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Role of Methionine in Cephalosporin Synthesis

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ABSTRACT

CALTRIDER, P. G. (Eli Lilly and Co., Indianapolis, Ind.), AND H. F. NISS. Role of methionine in cephalosporin synthesis. Appl. Microbiol. **14**:746–753. 1966.—Methionine has an almost unique stimulatory effect on biosynthesis of cephalosporins (by *Cephalosporium acremonium*). No other sulfur-containing compound tested, except DL-methionine-DL-sulfoxide, replaced methionine. DL-Methionine stimulated the synthesis of cephalosporins when added after the growth phase. The utilization of inorganic sulfate was repressed by methionine. Experiments with L-methionine-S^{35} showed that essentially all the sulfur in the cephalosporins was derived from methionine. Sulfur-labeled compounds found in the soluble pool from cells grown with methionine-S^{35} were methionine, homocysteine, taurine, cystathionine, cysteic acid, glutathionine, and cysteine. DL-Serine-3-C^{14} was incorporated into the antibiotics, and its utilization was stimulated by methionine. L-Cysteine had a sparing effect on the incorporation of methionine-S^{35} and serine-C^{14} into the antibiotics. The data are consistent with the hypothesis that a cystathionine-mediated pathway is operative in the transfer of sulfur between methionine and cysteine.

Methionine markedly stimulates the synthesis of cephalosporin C and penicillin N by *Cephalosporium acremonium* (8, 13, 17). D-Methionine was more active than L-methionine. Inorganic sulfur compounds and organic compounds, including those related to methionine, were either less effective than methionine or inactive (8). Only DL-methionine-DL-sulfoxide and *S*-methyl-L-cysteine showed significant methionine-replacing activity. DL-Norleucine, a nonsulfur methionine antagonist, replaced methionine in a synthetic medium (9). Schemes proposed for the biosynthesis of cephalosporin C and penicillin N suggest an indirect role for methionine. L-Cysteine, a precursor of penicillin, is also a precursor of the cephalosporin C nucleus (23). The studies reported herein were undertaken to elucidate further the role of methionine in the synthesis of cephalosporins. Evidence will be presented to show that methionine plays a direct role by supplying sulfur for the formation of cephalosporins.

MATERIALS AND METHODS

Organism and culture conditions. A mutant strain (CW-19) of *C. acremonium* (C.M.I. 49,137, mutant 8650), obtained from the National Research and Development Corp., London, England, was used throughout this investigation, except in one experiment. Suspensions of lyophilized material were used to inoculate slants of a modified LePage and Campbell medium (16), which was prepared at one-tenth of the original strength except for the addition of agar at 2% and calcium chloride at 1% concentrations. Tubes, 1 inch (2.54 cm) in diameter, containing approximately 20 ml of medium were inoculated and incubated at 25 C for 10 days and then were stored at 4 C.

Growth from a 1-inch slant was suspended in 10 ml of nutrient broth and was used to inoculate a seed medium. The seed medium contained 1.5% nutrisoy flour 200D (Archer-Daniels-Midland Co., Minneapolis, Minn.), 0.1% ammonium sulfate, 0.3% calcium carbonate, 2.0% corn meal, 2.0% methyl oleate, and deionized water. The seed was propagated in 2-liter Erlenmeyer flasks containing 800 ml of medium for 72 hr at 28 C on a rotary shaker at 250 rev/min.

The medium used for cephalosporin production contained 3% beet molasses, 3% Viti-Pro 90, a meat protein (Rath Chemicals, Waterloo, Iowa), 0.5% corn steep liquor, 0.15% calcium carbonate, 0.5% DL-methionine, 2.0% methyl oleate, and deionized water. The *p*H was adjusted to 6.8 and then the medium was sterilized for 20 min at 120 C. Wide-mouth Erlenmeyer fermentor flasks (500-ml capacity) contained 60 ml of medium and 8% inoculum. Fermentations were at 28 C for approximately 96 hr on a rotary shaker at 250 rev/min. Antibiotic synthesis was complete after this period.

