ANALYSIS OF THE INHIBITION OF GROWTH PRODUCED BY CANAVANINE IN *ESCHERICHIA COLI*[1]

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L-Canavanine (L-α-amino-γ-guanidinoxylbutyric acid), a naturally occurring structural analogue of arginine, has been found to inhibit the growth of Neurospora (Horowitz and Srb, 1948) and of many bacterial species (Volcani and Snell, 1948). Walker (1953), Kihara and Snell (1957), and Kalyankar *et al.* (1958) have demonstrated that canavanine is a substrate for a number of enzymatic reactions involving arginine; Walker concluded that canavanine inhibits growth by interfering with the last step in the biosynthesis of arginine, the conversion of argininosuccinate to arginine.

In the course of further study of the control of arginine metabolism we have observed that under certain conditions canavanine converts the mode of growth of the bacteria from an exponential to a linear one, suggesting interference with the formation of one or more enzymes necessary for growth (Cohen, 1958). In support of this suggestion, inhibition of the formation of certain enzymes will be demonstrated. From the experiments we have concluded that canavanine inhibits growth not by interfering with the biosynthesis of arginine, but rather by preventing the subsequent utilization of arginine in protein synthesis.

MATERIALS AND METHODS

The strains of *Escherichia coli* used in this study are W, wild type (ATCC 9637), and auxotrophic mutants derived from it by B. D. Davis.

Cells were grown at 37 C with aeration in minimal medium A (Davis and Mingioli, 1950) with 0.5 per cent glucose or sodium lactate as carbon source, or in a defined, enriched medium, containing amino acids, purines, pyrimidines, and vitamins, but not arginine (Gorini and Maas, 1958). For solid media, agar was added to a final concentration of 2 per cent. Other additions were made as specified in the legends to tables and figures. Viable counts were performed in minimal medium A supplemented with 0.2 per cent NZ Case (Sheffield) and 0.2 per cent yeast extract (Difco). Growth in liquid media was measured turbidimetrically with a Beckman spectrophotometer at 490 mμ.

The inocula for all experiments were taken from exponentially growing cultures. Unless otherwise indicated, cells before inoculation were harvested by centrifugation and washed once with minimal medium A lacking a nitrogen source.

Ornithine transcarbamylase was assayed by the method of Jones *et al.* (1955) in toluene-treated cells at 37 C. Citrulline was determined by a modification of Archibald's method (1944). A unit of ornithine transcarbamylase is that amount of enzyme which produces 1 μmole of citrulline in 1 hr at 37 C. β-Galactosidase was assayed by a modification of the method of Monod *et al.* (1952) at 37 C in toluene-treated cells. The reaction was arrested by boiling for 5 min. A unit of β-galactosidase is that amount of enzyme which hydrolyzes 1 μmole of *o*-nitrophenol-β-D-galactoside in 1 min at 37 C.

All chemicals were obtained commercially, with the exception of carbamyl phosphate, which was synthesized by the method of Spector *et al.* (1957), and β-thiomethylgalactoside, which was a gift of W. Sistrom.

RESULTS

Site of canavanine action. The extent as well as the kind of growth inhibition brought about by canavanine depend not only on the type

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of medium in which the cells are exposed to the inhibitor, but also on the previous conditions of growth. At first, experiments will be described involving arginine-free medium, where the characteristics of the inhibition can be seen most clearly. In this medium, as will be discussed in a later section in detail, the intracellular level of arginine is presumably low and therefore the antagonism of the action of canavanine by endogenously produced arginine is minimized.

Wild-type cells incubated with canavanine in arginine-free medium grew at a linear rate, as seen in figure 1, curve A. In a control experiment without canavanine, growth was exponential (figure 1, curve C). Presumably linear growth results when the concentration of a catalyst (for example, an enzyme) necessary for growth remains constant in the culture. Viable counts showed that the cells were able to divide under these conditions (figure 1, curve B). Thus canavanine, although it interferes with increase in mass, does not appear to affect cell division primarily.

