucose from Eastman Kodak (Rochester, N.Y.). Verrucarin A was kindly provided by Ch. Tamm. All other chemicals were purchased from Merck (Darmstadt, Germany).

RESULTS

Purification and biochemical properties. Data on the purification of CAH are given in Table 1. The most critical step was the removal of unknown viscous compounds from the fermentation broth. Neither the addition of deoxyribonuclease nor dextranase resulted in a decrease of the viscosity of the broth. By means of a batch procedure using ion-exchange resin, appreciable amounts of the viscous material were removed. Due to this technique it was possible to use column chromatography in the successive purification steps. Owing to the low CAH activity in the extracellular fluid, it was necessary to start the purification by concentrating with ammonium sulfate (precipitation by organic solvents resulted in a poor recovery of the enzyme activity).

Figure 2 shows that only the first hydrolase peak is capable of deacetylating cephalosporin C significantly. However, the CAH activity eluted after the first peak contributes also to the total deacetylating activity. Fractions 47 through 53 (Fig. 2) were pooled and designated as CAH. The total purification of the CAH was 220-fold. After gel electrophoresis and activity staining only one hydrolase band is present.

The pH optimum for CAH was 7.6 (Fig. 3A). Owing to the instability of cephalosporin C at higher temperatures, the temperature optimum had to be measured with *p*-nitrophenylacetate as substrate (Fig. 3B). Above 50 C the enzyme is irreversibly inactivated. By ultracentrifugation in a sucrose gradient a sedimentation coefficient of 2.6 was found. With the approximation of Martin and Ames (22), using hemoglobin and myoglobin as standards, a molecular weight of about 25,000 was calculated. Measurements of the isoelectric point gave a value of 4.3 for CAH.

A rather low affinity of cephalosporin C for CAH was found which is expressed in a K_m value of 20 mM. Table 2 shows the influence of some known hydrolase inhibitors on CAH. On the basis of these results it seems unlikely that CAH is a protease with the capability of cleaving acetyl esters. This is in agreement with the failure to detect any protease activity (mea-

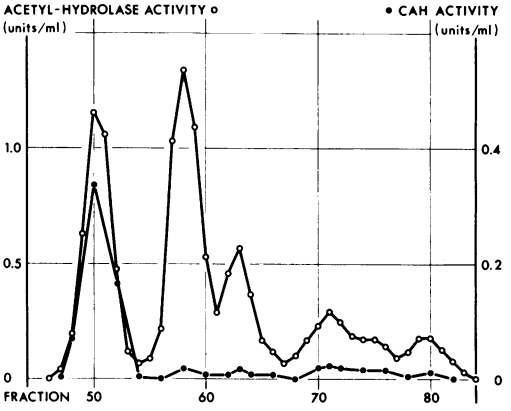

FIG. 2. *Elution pattern of diethylaminoethyl-Sephadex A-50 chromatography (for details see Materials and Methods).*

TABLE 1. *Purification of CAH*

Step	Protein (mg)	CAH (units)	Sp act (units/mg)	Recovery (%)	Purification (fold)
Medium	780	26.3[a]			
Amicon XM-50	410	21.7[a]			
Diethylaminoethyl-Sephadex A-50, fractions 47–53	102	15.1	0.148	80[b]	6.5[b]
Sephadex G-75	10	13.2	1.32	70	58
Hydroxyapatite	1.9	9.5	5.0	49	220

[a] Total CAH activity.
[b] Estimated values.

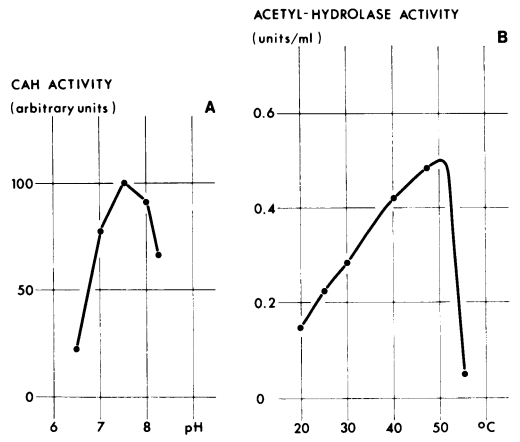

FIG. 3. *Effect of pH and temperature on CAH. (A) pH, measured by incubating cephalosporin C (2 mM) in 0.06 M phosphate buffer, pH 7.5, for 10 h at 25 C and determining cephalosporin C and deacetylcephalosporin C by high-pressure liquid chromatography. (B) Temperature, measured using p-nitrophenylacetate as substrate.*

