

Arginine Regulation of Gramicidin S Biosynthesis

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Received 17 April 1981/Accepted 9 July 1981

Several amino acids are known to affect the gramicidin S producer *Bacillus brevis* ATCC 9999 with respect to growth, soluble gramicidin S synthetase formation, antibiotic production, or a combination of these. Our studies confirmed that arginine has paradoxical effects on the *B. brevis* fermentation; it markedly increased growth and antibiotic production, yet decreased the soluble heavy gramicidin S synthetase activity. We found that arginine did not repress heavy gramicidin S synthetase. The amino acid stimulated growth and increased specific antibiotic production presumably by supplying a limiting precursor (ornithine) for gramicidin S synthesis. Although the amino acid decreased the specific activity of the soluble heavy gramicidin S synthetase, it markedly increased the particulate enzyme activity which persisted hours after the soluble heavy gramicidin S synthetase disappeared. One percent arginine was the optimum level for growth and gramicidin S production. After growth in 1% arginine, heavy synthetase activity in the particulate fraction more than doubled. We propose that arginine leads to the soluble enzyme becoming membrane bound and more stable in vivo. Although we found arginine capable of inhibiting the action of soluble heavy gramicidin S synthetase, this was not the mechanism involved in the lowering of soluble heavy gramicidin S synthetase specific activity.

Working with chemically defined medium F 3/6, Vandamme and Demain (7) found that *Bacillus brevis* initially grew fast at the expense of the amino acid mixture. L-Arginine and L-glutamine were first exhausted from the medium, serving as carbon and nitrogen sources. Gramicidin S (GS) was produced only after L-arginine and L-glutamine had been exhausted from the medium. D-Fructose and L-histidine were used as carbon and nitrogen sources during this phase.

The above results suggest that the exhaustion of L-glutamine and L-arginine determines the onset of synthetase formation, indicating that catabolite repression, effected by amino acid metabolism, might be a control mechanism in GS synthetase formation (8).

Nimi and Demain (5), working with F 3/6 medium, investigated the effect of amino acids on GS synthetase formation by using the assay which measures the overall activity of the synthetases (i.e., [¹⁴C]ornithine incorporation into GS). They found that arginine markedly increased both growth and antibiotic production. Surprisingly, they found that soluble synthetase activity decreased when *B. brevis* was grown in arginine. In addition, L-arginine had an inhibi-

tory effect on enzyme activity. These results indicated that the low soluble enzyme values might be caused by inhibition of enzyme activity or by repression of enzyme formation by L-arginine (or by both). However, it was unclear how arginine could have these activities and still stimulate GS production. This paradox formed the basis of our study.

MATERIALS AND METHODS

Cultures. The GS-producing strain, *B. brevis* ATCC 9999, and the GS assay strain, *Bacillus subtilis* ATCC 6051, were obtained from the American Type Culture Collection.

Media. All fermentations were carried out in a chemically defined medium, F 3/5, which contains fructose, four growth-stimulatory amino acids (L-glutamine, L-histidine, L-methionine, and L-proline), L-phenylalanine as a GS precursor, and inorganic salts. Medium F 3/5 is medium F 3/6 (7) minus arginine. Arginine was added to medium F 3/5 at different concentrations.

The seed medium for all fermentations was F 3/5 medium. To stimulate the germination of the spores, 0.005% vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) and 0.0002% yeast extract (Difco) were added to the medium.

Sporulation medium consisted of nutrient broth (8 g/liter; Difco) supplemented with 1.0 mM MgCl₂, 0.7 mM CaCl₂, 0.05 mM MnCl₂, and 0.001 mM FeCl₃.

Spore preparation. Cultures were incubated for 4

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days in sporulation medium on a rotary shaker at 220 rpm at 37°C. The spores were harvested by centrifugation (20,000 × *g* for 15 min), washed twice with distilled water, suspended, and heated at 80°C for 15 min to denature proteins and inactivate nonsporulated cells. Spores were washed again, suspended in a small volume of sterile distilled water, and stored at 4°C.