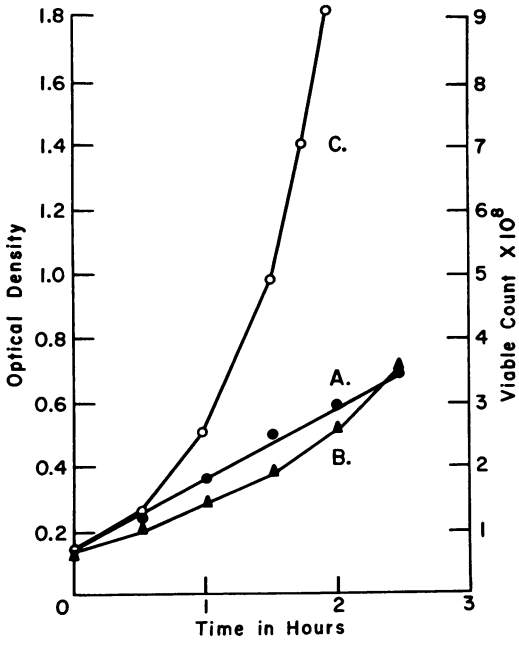

Figure 1. Linear growth in canavanine. The inoculum was grown for over 10 generations in arginine-free medium with 0.5 per cent glucose, and diluted into fresh medium of the same composition at the start of the experiment. *A:* 100 μg/ml canavanine were added. No addition was made to *C*. Curve *B* represents viable counts of *A*.

The observed linear growth suggests that canavanine inhibits the utilization rather than the synthesis of arginine. Figure 2 presents further evidence for this suggestion. When arginine was added to a culture which had been grown in the presence of canavanine for some time, there was a long delay before normal growth was resumed. When arginine was added to the culture before growth in canavanine had taken place, the subsequent addition of canavanine did not result in inhibition. It has been shown previously that the concentration of canavanine used in the present experiment (50 μg/ml) does not interfere appreciably with the entrance of arginine into the cell, although higher concentrations of canavanine (500 μg/ml) do inhibit its entrance (Schwartz *et al.*, 1959).

It can be demonstrated that growth in canavanine is necessary if inhibition is to occur. Thus, an isoleucine auxotroph deprived of its required amino acid was incubated in the presence of canavanine. After 90 min, both isoleucine and arginine were added; growth began immediately. On the other hand, a delay in the reversal, similar to that observed in the wild type, was found in the auxotroph upon the addition of arginine after several hours of growth with isoleucine and canavanine.

Effect of previous growth in arginine on canavanine inhibition. The phenomenon of enzyme repression provides a method for altering the capacity of cells to synthesize arginine at the time of their exposure to canavanine. After growth in arginine, cells possess a low level of some of the enzymes required to produce arginine (Gorini and Maas, 1958). Consequently such cells, after removal of the arginine by washing, initially make less arginine than do cells which have not been repressed. Because of their reduced ability to synthesize arginine, repressed cells should be more sensitive to canavanine inhibition than cells which have not been repressed. This expectation was found to be correct. Cells which had been grown in arginine did not grow at all when exposed to canavanine in an arginine-free medium, whereas linear growth was observed after growth without arginine.

The complete inhibition of cells in an arginine-free medium after growth in arginine suggests that canavanine might interfere with the synthesis of one or several of the enzymes concerned with the production of arginine. A direct demon-

