Biosynthesis of Cephalosporin C

I. Factors Affecting the Fermentation

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ABSTRACT

Ott, J. L. (Eli Lilly and Co., Indianapolis, Ind.), C. W. Godzeski, D. Pavey, J. D. Farran, and D. R. Horton. Biosynthesis of cephalosporin C. I. Factors affecting the fermentation. Appl. Microbiol. **10**:515–523. 1962.—A series of complex and synthetic media have been developed that are suitable for the production of cephalosporin C and cephalosporin N by a mutant strain of *Cephalosporium* (C.M.I. 49,137). DL-Methionine increased the yield of both antibiotics, with more effect on cephalosporin N. L-Cystine had a greater enhancing effect on formation of cephalosporin C than on formation of cephalosporin N in synthetic media; serine increased yields of cephalosporin C under certain conditions. Disaccharides or polysaccharides appeared to be the best source for carbohydrates. No evidence was found for precursor action such as is found in penicillin fermentations. The ability of resting cells to produce antibiotic was demonstrated.

Cephalosporin C was first reported by Newton and Abraham (1955) in crude preparations of cephalosporin N. Abraham and co-workers have described the isolation, properties, and structure of cephalosporin C (Newton and Abraham, 1956; Abraham and Newton, 1956*a*, *b*, 1961; Jeffery, Abraham, and Newton, 1960). Cephalosporin N and a series of other solvent-extractable antibiotics designated as cephalosporin P were reported by Burton and Abraham (1951) to be produced by a strain of *Cephalosporium* (C.M.I. 49,137). Another culture, identified as *C. salmosynnematum* Roberts, was shown to produce synnematin B (Gottshall et al., 1951; Olson, Jennings, and Junek, 1953). This antibiotic was later shown to be identical with cephalosporin N (Abraham et al., 1953, 1955). The perfect stage of this fungus was demonstrated by Grosklags and Swift (1957), and was classified as *Emericellopsis salmosynnemata*. They also reported that many species produced antibiotic activity similar to cephalosporin N. Kavanagh, Tunin, and Wild (1958*a*) confirmed these observations of the production of cephalosporins N and P.

Fermentation studies on the production of cephalosporin N have been reported by a number of workers (Gottshall et al., 1951; Pisano, Olson, and San Clemente, 1954; Bhuyan and Johnson, 1958; Kavanagh, Tunin, and Wild, 1958*b*; Harvey and Olson, 1958; Nara and Johnson, 1959). Very little has been published on *Cephalosporium* C.M.I. 49,137, from which cephalosporin C was isolated (Crawford et al., 1952; Florey et al., 1956; Kavanagh et al., 1958*a*, *b*).

The studies reported here were carried out with a mutant strain from *Cephalosporium* C.M.I. 49,137, which produced cephalosporins C, N, and P. Several media suitable for the production of cephalosporins C and N will be described.

Materials and Methods

A strain of *Cephalosporium* (C.M.I. 49,137; mutant 8650), obtained from E. P. Abraham, was used throughout this investigation. The mutant produced cephalosporin N, cephalosporin C, and antibiotics of the cephalosporin P type.

The culture was preserved by lyophilization in bovine serum. Stock slants were prepared from lyophilized cells on a modified LePage and Campbell (1946) medium. The medium was prepared at one-tenth the original strength, except for use of 2% agar and the addition of 1% calcium chloride; slants were prepared with 20 ml of medium in 29 × 162 mm tubes. Slants were incubated at 25 C for 14 days and remained productive after several months at 4 C. The surface growth from a slant was suspended in 12 ml of sterile water; each flask of seed medium was inoculated with 2.0 ml of this suspension.

Fermentations were carried out in 250-ml wide-mouth Erlenmeyer flasks with cotton stoppers. Separate flasks were used for each time period of sampling. In most cases, duplicate or triplicate flasks were taken for assay.

A rough estimation of mycelial growth was obtained by measuring packed solids after centrifugation of a fermentation sample at 1,500 × *g* for 10 min in a graduated conical centrifuge tube. Mycelial weights were not determined. The pH of the fermentations was determined with a pH meter. Data on packed solids and pH are not reported with every experiment. The pH of the fermentations varied from 6.5 to 8.2 at the time of harvest. With all supplements tested, the packed solids and pH of the fermentation flasks were quite similar to those of control flasks unless otherwise noted.

Antibiotic assay. Antibiotic activity was determined by

a filter paper disc plate diffusion assay (Loo et al., 1945) against *Salmonella gallinarum*. The assay was modified in that discs were dipped into the samples and placed on the plates, rather than pipetting 0.1 ml onto the discs. A pure sample of the sodium salt of cephalosporin C was used as the standard. Cephalosporin N can be destroyed by treatment with penicillinase or by holding the sample below pH 4.0 for a period of time (Abraham et al., 1953). The amount of cephalosporin C in fermentation samples was determined after the antibiotic activity of cephalosporin N was destroyed by either (i) treatment of the sample with penicillinase (Ricker Laboratories, Northridge, Calif.) at 37 C for 60 min, or (ii) adjustment of the sample to pH 2.2 with acid, incubation at 37 C for 90 min, and careful neutralization to pH 6.0 to 7.0. Both methods gave comparable results. The penicillinase preparation used had no effect on the antibiotic activity of cephalosporin C. Cephalosporin N is 3.5 times as active as cephalosporin C against *S. gallinarum*, but the assay slopes are similar for both antibiotics. The quantity of cephalosporin N present in a fermentation sample was calculated by the following formula: (total antibiotic activity as μg per ml of cephalosporin C minus μg per ml of cephalosporin C in treated samples)/3.5. Antibiotics of the cephalosporin P type were not active against *S. gallinarum* even at much higher concentrations than were found in fermentation samples.