TABLE 2. *Effect of various substances (1 mM) on CAH*[a]

Substance	Relative CAH activity (%)
None	100
Sodium chloride	100
Calcium chloride	99
Sodium fluoride	67
Diisopropylfluorophosphate	5
Eserine sulfate	95
p-Chloromercuribenzoate	91
Phenylmethylsulfonylfluoride	98

[a] Measurements were done by the acidimetric test (see Materials and Methods).

sured according to reference 27) in the purified CAH extract.

Physiological properties of CAH. Figure 4A shows the course of the fermentation of mutant C462 in the production medium. Until 120 h glucose was added daily to prevent a decrease of the carbon source below 5 g/liter. The maximum cephalosporin C titer is reached at about 144 h. After this time the antibiotic disappears rapidly. Simultaneously, the deacetylcephalosporin C titer rises and CAH activity appears in measurable amounts in the broth. Figure 4B demonstrates that the addition of glucose until the end of the fermentation prevents the appearance of CAH activity in the broth. As a consequence the cephalosporin C level remains high.

In view of the obvious effect of glucose on CAH the question arose whether other carbon sources may have the same effect in suppressing the hydrolytic activities. Maltose and sucrose, two disaccharides readily converted to glucose, and glycerol and succinate, two more distant metabolites derived from glucose, were tested. We have found that, in addition to glucose, maltose and sucrose have the same effect in protecting cephalosporin C from rapid degradation (Table 3). In polyacrylamide gels (Fig.

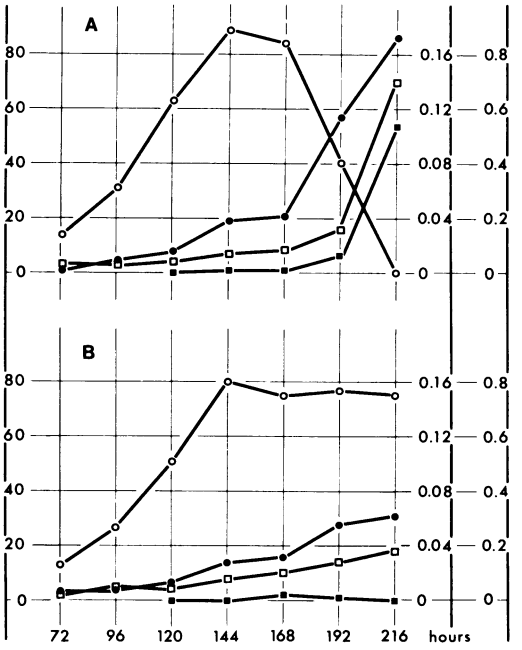

FIG. 4. *Course of the fermentation of mutant strain C462 in the production medium. (A) Glucose was added to maintain its concentration above 5 g/liter until 120 h. (B) Glucose was added until the end of the fermentation. Symbols: β-lactam antibiotic (arbitrary units) — ○, cephalosporin; ●, deacetylcephalosporin; □, acetylhydrolase activity (units per milliliter); ■, CAH activity (units per milliliter).*

TABLE 3. *Effect of different compounds on cephalosporin C titer*[a]

Addition	Cephalosporin C (mg/liter)			
	72 h	96 h	120 h	144 h
None	580	2,050	950	230
Glucose	600	2,010	3,200	4,100
Maltose	605	2,100	3,070	4,020
Sucrose	590	1,980	2,970	3,700
Glycerol	620	2,020	2,100	1,090
Succinate	615	1,930	1,030	630
1-Thio-D-glucose	610	1,380	1,100	980
Verrucarin A	605	1,080	1,010	900

[a] The production medium was used, and from 72 h on the supplementations were begun. Glucose, maltose, sucrose, glycerol, and succinate were added to maintain concentrations of above 5 g/liter. 1-Thio-D-glucose was added once at 72 h (10 g/liter). Verrucarin A was added in amounts of 40 mg/liter each day. Cephalosporin C titers were measured by the biological assay.

