EFFECT OF METHIONINE, NORLEUCINE, AND LYSINE DERIVATIVES ON CEPHALOSPORIN C FORMATION IN CHEMICALLY DEFINED MEDIA

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Received for publication 27 August 1962

ABSTRACT

DEMAIN, A. L. (Merck Sharp & Dohme Research Laboratories, Rahway, N.J.), JOANNE F. NEWKIRK, AND D. HENDLIN. Effect of methionine, norleucine, and lysine derivatives on cephalosporin C formation in chemically defined media. J. Bacteriol. **85:** 339–344. 1963.—Chemically defined media were developed for production of cephalosporin C by *Cephalosporium* sp. In such media, the requirement for methionine can be satisfied by norleucine. Further stimulation of antibiotic production was obtained with the lysine derivatives ϵ-*N*-acetyl-L-lysine and ϵ-aminocaproic acid but not with lysine itself. Also inactive were α-aminoadipic and ketoadipic acids. Other lysine derivatives were found to inhibit cephalosporin C production at 0.01 M. The final medium supported the production of approximately 0.5 g of cephalosporin C per liter of medium.

We previously reported on the formation of cephalosporin C (Abraham and Newton, 1961) in a crude medium (Demain and Newkirk, 1962). While these experiments were in progress, an attempt was made to devise a chemically defined medium in which refined biochemical studies could be carried out on the biosynthesis of cephalosporin C. As a result, a synthetic medium capable of supporting the production of approximately 500 μg of cephalosporin C per ml was developed. The present paper describes the experiments which led to its formulation. In addition, it has been found that norleucine is capable of replacing methionine and that certain compounds structurally related to lysine can stimulate cephalosporin C biosynthesis in a methionine-containing medium.

MATERIALS AND METHODS

Initially, mutant 8650 derived from *Cephalosporium* sp. strain CMI 49137 was used. It was obtained from the National Research Development Corporation, London, England. Later in the investigation, mutant C-91 replaced the British culture. Strain C-91, a higher-yielding mutant, was obtained in Rahway, by M. Jackson, by ultraviolet irradiation of mutant 8650. Both cultures were maintained as previously described (Demain and Newkirk, 1962).

Conditions for inoculum development and production of cephalosporin C as well as methods for the assay of cephalosporin C, penicillin N (cephalosporin N: the name penicillin N is preferred over cephalosporin N since the molecule contains a 6-aminopenicillanic acid nucleus, not a 7-aminocephalosporanic acid nucleus), mycelial dry weight, and paper chromatography were the same as previously used (Demain and Newkirk, 1962). Preparation of the broth for paper chromatography was modified, however, to eliminate interference by the inorganic salts present in the synthetic medium. After centrifugation of whole broth, 2.5 volumes of cold methanol were added to the supernatant. The tube was placed in ice for 30 min and then centrifuged to remove the alcohol precipitate. The clear supernatant fluid was evaporated under vacuum to 10% of the original broth volume; 10 μliters of this solution were spotted on sheets of Whatman no. 1 paper and developed as previously described.

All concentrations of cephalosporin C are in terms of the sodium salt used as the assay standard. L-Lysine was determined by use of lysine decarboxylase in a Technicon Auto-Analyzer (Schaiberger and Ferrari, 1960).

RESULTS

Preliminary experiments. Using inoculum developed in a crude seed medium (Demain and Newkirk, 1962), we attempted to produce cephalosporin C in the synthetic medium of Bhuyan and Johnson (1958), which they had developed for penicillin N production by *C. salmosynnematum*. When this failed, both crude and

chemically defined supplementation of that medium were tried, but no activity was detected.

Finally, after numerous experiments, activity was noted in a medium containing sucrose, glucose, glycerol, ammonium citrate, ammonium acetate, ammonium sulfate, DL-methionine, oleic acid, linoleic acid, $CaCO_3$, phosphate, and other mineral salts. Further experimentation showed that glycerol, ammonium citrate, ammonium acetate, and linoleic acid could be eliminated without adverse effect. By increasing the phosphate concentration, we were able to omit $CaCO_3$, thus obtaining a clear medium. This medium, designated no. 3, contained the following components (per cent by weight): sucrose, 3.6; glucose, 2.7; DL-methionine, 0.5; oleic acid, 0.15; $(NH_4)_2SO_4$, 0.75; KH_2PO_4, 1.5; K_2HPO_4, 2.1; Na_2SO_4, 0.075; $MgSO_4$, 0.018; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.015; $CaCl_2$, 0.006; $MnSO_4$, 0.003; $ZnSO_4 \cdot 7H_2O$, 0.003; and $CuSO_4 \cdot 5H_2O$, 0.00075. Glucose was autoclaved separately. The initial pH was 7.4.