Chromatographic separation of cephalosporins C, N, and P and derivatives resembling penicillin V. Cephalosporins C and N were separated by descending chromatography on Whatman no. 1 chromatography paper buffered at pH 4.0 with 0.75 M phosphate. The chromatograms were developed with methanol–*n*-propanol–water (60:20:10) for 18 to 20 hr. Antibiotics of the cephalosporin P type ran off the end of the chromatograms in this system. Zones of antibiotic activity were detected by plating on *S. gallinarum* or *Bacillus subtilis*.

For detection of modified cephalosporin antibiotics resembling penicillin G or V, descending paper chromatography was carried out on Whatman no. 1 chromatography paper with 70% *n*-propanol as the solvent (Kavanagh et al., 1958*b*). Cephalosporins C and N moved as a single zone but were well separated from penicillin G or V and antibiotics of the cephalosporin P type. Zones of activity were detected by developed bioautographs on *B. subtilis* agar plates.

TABLE 1. *Effect of temperature on production of cephalosporins C and N**

Incubation temp	Time of sample	Antibiotic relative to that formed at 25 C	
		Ceph N	Ceph C
C	hr	%	%
20	48	57	117
	72	61	95
	96	61	91
	120	22	62
30	48	126	133
	72	146	86
	96	168	100
	120	138	81

* Medium no. 1: lactose, 3.0%; corn steep solids, 3.8%; $CaCO_3$, 1.0%. Inoculum was a 72-hr seed culture in same medium. The 250-ml flasks contained 60 ml of medium; incubated at 350 rev/min on rotary shaker describing a circle of 1 in. diam. All flasks were incubated at 25 C for 17 hr and then moved to their respective experimental temperature. The yields of cephalosporin N (ceph N) at 25 C were 418, 363, 248, and 219 μg/ml at the 48, 72, 96, and 120 hr sampling periods. The corresponding yields of cephalosporin C (ceph C) were 48, 84, 88, and 84 μg/ml.

RESULTS

Complex media. The effect of temperature of incubation on antibiotic production was determined in a corn steep medium at 20, 25, and 30 C. Flasks were incubated at 25 C for 17 hr after inoculation and then were placed at the experimental temperatures. The results are presented in Table 1. In a similar experiment, flasks were maintained from the time of inoculation at 25 and 30 C. The higher temperature under these conditions gave decreased yields of cephalosporin C. Lowered yields of cephalosporin C at 30 C from zero time, and stimulation of production of cephalosporin N at 25 C for 17 hr followed by 30 C incubation without increased production of cephalosporin C, led to use of 25 C for subsequent experiments.

A series of media were tested for satisfactory formation of a seed inoculum for production flasks. Yields of cephalosporins C and N in a series of production media were used as the criterion for a satisfactory seed stage. Of 14 media tested, the following, designated medium no. 2, gave the best results: corn meal, 2%; Bakers 200D nutrisoy flour (Archer-Daniels-Midland Co., Minneapolis, Minn.), 1.5%; $(NH_4)_2SO_4$, 0.1%; $CaCO_3$, 0.3%; and methyl oleate, 1.6%.

Experiments were carried out to determine whether a second seed stage before final inoculation of production flasks would increase yields of antibiotic. Medium no. 2 was used for the first seed stage. In no case was a significantly greater yield of antibiotic produced with a second seed stage (14 media tested). Since a large volume of inoculum was not required in the experiments reported, a single seed stage was used for all inoculations of production media. The optimal time of incubation for the seed stage was 72 hr.

Effect of various additions to complex media. The stimulatory effect of methionine had been found much earlier on other strains of *Cephalosporium* and *Emericellopsis* by one of us (C.W.G.), as reported by Kavanagh et al. (1958*b*). With the mutant strain under study here, the addition of 0.1% DL-methionine after 48 hr to medium no. 1 increased the total antibiotic yield 1.5 times. Addition of 0.1% ethionine under the same conditions decreased total activity to one-third of the control yield. When both DL-methionine and ethionine were added at 48 hr, the stimulatory effect was noted. Addition of oleic acid at

the time of inoculation in medium no. 1 stimulated the yield of cephalosporin C to 175% of the control value; when added at 24 hr, no effect was found. In this medium, 0.5% methyl oleate had no stimulatory effect on yields of cephalosporin C.

The effects of additions to another corn steep medium, medium no. 3, with a different carbohydrate source are given in Table 2. The effects of several carbohydrates on production of cephalosporins C and N in a supplemented corn steep medium are given in Table 3.

The use of a peanut meal complex medium gave better yields of cephalosporins C and N than corn steep medium (Elander, *personal communication*). The stimulatory effect of DL-methionine in this type of medium and the effects of certain inhibitors are shown in Table 4. Other potential inhibitors that showed no effect on antibiotic formation unless growth was adversely affected were sodium fluoride, potassium cyanide, and iodoacetate. The addition of sodium oxalate to a different peanut meal medium stimulated cephalosporin N formation, but lowered that of cephalosporin C (Table 4). Other related compounds such as longer chain dicarboxylic acids and tricarboxylic acid cycle intermediates had little effect on the yield or ratio of cephalosporins C and N.