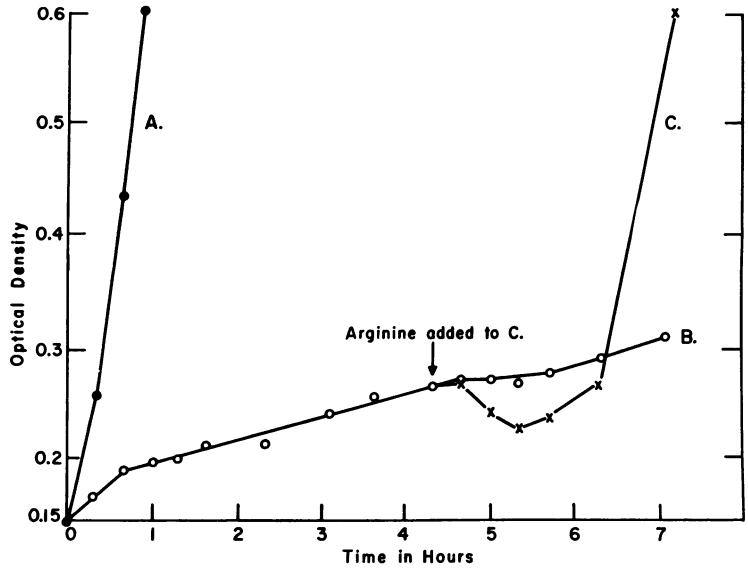

Figure 2. Delay in the reversal of canavanine inhibition by arginine. The inoculum was grown for over 10 generations in arginine-free medium with 0.5 per cent lactate. At the start of the experiment, it was diluted in fresh medium of the same composition. *A:* 100 μg/ml arginine and 50 μg/ml canavanine were added at zero time. *B:* 50 μg/ml canavanine alone were added. At the time indicated by the *arrow*, culture *B* was divided into two portions, and, to one (*C*), 100 μg/ml arginine were added.

stration of such an effect is shown in table 1; during linear growth in canavanine, cells did not synthesize active ornithine transcarbamylase. However, it is not probable that the inhibition of ornithine transcarbamylase formation by canavanine is similar in mechanism to repression by arginine, since with arginine the inhibition of enzyme formation is specific for enzymes in the arginine pathway, but canavanine also inhibits the synthesis of β-galactosidase (table 1).

Effect of constituents of the growth medium. On structural grounds it seems likely that arginine antagonizes canavanine inhibition competitively. However, a possible competitive relation cannot be studied unambiguously by varying the external concentrations of either the inhibitor or the antagonist because at least one of them, arginine, is concentrated by the cell (Schwartz *et al.*, 1959). Exogenous arginine supplied in concentrations as low as 1 μg/ml will completely prevent inhibition by any concentration of canavanine below 500 μg/ml until the arginine is exhausted from the medium (provided the cells have not been grown with canavanine alone as described above).

It has been shown in the preceding sections that the inhibition by canavanine can be demonstrated clearly in an enriched, but arginine-free, medium. Judging from the degree of repression of ornithine transcarbamylase, the level of intracellular arginine is relatively low under these conditions (Gorini and Maas, 1958). In minimal medium, judging by the same criterion, the arginine level is higher. In this medium one cannot demonstrate inhibition by canavanine except by first growing the cells in the presence of arginine; even then the growth rate cannot be reduced by more than 50 per cent. However, this reduction persists for at least 3 divisions. Under these conditions growth was exponential, and viable counts paralleled turbidity determinations.

The effects of changing the composition of the medium and of growing with or without arginine are summarized in table 2. There, the conditions of the experiments are arranged in order of decrease in presumed intracellular arginine concentration (judged by the degree of repression, as mentioned above) at the time of exposure to canavanine. It can be seen that the strength of the inhibition is inversely related to this intracellular arginine concentration. Although no quantitative relation between canavanine and arginine concentration has been established, these results nevertheless support the hypothesis that the relationship is competitive.

TABLE 1

*Synthesis of ornithine transcarbamylase and β-galactosidase during linear growth in canavanine**

ΔOD	Ornithine Transcarbamylase (units/ml culture)		β-Galactosidase (units/ml culture)	
	(1) Canavanine	(2) No canavanine	(3) Canavanine	(4) Canavanine and arginine
0	0.82	0.82	0 (<2)	0
0.150	0.54	2.70	0	17
0.320	0.86	3.42	0	600
1.00	—	—	580	5000

* The inocula, grown in an arginine-free medium with 0.5 per cent lactate for 10 generations, were diluted in fresh medium of the same composition to an optical density of 0.150. Subsequent growth was followed turbidimetrically (ΔOD). *Ornithine transcarbamylase:* To one flask, 100 μg/ml canavanine were added (1); to the other (2), no addition was made. Samples were toluenized at times corresponding to the tabulated increase in optical density. *β-Galactosidase:* Two flasks containing 0.01 M β-thiomethylgalactoside, as inducer, and 100 μg/ml canavanine. To one of these (4), 100 μg/ml arginine were added. Samples were treated with toluene at times corresponding to the tabulated increase in optical density. In both experiments, the form of the growth curves were similar to those shown in figure 1: (1) and (3) resembled curve A, and (2) and (4), curve C.