5), the band responsible for CAH is rather weak. Although the band is present, the low efficiency of the enzyme in degrading cephalosporin C is not high enough to deacetylate larger amounts of the antibiotic. On the other hand, the addition of glycerol or succinate does not prevent the deacetylation.

The question as to whether the protection from deacetylation depends on protein synthesis has been tested. Indeed, verrucarin A, a potent inhibitor of protein synthesis in eukaryotic cells (5), suppressed CAH (Fig. 6).

To investigate further the effect of glucose on CAH, a glucose analogue, 1-thio-D-glucose, was added to the medium. Figure 6 shows that the glucose analogue has a similar effect on the hydrolase pattern as verrucarin A. However, 1-thio-D-glucose does not inhibit growth of *C. acremonium* in the complex medium. As expected, verrucarin A and 1-thio-D-glucose do not lead to a significant decrease in the cephalosporin C titers (Table 3).

It is evident from our results that CAH is an extracellular enzyme and not an enzyme excreted into the medium by cell lysis. We have failed to determine any intracellular CAH activity. Cell-free extracts were analyzed by gel electrophoresis, and no CAH could be detected after activity staining (Fig. 5).

DISCUSSION

C. acremonium mutant C462 produces extracellular hydrolases. At least one of these hydrolases is able to hydrolyze cephalosporin C to deacetylcephalosporin C. Huber et al. (15) could not detect any CAH activity in fermentation broths of *C. acremonium*. They have concluded that the deacetylation of cephalosporin C is of a non-enzymatic nature. The discrepancy between their results and ours may be due to the fermentation conditions or strains used. We only find appreciable amounts of CAH activity after 120 h, and then its appearance is paralleled by the exhaustion of glucose in the me-