Antibiotic assay. The total concentration of β-lactam-containing compounds (cephalosporin C, desacetyl cephalosporin C, and penicillin N) was deter-

mined chemically by the hydroxylamine assay of Boxer and Everett (4) with cephalosporin C as a standard. Cephalosporin C activity (which included the desacetyl derivative) was determined after penicillin N was destroyed by treatment with penicillinase (Riker Laboratories, Northridge, Calif.). Throughout these studies, any reference to cephalosporin C includes the desacetyl derivative. Penicillin N was calculated as the difference between untreated and penicillinase-treated samples.

Radioactive tracers. L-Methionine-S^{35} (Schwartz BioResearch, Inc., Orangeburg, N.Y.) and DL-serine-3-C^{14} (Volk Radiochemical Co., Chicago, Ill.) were used for studies on the incorporation of methionine sulfur in cephalosporins. The methionine used in the radioisotope experiments was sterilized separately and was added at the time of inoculation. Radioactive cephalosporin C, desacetyl cephalosporin C, and penicillin N in the fermentor broth were separated by paper chromatography. A 50-μliter amount of broth diluted 1:5 was applied to acetate-buffered (*p*H 4.8) Whatman 3 MM paper and developed by descending chromatography in methanol-*n*-propanol-water (6:2:1) for approximately 18 hr at room temperature. The labeled antibiotics were detected with a Vanguard model 880 scanner. The spots were cut out and eluted with 20% aqueous methanol; radioactivity was then determined in a Packard Tri-Carb liquid scintillation spectrometer. Radioactivity was determined in absolute units (disintegrations per minute, dpm) by employing the internal-standard technique.

The amino acid pool from cells grown in the presence of methionine-S^{35} was extracted according to the method of Bent and Morton (3). After the protein was removed by precipitation with trichloroacetic acid, the samples were applied to Whatman no. 1 paper and developed in a descending fashion in three different solvent systems. These systems were methanol–*n*-propanol–water (6:2:1), *n*-butanol–acetic acid–water (3:1:1), and phenol saturated with water. After removal of solvent by drying in air, the chromatograms were cut in strips and the radioactivity was detected as before. Identification of the S^{35}-labeled compounds was made by co-chromatography with known standards.

Sulfate and methionine analysis. Inorganic sulfate in filtered broths was determined by precipitation with benzidine from a trichloroacetic acid supernatant fluid according to Raistrick and Vincent (18). The benzidine sulfate precipitate was dissolved with dilute hydrochloric acid, and the sulfate was determined turbidimetrically by use of the barium chloride-Tween 80 reagent of Garrido (12). Methionine was determined according to the method of LaRue (15).

RESULTS

Methionine replacement. Stimulation of cephalosporin synthesis by methionine is illustrated in Fig. 1. At all levels tested, D-methionine was equivalent to DL-methionine, and neither produced more than 10% better results than L-methionine. Similar results were obtained in these experiments with methionine from two suppliers (Nutritional Biochemicals, Corp., Cleveland, Ohio, and Mann Research Laboratories, Inc., New York, N. Y.).

Of the sulfur-containing compounds tested, DL-methionine-DL-sulfoxide and DL-methionine-DL-sulfone gave, respectively, 90 and 67% of the yields obtained in controls supplied with DL-methionine (Table 1). All the other sulfur-containing compounds were substantially less effective. S-Methyl-L-cysteine, L-cysteine, taurine, or DL-homocysteine, L-cysteic acid, DL-allo-cysta-