Experiments with medium 3. Paper chromatographic analysis indicated that the same antibiotics were produced in this medium as were formed in a crude medium, i.e., cephalosporin C, penicillin N, and "cephalosporin" P. Instead of using a complex seed medium, medium 3 was also used to develop inocula for all subsequent experiments. Thus, it served as both a seed and a production medium. No other carbohydrates or their derivatives tested could substitute for the sucrose or the glucose. Optimal production of cephalosporin C was obtained when the capacity of the Erlenmeyer flask was five times the medium volume. Most of the subsequent experiments were done in 50-ml flasks containing 10 ml of medium 3. Production of cephalosporin C by mutant C-91 ranged from 250 to 450 μg per ml.

Methionine requirement and replacement by norleucine. The omission of DL-methionine from medium 3 markedly reduced the production of cephalosporin C. As previously observed in crude medium (Demain and Newkirk, 1962), D-methionine was more active than the L form. Figure 1 indicates that the response to DL-methionine approximates the sum of the individual effects of the D and L isomers.

In medium 3, a number of compounds were tested as replacements for methionine. All were studied at 0.01 M, and some were further examined at higher and lower concentrations. To determine whether the role of methionine was concerned with the side-chain of cephalosporin C, α-aminoadipic acid, lysine, and compounds either structurally or metabolically related to these were tested, but none had activity. These included DL- and D-α-aminoadipic acid, ketoadipic acid, DL- and L-lysine, L-lysine methyl ester, DL-pipecolic acid, α,ϵ-diaminopimelic acid, and DL-ornithine. Other inactive nonsulfur compounds were DL-homoserine, L-valine, choline chloride, and betaine. Sulfur-containing compounds were examined next. DL-Homocysteine and L-cysteic acid were completely inactive. Slight stimulation of cephalosporin C production was observed with the L and DL forms of cysteine and cystine. The α-hydroxy analogue of methionine (calcium α-hydroxy-γ-methiolbutyrate) was somewhat more

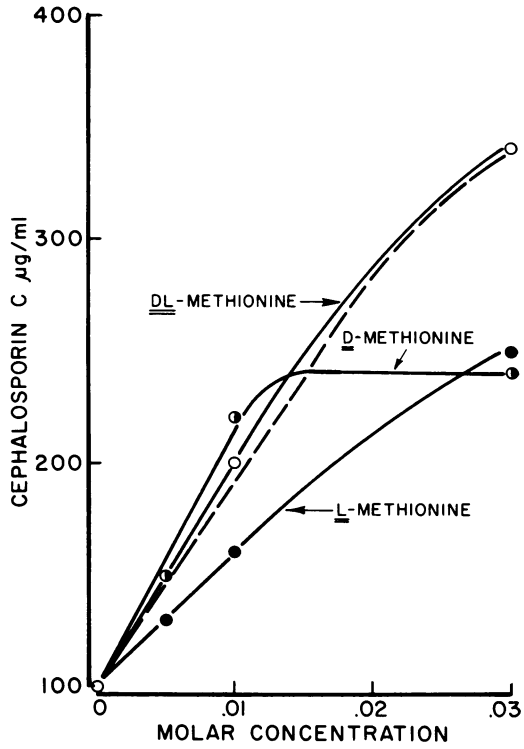

FIG. 1. *Effect of isomers of methionine on cephalosporin C biosynthesis in medium 3 without methionine. The culture was mutant 8650. The assay values represent averages of results obtained after 120 and 144 hr. Broths were assayed with Escherichia coli W-208 in the presence of penicillinase to destroy penicillin N. The dotted curve is the theoretical curve for DL-methionine, assuming an additive response for D- and L-methionine.*

active. DL-Methionine-DL-sulfoxide exhibited partial methionine-replacing activity at low concentrations but became inhibitory to cephalosporin C production at concentrations greater than 0.01 M. Likewise, S-methyl-L-cysteine became inhibitory but, at low concentrations, it was as potent as L-methionine. The DL and D forms of S-methyl cysteine were not available for comparison with DL- and D-methionine.