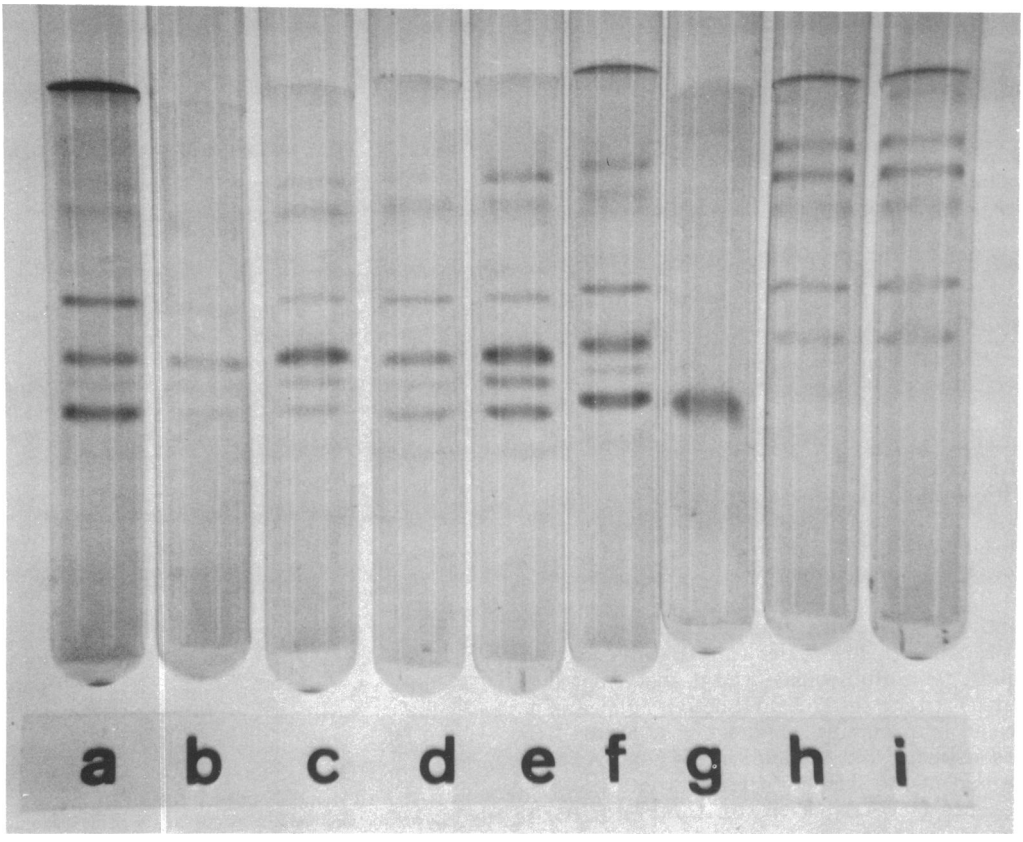

FIG. 5. *Hydrolase spectrum after polyacrylamide gel electrophoresis and activity staining. Samples were taken from the 144-h cultures, supplemented with the following carbon sources (see Table 3): (a) none; (b) glucose; (c) maltose; (d) sucrose; (e) glycerol; (f) succinate; (g) CAH standard; (h) and (i) cell-free extracts with no addition and glucose addition, respectively.*

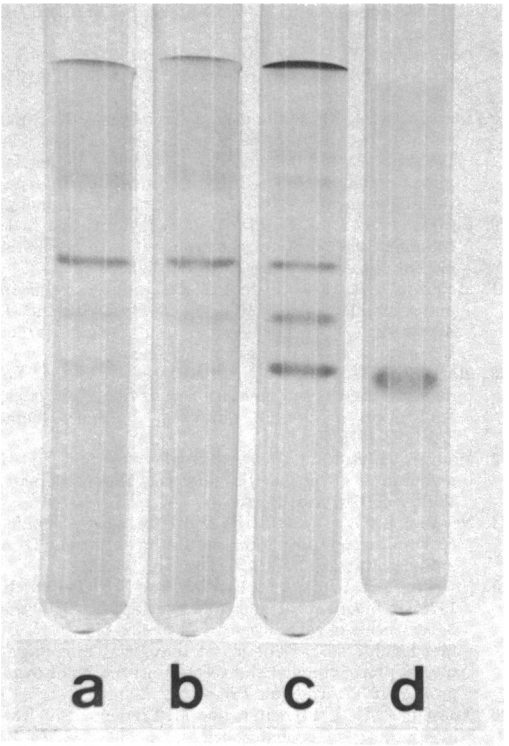

FIG. 6. *Hydrolase spectrum after polyacrylamide gel electrophoresis and activity staining. Samples were taken from the 144-h cultures. The following substances were added (see Table 3): (a) 1-thio-D-glucose; (b) verrucarine A; (c) none; (d) CAH standard.*

dium. Furthermore, CAH activity is very low (0.1 unit/ml), which is expressed in the low cephalosporin C deacetylation rate in the broth. Excluding enzymatic hydrolases, the daily decrease of cephalosporin C titer is 12% ($k = 5.2 \times 10^{-3}$/h; 18), taking into account deacetylation and hydrolysis of the β-lactam ring. Upon carbon starvation this value rises to about 50% in our system. Thus, a fourfold increase, mainly due to deacetylation, can be attributed to the hydrolases in the broth. In spite of the relatively low enzymatic activity, this is enough to create serious problems in the cephalosporin C fermentation process.

Recently Fujisawa et al. (10) have obtained mutants of strain 8650, which accumulate deacetylcephalosporin C in the culture medium. One of these mutants was found to have extracellular CAH activity. The other strains were impaired in the last step of the biosynthesis, the conversion of deacetylcephalosporin C to cephalosporin C. Their findings support our assumption that *C. acremonium* has the genetic potential to produce hydrolases capable of deacetylating cephalosporin C. In the course of different development programs, some mutants might have lost or acquired the possibility to express this genetic information. Our own preliminary results show that mutagenic treatment of strain C462 can lead to mutants with highly reduced CAH activity (H. J. Treichler, personal communication). As a consequence, such mutants retain a high titer of cephalosporin C under conditions of glucose starvation.