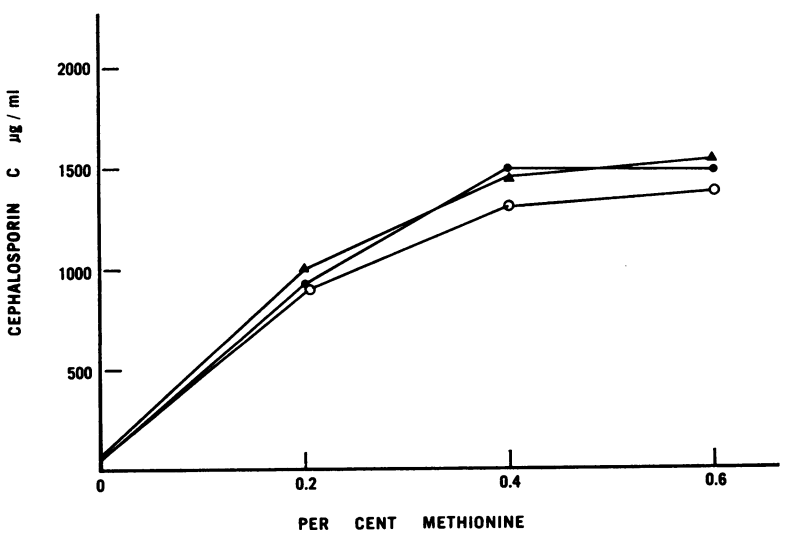

FIG. 1. *Effect of methionine on cephalosporin synthesis. Symbols:* ●, *DL-methionine;* ▲, *D-methionine;* ○, *L-methionine.*

thionine, and sodium sulfate were 22 to 37% as effective as DL-methionine. DL-Norleucine (a methionine antagonist) and DL-norvaline were not stimulatory.

Sulfur requirement for antibiotic synthesis. The total sulfur present in the basal medium is sufficient for growth but deficient with respect to antibiotic synthesis. Data in Table 1 show that, when excess sulfur was supplied to the basal medium, none of the sulfur-containing compounds was equal to methionine in stimulating cephalosporin synthesis. To demonstrate further the unique role of methionine in antibiotic synthesis, the effect of methionine was studied in a medium with excess sulfate. The results of this experiment are shown in Table 2. The basal medium with sulfate had 40% of the potency of the control, and the addition of sulfate to the methionine-containing control medium resulted in potencies which were equal to the control potencies.

TABLE 1. *Effect of sulfur-containing compounds on the synthesis of cephalosporins*

Sulfur compound[a]	Cephalosporins (96 hr)	Percentage of control
	μg/ml	
DL-Methionine (control)	1,410	100
DL-Methionine-DL-sulfone	900	67
DL-Methionine-DL-sulfoxide	1,270	90
S-Methyl-L-cysteine	385	28
L-Cysteine	480	34
Taurine	410	29
DL-Homocysteine	365	26
S-Ethyl-L-cysteine	450	32
L-Cysteic acid	310	22
DL- plus allo-cystathionine	480	34
DL-Norleucine	210	15
DL-Norvaline	155	11
Sodium sulfate	520	37
None	185	13

[a] Sulfur compounds were sterilized separately and added to the basal medium at the time of inoculation to give a final concentration of 0.026 M.

TABLE 2. *Effect of methionine on cephalosporin synthesis in the basal medium with sodium sulfate*[a]

Time	Cephalosporin (*μg/ml*)		
	Basal medium plus methionine	Basal medium plus 0.2% sodium sulfate	Basal medium plus 0.2% sodium sulfate and methionine
hr			
0	—	—	—
24	—	—	—
48	1,175	500	1,125
72	1,700	700	1,750
96	1,600	685	1,525

[a] The basal medium had 185 μg/ml of cephalosporins at 96 hr.

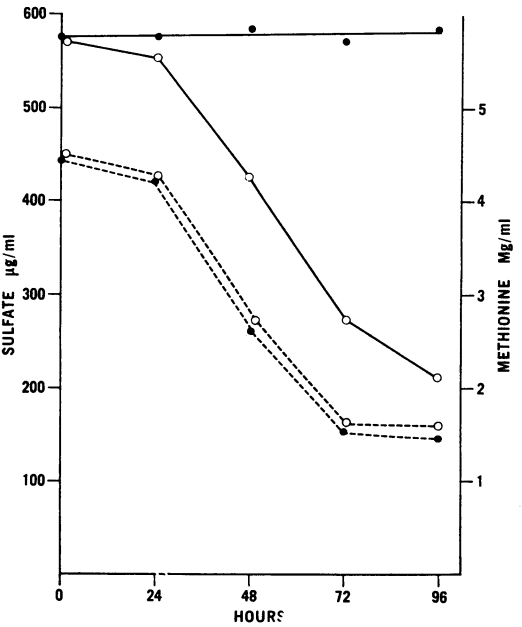

FIG. 2. *Utilization of sulfate and methionine by Cephalosporium acremonium. Symbols:* ● *with solid line, sulfate utilization in the basal medium plus methionine and sulfate;* ○ *with solid line, sulfate utilization in the basal medium plus sodium sulfate;* ● *with broken line, methionine utilization in the basal medium plus sodium sulfate and methionine;* ○ *with broken line, methionine utilization in the basal medium plus methionine.*