Synthetic media. The effect of various substrates on antibiotic production can be judged somewhat more accurately in a medium of known composition. Previous work on other *Cephalosporium* strains, particularly *C. salmosynnematum* (Pisano et al., 1954; Bhuyan and Johnson, 1958; Bhuyan, Mohberg, and Johnson, 1958), concerned only production of cephalosporin N and had shown that ammonium sulfate could serve as a source of nitrogen for antibiotic production. Our preliminary experiments indicated that DL-methionine increased yields of antibiotic activity as shown in complex media. Other amino acids influencing the total antibiotic yield are listed in Table 5. The effect of carbohydrates was determined in an ammonium sulfate medium supplemented with 0.12% DL-methionine (Table 6). The combination of 3.2% sucrose and 0.8% cerelose was chosen for further study since better yields of both antibiotics were attained with this carbohydrate source. It will be noted that decreased yields of antibiotic were obtained at 114 hr with 6% soluble starch (Difco) and 2% cerelose plus 4% lactose. For this reason these carbohydrates were not tested further in this medium.

Effect of supplements. The effects of additions to the

TABLE 2. *Effect of additions to corn steep medium**

Additions	Antibiotic at 114 hr relative to control	
	Ceph N	Ceph C
	%	%
0.1% $(NH_4)_2SO_4$	125	66
0.1% L-Cystine·HCl	111	109
0.4% DL-Methionine	925	126
1.7% Methyl oleate	248	112
2.0% Bakers 200D nutrisoy flour	134	114
0.1% L-Cystine·HCl + 0.4% DL-methionine	1,038	156
0.1% L-Cystine·HCl + 0.4% DL-methionine + 0.1% $(NH_4)_2SO_4$	1,332	171
0.1% L-Cystine·HCl + 0.4% DL-methionine + 1.7% methyl oleate	1,222	190
0.1% L-Cystine·HCl + 0.4% DL-methionine + 0.1% $(NH_4)_2SO_4$ + 1.7% methyl oleate	1,042	204
0.1% L-cystine·HCl + 0.4% DL-methionine + 2.0% Bakers 200D nutrisoy flour	1,530	178
0.1% L-Cystine·HCl + 0.4% DL-methionine + 0.1% $(NH_4)_2SO_4$ + 2.0% Bakers 200D nutrisoy flour	1,595	198
0.1% L-Cystine·HCl + 0.4% DL-methionine + 0.1% $(NH_4)_2SO_4$ + 2.0% Bakers 200D nutrisoy flour + 1.7% methyl oleate	2,130	335
2.0% Corn steep liquor	203	140
2.0% Corn steep liquor + 0.1% L-cystine·HCl	302	149
2.0% Corn steep liquor + 0.4% DL-methionine	1,198	164
2.0% Corn steep liquor + 1.7% methyl oleate	454	142
2.0% Corn steep liquor + 0.1% L-cystine·HCl + 0.4% DL-methionine	1,460	204
2.0% Corn steep liquor + 0.1% L-cystine·HCl + 0.4% DL-methionine + 1.7% methyl oleate	1,700	244

* Control values: 63 μg/ml of ceph N; 94 μg/ml of ceph C. Medium no. 3: corn steep liquor, 3.0%; cerelose, 1.0%; lactose, 5.0%; $CaCO_3$, 0.2%; pH was adjusted to 7.0 before sterilization; additions as indicated were made before sterilization; 30 ml of medium per 250-ml flask. Incubation at 25 C on a rotary shaker describing a circle of 1.5 in. diam at 250 rev/min. Inoculum was a 72-hr seed culture in medium no. 2.

TABLE 3. *Effect of carbohydrates on antibiotic formation in corn steep medium**

Carbohydrate	Antibiotic at 114 hr reative to control	
	Ceph N	Ceph C
	%	%
1% Cerelose	9	7
2% Cerelose	28	7
4% Cerelose	33	76
2% Cerelose + 5% lactose	50	79
2% Cerelose + 4% lactose	54	78
3% Cerelose + 3% lactose	61	86
1% Cerelose + 5% mannitol	29	59
1% Cerelose + 5% xylose	4	23
1% Cerelose + 5% ribose	—†	—
4% Maltose	36	69
6% Mannitol	29	52
6% Xylose	—	12
6% Ribose	—	—
6% Glycerol	34	14
6% Stadex‡	24	37

* Control medium had 1% cerelose + 5% lactose as carbohydrate; 1,410 μg/ml of ceph N and 310 μg/ml of ceph C were formed. Medium no. 4: $(NH_4)_2SO_4$, 0.1%; L-cystine·HCl, 0.1%; DL-methionine, 0.4%; corn steep liquor, 3.0%; Bakers 200D nutrisoy flour, 2.0%; $CaCo_3$ 0.2%; pH was adjusted to 6.0 before sterilization. The other conditions were same as in Table 2.

† No antibiotic formed.

