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Utilization of Ornithine and Arginine as Specific Precursors of Clavulanic Acid

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Ornithine and arginine (5 to 20 mM), but not glutamic acid or proline, exerted a concentration-dependent stimulatory effect on the biosynthesis of clavulanic acid in both resting-cell cultures and long-term fermentations of *Streptomyces clavuligerus*. Ornithine strongly inhibited cephamycin biosynthesis in the same strain. [1-^{14}C]-, [5-^{14}C]-, or [U-^{14}C]ornithine was efficiently incorporated into clavulanic acid, whereas the incorporation of uniformly labeled glutamic acid was very poor. [U-^{14}C]arginine was also well incorporated into clavulanic acid, but [guanido-^{14}C]arginine and [carboxyl-^{14}C]citrulline were not incorporated at all. Mutant *nca-1*, a strain that is blocked in clavulanic acid biosynthesis, did not incorporate arginine into clavulanic acid. *S. clavuligerus* showed arginase activity, converting arginine into ornithine, but not amidinotransferase activity. Both arginase activity and clavulanic acid formation were enhanced simultaneously by supplementing the production medium with 10 mM arginine.

Clavulanic acid, produced by *Streptomyces clavuligerus* NRRL 3585 (8), is a potent inhibitor of β-lactamases of gram-positive and gram-negative bacteria (15). It has a fused nucleus containing a β-lactam and an oxazolidine ring (Fig. 1).

The biosynthesis of clavulanic acid has been investigated by feeding labeled precursors to the fermentation (3, 4). The three carbon atoms (C-5, C-6, and C-7) of the β-lactam ring are derived from glycerol apparently via a β-hydroxypropionate intermediate (6, 19). Elson and co-workers (3, 4) provided evidence that carbons 2 and 8 of the molecule originated from glutamic acid. Carbons 3 and 10 appear to derive also from glutamate, and therefore it was proposed that the five carbon atoms of glutamate are incorporated into five carbon atoms (C-2, C-3, C-8, C-9, and C-10) of the clavulanic acid molecule (Fig. 1) (4, 17).

However, we have shown in a recent study on the dissociation of clavulanic acid and cephamycin production by *S. clavuligerus* (16) that glutamic acid negatively affects the formation of clavulanic acid presumably owing to nitrogen catabolite regulation. Moreover, the δ-carboxyl group of glutamic acid is more oxidized than the δ-hydroxyl group that appears in carbon 9 of clavulanic acid, and therefore a reduction step of this carbon atom would be required (Fig. 1). These considerations prompted us to study the possible utilization of five-carbon compounds metabolically related to glutamic acid as clavulanic acid precursors. We found that arginine and ornithine, but not proline, are direct precursors of clavulanic acid. While this paper was in preparation a report appeared suggesting that ornithine was a clavulanic acid precursor (18). However, the origin of ornithine and the influence of ornithine or other precursors on clavulanic acid and cephamycin biosynthesis remained unclear. In this paper we provide evidence of the utilization of arginine (in addition to ornithine) rather than glutamic acid as a source of the five-carbon moiety of clavulanic acid. Evidence of the conversion of arginine into ornithine is also shown.

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MATERIALS AND METHODS

Microorganisms. A single clone isolated from the clavulanic acid-producer *S. clavuligerus* NRRL 3585 (8) was used throughout this study. *S. clavuligerus nca-1* is a nonproducing mutant, isolated in our laboratory, which is blocked in clavulanic acid biosynthesis but produces normal amounts of cephamycin C (J. Romero, P. Liras, and J. F. Martín, unpublished data).

Media and growth conditions. *S. clavuligerus* was grown for clavulanic acid production in GSPG medium (containing in grams/liter: glycerol, 15; sucrose, 20; proline, 2.5; glutamic acid, 1.5; NaCl, 5; K_2HPO_4, 2; $CaCl_2$, 0.4; $MnCl_2 \cdot 4H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.1; $ZnCl_2$, 0.05; $MgSO_4 \cdot 7H_2O$, 1; and distilled water at pH 7) as described previously (16). GSPO or GSPA media, which were identical to GSPG except that they contained 10 mM ornithine or arginine, respectively, in place of glutamic acid, were used in some experiments to study the role of ornithine and arginine as precursors. Phosphate-limited resting-cell systems were prepared as reported before (16).