Seed preparation. Seed cultures were prepared by inoculating 0.1 ml of stock spore suspension into 80 ml of F 3/5 medium. The inoculum flasks (500 ml, unbaffled) were incubated overnight at 37°C on a rotary shaker (220 rpm) until growth had reached 300 to 600 Klett units (15 h). These exponentially growing cells were used directly to inoculate fermentation cultures.

Fermentations. Initially, a series of six 500-ml unbaffled flasks, each containing 80 ml of F 3/5 medium supplemented with L-arginine at concentrations from 0 to 2%, were inoculated with 5 ml of seed culture and incubated on a rotary shaker (220 rpm) at 37°C. For growth and GS determinations, a small amount of whole broth was removed from the flasks. For synthetase determinations, an entire flask was sacrificed at each time point.

To minimize flask-to-flask variation, the fermentation was scaled up to 2.8-liter Fernbach flasks to allow all samples to be taken from the same flask. At various times, samples of 40 ml were removed from each of the duplicate flasks, combined, and treated.

Growth determinations. Growth was determined by optical density in a Klett-Summerson photoelectric colorimeter with a red filter. Samples were diluted to read between 30 and 150 Klett units. One gram of dry cell weight per liter corresponded to 280 Klett units.

GS bioassay. GS was bioassayed with *B. subtilis* ATCC 6051 by the agar diffusion technique (7).

Preparation of crude cell-free extracts. Cells were harvested in a refrigerated centrifuge at 12,000 × *g* for min; the pellets were washed twice with cold buffer and stored in the freezer until used. Heavy GS synthetase was stable under these conditions for at least 4 weeks.

Preparation of enzyme extracts was done in the cold. The enzyme was liberated by lysozyme treatment. This consisted of suspending 1 g (wet weight) of frozen cell paste in 3 ml of buffer A [10 mM tris(hydroxymethyl)aminomethane-ethanolamine, 10 mM magnesium chloride, 0.75 mM ethylenediaminetetraacetic acid, pH 7.6] that contained 6 mg of lysozyme. After incubation at 30°C for 20 min, the suspension was centrifuged at 20,000 × *g* for 30 min, yielding a supernatant fluid that contained the synthetase. Extracts were stored at -20°C, at which temperature enzyme activity was stable for at least 4 weeks.

Preparation of insoluble cell fraction. After the lysozyme treatment and centrifugation, the supernatants (crude cell extract) and pellets were kept frozen. The pellets were washed twice with cold buffer A to get rid of any soluble enzyme fraction. They were then resuspended in buffer A, and this suspension, containing insoluble, membrane-bound enzymes, was used for the GS synthetase assay (E. J. Vandamme, D.Sc. thesis, University of Ghent, Ghent, Belgium, 1977).

Extraction of GS synthetase from insoluble fraction by Triton X-100. The pellets were washed twice in cold buffer A and resuspended in buffer after

the addition of 0.1% Triton X-100. The suspension was incubated at 4°C for 5 h, after which the cellular debris was removed by high-speed centrifugation (20,000 × *g* for 30 min) (Vandamme, D.Sc. thesis). The supernatants containing the solubilized enzyme fractions were kept frozen (-20°C) until they were used.

Protein determination. Protein concentrations of the crude cell-free extract, the insoluble cell fraction, and the Triton X-100-solubilized fraction were determined by the biuret method of Gornall et al. (2). Bovine serum albumin was used as the standard.

Determination of GS synthetase activity. The overall biosynthetic activity of GS synthetase, based on the incorporation of L-[³H]ornithine, was carried out in a manner similar to that of Friebel and Demain (1). The assay of the heavy GS synthetase was done with the ornithine-dependent adenosine triphosphate-inorganic pyrophosphate exchange reaction (4).

Determination of L-arginine. L-Arginine was determined by colorimetry by the method of Rosenberg et al. (6).

RESULTS

Arginine effect on soluble GS synthetase in vivo. In a preliminary study of the effect of arginine on the GS fermentation, Nimi and Demain (5), using the assay which measures the total synthetase activity (i.e., [¹⁴C]ornithine incorporation into GS), reported on the action of arginine as a possible repressor of enzyme formation. They found that arginine markedly increased both growth and antibiotic production, but decreased specific activity of the soluble GS synthetase. We checked these observations by using the ornithine-dependent adenosine triphosphate-inorganic pyrophosphate exchange assay for the heavy GS synthetase.