‡ Partially hydrolyzed product of corn starch from A. E. Staley Manufacturing Co., Decatur, Ill.

ammonium sulfate medium are shown in Table 7. Results are reported for samples taken after 114 hr of fermentation. Yields at this time were maximal except with addition of 0.3% choline, 0.4% ammonium acetate, 0.1% methionine plus 0.2% valine, 0.12% methionine plus 0.5% oleic acid, and 0.2% methionine plus 0.1% ethionine, which showed significantly more cephalosporin N at 90 hr. Only with 0.12% methionine plus 0.5% oleic acid was cephalosporin C significantly higher at 90 hr than at 114 hr. Some experiments were carried out with glycine or glutamic acid as the principal nitrogen source. DL-Methionine or L-cystine were required for the production of cephalosporin C. With 0.5% glycine, no antibiotic was formed without supplementation with 0.1% DL-methionine, 0.1% L-cystine, or 0.1% ammonium sulfate. When glycine was increased to 1.0%, cephalosporin N was found, but no cephalosporin C appeared without the supplements mentioned above as required in the presence of 0.5% glycnie. The carbohydrate source for best yields was different in glycine medium than that found with ammonium sulfate medium. In medium supplemented with 0.2% DL-methionine, 6% soluble starch was much better than other sources of carbohydrate. Glutamic acid medium gave results similar to those obtained with glycine, but with lower yields of cephalosporins C and N. Betaine, choline, and ethionine served to stimulate cephalosporin N formation in 1% glycine medium, as they did in the ammonium sulfate medium.

A synthetic medium with part of the ammonium sulfate replaced by nitrate was investigated. This medium had been used in another laboratory in connection with other investigations (Kory, *personal communication*). The composition of the medium in which the effects of carbohydrate sources were studied is presented in Table 8. This medium was of interest because almost twice as much cephalosporin C as cephalosporin N was formed in the basal medium. To avoid addition of corn meal and soybean flour in the seed inoculum from medium no. 2, all experiments with medium no. 8 and no. 9 were carried out using medium no. 9 for the seed stage. Optimal incubation time for the seed culture in this synthetic medium was 96 hr

TABLE 4. *Effect of methionine and inhibitors on antibiotic production**

Additions	Antibiotic at peak relative to control	
	Ceph N	Ceph C
	%	%
To medium no. 5		
100 μg/ml Sulfanilamide	51	73
100 μg/ml Sulfathiazole	120	39
1 μg/ml Fluoroacetate	91	54
0.3% DL-Methionine	405	213
0.3% DL-Methionine + 100 μg/ml sulfanilamide	304	194
0.3% DL-Methionine + 100 μg/ml sulfathiazole	480	147
To medium no. 12		
0.05% Phenol	84	70
0.1% Phenol	35	40
0.01% Oxalate	108	84
0.05% Oxalate	146	86
0.1% Oxalate	205	44
0.2% Oxalate	170	<18
0.3% Oxalate	135	61
0.4% Oxalate	122	72

*Medium no. 5: Dextrin 700 (Morningstar Paisley, Inc., New York, N.Y.), 3.0%; cerelose, 3.0%; $CaCO_3$, 0.5%; Na_2SO_4, 0.3%; peanut meal, 3.0%; methyl oleate, 0.5%; adjust to pH 6.8 to 7.0 before sterilization. Medium no. 12: peanut meal, 3.0%; beet molasses, 2.0%; corn meal, 2.0%; $CaCO_3$, 0.5%; DL-methionine, 0.2%; $Na_2S_2O_3 \cdot 5H_2O$, 0.1%; borax, 0.05%; hot tap water; add 0.67% methyl oleate to medium in flask prior to sterilization. Experimental conditions: The flasks contained 60 ml of medium and were inoculated with a 72-hr seed culture in medium no. 1. Incubated at 25 C on a rotary shaker describing a circle of 1 in. diam at 350 rev/min. The control values were: medium no. 5, 736 μg/ml cephalosporin N and 121 μg/ml cephalosporin C; medium no. 12, 1,059 μg/ml cephalosporin N and 785 μg/ml cephalosporin C.

TABLE 5. *Effect of amino acid additions on antibiotic production in synthetic medium**

Amino acid added	Antibotic at peak relative to control (total ceph C and N)
	%
DL-Glutamic acid	121
DL-Serine	145
L-Lysine	124
DL-Aspartic acid	137
DL-Threonine	156
DL-Valine	100
L-Glutamine	78

*Medium no. 13 (Bhuyan and Johnson, 1958): glucose, 4.0%; $(NH_4)_2SO_4$, 0.8%; $CaCO_3$, 1.2%; biotin, 10 μg/100 ml; salts solution [(g/liter): KH_2PO_4, 10.9; $MgSO_4 \cdot 7H_2O$, 8.12; Na_2SO_4, 4.45; $CaCl_2 \cdot H_2O$, 2.42; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.28; $CuSO_4 \cdot 5H_2O$, 0.033; $MnSO_4 \cdot H_2O$, 0.02], 10.0%; adjust to pH 6.0 after sterilization. All additions were to a final concentration of 0.2%. Other conditions same as in Table 4. Control value was 310 μg/ml total antibiotic as cephalosporin C.

TABLE 6. *Effect of carbohydrates on antibiotic production in ammonium sulfate medium**

Carbohydrate	Antibiotic produced (μg/ml)			
	90 hr		114 hr	
	Ceph N	Ceph C	Ceph N	Ceph C
2% Cerelose + 4% lactose	166	85	40	<20
4% Maltose	198	132	149	180
6% Soluble starch	122	182	57	34
0.8% Cerelose + 3.2% sucrose	452	161	542	173

*Medium no. 6: $(NH_4)_2SO_4$, 0.75%; DL-methionine, 0.12%; KCl, 0.038%; $MgCl_2 \cdot 6H_2O$, 0.033%; $NaH_2PO_4 \cdot H_2O$, 0.02%; $FeSO_4 \cdot 7H_2O$, 0.0025%; $ZnSO_4 \cdot 7H_2O$, 0.002%; $CuSO_4 \cdot 5H_2O$, 0.000032%; adjust to pH 7.0 before sterilization; add $CaCO_3$ to 0.5% before inoculation. Other conditions same as in Table 2.