In support of this notion are the effects of arginine precursors. These precursors influence canavanine inhibition only under conditions where they can be converted to arginine at a sufficiently rapid rate. Thus, in minimal medium both citrulline and ornithine completely prevented canavanine inhibition. In arginine-free medium, the inhibition of previously nonrepressed cells was mitigated to a large degree by citrulline, but not at all by ornithine. It has been shown previously that in this medium the addition of ornithine does not increase the rate of arginine production, in spite of a high level of ornithine transcarbamylase, because the conversion of ornithine to citrulline is limited by the rate of carbamyl phosphate synthesis (Gorini and Maas, 1958). Finally, with cells in an arginine-free medium, which have been repressed by previous growth in arginine, neither ornithine nor citrulline but only arginine prevented the complete inhibition brought about by canavanine.

DISCUSSION

We have shown that canavanine inhibits growth by interfering with the utilization of arginine in protein synthesis, preventing the formation not only of enzymes in the arginine biosynthetic pathway, but also of an enzyme unrelated to arginine biosynthesis. Structural analogues of a number of amino acids (methionine, tryptophan, phenylalanine, and isoleucine) have been shown to inhibit protein synthesis (Cohen, 1958; Gross and Tarver, 1955; Munier and Cohen, 1956; Pardee and Prestidge, 1958). Some of these are incorporated into proteins in place of their related, normal amino acid. Other analogues have been shown to inhibit protein synthesis without replacing the normal amino acid in proteins. Labeled canavanine is not now available, and the chemical methods we have used to test canavanine incorporation into protein were only sensitive enough to

TABLE 2

*Effect of previous growth conditions and of different media on canavanine inhibition**

Conditions of Experiments		Growth Inhibition
Previous growth condition	Exposure medium	
Minimal medium	Minimal medium	None
Minimal medium + arginine	Minimal medium	Maximal inhibition: 50% (exponential growth)
Arginine free	Arginine free	Linear growth
Arginine free + arginine or minimal + arginine	Arginine free	Maximal inhibition: 100%

* Previous growth condition refers to the medium in which the inoculum has been grown. Exposure medium is the medium in which the cells are exposed to canavanine to determine the extent of inhibition. The last column describes this inhibition. See text for details of the experiments.

detect a replacement of 20 per cent of the arginine. With these methods we found no canavanine in cellular protein.

Our original interest in canavanine arose from our studies of repression. We were looking for an analogue of arginine that would repress enzyme formation without being used up during growth. Although canavanine does inhibit the formation of enzymes in the arginine biosynthetic pathway it does not fit the specifications of a repressor since it also inhibits formation of other unrelated enzymes.

Our study shows how differences in growth conditions and differences in the previous history of the cells influence canavanine inhibition. Several strains of *E. coli* were thought to be resistant to canavanine, since no inhibition occurred in minimal medium (Volcani and Snell, 1948). Yet we have shown that under certain conditions canavanine inhibits the growth of *E. coli* completely. Sensitivity to an inhibitor clearly might depend on the intracellular concentration of a metabolite which antagonizes the inhibitor. Conditions which alter the intracellular concentration of the metabolite will then alter the vulnerability of the cell to the inhibitor. Indeed, one of the rewards of studying the action of a weak inhibitor such as canavanine is the opportunity it provides for recognizing the very conditions which alter the intracellular concentration of the antagonizing metabolite.

SUMMARY

Evidence is presented that canavanine inhibits the growth of *Escherichia coli* strain W by interfering with the utilization of arginine in protein synthesis. The extent and kind of growth inhibition appear to depend on the intracellular concentration of arginine at the time of exposure to canavanine.

In the experiments described, the endogenous formation of arginine has been varied by the use of different media and by varying the state of repression of arginine-synthesizing enzymes in the cells at the time of inoculation. By these means, it has been possible to obtain in the presence of the same concentration of canavanine either complete inhibition of growth, linear growth, slowed growth, or unimpaired growth. The varying degrees of inhibition can be reversed by arginine, or by ornithine and citrulline under circumstances that permit these compounds to be readily converted to arginine. During the linear growth certain enzymes are no longer synthesized in active form.

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