To eliminate inactivation problems during the purification steps, we used crude, unfractionated extracts of the enzyme assay. We first determined that the use of these crude cell extracts would not interfere with the enzyme assay.

Fermentations were carried out in F 3/5 with and without 0.3% L-arginine (Fig. 1). GS formation began at the same time in both media, i.e., 8 h after inoculation, and continued to increase over a period of 25 h. The soluble heavy GS synthetase appeared in cell extracts at the end of the logarithmic growth phase, reached a peak, and then disappeared. When arginine was present in the medium, growth and antibiotic production increased, whereas the specific activity of the soluble GS synthetase decreased.

With a higher arginine concentration (1%), growth and antibiotic production increased (Fig. 2). Simultaneously, the maximum soluble heavy GS synthetase specific activity decreased from 28.7 U/mg of protein in medium F 3/5 to 11.6 U/mg of protein in the 1% arginine-supplemented medium.

Arginine inhibition of soluble GS synthetase activity. With all three extracts of the previous experiment, 20 mM arginine inhibited

in vitro soluble heavy GS synthetase activity by 35 to 50% (Fig. 3); higher concentrations had no further effect.

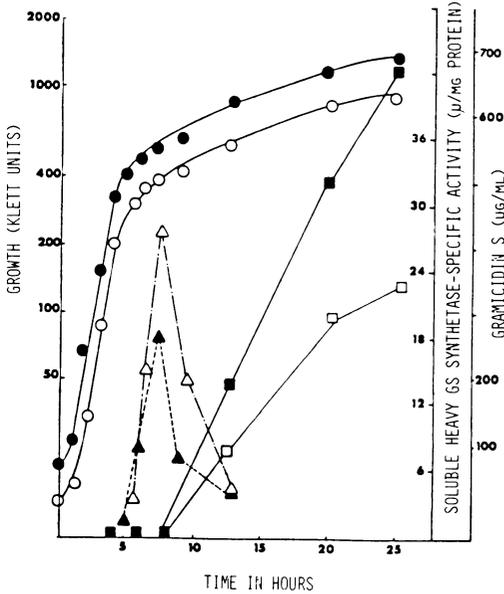


FIG. 1. Formation of soluble heavy GS synthetase in F 3/5 (0% arginine; open symbols) and F 3/6 (0.3% arginine; solid symbols) media. Symbols: ○ and ●, = growth; △ and ▲, synthetase specific activity; □ and ■, GS.

Since the arginine concentrations in the crude cell extracts were unknown, it is possible that the previously observed decrease in soluble heavy GS synthetase specific activity was merely due to arginine carryover into the extracts and inhibition of enzyme activity (rather than of enzyme formation). However, the concentrations of arginine in the three extracts were found to be extremely low, i.e., less than 0.05 mM.

Determination of the optimum concentration of arginine for growth and GS production. In determining the optimum concentration of arginine, we also tested ornithine since it is a precursor of arginine, a product of arginine catabolism, and one of the five amino acids which make up the GS molecule. The results (Fig. 4) showed that (i) both arginine and ornithine markedly increased growth and GS production; (ii) 1% arginine and 1% ornithine were optimal; and (iii) ornithine appeared more active than arginine for GS production.

If the amino acid pool of *B. brevis* contains L-ornithine in limiting amounts for GS synthesis in F 3/5 medium, the stimulatory effect of L-ornithine could be due to this amino acid playing a direct precursor role. Assuming that GS synthesis is limited by an inadequate supply of L-