The suppression of the CAH activity by the addition of glucose or disaccharides readily converted to glucose supports our hypothesis that the synthesis of CAH and probably other hydrolases is regulated by carbon catabolite repression. Although the mechanism of carbon catabolite repression in bacteria is now largely understood (6, 8, 21, 28), relatively little is known about such systems in eukaryotes (1, 2, 16, 17). Our findings show that suppression of CAH activity is exerted by glucose, maltose, and sucrose, whereas glycerol and succinate have no effect. This is very similar to the results obtained with bacteria (28). We have not been able to release repression by adding cyclic adenosine 3',5'-monophosphate to the culture medium, but there is no evidence that this compound actually enters the cells.

No significant amounts of CAH have been found in a chemically defined medium. This has led us to the assumption that, under such conditions, an additional mechanism might regulate CAH synthesis. Absence of induction or a type of repression other than carbon catabolite repression are two possibilities. Cephalosporin C itself could act as inducer since this compound is formed in six- to eightfold amounts in the complex medium compared to the defined one. Attempts to show this by adding cephalosporin C (10 g/liter) to the chemically defined medium were not successful.

We have tried to explain our results on the basis of transcription control since analogies to well-known bacterial systems are evident. Of course, other explanations are possible. Posttranslational control by inactivating enzymes is an attractive possibility (13, 14). Although *C. acremonium* produces extracellular proteases (32; personal observations), we have no evidence that would support such a mechanism. We hope to obtain more insight into our eukaryotic system by developing chemically defined media in which *C. acremonium* exhibits the same regulatory phenomena as in the complex fermentation system.

ACKNOWLEDGMENTS

We would like to thank J. A. L. Auden, M. Küenzi, M. Liersch, and H. J. Treichler for helpful discussions and critical reading of this manuscript.