Antibiotic assays. Clavulanic acid and cephamycin C were determined by bioassay as described previously (1, 16). Clavulanic acid was purified by thin-layer chromatography (16). A pure sample of clavulanic acid (Beecham Laboratories, Betchworth, U.K.) was used as a standard.

Radiochemicals. DL-[1-^{14}C]ornithine hydrochloride (50 mCi/mmol), DL-[5-^{14}C]ornithine hydrochloride (50 mCi/mmol), L-[U-^{14}C]ornithine hydrochloride (250 mCi/mmol), L-[U-^{14}C]arginine monohydrochloride (300 mCi/mmol), L-[guanido-^{14}C]arginine monohydrochloride (40 mCi/mmol), L-[carbamoyl-^{14}C]citrulline (55 mCi/mmol), L-[U-^{14}C]glutamic acid (250 mCi/mmol), and [U-^{14}C]leucine (330 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England.

Incorporation of labeled precursors into clavulanic acid. The incorporation of labeled precursors into clavulanic acid was carried out in carbon-, nitrogen-, and phosphate-limited resting-cell systems. The resting cells were preincubated for 6 h at 28°C in MOPS (morpholinepropanesulfonic acid)

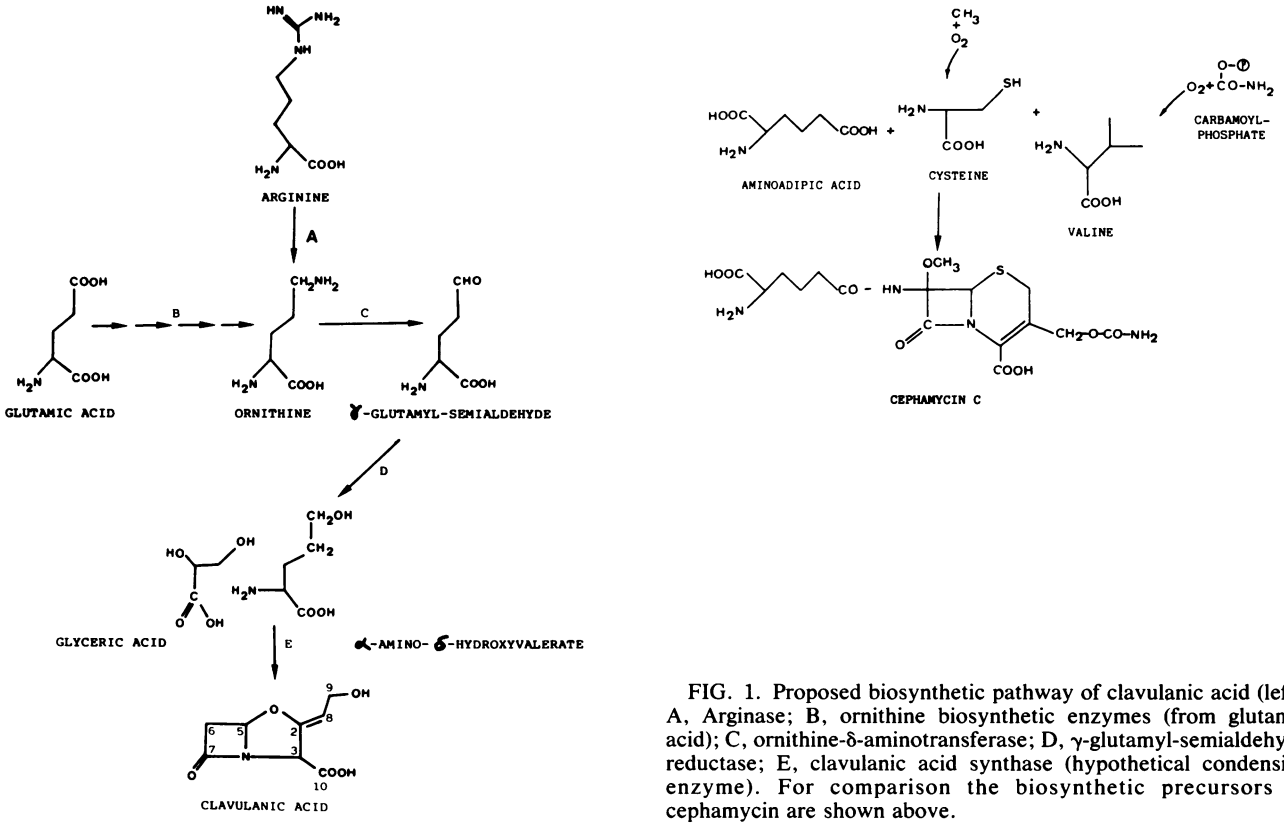

FIG. 1. Proposed biosynthetic pathway of clavulanic acid (left). A, Arginase; B, ornithine biosynthetic enzymes (from glutamic acid); C, ornithine-δ-aminotransferase; D, γ-glutamyl-semialdehyde reductase; E, clavulanic acid synthase (hypothetical condensing enzyme). For comparison the biosynthetic precursors of cephamycin are shown above.

buffer to equilibrate their metabolism to the nutrient-limited conditions. They were then supplemented with 0.1 mM unlabeled precursor, followed by incubation for 60 min. Radioactive precursors were then added at a concentration of 0.15 μCi/ml (0.30 μCi/ml when the amino acid was in the DL form), and incorporation into clavulanic acid was allowed for another 60 min.

Clavulanic acid was separated from the precursor by thin-layer chromatography of samples of the culture (after 60 min of incubation) on Silica Gel G plates (0.1 mm) to determine the incorporation of radioactivity into the antibiotic. Duplicate samples (60 μl) of the culture supernatant obtained by centrifugation at 15,000 × *g* for 10 min were applied and developed in *n*-butanol–acetic acid–water (12:3:5, vol/vol). The R_f of pure clavulanic acid under these conditions was 0.63, while arginine, glutamic