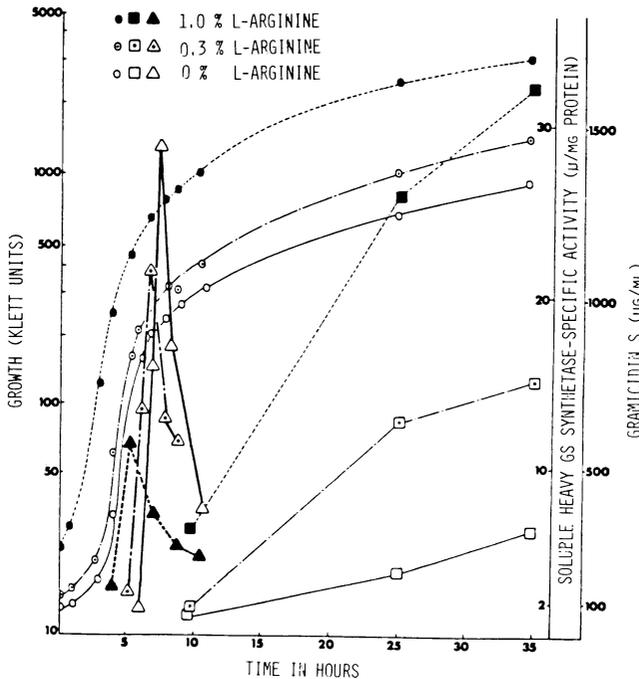


FIG. 2. Effect of arginine concentration on the formation of soluble heavy GS synthetase. Symbols: ○, ⊙, and ●, growth; △, Δ, and ▲, synthetase specific activity; □, ⊞, and ■, GS.

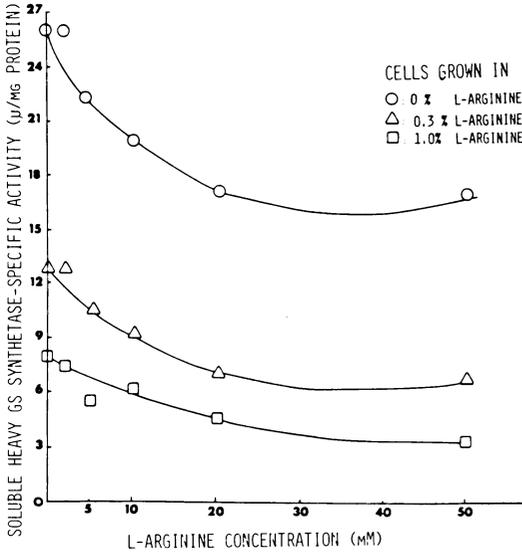


FIG. 3. Inhibition of soluble heavy GS synthetase activity by arginine.

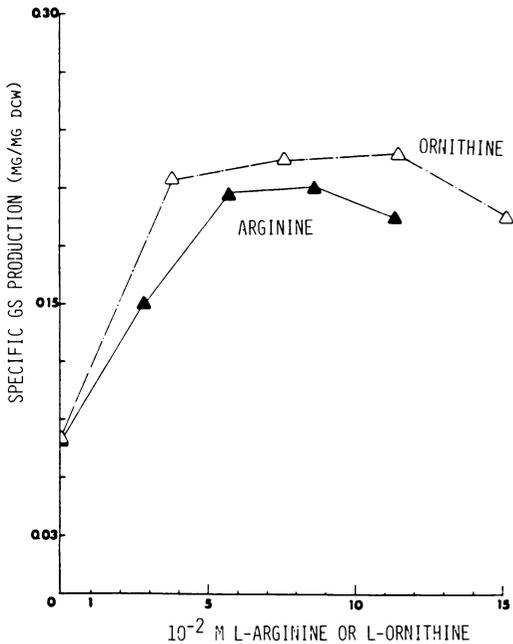


FIG. 4. Effect of growth in arginine or ornithine on GS specific production according to the molarity of the amino acid.

ornithine, L-arginine (via conversion to ornithine) could overcome this and stimulate production as an indirect precursor of the GS molecule.

A likely explanation of these data is that arginine is used both as a limiting precursor of protein and as an indirect precursor (via ornithine) of GS production. The latter would ex-

plain the somewhat greater effect of ornithine as compared with that of arginine on specific GS production.

