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Effect of Methionine and Sulfate on the Metabolism of *Cephalosporium acremonium*

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The metabolism of *Cephalosporium acremonium* grown in a complex medium supplemented with DL-methionine or inorganic sulfate was studied. More growth occurred in a sulfate medium than in a methionine medium. Methionine-grown cells had an increased rate of respiration, a higher rate of catabolism with acetate and glucose as substrate, and higher specific activities of certain respiratory enzymes than sulfate-grown cells. Labeled acetate and glucose were assimilated at a faster rate by methionine-grown cells than sulfate-grown cells. Taurine, cystathionine, and small quantities of four acidic compounds were present in the amino acid pool of methionine-grown cells, but they were not detected in the pool of sulfate-grown cells. The differences in metabolic activity of sulfate and methionine-grown cells are discussed in regard to cephalosporin C synthesis.

The stimulatory action of methionine on cephalosporin C biosynthesis has been well documented (3, 6, 15, 20). Other inorganic and organic sulfur-containing compounds were shown to be markedly less stimulatory than methionine. This observation has been partially explained in that the amino acid plays a direct biosynthetic role by supplying the sulfur for the cephalosporin C molecule (3). However, the methionine concentrations for maximal antibiotic yields were in excess of that needed to supply sulfur for cephalosporin synthesis. These results suggest that methionine also plays a secondary role in the biosynthesis of cephalosporin C. Such a role may involve the induction of specific metabolic changes conducive to cephalosporin C synthesis. This investigation was undertaken to determine if methionine induced such changes in *Cephalosporium acremonium*.

MATERIALS AND METHODS

Cultural conditions. Both strain and cultural conditions were identical to those previously described (3). When sulfate was the sulfur source, 0.20% sodium sulfate was substituted for DL-methionine in the fermentation medium. Cephalosporin C was determined by the hydroxylamine assay of Boxer and Everett (2).

Respiratory studies. Standard manometric techniques were employed for respiratory determinations (21). *C. acremonium* was grown for 24, 48, 72, and 96 hr in the fermentation medium with exogenous methionine or sulfate, centrifuged (1,000 × *g*) for 15 min, washed twice with 0.05 M potassium phosphate buffer (*p*H 7.0), and added to the Warburg flasks. The Warburg flasks contained potassium phosphate buffer (*p*H 7.0), 100 μmoles; magnesium chloride, 10 μmoles; glucose, 50 μmoles; cells; and deionized water to 3.2 ml. For the determination of Q_{O_2}, 0.2 ml of 20% sodium hydroxide was added to the center well of the vessel.

Total carbohydrate and lipid determinations. Total carbohydrate was determined by the anthrone method as described by Morris (14). Neutral lipid was determined by extraction of neutralized broth with chloroform (v/v) and concentration of the organic phase to dryness in vacuo. These residues were measured gravimetrically and assumed to be neutral lipid.

Preparation of cell-free extracts. Cells (48 hr) were collected by filtration and washed with deionized water. Wet mycelia (10 g) were ground with sand (20 g) in a precooled mortar and pestle with 40 ml of 0.05 potassium phosphate buffer (*p*H 7.0) for 15 min at 0 C. The resulting brei was centrifuged at 1,000 × *g* for 20 min at 0 C, and the supernatant fluid was used for the enzyme assays.

Enzyme assays. All enzymes were assayed spectrophotometrically with a Hitachi Perkin-Elmer spectrophotometer model 139 coupled with a Varicord model 43 recorder. Assays were conducted at ambient temperature, and the reactions were initiated by the addition of enzyme. The protein content of the extracts was determined by the method of Lowry et al. (13) with bovine serum albumin as the standard. Hexokinase activity was determined by observing the increase in optical density at 340 nm in a system containing potassium phosphate buffer (*p*H 7.0), 30 μmoles; glucose, 10 μmoles; glucose-6-phosphate dehydrogenase, 50 μg; adenosine triphosphate, 10 μmoles; magnesium chloride, 10 μmoles; nicotinamide adenine dinucleotide phosphate (NADP), 1.0 μmole; cell-free extract; and deionized water to 3.0

