THE EFFECTS OF CANAVANINE, ARGinine, AND RELATED
COMPONdS ON THE GROWTH OF BACTERIA*

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The physiological properties of canavanine, a naturally occurring, structural analogue of arginine, have not been extensively investigated. Recently, Horowitz and SrB (1) found that canavanine inhibited growth of wild type Neurospora, and that the inhibition was competitively alleviated by arginine. Lysine and methionine were also effective in alleviating canavanine inhibition for certain strains of Neurospora, though not so effective as arginine. The sensitivity of various Neurospora types to inhibition by canavanine varied considerably, and appeared to be genetically determined. These authors review the limited previous work dealing with the physiological response of various organisms to canavanine.

As an extension of this work, the present article describes the effects of canavanine on growth of several lactic acid bacteria. In contrast to wild type Neurospora, most of these organisms require arginine for growth. For comparative purposes, two strains of Escherichia coli have been used, a parent strain which grows without added arginine, and a arginine-requiring mutant derived from the parent strain by treatment with mustard gas.1

**Procedure**

*Cultures and Media—Cultures of Lactobacillus arabinosus 17-5, L. casei, L. delbrueckii 3, L. fermenti 36, Leuconostoc mesenteroides P-60, and Streptococcus faecalis R were carried as stab cultures in yeast extract-glucose-agar. Inocula were grown by transfer from these to a complete liquid

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1 We are indebted to Dr. J. Lederberg and Dr. E. L. Tatum for cultures of these organisms.
semisynthetic medium similar in composition to that of MacLeod and Snell (2) but with two-thirds of the enzymatically digested casein replaced with acid-hydrolyzed casein. For *L. fermenti*, 30 μg of extra thiamine were added per 10 cc. After 24 hours incubation, cells were centrifuged and resuspended in 10 cc. of sterile 0.9 per cent sodium chloride solution. This heavy suspension was diluted 1:10 with sterile saline, and 1 drop of this dilute suspension was used to inoculate each experimental culture of 2 cc. For assay, the medium of Henderson and Snell (3), with arginine omitted, was used. For *L. fermenti*, this medium was modified by replacing the sodium citrate with an equal weight of sodium acetate. The initial pH was 6.4.

*Escherichia coli* Y109 (the parent strain) and *Escherichia coli* Y117 (the arginine-requiring mutant) were carried as slant cultures on yeast extract-glucose-agar. For inoculum, a small amount of surface growth from a 24 hour culture was suspended to barely visible turbidity in sterile 0.9 per cent sodium chloride solution, and 1 drop of this suspension was used to inoculate each experimental culture of 2 cc. The basal medium used was that of Tatum and Lederberg (4), which contains inorganic salts, glucose, and asparagine.

Additions of arginine, canavanine, and other compounds were made to 1 cc. of the double strength medium, the cultures were diluted to 2 cc. with distilled water, capped, autoclaved at 15 pounds pressure for 10 minutes, cooled, inoculated, and incubated at 37° for 24 to 38 hours. Cultures were then diluted to 10 cc. with water, and turbidities compared visually or quantitatively in the photoelectric colorimeter.

**Results**

Response of Cultures to Arginine and Related Compounds—The effects of arginine, citrulline, ornithine, and canavanine on the growth of the various test organisms are compared in Table I. Under the conditions used, each of these organisms except *Escherichia coli* Y109 requires arginine or one of its precursors for growth. *Lactobacillus fermenti* and *Escherichia coli* Y117 can utilize either ornithine or citrulline in place of arginine; *L. arabinosus*,\(^2\) *L. casei*, and *L. delbrueckii* utilize citrulline, but not ornithine, in place of arginine. Finally, organisms such as *Streptococcus faecalis* and *Leuconostoc mesenteroides* utilize neither ornithine nor citrulline, but require preformed

\(^2\) Lyman et al. (5) reported that their culture of *Lactobacillus arabinosus* did not require arginine for growth when the medium contained ample vitamin B₆, and the carbon dioxide tension was raised. Under conditions used in this work, no growth occurred without arginine or citrulline during 48 hours of incubation. On prolonged incubation, however, the organism eventually grew without added arginine, and by subculturing from such a culture, a strain of *Lactobacillus arabinosus* was readily derived which grew rapidly without either ornithine, citrulline, or arginine.
arginine. Such data indicate that arginine synthesis in lactic acid bacteria, as in Neurospora (6), occurs via the ornithine cycle, and that an apparent requirement for arginine may result from loss of ability to catalyze any one of the several consecutive reactions involved in, or preceding, this cycle.

None of the organisms could effectively utilize canavanine in place of arginine, although high levels of this compound permitted very slight growth of Leuconostoc mesenteroides, Streptococcus faecalis, and Lactobacillus fermenti. The growth-promoting activity here was considerably less than 1 per cent of that of arginine, and may possibly result from the presence of traces of arginine as an impurity in the canavanine. As appears from the

### Table I
Comparative Effects of Arginine and Related Compounds on Growth* of Bacteria in Arginine-Free Media

<table>
<thead>
<tr>
<th>Organism</th>
<th>Additions† amount per 2 cc.</th>
<th>DL-Ornithine, 200 γ</th>
<th>DL-Citrulline, 200 γ</th>
<th>L-Arginine, 100 γ</th>
<th>L-Canavanine, 1000 γ</th>
<th>L-Arginine, 10 γ + L-canavanine, 1000 γ</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. arabinosus</em></td>
<td></td>
<td>- -</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>“ casei</td>
<td></td>
<td>- -</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>“ delbrueckii</td>
<td></td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>“ fermenti</td>
<td></td>
<td>- + + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>“ mesenteroides</td>
<td></td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>-</td>
</tr>
<tr>
<td><em>S. faecalis</em></td>
<td></td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> Y109</td>
<td></td>
<td>+ +</td>
<td>+ +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>-</td>
</tr>
<tr>
<td>“ Y117</td>
<td></td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>-</td>
</tr>
</tbody>
</table>

* - , no growth; ±, barely visible growth; +, easily visible growth; ++, good growth; ++++, heavy growth.