Inability of ornithine to reverse the arginine depression of soluble heavy GS synthetase specific activity. At this point, it was still unclear how arginine could increase GS production and simultaneously repress soluble heavy GS synthetase. A possible explanation was that under conditions of arginine addition, its concentration might increase in the amino acid pool and repress or inhibit (or both) its own biosynthetic pathway. If so, it would interfere with the early steps of ornithine synthesis. Ornithine would then be missing as a substrate for GS synthetase, and this substrate deficiency might destabilize the enzyme and be responsible for the decrease in soluble synthetase activity. If this hypothesis was correct, growth in the presence of ornithine plus arginine should have reversed the decrease in soluble heavy GS synthetase specific activity caused by arginine.

We therefore investigated the effect of ornithine on soluble synthetase activity. Fermentations were run in: F 3/5 medium (control); F 3/5 plus 1% arginine; and F 3/5 plus 1% arginine and 1% ornithine. Our results (Table 1) showed that ornithine did not reverse the depressive effect of arginine. Furthermore, arginine did not appear to be a repressor of synthetase formation, since it increased antibiotic production per cell and also caused no delay in the onset of antibiotic production when it was added.

Studies of particulate heavy GS synthetase. One possible explanation of these paradoxical effects of arginine is that the soluble GS synthetase activity is only part of the complete GS biosynthetic activity and that an insoluble form is even more important; perhaps the soluble enzyme is the precursor of the insoluble activity. Vandamme and Demain (7) noted in preliminary experiments that there was some enzyme activity in cell pellets after lysozyme treatment.

The heavy GS synthetase activity of the insoluble cell fractions from the previous experiment was examined. The pellets, either untreated or extracted with Triton X-100, were assayed by the adenosine triphosphate-inorganic [³²P]pyrophosphate exchange reaction. Enzyme activities were calculated on the basis of specific activity (units per milligram of protein) and of total activity per flask (units per 80 ml of whole broth).

We found much more activity in the pellets from cells grown in the arginine-supplemented medium than in the pellets from the control medium (F 3/5) (Table 2). Although soluble fraction specific activity decreased by 75% when

cells were grown with arginine, the specific activity of the pellet fraction almost tripled. Treatment of the cell pellets with Triton X-100 resulted in extraction of most of the insoluble GS synthetase activity. The treatment of pellets from arginine-supplemented medium resulted in a major increase in the specific activity of the enzyme. Addition of arginine to the control medium resulted in an increase of the total heavy GS synthetase activity of the insoluble cell fractions, both untreated or treated with Triton X-100.

The activity distribution between insoluble and soluble fractions suggests that growth in arginine favored the location of synthetase activity in the membrane fractions of the cell where it was possibly more stable than in the soluble portion of the cell (Fig. 5). Furthermore, the addition of arginine to the medium increased the total activity distributed in the soluble and pellet fractions (Fig. 6 and Table 2). Although arginine decreased the activity of the soluble heavy GS synthetase, it markedly increased the activity of the pellet fractions, so that overall, arginine had a positive effect on the activity of GS synthetase.

Another important point revealed by the data was that in each medium (with or without arginine), there was GS synthetase activity in pellets prepared from late-stage cells (25 h of fermentation), yet at that time there was no soluble GS

synthetase activity. This suggested that the membrane-bound form of the enzyme was more resistant to *in vivo* degradation or inactivation (1) than was the soluble form; this membrane-bound fraction would be responsible for late GS production after the soluble form was inactivated. The residual activity after 25 h of fermentation in pellet fractions (either untreated or treated with Triton X-100) was 2 to 3 times higher in the arginine medium than in the control medium (Fig. 5). Again, this showed that arginine increased the particulate enzyme activity which persisted hours after the soluble GS synthetase disappeared.

DISCUSSION

The finding of high heavy GS synthetase activity in the pellet fractions and its increase after growth in arginine constituted the major findings of our study. We are now able to explain the paradoxical effects of arginine. Nimi and Demain (5) first reported that arginine stimulates growth and GS production, but at the same time decreases soluble GS synthetase formation. Our results suggested the following. (i) Arginine does not repress heavy GS synthetase formation; although it decreased the activity of the soluble GS synthetase, it markedly increased the insoluble fraction activity. (ii) Although arginine in-

TABLE 1. Effect of ornithine and arginine on soluble heavy GS synthetase specific activity

Medium	Maximum DCW ^a (g/liter)	Maximum GS		Maximum soluble heavy GS synthetase activity	
		(mg/liter)	(mg/mg of DCW)	(U/mg of protein)	(Total U)
F 3/5	4.2	430	0.10	20.2	395
F 3/5 + 1% arginine	10.1	2380	0.23	5.5	129
F 3/5 + 1% arginine + 1% ornithine	10.7	2805	0.26	5.3	119

^a DCW, Dry cell weight.