The only compound capable of supporting high cephalosporin C production in the absence of methionine was its structural analogue, DL-norleucine (Table 1). As with methionine, the D-isomer of norleucine was more active than the L form, although both had considerable activity. When both DL-methionine and DL-norleucine were added to the same flask at optimal concentrations of each, the response was no greater than to either alone. DL-Norvaline also had some methionine-replacing ability, but it was not as strong as norleucine.

Time requirement of methionine addition. The effect of the time of methionine addition on cephalosporin C production was studied by adding 30 mg of DL-methionine to flasks at various time intervals. Table 2 shows that it was essential to add methionine no later than 24 hr for maximal production of cephalosporin C. At this time, growth was in its early stages. If methionine was added at 48 hr, when growth was complete, no stimulatory effect was noted. Apparently, methionine has to be present during the growth phase, even though other experiments have shown that its presence in the synthetic medium does not increase the rate of growth nor the maximal yield of mycelium. The possibility that some other compound becomes limiting after growth has not been eliminated, however.

Activity of lysine derivatives. In medium 3 containing DL-methionine, addition of α-aminoadipic acid (DL or D) as side-chain precursor failed to stimulate production of cephalosporin C. In fact, in many experiments the compound partially inhibited antibiotic formation at 0.02 to 0.03 M concentration. Ketoadipic acid and lysine (DL or L) behaved in a similar manner. The ability of the culture to assimilate lysine was tested by adding L-lysine·HCl to medium 3 in a series of flasks at concentrations of 10, 20, 30, and 50 mg per 10 ml. After 5 days, the centrifuged broths were assayed for L-lysine. The only flask showing over 2.5 mg per 10 ml was the one that originally contained 50 mg per 10 ml. In this flask, 9.5 mg of lysine per 10 ml remained. Apparently, lysine was taken up by the fungus. In another experiment, the effect of serial addition of lysine and α-aminoadipic acid was studied; 40 mg of each compound were added to flasks containing 10 ml of medium, either initially or in four daily doses of 10 mg, starting 24 hr after inoculation. The results showed that daily feeding of lysine or α-aminoadipic acid was even more inhibitory to antibiotic production than addition at the time of inoculation.

Further studies revealed that two compounds structurally related to lysine stimulated the synthesis of cephalosporin C (and penicillin N).

TABLE 1. *Replacement of* DL-*methionine by* DL-*norleucine*[a]

Additive	Concn		Cephalosporin C
	mg/10 ml	*M*	*μg/ml*
None	—	—	150
DL-Methionine	10	0.007	290
	20	0.013	320
	30	0.020	360
DL-Norleucine	1	0.001	220
	10	0.008	290
	20	0.015	330
	30	0.023	300

[a] The culture was mutant C-91. Duration of experiment was 96 hr. The broths were assayed with *Escherichia coli* W-208 in the presence of penicillinase to destroy penicillin N.

TABLE 2. *Effect of the time of methionine addition on cephalosporin C biosynthesis*[a]

Time of addition	Mycelial wt at time of addition	Cephalosporin C	
		At time of addition	At 146 hr
hr	*mg/ml*	*μg/ml*	*μg/ml*
Not added	—	—	111
0	0	0	284
24	2.5	35	267
48	10.4	106	122
73	7.3	128	77
96	6.2	152	72

[a] The culture was mutant C-91. The broths were assayed with *Escherichia coli* W-208 in the presence of penicillinase to destroy penicillin N. DL-Methionine was used at 30 mg per 10 ml of medium in 50-ml Erlenmeyer flasks.

These were ε-*N*-acetyl-L-lysine and ε-amino-*n*-caproic acid (Table 3). The effect of acetyl lysine was not duplicated by acetate plus lysine. Likewise, caproic acid could not replace ε-amino-*n*-caproic acid. The yield of cephalosporin C was greater when both acetyl lysine and aminocaproic acid were added together than it was with either alone.