acid, and ornithine moved behind with R_f values of 0.26, 0.33, and 0.17, respectively. The silica gel was divided into 1-cm bands and scraped off, and the radioactivity was measured with a scintillation fluid containing PPO (2,5-diphenyloxazole) (3.5 g/liter) and POPOP [1,4-bis(5-phenyloxazolyl)benzene] (50 mg/liter) in toluene, in a Philips CW4700 liquid scintillation counter with automatic quenching correction. The values were corrected for the background counts.

Protein synthesis. Protein synthesis was determined by measuring the incorporation of [U-^{14}C]leucine, [U-^{14}C]ornithine, or [U-^{14}C]arginine into 5% trichloroacetic acid-insoluble material as described by Martín et al. (11). Labeled leucine was added at a final concentration of 1 μCi/ml to the resting-cell culture supplemented with 0.1 mg/ml of unlabeled leucine, and then 250-μl samples of the culture were taken into 250 μl of ice-cold 10% trichloroacetic acid.

Uptake of precursors. Samples (250 μl) to determine the cellular uptake of the labeled precursor were taken at intervals from cultures supplemented with the radioactive precursor, chased with 10 ml of a 1 mM solution of unlabeled precursor, filtered immediately through a Whatman GFA filter, and washed twice with 10 ml of the same unlabeled precursor solution. The intracellular pool of labeled precursors was calculated by subtracting, at a given time, the radioactivity existing in the trichloroacetic acid-precipitable material from the total cellular label.

Arginase and amidinotransferase activities. Arginase activity was measured in extracts of *S. clavuligerus* prepared by sonication for 1 min at 15-s intervals in 50 mM glycine buffer (pH 9.5). Determination of the product (ornithine) was as described by Harwood and Baumberg (7) except that cell extracts were put through Sephadex G-25 to remove the contaminating ornithine in the extract.

Amidinotransferase was determined in the same cell extracts, using hydroxylamine or glycine as the acceptor of the guanidino group (20).

RESULTS

Utilization of ornithine, arginine, and proline as sole carbon and nitrogen sources. *S. clavuligerus* uses proline, arginine, or ornithine as the sole nitrogen source. Proline can also be used as the sole carbon source but not ornithine or arginine.