TABLE 2. Effect of arginine on soluble and insoluble heavy GS synthetase activity

Expt	Medium	Maximum DCW ^a (g/liter)	Maximum GS		Maximum heavy GS synthetase specific activity (U/mg of protein)			Maximum heavy GS synthetase total activity (U/80 ml of whole broth)		
			(mg/liter)	(mg/mg of DCW)	Soluble fraction	Pellet	Triton X-100 extract	Soluble fraction	Pellet	Triton X-100 extract
A	F 3/5	4.2	430	0.10	20.2	6.7	8.1	395	369	203
	F 3/5 + 1% arginine	10.1	2,380	0.23	5.5	13.4	53.6	129	948	753
B	F 3/5	4.1	420	0.10	22.5	4.4	8.7	599	1,044	678
	F 3/5 + 1% arginine	8.2	2,020	0.25	6.8	13.3	36.5	207	2,090	2,090

^a DCW, Dry cell weight.

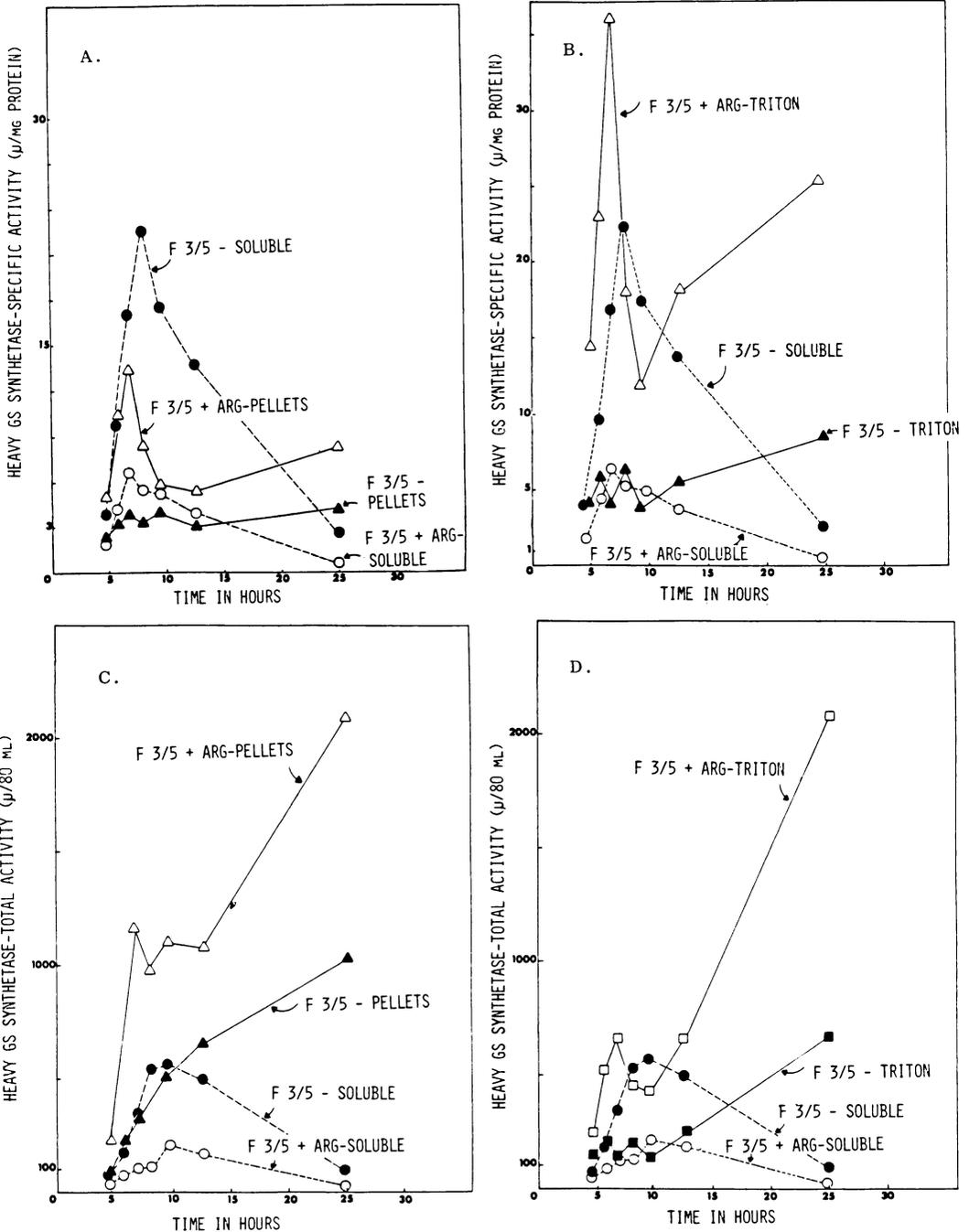


FIG. 5. Distribution of heavy GS synthetase between insoluble and soluble fractions, in control and arginine-supplemented media. (A) Specific activity in the soluble versus pellet fractions. (B) Specific activity in the soluble fraction versus the Triton X-100 extract of the pellet fraction. (C) Total units in the soluble versus pellet fractions. (D) Total units in the soluble fraction versus the Triton X-100 extract of the pellet fraction.

hibited soluble heavy GS synthetase activity, enzyme inhibition was not the control mechanism by which specific activity of soluble GS

synthetase was lowered in vivo. (iii) Exogenous arginine stimulated growth by supplying a limiting precursor (arginine) for protein synthesis.