The utilization of inorganic sulfate and methionine, when added singly or in combination, is shown in Fig. 2. Sulfate was utilized to a significant extent in the absence of exogenous methionine. However, no sulfate was utilized in the control medium with methionine, and it did not reduce the rate of methionine utilization.

Time requirement of methionine. DL-Methionine was added at different intervals during the fermentation to determine whether its presence during the growth phase was important to stimulation of subsequent synthesis of antibiotics. Figure 3 shows that a stimulation in cephalosporin synthesis occurred when methionine was added as late as 72 hr with either strain 8650 or CW-19. Under the conditions of these experiments, antibiotic syntheses was completed at 72 to 96 hr. Several nutrients probably become limiting

in the latter phase of the fermentation; thus, no attempt was made to prolong the fermentation in flasks to which methionine was added at 48 or 72 hr.

Growth was nearly the same for both strains, and it was essentially complete by 48 hr. Methionine depressed growth in both strains. The dry weight at 96 hr in strain 8650 was reduced 8% by methionine and in strain CW-19 it was reduced 22%.

To study further the time requirement of methionine, washed cells grown in the presence and absence of added methionine were tested for their ability to synthesize cephalosporins. The basal medium without added methionine after inoculation contained 0.03% methionine. Under all conditions, methionine-grown cells had a greater synthetic capacity than cells grown without methionine (Table 3). If the endogenous synthesis was subtracted, however, the differences in synthesis were less pronounced. Apparently, methionine-grown cells contain biosynthetic intermediates which are not present in control cells but which support a reasonable rate of synthesis. The *p*H in the resting-cell experiments was adjusted to 7.0, and the final *p*H normally ranged from 6.8 to 7.2. These short-term experiments with washed cells give additional evidence that methionine promotes synthesis in nongrowing cells.

Incorporation of methionine-S^{35} into cephalosporin C. S^{35}-labeled methionine was used to elucidate further the role of methionine in cephalosporin synthesis. Data in Table 4 show the incorporation of L-methionine-S^{35} into cephalosporins.

In these experiments, less methionine was added (0.4%) to maintain a high specific radioactivity in the methionine available to the organism. Only a small percentage of the total methionine was

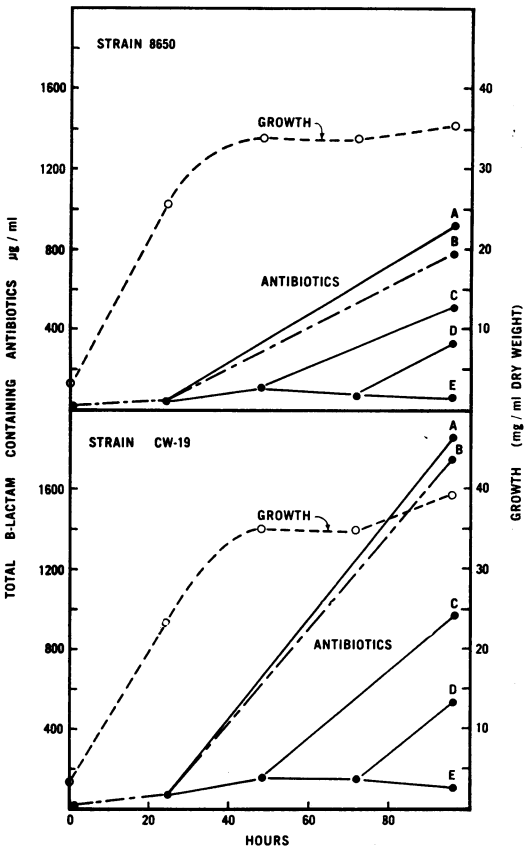

FIG. 3. *Effect of the addition of* DL-*methionine at intervals throughout the fermentation on cephalosporin synthesis by strains 8650 and CW-19. Curves A, B, C, and D show the time when methionine (0.4%) was added and the antibiotic concentration at 96 hr. Curve E represents the concentration of cephalosporin at the time methionine was added. Growth was determined in the control medium.*