LITERATURE CITED

1. **Arst, H. N., Jr., and D. W. Macdonald.** 1975. A gene cluster in *Aspergillus nidulans* with an internally located cis-acting regulatory region. Nature (London) 254:26–31.
2. **Bailey, C., and H. N. Arst, Jr.** 1975. Carbon catabolite repression in *Aspergillus nidulans*. Eur. J. Biochem. 51:573–577.
3. **Benz, F., M. Liersch, J. Nüesch, and H. J. Treichler.** 1971. Methionine metabolism and cephalosporin C synthesis. D-amino acid oxidase. Eur. J. Biochem. 20:81–88.
4. **Caltrider, P. G., and H. F. Niss.** 1966. Role of methionine in cephalosporin synthesis. Appl. Microbiol. 14:746–753.
5. **Cundliffe, E., M. Cannon, and J. Davies.** 1974. Mechanism of inhibition of eukaryotic protein synthesis by trichothecene fungal toxins. Proc. Natl. Acad. Sci. U.S.A. 71:30–34.
6. **deCrombrugghe, B., R. L. Perlman, H. E. Varmus, and I. Pastan.** 1969. Regulation of inducible enzyme synthesis in *Escherichia coli* by cyclic adenosine 3′,5′-monophosphate. J. Biol. Chem. 244:5828–5835.
7. **Demain, A. L., J. F. Newkirk, and D. Hendlin.** 1963. Effect of methionine, norleucine, and lysine derivatives on cephalosporin C fermentation in chemically defined media. J. Bacteriol. 85:339–344.
8. **Epstein, W. L., L. B. Rothman-Denes, and J. Hesse.** 1975. Adenosine 3′:5′-cyclic monophosphate as mediator of catabolite repression in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 72:2300–2304.
9. **Flynn, E. H. (ed.).** 1972. Cephalosporins and penicillins. Chemistry and biology. Academic Press Inc., New York.
10. **Fujisawa, Y., H. Shirafuji, M. Kida, K. Nara, M. Yoneda, and T. Kanzaki.** 1973. New findings on cephalosporin C biosynthesis. Nature (London) New Biol. 246:154–155.
11. **Gabriel, O.** 1971. Locating enzymes on gels, p. 578–604. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 22. Academic Press Inc., New York.
12. **Hayes, M. B., and D. Wellner.** 1969. Heterogeneity of L-amino acid oxidase. Separation of multiple compounds by polyacrylamide gel electrofocusing. J. Biol. Chem. 244:6636–6644.
13. **Holzer, H.** 1974. Role of proteinases and their inhibitors in the regulation of yeast metabolism, p. 325–333. *In* E. H. Fischer, E. G. Krebs, H. Neurath, and E. R. Stadtman (ed.), Metabolic interconversion of enzymes. Springer-Verlag, New York.
14. **Holzer, H., and W. Duntze.** 1971. Metabolic regulation by chemical modification of enzymes. Annu. Rev. Biochem. 40:345–374.
15. **Huber, F. M., R. H. Baltz, and P. G. Caltrider.** 1968. Formation of deacetylcephalosporin C in cephalosporin C fermentation. Appl. Microbiol. 16:1011–1014.
16. **Hulme, M. A., and D. W. Stranks.** 1970. Induction and the regulation of production of cellulase by fungi. Nature (London) 226:469–470.
17. **Hynes, M. J.** 1972. Mutants with altered glucose repression of amidase enzymes in *Aspergillus nidulans*. J. Bacteriol. 111:717–722.
18. **Konecny, J., E. Felber, and J. Gruner.** 1973. Kinetics of the hydrolysis of cephalosporin C. J. Antibiot. 3:135–141.
19. **Liersch, M., J. Nüesch, and H. J. Treichler.** 1976. Final steps in the biosynthesis of cephalosporin C, p. 179–195. *In* K. D. Macdonald (ed.), Genetics of industrial microorganisms: proceedings of the second international symposium 1974. Academic Press Inc., London.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
21. **Magasanik, B.** 1970. Glucose effects: inducer exclusion and repression, p. 189–219. *In* J. R. Beckwith and D. Zipser (ed.), The Lac operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Martin, R. G., and B. N. Ames.** 1961. Methods for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372–1379.
23. **Maurer, H. R.** 1971. Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis. Walter de Gruyter, Berlin.
24. **Newton, G. G. F., and E. P. Abraham.** 1955. Cephalosporin C, a new antibiotic containing sulfur and D-α-amino adipic acid. Nature (London) 175:548.
25. **Nüesch, J., A. Hinnen, M. Liersch, and H. J. Treichler.** 1976. A biochemical and genetical approach to the biosynthesis of cephalosporin C, p. 451–472. *In* K. D. Macdonald (ed.), Genetics of industrial microorganisms: proceedings of the second international symposium 1974. Academic Press Inc., London.
26. **Nüesch, J., H. J. Treichler, and M. Liersch.** 1970. The biosynthesis of cephalosporin C, p. 309–334. *In* Z. Vanek, Z. Hostalek, and J. Cudlin (ed.), Genetics of industrial microorganisms. Actinomycetes and fungi. Academia, Prague.
27. **Oakley, C. L., G. H. Warrack, and W. E. van Heyningen.** 1946. The collagenase of Cl. welchii type A. J. Pathol. Bacteriol. 58:229–235.
28. **Pastan, I., and R. Perlman.** 1970. Cyclic adenosine monophosphate in bacteria. Science 169:339–344.
29. **Pocker, Y., and J. T. Stone.** 1967. The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalyzed hydrolysis of p-nitrophenyl acetate. Biochemistry 6:668–678.
30. **Rubin, F. A., and D. H. Smith.** 1973. Characterization of R factor β-lactamase by the acidimetric method. Antimicrob. Agents Chemother. 3:68–73.
31. **Smith, B., S. C. Warren, G. G. E. Newton, and E. P. Abraham.** 1967. Biosynthesis of penicillin N and cephalosporin C antibiotic production and other features of the metabolism of a *Cephalosporium sp.* Biochem. J. 103:877–890.
32. **Yagi, J., T. Yana, Y. Kubochi, S. Hattori, M. Ohashi, H. Sakai, K. Jomon, and M. Ajisaka.** 1972. Studies on alkaline proteinase from *Cephalosporium*. (I) Purification of the enzyme. J. Ferment. Technol. 50:592–599.