ml. Glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and isocitric dehydrogenase activities were determined by observing the rate of NADP reduction at 340 nm. The assay mixture contained potassium phosphate buffer (*p*H 7.5), 30 μmoles; magnesium chloride, 10 μmoles; substrate, 10 μmoles; NADP, 1.0 μmole; cell-free extract and deionized water to 3.0 ml. Glyceraldehyde-3-phosphate dehydrogenase was assayed according to the method of Krebs (12). Lactic acid and alcohol dehydrogenases were measured by the methods described by Kornberg (11) and Racker (17), respectively. Aconitase and fumarase were determined by the method of Racker (16). Esterase and isocitratase activities were measured by the methods of Keay and Crooke (10) and Dixon and Kornberg (7), respectively. α-Glycerol phosphate dehydrogenase activity was assayed by observing the decrease in optical density at 340 nm caused by the oxidation of reduced nicotinamide adenine dinucleotide (NADH) with glyceraldehyde-3-phosphate as the substrate. The assay system contained potassium phosphate buffer, (*p*H 7.5), 30 μmoles; glyceraldehyde-3-phosphate, 3 μmoles; cell-free extract; and distilled water to 3.0 ml. Malic dehydrogenase activity was determined by the same technique, except that freshly prepared oxalacetic acid (10 μmoles) was used as the substrate. All assays were replicated twice with different cell-free extracts. The values reported are the average of two determinations.

Tracer experiments. All radioisotope experiments were performed in Warburg vessels. Each system contained fermentation broth (2.3 ml) and substrate. A 20% sodium hydroxide solution (0.2 ml) was added to each center well. After temperature equilibration, either glucose-U-^{14}C (25 μmoles), sodium acetate-*1*-^{14}C (83 μmoles), or phenylalanine-*1*-^{14}C (20 μmoles) was added to the main compartment of the flask. Total radioactivity per flask approximated 1 μc. After incubation for 90 min, the cellular material was separated from the broth by centrifugation (1,000 × *g*), and cells, broth, and sodium hydroxide were assayed for radioactivity. After extensive washing with deionized water, the cells were dried overnight in vacuo (72 C), pulverized, and combusted. The resulting radioactive carbon dioxide was determined by the method of Dobbs (8). The radioactivity of the various samples was determined by liquid scintillation counting on a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Efficiency of counting was determined by the internal standard technique. All labeled substrates were obtained from Calbiochem, Los Angeles, Calif.

Amino acid analysis. Mycelia from 90-hr fermentations were collected, filtered, and washed. The amino acid pools were extracted according to the method of Bent and Morton (1). After protein was removed by precipitation with trichloroacetic acid, the samples were treated with performic acid and analyzed on a Beckman model 120 B amino acid analyzer according to the method of Spackman et al. (19). Concentration of amino acids was expressed as μmoles per gram (dry weight) of cells.

RESULTS

The synthesis of cephalosporin C in the basal medium supplemented with either methionine or sodium sulfate is shown in Fig. 1. Antibiotic production in the methionine-containing medium was twice that obtained in the sulfate medium. Furthermore, the synthetic rate of the organism was substantially faster when methionine was used. Growth in the sulfate medium was greater after 96 hr, and it occurred at a faster rate than in the methionine medium (Fig. 2). Morphologically, the mycelia from the sulfate medium were generally filamentous. Mycelia grown in the methionine medium were swollen, irregular, more highly fragmented, and sporulated to a greater degree.

No difference was observed in the rate of sugar utilization when *C. acremonium* was grown on either medium (Fig. 3A). At 72 hr, all carbohydrate had disappeared from the medium. Neutral lipid was utilized at a significantly higher rate in the sulfate medium than in the methionine-containing medium (Fig. 3B). Depletion of neutral lipid in sulfate and methionine media occurred at approximately 72 and 96 hr, respectively.