Effect of ornithine on clavulanic acid and cephamycin production in long-term cultures. The effect of ornithine on clavulanic acid and cephamycin production was tested both in long-term fermentation and in short-term resting-cell cultures. When glutamic acid was substituted in the GSPG

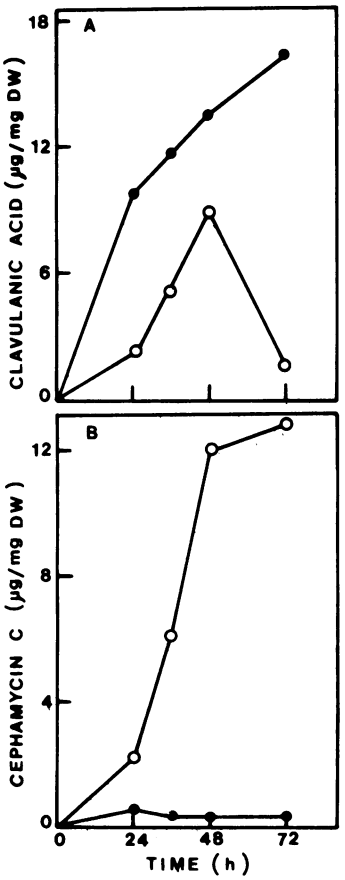

FIG. 2. Effect of ornithine on clavulanic acid (A) and cephamycin (B) production in long-term cultures of *S. clavuligerus*. Symbols: ○, GSPG; ●, GSPO (10 mM ornithine). DW, Dry weight.

medium by the same concentration of ornithine, the relative proportions of clavulanic acid and cephamycin produced changed drastically (Fig. 2). Clavulanic acid biosynthesis was doubled by adding ornithine (Fig. 2A), but cephamycin C formation was completely inhibited (Fig. 2B). Under these conditions (GSPO medium), it was thus possible to dissociate the biosynthesis of clavulanic acid from that of cephamycin.

The addition of ornithine to GSPG medium doubled the production of clavulanic acid only when added before 30 h of fermentation. Late additions of ornithine produced only a slight increase in clavulanic acid formation but still reduced the biosynthesis of cephamycin C. The greatest stimulation of clavulanic acid biosynthesis in long-term fermentations was obtained when an additional 10 mM ornithine was added before 30 h of incubation to GSPO medium (i.e., final ornithine content of 20 mM).

Ornithine stimulation of clavulanic acid biosynthesis by resting cells of *S. clavuligerus*. Resting-cell systems prepared with washed cells collected from GSPG medium supplemented with 10 mM ornithine produced about the same amounts of cephamycin C (11 µg/mg [dry weight]) and clavulanic acid (10 µg/mg [dry weight]) as resting cells grown under control (unsupplemented) conditions. The addition of ornithine (5 to 10 mM) directly to resting-cell cultures produced a concentration-dependent stimulation of the biosynthesis of clavulanic acid (up to 24 µg/mg [dry weight]). These results suggest that ornithine was not inducing the clavulanic acid-synthesizing enzymes but rather acted as a precursor needed for clavulanic acid production.

TABLE 1. Effect of different compounds metabolically related to glutamic acid on clavulanic acid and cephamycin C formation by *S. clavuligerus* NRRL 3585

Addition (10 mM)	µg/mg (dry wt) (%)	
	Clavulanic acid[a]	Cephamycin C[a]
None	13.5 (100)	5.7 (100)
L-Glutamic acid	9.8 (75)	4.2 (73)
N-Acetyl-L-glutamic acid	11.5 (85)	4.7 (82)
N-Acetyl-L-ornithine	13.5 (100)	3.5 (61)
L-Ornithine	19.0 (140)	0.3 (5)
L-Citrulline	13.5 (100)	4.7 (82)
L-Arginine	16.0 (118)	4.0 (70)
L-Proline	13.5 (100)	5.0 (88)

[a] Production after 30 h of incubation in resting-cell cultures grown previously in GSPG medium.