TABLE 7. *Effect of various additions on antibiotic production in ammonium sulfate medium**

Additions	Antibiotic at 114 hr relative to control	
	Ceph N	Ceph C
	%	%
0.1% DL-Methionine	410	212
0.2% DL-Methionine	652	240
0.3% DL-Methionine	670	239
0.4% DL-Methionine	753	160
0.5% DL-Methionine	820	175
0.6% DL-Methionine	760	150
0.1% L-Cysteine	86	162
0.1% L-Cystine·HCl	110	146
0.1% Glutamic acid	82	151
0.1% Glutamine	109	121
0.1% Aspartic acid	76	116
0.1% Asparagine	90	130
0.1% DL-α-Aminoadipic acid	114	95
0.1% Adipic acid	99	84
0.2% DL-Serine	165	118
0.4% DL-Serine	214	114
0.2% DL-Valine	112	95
0.1% Betaine	173	158
0.2% Betaine	169	132
0.3% Betaine	202	216
0.1% Choline	142	190
0.2% Choline	158	156
0.3% Choline	147	219
0.1% Ethionine	178	106
0.1% α-Ketoglutaric acid	99	78
0.1% Malonic acid	102	92
0.1% Oxalic acid	100	89
0.1% Malic acid	65	113
0.1% Sodium acetate	138	103
0.4% Sodium acetate	139	94
0.1% Ammonium acetate	122	168
0.4% Ammonium acetate	178	79
0.5% Methyl oleate	129	170
1.7% Methyl oleate	81	117
0.5% Oleic acid	57	130
0.5% Ethyl oleate	124	161
0.25% KNO_3	109	104
0.1% DL-Methionine + 0.2% betaine	488	109
0.1% DL-Methionine + 0.2% DL-valine	<58	84
0.1% DL-Methionine + 0.1% DL-valine + 0.1% L-cystine·HCl	355	206
0.1% DL-Methionine + 0.1% DL-valine + 0.1% L-cystine·HCl + 0.1% sodium acetate + 0.1% α-ketoglutaric acid	338	200
0.12% DL-Methionine + 0.5% oleic acid	620	552
0.2% DL-Methionine + 0.1% betaine	902	306
0.2% DL-Methionine + 0.2% betaine	820	143
0.2% DL-Methionine + 0.1% choline	1,130	457
0.2% DL-Methionine + 0.1% ethionine	515	233
0.2% DL-Methionine + 0.2% DL-serine	478	123
0.2% DL-Methionine + 0.4% DL-serine	595	143
0.4% DL-Methionine + 0.1% betaine	1,090	96
0.1% Ethionine + 0.1% betaine	445	150
0.1% Ethionine + 0.1% choline	151	<37

* Medium no. 7: same as medium no. 6, without 0.12% DL-methionine and with 0.8% cerelose plus 3.2% sucrose. Other conditions same as in Table 2. Control medium produced 83 μg/ml of ceph N and 72 μg/ml of ceph C.

at 25 C. Again, the best carbohydrate source for production of cephalosporin C was different from that found in media previously reported.

Supplementation of the ammonium sulfate-potassium

TABLE 8. *Effect of carbohydrates on antibiotic production in synthetic ammonium sulfate-potassium nitrate medium**

Carbohydrates	Antibiotic produced at 114 hr	
	Ceph N	Ceph C
	μg/ml	μg/ml
5% Dextrin 700 + 0.5% sucrose	36	69
5% Dextrin 700	44	38
6% Soluble starch	21	24
5% Sucrose	30	21
3.2% Sucrose + 0.8% glucose	29	45
5% Maltose	38	25

* Medium no. 8: $(NH_4)_2SO_4$, 0.25%; KNO_3, 0.5%; $CaCO_3$, 0.5%; $MgSO_4$, 0.02%; KH_2PO_4, 0.02%; trace mineral solution [(g/liter): $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$, 2.7; $ZnSO_4 \cdot 7H_2O$, 2.8; $CuSO_4 \cdot 7H_2O$, 0.125; $MnSO_4 \cdot H_2O$, 1.0], 1.0%; adjust to pH 7.0 before sterilization; 50 ml of medium used per 250-ml flask. Seed medium: medium no. 9, same as medium no. 8 with 5% Dextrin 700 plus 0.5% sucrose added; growth for 96 hr at 25 C. Other conditions same as in Table 2.