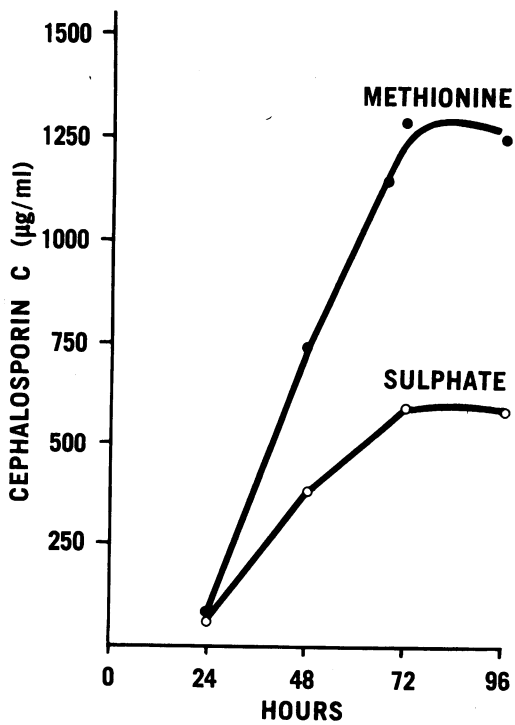

FIG. 1. *Synthesis of cephalosporin C in a methionine or sodium sulfate medium.*

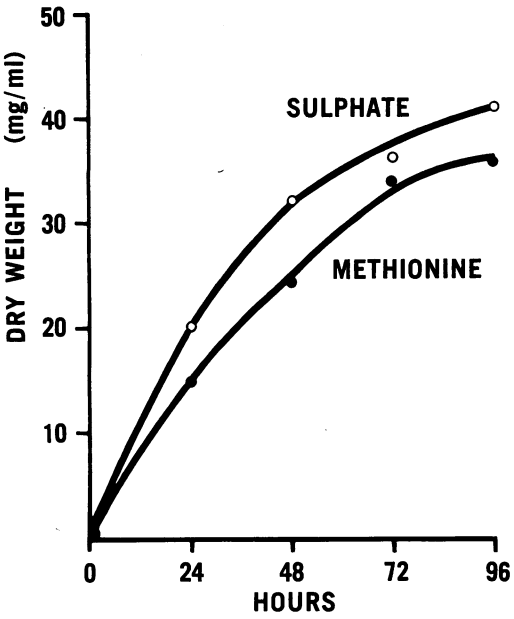

FIG. 2. *Growth of C. acremonium on a methionine or sulfate medium.*

The rates of oxygen consumption (Q_{O_2}) and carbon dioxide liberation (Q_{CO_2}) were determined for methionine- and sulfate-grown cells at daily intervals from 24 to 96 hr (Fig. 4A, B). Methionine-grown cells had a significantly higher Q_{O_2} and Q_{CO_2} at 24, 48, and 72 hr, but these values were equivalent to those of sulfate-grown cells at 96 hr. Although the methionine-grown cells had higher respiratory rates, the respiratory quotient was no different from that of the sulfate-grown cells.

Before conducting various dehydrogenase assays, it was established that neither NADH nor NADPH oxidase activities were present in the extracts. Therefore, in calculating the specific activities of the various dehydrogenases, no corrections were made for these reactions. A comparison of the specific activities of enzymes in extracts from methionine-grown cells and from sulfate-grown cells is presented in Table 1. In general, the enzymes from cells grown in the methionine medium had higher specific activities than those of cells grown in the sulfate medium. The activities of glyceraldehyde-3-phosphate dehydrogenase, α-glycerol phosphate dehydrogenase, malic dehydrogenase, aconitase, and isocitritase in cell-free extracts of methionine-grown cells were approximately twice those in extracts of sulfate-grown cells. Both lactic and alcohol dehydrogenase activities were slightly higher in extracts from sulfate-grown cells.

To study further the effect of methionine on metabolism, ^{14}C recovery data were obtained from the utilization of glucose-U-^{14}C, acetate-*1*-^{14}C, and phenylalanine-*1*-^{14}C (Table 2). In such experiments, the cells were not washed, but rather whole fermentation broth was added directly to the Warburg vessel. The methionine cells incorporated twice the amount of glucose, three times the amount of acetate, and an equivalent amount of phenylalanine compared to the sulfate-grown cells. Relatively low levels of phenylalanine were incorporated into cells of either type. More $^{14}CO_2$ was liberated from labeled substrates by methionine-grown cells, and these cells utilized more of the labeled substrates than sulfate-grown cells. In this experiment, the Q_{O_2} for the methi-