The optimal ornithine concentration in resting-cell cultures for clavulanic acid biosynthesis was approximately 10 mM. At 20 mM, the stimulatory effect of ornithine was smaller than at 10 mM. The inhibitory effect of ornithine on cephamycin C production by resting cells was also concentration dependent. The synthesis of cephamycin by resting cells was reduced 75% with respect to the control by 10 mM ornithine and completely inhibited by 20 mM.

Effect of ornithine-related compounds on clavulanic acid and cephamycin biosynthesis. Several five-carbon compounds (glutamic acid, proline, ornithine), arginine, and other intermediates of the arginine biosynthetic pathway were tested as possible precursors of clavulanic acid and as possible inhibitors of cephamycin formation.

Arginine (5 to 10 mM) exerted a smaller stimulatory effect than ornithine on clavulanic acid production in long-term cultures (data not shown). In resting-cell cultures (Table 1), arginine also produced a lower degree of stimulation of clavulanic acid biosynthesis than ornithine; its inhibitory effect on cephamycin biosynthesis was also less intense than the effect of ornithine (Fig. 3).

Proline, citrulline, *N*-acetylornithine, and *N*-acetylglutamic acid did not stimulate clavulanic acid formation,

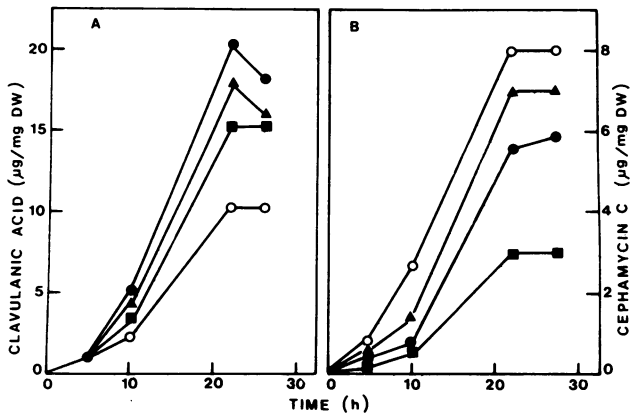

FIG. 3. Stimulation of clavulanic acid (A) and inhibition of cephamycin C biosynthesis (B) by arginine in resting-cell systems of *S. clavuligerus*. Symbols: ○, control; supplemented with arginine, 5 mM (▲), 10 mM (●), 20 mM (■). DW, Dry weight.

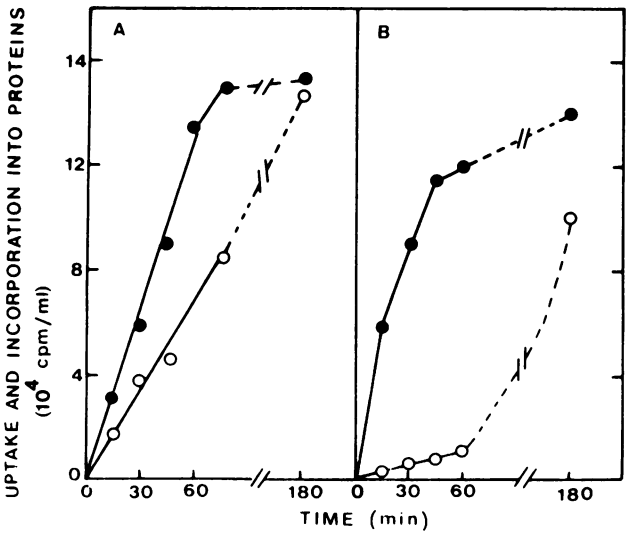

FIG. 4. Cellular uptake (●) and incorporation into protein (○) of [^{14}C]arginine (A) and [^{14}C]ornithine (B) by resting cells of *S. clavuligerus*.

whereas glutamic acid was clearly inhibitory (Table 1) as described previously (16).