TABLE 9. *Effect of addition of amino acids on antibiotic production in ammonium sulfate-potassium nitrate medium**

Additions	Antibiotic at 114 hr relative to control	
	Ceph N	Ceph C
	%	%
0.05% DL-Methionine	392	256
0.2% DL-Methionine	532	206
0.4% DL-Methionine	885	241
0.6% DL-Methionine	1,410	229
0.1% L-Cystine·HCl	145	274
0.2% L-Cystine·HCl	175	242
0.4% L-Cystine·HCl	186	223
0.2% DL-Serine	134	115
0.4% DL-Serine	132	127
0.6% DL-Serine	162	197
0.1% DL-Valine	120	120
0.2% DL-Valine	118	86
0.4% DL-Valine	88	89
0.6% DL-Valine	85	97
0.2% L-Cystine·HCl + 0.2% DL-serine	302	294
0.2% L-Cystine·HCl + 0.2% DL-valine	89	323
0.05% DL-Methionine + 0.2% DL-valine	186	193
0.05% DL-Methionine + 0.4% DL-valine	258	226
0.05% DL-Methionine + 0.2% L-cystine·HCl	488	319
0.05% DL-Methionine + 0.4% L-cystine·HCl	442	331
0.05% DL-Methionine + 0.2% L-cystine·HCl + 0.2% DL-valine	269	316

* Seed and production flasks contained medium no. 9; production flasks with additions indicated. Other conditions same as in Table 8. Control values: 36 μg/ml of ceph N and 69 μg/ml of ceph C.

TABLE 10. *Effect of nitrogen source on antibiotic formation in synthetic medium**

Omitted from control medium	Added nitrogen	Antibiotic at 114 hr relative to control	
		Ceph N	Ceph C
		%	%
$(NH_4)_2SO_4$ and KNO_3	0.4% DL-Methionine	155	54
	0.4% L-Glutamine	290	145
	0.4% L-Asparagine	94	88
	0.4% L-Cystine·HCl	238	29
	0.4% DL-Serine	72	38
	0.4% Glycine	160	32
	0.4% L-Glutamate	97	26
	0.4% DL-Valine	—†	—
	0.4% L-Lysine	—	—
	0.4% DL-Aspartic acid	—	—
	None	—	—
$(NH_4)_2SO_4$	None	—	—
	0.4% DL-Methionine	284	118
	0.4% L-Cystine·HCl	175	136
	0.4% DL-Valine	—	—
	0.4% DL-Serine	—	—
KNO_3	None	—	—
	0.4% DL-Methionine	150	88
	0.4% DL-Serine	70	93
	0.4% DL-Valine	—	—
	0.4% L-Cystine·HCl	—	—
Nothing	0.4% DL-Methionine	885	241
	0.4% L-Cystine·HCl	186	223
	0.4% DL-Serine	132	127
	0.4% DL-Valine	88	89

* Medium no. 9 with omissions and additions as indicated. Other conditions same as Table 8. Control values as in Table 9.
† No antibiotic formed.

TABLE 11. *Effect of minerals on antibiotic formation in synthetic medium**

Omitted from control medium	Added to medium	Antibiotic at 114 hr relative to control	
		Ceph N	Ceph C
		%	%
Nothing	0.4% DL-Methionine	885	241
Nothing	0.4% DL-Methionine and $CaCO_3$ before inoculation	950	545
KH_2PO_4	0.4% DL-Methionine	530	112
$MgSO_4$	0.4% DL-Methionine	1,030	121
KH_2PO_4 and $MgSO_4$	0.4% DL-Methionine	570	89
Trace mineral solution†	0.4% DL-Methionine	905	274
KH_2PO_4	Nothing	—‡	—
$MgSO_4$	Nothing	—	—
KH_2PO_4 and $MgSO_4$	Nothing	—	—
Trace mineral solution†	Nothing	72	189

* Medium no. 9 with omissions and additions as indicated. Other conditions as in Table 8. Control values as in Table 9.
† Same as in Table 8.
‡ No antibiotic formed.

nitrate medium gave results similar to those recorded previously. Data are presented in Table 9.

Effect of nitrogen source. The results of attempts to replace the nitrogen sources in medium no. 9 either wholly or in part by various amino acids are presented in Table 10.

Effect of inhibitors. The inhibition of formation of cephalosporins C and N by fluoroacetate in complex medium (Table 4) was also evident in synthetic medium (medium no. 6 with 6% soluble starch, 0.1% DL-methionine, and 0.5% oleic acid). In the latter case, fluoroacetate caused a 3-fold reduction in production of cephalosporin N and a 13-fold reduction in the amount of cephalosporin C formed. Sodium azide, 2,4-dinitrophenol, potassium cyanide, and iodoacetate did not affect antibiotic production unless added in sufficient quantity to decrease growth.

Effect of minerals. The effects of omission of magnesium, phosphate, and trace minerals from medium no. 9 and of supplementation of such media with DL-methionine are presented in Table 11.

Side-chain precursors. The ability of strains of *Penicillium* to incorporate various substances (precursors) as the side chain and thereby form a variety of penicillins has been known for many years (Moyer and Coghill, 1947;