TABLE 3. *Synthesis of cephalosporins by washed cells grown in the control and basal medium*[a]

Expt	Suspending medium	"Methionine" cells	"Methioneless" cells	Ratio: plus methionine cells/minus methionine cells	
				Plus endogenous	Minus endogenous
1	Water alone	185	60	3.08	—
	DL-Methionine, 0.4%	365	195	1.87	1.33
2	Water alone	210	70	3.00	—
	DL-Methionine, 0.4%	475	320	1.48	1.06

[a] Cells were grown in the fermentor medium for 48 hr, centrifuged, and washed twice with water. Approximately 0.5 g (dry weight) was dispensed to each flask, and the flasks were then shaken on a rotary shaker at 28 C for 18 hr. "Methionineless" cells were grown in the basal medium.

incorporated into the cephalosporins (9.7%). This increased only slightly if larger quantities of methionine (0.5 to 0.6%) were added to the medium. Even though only a small percentage of the added methionine was incorporated into the cephalosporins, the molar dilutions of the specific radioactivity in the antibiotics showed that virtually all the sulfur was derived from methionine.

The incorporation of cysteine into the β-lactam ring of cephalosporin C and penicillins has been demonstrated (23). In our experiments, the addition of L-cysteine reduced the incorporation of methionine-S^{35} into cephalosporins, with a concomitant increase in molar dilution of radioactivity (Table 4). Cysteine also had a sparing action on methionine utilization, as shown by the increase in residual methionine from 14% in the control to 18% where cysteine was added. Inorganic sulfate had no sparing action on the incorporation of methionine-S^{35} into the antibiotics. The amount of cephalosporins produced was not affected by the addition of cysteine or sulfate.

Most of the radioactivity in filtrates from 96-hr fermentor flasks, as detected by paper chromatography, was in cephalosporin C, penicillin N, desacetyl cephalosporin C, methionine, and an unidentified spot near the origin (R_F, 0.03). Filtrates from a 48-hr fermentation, or filtrates from a 96-hr fermentation to which methionine was added at 72 hr, possessed radioactivity in the above compounds plus homocysteine and two unidentified spots with a R_F of 0.09 and 0.89.

Radioactive compounds, identified by paper chromatography, in the soluble pool of washed cells were methinoine, homocysteine, taurine, cystathionine, cephalosporin C, desacetyl cephalosporin C, penicillin N, cysteic acid, glutathione, and cysteine. No attempt was made to quantitate these sulfur-containing compounds. Taurine and cystathionine were detected in substantial quantities by a Beckman model 120 B amino acid analyzer in cells grown in a medium with added methionine, but not in cells grown without added methionine.

Incorporation of DL-*serine-3-*C^{14} *into cephalosporins.* The marked stimulation of cephalosporin syntheses by methionine, and the sparing effect of cysteine on the incorporation of methionine-S^{35}, may imply a trans-sulfuration of methionine sulfur to form cysteine by a cystathionine pathway. Incorporation of serine-C^{14} into cephalosporins in the same order of magnitude as methionine sulfur would provide further evidence that methionine is metabolized by this pathway. Table 5 shows data for the incorporation of DL-serine-3-C^{14} into cephalosporins. The addition of methionine influenced the metabolism and incorporation of serine into cephalosporins. Without added methionine, 3.55% of the serine-C^{14} was incorporated into cephalosporins with a dilution of molar radioactivity of 1.49. When methionine was added, however, the percentage of incorporation nearly doubled, and the dilution of molar radioactivity increased. Residual serine-C^{14} was detected in broths from cultures not supplemented with methionine, but it was not detected in broths from methionine-supplemented cultures. Fermentations harvested at 41 hr had the same