Incorporation of labeled glutamic acid, ornithine, and arginine into clavulanic acid. Labeled ornithine was efficiently taken up by cells of *S. clavuligerus* in contrast to glutamic acid which was very poorly incorporated (see Discussion). About 60% of the precursor in the broth was incorporated into the cells by 30 min. However, incorporation of [^{14}C]ornithine into protein was delayed with respect to uptake (Fig. 4); on the contrary, the incorporation of arginine into protein (about 50% at all times) was not delayed. These results suggest that under clavulanic acid production conditions ornithine is not readily converted into arginine and therefore that a higher pool of ornithine is available for incorporation into clavulanic acid.

Less than 0.4% of uniformly labeled glutamic acid in the pool was incorporated into clavulanic acid (Table 2). [^{14}C]ornithine labeled in carbon 1, carbon 5, or uniformly was efficiently incorporated into clavulanic acid. Between 1.9 and 3.7% of the ornithine in the pool was converted into clavulanic acid in a number of experiments. Arginine appeared to be incorporated into clavulanic acid with a slightly higher efficiency than ornithine. Mutant *nca-1*, a strain that is blocked in clavulanic acid biosynthesis, did not incorporate any significant amount of [^{14}C]arginine into clavulanic acid in three different experiments. The carbamoyl group of citrulline was essentially not incorporated into clavulanic acid. Similarly, the guanidine group of arginine did not seem to be incorporated into clavulanic acid.

Conversion of arginine into ornithine *in vitro*. Extracts of *S. clavuligerus* were tested for their ability to convert arginine into ornithine via arginase and amidinotransferase. Arginase activity was low in cells grown in GSPG medium (containing glutamic acid), but it was higher in GSPO and particularly in GSPA media (containing ornithine and arginine, respectively) (Table 3). Since the level of arginase at 33 h in GSPA and in GSPG supplemented with arginine was clearly higher than in GSPG medium, arginase is probably induced by arginine in *S. clavuligerus* as it is in many other microorganisms. No amidinotransferase was detected under any experimental condition used.

DISCUSSION

Arginine very rarely occurs in peptide antibiotics, but it is the precursor of the ornithine component existing in bacitracin and gramicidin S (14). [1-^{14}C]-, [5-^{14}C]-, or uniformly labeled [^{14}C]ornithine is efficiently incorporated into clavulanic acid (Table 2), indicating that it is a direct precursor of this antibiotic. Uniformly labeled arginine was also very well incorporated into clavulanic acid, whereas [U-^{14}C]glutamic acid had very poor incorporation (30- to 40-fold lower). Most of glutamic acid was rapidly incorporated into proteins (Table 2). Our results agree with those of Townsend and Ho (18) who reported a better incorporation of ornithine than of glutamic acid into clavulanic acid.

Ornithine stimulated clavulanic acid biosynthesis in long-term fermentation and in resting-cell systems. Glutamic acid, previously proposed as a precursor (4), or proline, another five-carbon amino acid derived from glutamic acid, did not enhance clavulanic acid biosynthesis. Our results indicate that ornithine is a limiting precursor in clavulanic

TABLE 2. Incorporation of [^{14}C]ornithine and related compounds into clavulanic acid by resting cells of *S. clavuligerus* NRRL 3585 and the nonproducer mutant *S. clavuligerus nca-1*

Precursor	Uptake (cpm/ml)	Trichloroacetic acid-precipitable material (cpm/ml)	Intracellular pool (cpm/ml)	[^{14}C]clavulanic acid sp act (cpm/μg)[a]	% of pool incorporated into clavulanic acid[b]
S. clavuligerus NRRL 3585					
L-[U-^{14}C]glutamic acid	229,680	159,510	70,170	52	0.37
DL-[1-^{14}C]ornithine	221,300	73,900	147,400	751	2.55
DL-[5-^{14}C]ornithine	219,500	48,250	171,250	1,674	4.89
L-[U-^{14}C]ornithine	164,450	80,560	83,890	605	3.61
L-[U-^{14}C]arginine	385,050	203,450	181,600	2,262	6.23
L-[U-^{14}C]arginine	222,230	80,700	141,530	1,811	6.40
[U-^{14}C]carbamoyl citrulline	65,500	20,440	45,110	0	0
S. clavuligerus nca-1 ([U-^{14}C]arginine)[c]	362,366	271,530	90,836	0	0

[a] The total production of clavulanic acid in these experiments was 5 μg/ml at the beginning of the incorporation of labeled precursors. The increase of clavulanic acid during the 60 min of incorporation was about 0.3 μg/ml.