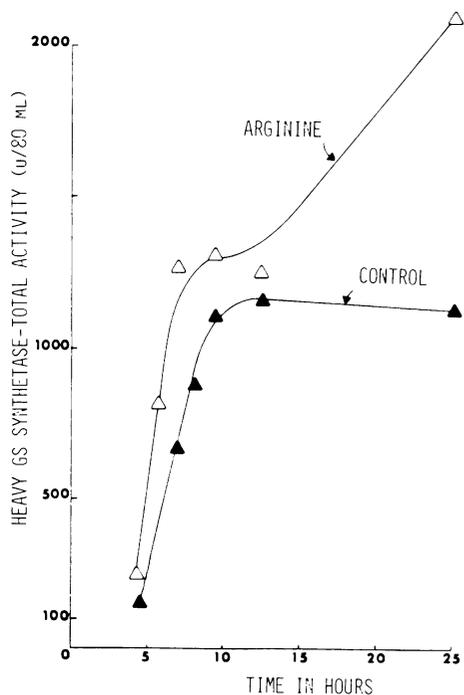


FIG. 6. Effect of growth in arginine on total heavy GS synthetase activity.

(iv) Exogenous arginine stimulated antibiotic production by supplying a limiting precursor (ornithine) for GS synthesis. (v) Exogenous arginine stimulated antibiotic production by increasing the total heavy GS synthetase activity of the culture; this appears to be due to the soluble activity becoming membrane bound and more stable in vivo. (f) One percent (wt/vol) arginine was the optimum level for growth and GS production; 1% ornithine was capable of re-

placing arginine. An alternative hypothesis, that the arginine stimulation of antibiotic potency is due to substitution of the two ornithine residues by arginine, thus making a new, more active antibiotic, is untenable, since such a compound has been made synthetically and is no more active than GS itself (3).

ACKNOWLEDGMENTS

A.P. was partially supported by a grant from the Ministère des Affaires Étrangères, France. Research by A.L.D. on gramicidin S is partially supported by U.S. Army Research Office Contract DAAG29-78-C-0015.

Gramicidin S was generously provided by T. Wakazawa of Meiji Seika Kaisha Ltd. We thank Jacqueline Piret and Erick Vandamme for advice.

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