TABLE 4. *Incorporation of* L-*methionine-*S^{35} *into cephalosporins*

Medium[a]	Per cent incorporation	Dilution of radioactivity	Per cent residual methionine
Control	9.7	0.97	14.2
Control plus 0.2% L-cystein at 24 hr	6.5	1.14	18.1
Control plus 0.2% sodium sulfate at 24 hr	9.3	1.05	14.8

[a] The initial concentration of methionine was 1,130 μg/ml. Methionine in the medium after inoculation was calculated on the basis of the L-methionine-S^{35} (9.9 μc per flask), unlabeled DL-methionine (0.4%), and that present in the basal medium.

TABLE 5. *Incorporation of* DL-*serine-3-*C^{14} *into cephalosporins*

Medium[a]	Time of harvest	Cephalosporins	Per cent incorporation	Dilution of radioactivity
	hr	*μg/ml*		
Basal medium	96	175	3.55	1.49
Basal medium and 0.2% methionine	96	870	6.54	3.90
Control	96	1,240	6.78	5.30
Control	41	440	7.18	1.40
Control plus 0.01 M DL-serine at 24 hr	96	1,005	7.75	1.44
Control plus 0.01 M L-cysteine at 24 hr	96	1,315	6.64	6.0

[a] DL-Serine-3-C^{14} (0.261 μc/ml) added at 24 hr; basal medium serine was calculated from that present in Viti-Pro 90.

percentage of incorporation of serine-C^{14} into cephalosporins as 96-hr fermentations, but the dilution of molar radioactivity was 1.4 rather than 5.3. These data suggest that, with abundant methionine, serine-C^{14} was metabolized rapidly and was exhausted early in the fermentation. Consequently, unlabeled cephalosporins synthesized after approximately 40 hr diluted the specific radioactivity of cephalosporins. Further evidence for this was the significant reduction in dilution of molar radioactivity with increased levels of exogenous serine.

The addition of L-cysteine to compete with labeled serine increased the dilution of molar radioactivity of C^{14} in cephalosporins. These results support the hypothesis that methionine sulfur is used to form cysteine, which is subsequently incorporated into cephalosporins.

DISCUSSION

Previously suggested biosynthetic schemes for these antibiotics do not include methionine as a direct precursor. Methionine was thought to function by repressing the formation of an enzyme that degrades cysteine to pyruvate, thus giving an increased level of cysteine in the cells (8, 9). The bases for this concept were: (i) the ability of norleucine (a methionine antagonist) to replace methionine, (ii) the failure of intermediates of methionine metabolism and other sulfur-containing compounds to replace methionine, and (iii) the requirement for methionine during the growth phase.

The results of the studies reported here indicate that methionine plays a direct role in the synthesis of cephalosporins. A significant stimulation in cephalosporin synthesis occurred with the addition of methionine after growth had stopped (48 to 72 hr). Likewise, methionine stimulated nearly the same level of synthesis in washed cells grown with or without methionine. The differences in these results from those previously reported (6, 7 9) may be attributed to fermentation conditions, since the same mutant strain (8650) was used in some experiments. The soluble complex medium used in our investigations gave approximately three times as much growth and antibiotic synthesis as Demain's synthetic medium. Moreover, the extensive lysis of the cells which occurred in the synthetic medium did not occur in the complex medium. The inordinately high phosphate concentration in the synthetic medium was very inhibitory to antibiotic synthesis in our complex medium.

One fundamental and direct role for methionine in the cephalosporin fermentation is to supply sulfur. Experiments employing methionine-S^{35} provide conclusive evidence that virtually all the sulfur in cephalosporins is derived from methionine. Methionine-S^{35} was found by Albu and Thomas (2) to contribute sulfur to naturally occurring penicillins synthesized by washed cells of *Penicillium chrysogenum*. When both sulfate and methionine were present in the medium in substrate amounts, one organism preferentially utilized methionine. Segal and Johnson (21) reported similar findings for *P. chrysogenum*.