TABLE 12. *Substrates tested for precursor activity**

Methionine	*N*-(2-hydroxyethyl)-phenoxyacetamide
α-Aminoadipic acid	*p*-Carboxyphenylacetic acid
Glutamic acid	*p*-Carboxyphenylacetoethylamide
Serine	*N*-(2-hydroxyethyl)-8-quinolinoacetamide
Lysine	Kinetin
Glycine	Hydroxyphenylacetic acid
Aspartic acid	2-Benzofuranacetic acid
Threonine	Indole-2-propionic acid
Valine	*p*-Dimethylaminophenyl acetic acid
Cystine	Phenoxyacetyl chloride
Leucine	Ethyl oxalacetic acid
Ethionine	Palmitic acid
Isoleucine	Methyl oleate
Homocystine	Sulfanilamide
L-Cystinyl-diglycine	Sulfathiazole
Betaine	Catechol
Phenoxyglycine	Benzene
Tyrosine	Phenol
N,*N*′-diphenoxyacetyl-L-cystine†	Malonic acid
N-phenoxyacetyl-L-cysteine†	Dihydroxyacetone
N-phenoxyacetyl-DL-serine†	Glycerol
N-phenoxyacetyl-S-ethyl-cystine†	Dihydroxyacetone phosphate
Glutamine	Malonamide
Asparagine	DL-Adipic acid
Acetic acid	α-Ketoglutaric acid
Acetamide	Phenylacetic acid
Oxalic acid	Phenoxyacetic acid
Oxamide	Phenylacetamide
Propionic acid	Phenoxyacetamide
Propionamide	
L-Malic acid	

* All materials were added to a final concentration of 0.1%. For other conditions see text.
† Prepared by E. B. Herr, Jr., Eli Lilly and Co.

Behrens et al., 1948). We have attempted to find antibiotics of the cephalosporin C type produced by *Cephalosporium* that contain a side chain other than D-α-aminoadipic acid (Newton and Abraham, 1955). Chromatographic methods were used as described in Materials and Methods. The precursors tested are listed in Table 12. Additions were made at 0, 24, 48, and 72 hr in a variety of complex and synthetic media. Agents thought to have a possible effect on cellular permeability to phenoxyacetic acid were also tested (Table 13). In no case has evidence been found for the formation of an antibiotic with a side chain differing from D-α-aminoadipic acid.

TABLE 13. *Reagents tested for possible effect on permeability to phenoxyacetic acid**

Reagent	Concn tested
	%
Ethanol	1, 3.3, 5, 6.7, 10, 15
Acetone	1, 5, 10
Toluene	1, 5, 10
Benzene	1
Deoxycholate	0.001
n-Butanol	1, 4, 5, 10
Methylethyl ketone	1, 3.3, 6.7
n-Propanol	1, 3.3, 6.7
Cetyltrimethyl ammonium bromide	0.001 and 0.01 M
Tween 21	1, 2, 5
Tween 40	1, 2, 10
Tween 80	1, 2, 5, 10

* All reagents, except for the Tweens (polyoxyethylene sorbitan monooleate, Atlas Powder Co., Wilmington, Del.), included in the medium make up were added at various times after inoculation: 0, 24, 48, or 72 hr. Various media were used (see text).

TABLE 14. *Antibiotic production with resting cells**

Sample time	Antibiotic assay for total activity (μg/ml)			
	Cells from medium no. 11		Cells from medium no. 10	
	44-hr cells	66-hr cells	72-hr cells	72-hr cells†
hr				
0	4	0	0	0
1	10	0	40	18
2	26	0	68	18
3	38	0	90	34
4				36
6				50
20	111	28	150	

* Incubation at 25 C as described in text. Flash-heated cells showed no antibiotic formation even with 20 hr of incubation. Medium no. 11: meat meal, 0.43%; fish meal, 0.92%; corn steep solids, 0.17%; $(NH_4)_2SO_4$, 0.24%; sucrose, 1.14%; cerelose, 0.43%; DL-methionine, 0.64%; adjusted to pH 7.0 before sterilization. Medium no. 10: soluble starch, 6%; $(NH_4)_2SO_4$, 0.75%; KCl, 0.038%; $MgCl_2 \cdot 6H_2O$, 0.033%; $NaH_2PO_4 \cdot H_2O$, 0.02%; $FeSO_4 \cdot 7H_2O$ 0.0025%; $ZnSO_4 \cdot 7H_2O$, 0.0020%; $CuSO_4 \cdot 5H_2O$, 0.00003%; DL-methionine, 0.01%; L-cystine·HCl, 0.01%; L-valine, 0.01%; L-lysine, 0.01%; L-glutamic acid, 0.01%; adjusted to pH 7.0 before sterilization; 0.5% $CaCO_3$ and 0.01% yeast extract added before inoculation.

† Cells had been held in refrigerator (4 C) for 24 hr before testing.

Phenoxyacetic acid has little effect on growth and antibiotic production at levels from 0.0167 to 0.167%. Addition at a concentration of 0.4%, however, reduced cephalosporin N by 40% and cephalosporin C by 70%. Other precursors showed no inhibitory effect at the level used.

Intracellular antibiotic and nucleus. Attempts were made to detect cephalosporins C and N, 6-amino-penicillanic acid (6-APA; Batchelor et al., 1959), and 7-amino-cephalosporanic acid (7-ACA; Loder, Newton, and Abraham, 1961) within the cells of the *Cephalosporium* strain under study. Cells were grown on a complex medium (no. 10) with the following composition: soybean meal extract (100 g of soybean meal in 1,500 ml of water, boiled for 30 min and filtered), 40%; soluble starch, 4.0%; corn steep liquor (50% solids), 3.0%; $CaCO_3$, 1.0%; and $(NH_4)_2SO_4$, 0.1%. Cells collected on the 2nd, 3rd, and 4th days of the fermentation were thoroughly washed, resuspended, and subjected to sonic disintegration in a Raytheon 10-kc sonic oscillator. The treated material was centrifuged, and the supernatant solution was tested for the presence of antibiotic activity by diffusion plate assay and paper chromatography. The possible presence of either 6-APA or 7-ACA was tested by acylation with γ-phenoxypropionyl chloride (Newton and Abraham, 1954).