Certain other derivatives of lysine inhibited production of cephalosporin C to a greater degree than did lysine or α-aminoadipic acid. These were ε-hydroxy-DL-α-amino-*n*-caproic acid (hexahomoserine), DL-4-oxalysine, L-canavanine, ε-benzoyl-DL-lysine, α-methyl-DL-lysine, and carbobenzoxy-L-lysine. All were autoclaved with the medium, and they inhibited cephalosporin C production at 0.01 M. Carbobenzoxy-L-lysine and α-methyl-DL-lysine were the most inhibitory (80 to 100% inhibition); the others reduced cephalosporin C formation to approximately half of the control value.

Other compounds related to lysine were tested and were found to have no marked effect on biosynthesis of cephalosporin C. These included DL plus allo-δ-hydroxy lysine, L-lysine methyl ester, DL-pipecolic acid, α,ε-diaminopimelic acid, DL-ornithine, and L-arginine.

Table 4 lists the ingredients of the final medium developed for the production of cephalosporin C. Cysteine is included because it stimulated production in certain experiments, although the effect was not consistent. The methionine content is somewhat lower than in the previous media because culture C-91, used in the later part of the work, requires less methionine than strain 8650. The final medium supported the production of approximately 0.5 g of cephalosporin C per liter of medium.

TABLE 3. *Stimulation of cephalosporin C synthesis by ε-N-acetyl-L-lysine and ε-amino-n-caproic acid*[a]

Additive	Concn		Cephalosporin C
	mg/10 ml	*M*	*μg/ml*
None	—	—	400
ε-*N*-Acetyl-L-lysine	9.5	0.005	460
	19	0.01	580
	57	0.03	560
ε-Amino-*n*-caproic acid	6.5	0.005	500
	13	0.01	570
	39	0.03	590

[a] The culture was mutant C-91. Duration of experiment was 120 hr. The broths were assayed with *Escherichia coli* W-208 in the presence of penicillinase to destroy penicillin N.

TABLE 4. *Final medium developed for cephalosporin C production*[a]

Component	Amt/liter
Sucrose	36.0 g
Glucose[b]	27.0 g
$(NH_4)_2SO_4$	7.5 g
DL-Methionine	3.0 g
ε-Amino-*n*-caproic acid	2.0 g
ε-*N*-Acetyl-L-lysine	2.0 g
L-Cysteine·HCl	1.6 g
Oleic acid[c]	1.5 g
K_2HPO_4[d]	21.0 g
KH_2PO_4[d]	15.3 g
Na_2SO_4[d]	750.0 mg
$MgSO_4$[d]	180.0 mg
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	150.0 mg
$CaCl_2$[d]	57.0 mg
$MnSO_4 \cdot H_2O$[d]	30.0 mg
$ZnSO_4 \cdot 7H_2O$[d]	30.0 mg
$CuSO_4 \cdot 5H_2O$[d]	7.5 mg

[a] The pH was adjusted to 7.4.

[b] Autoclaved separately.

[c] Added to flasks after subdivision of rest of medium, before autoclaving.

[d] Added as a concentrated mixture to medium before autoclaving.

DISCUSSION

Previous studies on the biosynthesis of cephalosporin C in a crude medium suggested to us that the stimulatory effect of methionine was due to the sparing of intracellular cysteine, a probable precursor of the cephalosporin C nucleus, 7-amino-cephalosporanic acid (Demain and Newkirk, 1962). Methionine was thought to function by repressing the formation of an enzyme that degrades cysteine to pyruvate, as occurs in *E. coli* (Rowbury and Woods, 1961*a*). Such repression would allow for an increased level of cysteine in the cells. The results reported in this paper add further support to this hypothesis, although they do not prove it.

First, the ability of norleucine, a known methionine antagonist (Harris and Kohn, 1941; Porter and Meyers, 1945), to replace methionine demon-

strates that incorporation of methionine sulfur into cephalosporin C probably does not occur. In this instance, norleucine may be acting as a repressor of the cysteine-degrading enzyme. Rowbury and Woods (1961*b*) demonstrated that norleucine, like methionine, represses the formation of homocysteine methylase. Although they did not report on whether norleucine could replace methionine for repression of the cysteine-degrading enzyme, Porter and Meyers (1945) demonstrated that norleucine inhibited the production of H_2S from cystine in *Proteus morganii*.