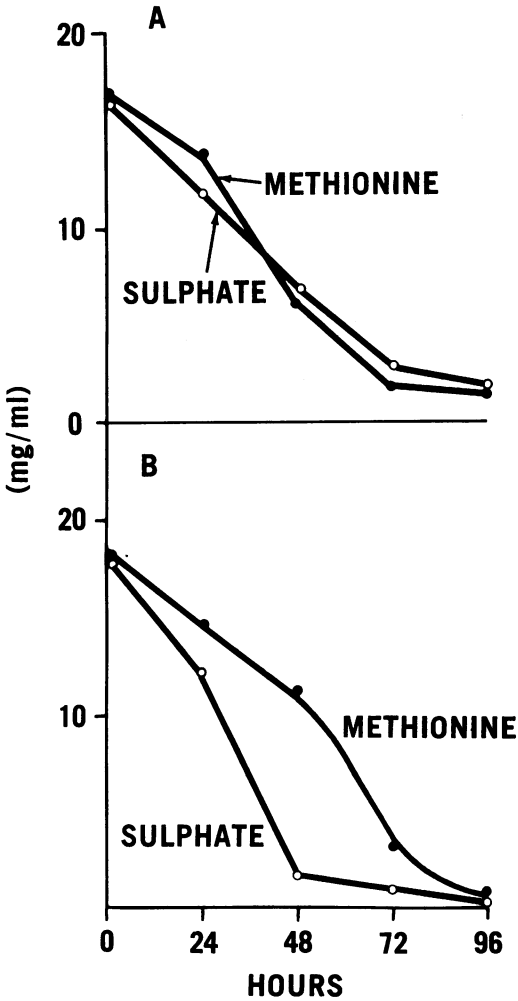

FIG. 3. *Influence of methionine and sulfate on the utilization of sugar (A) and neutral lipid (B).*

onine-grown cells with the various substrates was nearly twice that for sulfate-grown cells.

The concentration of some of the amino acids in the intracellular pool of mycelium of methionine and sulfate-grown cells is given in Table 3. The amino acids present in highest concentrations were hydroxyproline, proline, glutamic acid, and alanine. Lysine, serine, valine, α-aminobutyric acid, and leucine were present in lower concentration than those listed above, but they were present at higher concentrations than other amino acids.

The major differences in the amino acids in the intracellular pools were in the concentration of amino acids derived from methionine metabolism. Taurine and cystathionine were present in substantially higher concentration in methionine-grown cells. In addition, small quantities of four acidic compounds which were not identified were present in pools of methionine-grown cells, but they were not detected in sulfate-grown cells.

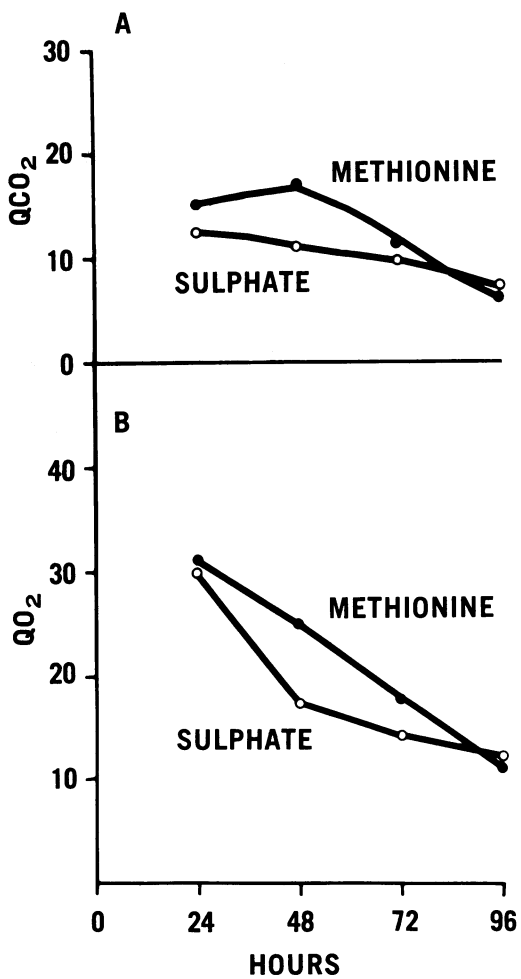

FIG. 4. *Influence of methionine and sulfate on oxygen uptake (A) and carbon dioxide liberation (B).*

TABLE 1. *Specific activities of enzymes in cell-free extracts of methionine- and sulfate-grown cells*

Enzyme	Specific activity[a]	
	Methionine-grown	Sulfate-grown
Hexokinase	7	6
Glucose-6-phosphate dehydrogenase	50	60
6-Phosphogluconic dehydrogenase	25	15
α-Glyceraldehyde-3-phosphate dehydrogenase	68	18
α-Glycerol-phosphate dehydrogenase	6.2	3.7
Alcohol dehydrogenase	15	25
Lactic dehydrogenase	31	43
Malic dehydrogenase	788	352
Aconitase	0.107	0.061
Fumarase	0.011	0.020
Isocitratase	0.029	0.011
Esterase	0.030	0-033

[a] Activities of aconitase, fumarase, isocitratase, and esterase are expressed as change in optical density per minute per milligram of protein. Others are expressed as nanomoles of pyridine nucleotide oxidized or reduced per minute per milligram of protein.