No active material, preformed antibiotic or acylatable nucleus, appeared to be present within the *Cephalosporium* cells. This is in contrast to cells of *Penicillium* from the normal penicillin fermentation, which contain no penicillin but do contain significant amounts of 6-APA.

The *Cephalosporium* culture studied here does not appear to produce an enzyme for splitting the D-α-aminoadipic acid from 6-APA or 7-ACA, since no evidence of either nucleus has been found in fermentations.

Resting-cell experiments. Cells from *Cephalosporium* are able to synthesize cephalosporins N and C in phosphate buffer. The effects of the medium used for growth, the age of the cells, and the storage before testing on antibiotic production are shown in Table 14. The cells were harvested at the times indicated, thoroughly washed in distilled water, resuspended in 0.1 M phosphate buffer (pH 7.0), and sampled at various times for antibiotic activity. Flasks were incubated at 25 C on a rotary shaker at 350 rev/min describing a circle 1 in. in diam. Flash-heated cells were used as a control to detect possible leakage of bound antibiotic from the cell suspension.

Discussion

DL-Methionine was the only substrate tested that stimulated antibiotic production in all of the complex and synthetic media reported. The stimulation of production of cephalosporin N, particularly with D-methionine, has been reported (Kavanagh et al., 1958*b*). Methionine increased production of cephalosporin N to a much greater extent than that of cephalosporin C. Betaine and

choline showed the same tendency, particularly when added with DL-methionine. The involvement of methyl group transfer in the biosynthesis of both antibiotics, especially in cephalosporin N synthesis, can be inferred from the stimulatory effects of methionine, betaine, and choline, the inhibition of synthesis by the sulfa drugs, the reversal of this inhibition by methionine, and the inhibition at early stages of antibiotic formation by ethionine.

L-Cystine had a greater stimulatory effect on formation of cephalosporin C than of cephalosporin N in synthetic medium. In corn steep medium, L-cystine showed the stimulation only on additional supplementation with methionine or methionine plus ammonium sulfate. Cystine and cysteine appeared to be equivalent in synthetic medium.

The stimulation of production of cephalosporin C by DL-serine in synthetic medium (Table 9) may imply the function of the cystathionine synthetic pathway from serine and methionine (through homocysteine) to form cysteine. Further evidence for this pathway is found in the ability of serine to serve as a nitrogen source when ammonium sulfate is present, but not when potassium nitrate serves as the other nitrogen source (Table 10).

Valine did not stimulate antibiotic formation, and in some cases it seemed to suppress the stimulation by methionine. The addition of cystine and valine, on the other hand, stimulated yields of cephalosporin C, but had little effect on production of cephalosporin N (Table 9).

All of the results with amino acid additions may involve much more than the simple speculations made above, namely, amino acid interactions and balance between nitrogen and carbon sources. The fact that the best yields of cephalosporins resulted from different carbon sources, depending on the other constituents of the medium, may indicate that the carbon-nitrogen balance for this organism is quite critical. The general conclusion may be drawn that disaccharides or polysaccharides (starch or dextrins) are more effective than simple monosaccharides in supporting the production of cephalosporins C and N in the media reported. These conclusions agree with the findings of Harvey and Olson (1958) with respect to synnematin B formation by *C. salmosynnematum*. Glucose may be the form of sugar actually utilized, the polysaccharides serving as a source for the slow feeding of glucose. When glucose was used, the pH of the fermentation tended to rise when small amounts were added and to fall rather rapidly when larger amounts were used. The stimulatory effects of methyl oleate, ethyl oleate, and oleic acid may be involved in carbon metabolism or may imply an importance of fatty acid metabolism in this organism for the formation of the antibiotic.

The relation of mineral metabolism of the organism to antibiotic production requires further investigation. Calcium carbonate added to most media to help control the pH may be involved in the formation of insoluble complexes of toxic or required minerals. The importance of mineral metabolism can be implied from earlier work on other strains, in which a variety of salt mixtures were used in combination with different carbon and nitrogen sources (Pisano et al., 1954; Harvey and Olson, 1958; Bhuyan and Johnson, 1958; Bhuyan et al., 1958; Kavanagh et al., 1958*b*).

The inhibition of antibiotic formation by fluoroacetate, and observations of stimulation of production of cephalosporin N accompanied by suppression of cephalosporin C formation by oxalate, suggest the importance of two-carbon compounds in synthesis. The differential effect on the two antibiotics may indicate that the syntheses of cephalosporins C and N differ in the way two-carbon fragments and probably one-carbon fragments (methionine, betaine, choline, and sulfa drugs) are used by the organism. Further investigations with resting cells are indicated.

The synthesis of cephalosporin N (D-α-aminoadipyl-6-aminopenicillanic acid) by *Cephalosporium* (C.M.I. 49,137, mutant 8650) by a different mechanism than that used by *Penicillium* in the formation of other penicillins may be indicated by the inability of precursors to induce formation of other penicillin antibiotics, and by the absence of 6-aminopenicillanic acid within the cells. The route of synthesis of both cephalosporin N and cephalosporin C may differ in a major way from the route of penicillin synthesis in *Penicillium*. Alternatively, the differences may be due to lack or inhibition of enzymes in *Cephalosporium* capable of cleaving or transferring D-α-aminoadipic acid from cephalosporins C and N.

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