Second, the demonstration that the methionine effect occurs only when it is added during the growth phase of the organism suggests that it plays an indirect role. If direct methionine incorporation were involved, then stimulation of cephalosporin C formation should have occurred even when methionine was added at 48 hr. At this time, growth was at its maximum, and the antibiotic level was only at one-third maximum.

Studies were also directed toward stimulation of cephalosporin C production by addition of probable side-chain precursors. Because the antibiotic contains a D-α-aminoadipic acid side-chain, we tested this amino acid and related compounds. Both DL- and D-α-aminoadipic acid failed to stimulate production and, in fact, were weak inhibitors. This was not too surprising, in view of the experience of previous investigators with penicillin N formation. The production of this antibiotic, which also has an α-aminoadipyl side-chain, was not stimulated by α-aminoadipic acid in a synthetic medium (Bhuyan and Johnson, 1958) nor in complex media (Kavanagh, Tunin, and Wild, 1958; Nara and Johnson, 1959). Lysine was also tested by these workers and, although Bhuyan and Johnson (1958) found it to be inactive in synthetic medium, Nara and Johnson (1959) noted stimulation of penicillin N formation in a complex medium. In the present studies, lysine failed to stimulate production of cephalosporin C. Furthermore, other compounds considered to be either lysine precursors in fungi, i.e., ketoadipic acid and ϵ-hydroxy-α-amino-*n*-caproic acid, or products of lysine metabolism, i.e., pipecolic acid (Work, 1955), were inactive in stimulating cephalosporin C production. In some instances, they proved to be weak inhibitors of antibiotic formation. Two compounds structurally related to lysine were active, however: ϵ-*N*-acetyl-L-lysine and ϵ-amino-*n*-caproic acid. The activity of ϵ-*N*-acetyl-lysine and the inactivity of lysine is reminiscent of the early work of Neuberger and Sanger (1944), who found that ϵ-*N*-acetyl-lysine but not lysine could be attacked by mammalian L- and D-amino acid oxidases. Also, Meister (1954) showed that ϵ-*N*-substituted α-ketolysine, but not the unsubstituted ketolysine, will transaminate in vitro with glutamine. Thus, acetylation of the ϵ-amino group makes the α-amino group of lysine more susceptible to removal, a step which appears to be necessary for its further metabolism. Work (1955) suggested that lysine is unique in that its six-carbon structure is susceptible to ring closure between keto and amino groups and that *N*-acetylation prevents such closure. In this regard, Schweet, Holden, and Lowy (1955) isolated a labeled compound tentatively identified as α-hydroxy-ϵ-*N*-acetylaminocaproic acid from *Neurospora* after metabolism of C^{14}-lysine. Thus, one might speculate that while *Cephalosporium* could use lysine for protein synthesis, the ϵ-*N*-acetylated lysine would be more available for metabolic conversion to an intermediate, usable for synthesis of cephalosporin C.

The reason for the activity of ϵ-amino-*n*-caproic acid remains unexplained, since virtually nothing is known of its metabolism.

That a lysine derivative is somehow involved in cephalosporin C biosynthesis is also suggested by the inhibitions observed with compounds known to be lysine antagonists in other organisms. These included canavanine (Horowitz and Srb, 1948; Walker, 1955), oxalysine (McCord et al., 1957), and ϵ-hydroxy-α-amino-*n*-caproic acid (Pagé, Gingras, and Gaudry, 1949). Other inhibitors of cephalosporin C formation were ϵ-benzoyl-DL-lysine, α-methyl-DL-lysine, and carbobenzoxy-L-lysine. It remains to be determined, however, whether these inhibitors are acting on growth, on cephalosporin C synthesis, or on both.

The final chemically defined medium developed during these studies is capable of supporting yields of cephalosporin C almost as high as those obtained in complex media. It should therefore be of use for refined studies on the mechanism of biosynthesis of cephalosporin C by use of isotopic tracers or washed-cell suspensions, or both.

ACKNOWLEDGMENTS

The authors wish to thank the following for supplying compounds used in these experiments:

John A. Brockman, Jr., of the Lederle Laboratories for ketoadipic acid, C. G. Skinner of the University of Texas for DL-4-oxylysine, E. Inamine for cephalosporin C, and E. Winkler for the D isomer of α-aminoadipic acid. We are also indebted to T. Jacob for fruitful discussions on the chemistry of cephalosporin C. Microbiological assays were carried out by H. Wallick and his associates.

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