[b] Data are given as the percentage of the radioactivity in the pool that is incorporated into clavulanic acid in 60 min, since different amounts of ornithine and arginine are incorporated into protein or are available as free amino acids in the pool.

[c] Broth samples from cultures of this strain were supplemented with unlabeled clavulanic acid as carrier before chromatography.

TABLE 3. Arginase activity in cell extracts of *S. clavuligerus* NRRL 3585 grown in different culture media

Culture media	Time (h)	Dry wt (mg/ml)	Arginase (nkat/g of protein)
GSPG	24	2.5	4.4
	33	3.2	11.1
GSPO	24	1.5	11.1
	33	1.5	15.0
GSPA	24	1.5	9.9
	33	2.2	17.5
GSPG + arginine (10 mM)	24	3.0	8.3
	33	4.2	20.0

acid biosynthesis in the glutamic acid- and glycerol-based GSPG medium used for clavulanic acid production (9, 16). Similarly, ornithine is a limiting amino acid in bacitracin biosynthesis by *Bacillus subtilis* (12) and in gramicidin S formation by *Bacillus brevis* (13). Ornithine strongly inhibits cephamycin biosynthesis, another β-lactam antibiotic produced by the same strain, apparently owing to competition with lysine (J. Romero, P. Liras, and J. F. Martín, unpublished data), a well-known precursor of cephamycins (10).

A stimulation of clavulanic acid biosynthesis was also exerted by arginine (Fig. 3; Table 1). Arginine also stimulates gramicidin S biosynthesis in *B. brevis* by providing ornithine (13). The guanidino group of arginine and the amidino group of citrulline were not incorporated into clavulanic acid, suggesting that only the C-5 (ornithine) moiety of arginine is incorporated into the antibiotic.

Two enzymes are known to convert arginine into ornithine in microorganisms, i.e., arginase (arginine amidinohydrolase) and amidinotransferase. The splitting of arginine in both cases results in the formation of ornithine. In the reaction carried out by arginase, urea is the second product, whereas in the reaction catalyzed by amidinotransferase, the amidino group of arginine is transferred to an amino-containing receptor molecule (glycine, canaline, or hydroxylamine) forming a new guanidine reaction product. Amidinotransferases have been found in the *Streptomyces* species which produce streptomycin, bluensomycin, and viomycin (21). Purified amidinotransferase may possess arginase activity, as in the case of the viomycin-producer *Streptomyces griseus* (14). However, in most microorganisms arginase does not possess amidinotransferase activity. *S. clavuligerus* showed a normal arginase, but not an amidinotransferase, activity (Table 3). The arginase activity was greater when arginine was added to the medium, which suggests that arginase may be induced as in *Saccharomyces cerevisiae* (2) and *Klebsiella aerogenes* (5).

Ornithine is an intermediate in both anabolic and catabolic routes of arginine metabolism. Under clavulanic acid production conditions, the ornithine formed by catabolism of arginine rather than the ornithine synthesized from glutamate appears to be the main source of precursor for clavulanic acid. This hypothesis is supported by the poor incorporation of [^{14}C]glutamic acid into clavulanic acid and the lack of stimulation of clavulanic acid biosynthesis by glutamate. Furthermore, ornithine conversion into arginine appears to be limited in resting cells as deduced from the delay in the incorporation of labeled ornithine into proteins (via arginine) (Fig. 4), thus favoring the channelling of ornithine into clavulanic acid.

This work provides, therefore, evidence of the utilization of arginine and ornithine as precursors of clavulanic acid and confirms the results of Townsend and Ho (18). In addition, we showed that arginine and ornithine exert a strong stimulatory effect on clavulanic acid biosynthesis (i.e., they are limiting in the previously described media for clavulanic acid production) but inhibit cephamycin biosynthesis. The presence of arginase activity in *S. clavuligerus* suggests that arginine is converted into ornithine and that this C-5 amino acid, instead of glutamic acid, is the direct precursor of clavulanic acid.

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