L-Cysteine is a known precursor of cephalosporin C (23). Apparently, in *Cephalosporium*, methionine sulfur is used in the synthesis of cysteine, because sulfur-labeled cysteine and compounds derived from it, such as cysteic acid, taurine, and glutathione were found in the soluble pool of the mycelium. In addition, cysteine, but not inorganic sulfate, gave a sparing effect on the utilization of methionine-S^{35} and reduced its incorporation into cephalosporins. Methionine-*1*-C^{14} is incorporated into these antibiotics in trace amounts (1). Furthermore, previous investigations in our laboratories showed that virtually no methyl carbon or carbon 2-labeled methionine was incorporated (*unpublished data*).

The cystathionine pathway provides a known mechanism for the incorporation of methionine sulfur into cysteine. If this pathway is operative the molar dilution of radioactivity for serine-C^{14} and methionine-S^{35} would be equivalent. Not only was such an equivalence found, but the utilization of serine was methionine-dependent. Furthermore, homocysteine and cystathione, the expected labeled intermediates of the cystathionine pathway, were found in the soluble pool of cells grown in the presence of methionine-S^{35}. Although serine did not enhance synthesis in the complex medium, such stimulation has been re-

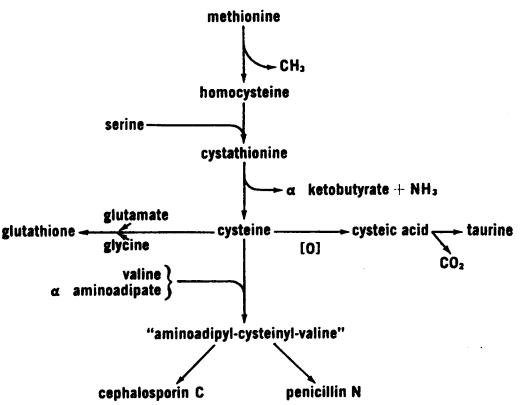

FIG. 4. *Proposed scheme for the relationship between methionine metabolism and the biosynthesis of cephalosporin C and penicillin N.*

ported (17). The scheme presented in Fig. 4 proposes a direct role for methionine metabolism in cephalosporin and penicillin N biosynthesis. This scheme is consistent with our data and with those data reported concerning trans-sulfuration in fungi (14) and cephalosporin biosynthesis (1, 6, 7).

In bacteria, methionine and some of its analogues (norleucine) repress the enzymes responsible for methionine synthesis (10, 20; Rowbury and Woods, Biochem. J. 79:36P, 1961). There is good evidence, however, that the formation of cysteine from methionine is not an irreversible process in fungi (5, 11, 14).

Although the effectiveness of methionine in the stimulation of cephalosporins and penicillin N synthesis is well documented, it is unclear why sulfate, cysteine, or intermediates of methionine metabolism will not replace methionine. One plausible explanation for the ineffectiveness of cysteine is that it is oxidized rapidly and is thus unavailable to the cells. Permeability is always a potential limiting factor.

Only a small amount of the methionine required for optimal synthesis was incorporated into cephalosporin and penicillin N (Table 4) and approximately 14% of the added methionine remained after net synthesis stopped. High levels of methionine may be required to maintain intracellular levels of cysteine which are optimal for antibiotic synthesis. Alternatively, besides serving as a sulfur source, methionine may cause metabolic changes in the cell conducive to cephalosporin synthesis. Stimulation of high levels of amino acid oxidase by D-methionine and transaminase by L-methionine has been reported in *Trigonopsis* (22). Active transamination would be required for the synthesis of high levels of cysteine, valine, and aminoadipic acid. Clark and Rowbury (5) reported that methionine in *Coprinus* reduces growth and stimulates overall amino acid synthesis, and excretes excess amounts of amino acids. In our experiments, fermentations with added methionine maintained a neutral *p*H, and the mycelium was swollen, irregular, and fragmented. Without added methionine, the mycelium was filamentous and much thinner, whereas the *p*H was much higher. Although it has been shown that methionine is the primary source of sulfur for cephalosporin synthesis, its specificity as a sulfur source for antibiotic synthesis and the mechanisms by which it acts remain to be explained.

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