DISCUSSION

The results presented in this paper indicate that methionine, in addition to supplying sulfur for antibiotic synthesis, plays a secondary role in the biosynthesis of cephalosporin C in *C. acremonium*. The metabolism of methionine-grown cells was significantly different from that of sulfate-grown cells. In general, methionine-grown cells had a different morphology, an increased rate of respiration, a higher rate of assimilation and catabolism of acetate and glucose, reduced growth, and higher specific activities of certain respiratory enzymes. The exact nature of the secondary role of methionine in cephalosporin C synthesis is not understood. One may postulate that methionine causes the accumulation of high concentrations of pool metabolites which are modified by shunt metabolism to form antibiotics. Clark and Rowbury (4) reported that methionine reduced growth and stimulated overall amino acid synthesis in *Coprinus*. Our data, however, with the exception of certain sulfur-containing amino acids, showed no excess of amino acids in methionine-grown cells. Smith et al. (18) reported that three amino

TABLE 2. *Metabolism of labeled substrates of C. acremonium grown on methionine and sulfate media*

Substrate	Q_{O_2}		Dry weight (counts per min, per mg)		Radioactivity in $^{14}CO_2$[a] (%)		Substrate utilized (%)	
	Methionine	SO_4	Methionine	SO_4	Methionine	SO_4	Methionine	SO_4
Glucose-U-^{14}C	11.2	6.6	4,772	2,506	0.95	0.13	56	50
Acetate-1-^{14}C	12.8	7.7	3,015	841	1.77	1.13	43	35
Phenylalanine-1-^{14}C	12.9	8.5	272	293	0.99	0.19	15	7

[a] Values presented are percentage of counts added as substrate.

TABLE 3. *Intracellular amino acids in cells grown in methionine and sulfate media*[a]

Amino acid	Methionine medium	Sulfate medium
Lysine	1.073	0.983
Histidine	0.251	0.371
Arginine	0.532	0.603
Hydroxyproline	10.47	13.67
Proline	8.137	6.123
Aspartic acid	0.452	0.768
Threonine	0.669	0.586
Serine	1.94	1.36
Glutamine, Asparagine	2.386	1.45
Glutamic acid	7.456	8.359
Glycine	0.899	0.818
Alanine	3.109	3.486
α-Aminobutyric	0.920	1.138
Valine	1.056	1.079
Methionine	0.439	0.420
Isoleucine	0.647	0.648
Leucine	1.406	1.60
Tyrosine	0.366	0.487
Phenylalanine	0.792	0.822
β-Alanine	+[b]	+
Glutathione	+	+
Taurine	0.626	+
Cystathionine	0.183	+
Cysteic acid	+	+

[a] Results expressed as μmoles per g (dry weight).
[b] Amino acid was present at relatively low concentrations.

acids, aminoadipic acid, cysteine, and valine, which are known precursors of cephalosporin C, were found in relatively low concentrations in the amino acid pool of *Cephalosporium* sp. 8650. Our data show no significant differences in the concentration of valine in the pools of methionine-grown and sulfate-grown cells. The increased levels of cystathionine and other sulfur-containing amino acids in the pool of methionine-grown cells may be significant in the stimulation of antibiotic synthesis. Although the intracellular cysteine pool remains stabilized, the increased demands for cysteine for antibiotic synthesis may be satisfied more readily with methionine. Evidence for formation of cysteine from methionine by a transsulfuration process has been described for fungi (3, 5, 9). In addition, studies on the regulation of sulfatase in *C. acremonium* show that the enzyme has high activity on methionine medium during the period of cephalosporin synthesis, and it is repressed at the cessation of antibiotic production. This suggests that methionine, as the sole sulfur source, is primarily directed toward cephalosporin synthesis, whereas additional sulfur requirements are supplied through breakdown of endogenous sulfur stores (D. D. Carver and D. W. Dennen, Bacteriol. Proc. p. 11, 1968).

Methionine-grown cells had higher Q_{O_2} and specific activities of various respiratory enzymes than sulfate-grown cells. This stimulation in metabolism may be directly related to increased antibiotic synthesis. If valine, aminoadipic acid, and cysteine are constantly removed from the cell pool, a higher rate of metabolism would be needed to maintain normal cell function. There is some evidence that the enzyme system for cephalosporin C synthesis may require high metabolic activity. Reduction of metabolic activity by decreasing the oxygen supply results in a reduction in cephalosporin C synthesis and an increase in penicillin N synthesis (18). The higher specific activities of alcohol and lactic dehydrogenase in sulfate-grown cells suggest a less oxidative system. The Q_{O_2} values of cells from the two systems were nearly equivalent after antibiotic synthesis ceased.

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