† Throughout this table and Tables II and III these compounds were added as DL-ornithine·HCl, DL-citrulline, L-arginine·HCl, and L-canavanine·H₂SO₄.

The last column in Table I, the growth response of these three organisms was unaffected by excess canavanine. In separate experiments, concentrations of canavanine sulfate as high as 5 mg. per 2 cc. failed to inhibit the growth response of these organisms to 10 γ of arginine; indeed, this response was enhanced by an amount equivalent to the slight growth obtained with the same quantity of canavanine alone. The behavior of these highly resistant organisms stands in direct contrast with that of the other organisms tested. With these, the growth response to arginine was prevented by simultaneous addition of sufficient canavanine. Escherichia coli Y109, which grows well without added arginine, was likewise inhibited by canavanine.
Further experiments were conducted with each of the organisms inhibited by addition of canavanine. Similar findings were made in each case; the inhibitory effects of canavanine were alleviated by addition of increased amounts of arginine. Only illustrative data, obtained with organisms of different types, will be given below.

Fig. 1 shows how growth of *Escherichia coli* Y109 is affected by variation of the concentration of arginine and canavanine. This organism grows maximally in the absence of arginine. In the absence of canavanine, additions of arginine do not affect growth. Less than 100 µ of canavanine sulfate per 2 cc. of medium completely prevent growth; simultaneous addition of increasing amounts of arginine hydrochloride permits growth even in the presence of large amounts of canavanine. The inhibition is competitive in nature; the ratio of canavanine to arginine which permits half maximum growth (galvanometer reading of 75) is 10:1, 12.5:1, and 10:1 respectively, at the three increasing levels of canavanine.

In Fig. 2, the effect of additions of arginine hydrochloride and canavanine sulfate on growth of the arginine-requiring mutant of *Escherichia coli* Y117 is shown. Here again canavanine inhibits growth, and the inhibition is alleviated by additional arginine. In this case, the ratio of canavanine to arginine which permits half maximum growth (galvanometer reading of 60) is approximately 23:1 at the lower concentration of canavanine (1000 µ per 2 cc.) and only 8:1 at the higher (2000 µ per 2 cc.). Thus, in this instance, arginine is less effective in counteracting canavanine inhibition as
the concentration of the latter is increased. This behavior was noted repeatedly.

*Escherichia coli* Y117 requires about 30 γ of arginine per 2 cc. to permit maximum growth (Curve 1, Fig. 2); addition of 2000 γ of canavanine does not completely prevent growth at this level of arginine (Curve 3, Fig. 2). The parent strain of *Escherichia coli* Y109 grows maximally without added arginine, since it synthesizes this amino acid. In the absence of arginine, however, less than 100 γ of canavanine completely inhibits its growth (Curve 2, Fig. 1). At equivalent levels of growth, therefore, the parent strain, which synthesizes its own arginine, is much more sensitive to the inhibitor. This is to be expected, since the inhibition is dependent upon the ratio of the concentrations of arginine and canavanine. In the arginine-synthesizing culture, the arginine is utilized for growth as formed, and presumably never accumulates in excess. Its concentration is always very low, and hence relatively low concentrations of canavanine suffice to prevent growth. When arginine is added to the medium, the sensitivity of the parent and mutant strain to canavanine inhibition is very similar (cf. Curve 4, Fig. 1, and Curve 3, Fig. 2).

Each of the lactic acid bacteria whose growth was inhibited by canavanine (Table I) was investigated in more detail. The findings in each case were similar to those recorded above for the arginineless mutant of *Escherichia coli*. In Table II, the arginine concentrations necessary to promote equal growth in the absence and in the presence of various levels of canavanine.
of canavanine are compared. These and many other data not tabulated show that the ratio of canavanine to arginine required to reduce growth to a given level is not constant at various levels of canavanine, but decreases as the canavanine level increases. As the concentration of canavanine is increased, relatively more arginine is required to overcome its inhibitory action. The explanation for these inconstant ratios is not yet known.

From day to day, considerable variation in the inhibition index at any one level of canavanine was seen. Previous experiments (7) with analogues of pantotenic acid showed that increasing the time of incubation decreased the effectiveness of the inhibitor; i.e., increased the inhibition index. This was true also in the present case. A more important factor leading to variation was the size of the inoculum. The last two lines of Table II compare the results obtained in parallel assays with Lactobacillus delbrueckii when the inoculum was varied over a 3-fold range. With the larger inoculum, the inhibitory properties of canavanine at this concentration have been very considerably decreased.

Effect of Precursors of Arginine on Canavanine Inhibition—From Table I it is evident that citrulline and ornithine serve as precursors of arginine. Their effect on the inhibitory properties of canavanine was therefore determined. Results obtained with Escherichia coli Y109 are shown in Table III. It is seen by inspection that citrulline and ornithine are approximately equally effective in counteracting canavanine inhibition, but are only about one-twentieth as active in this capacity as arginine. En-

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<table>
<thead>
<tr>
<th>Organism</th>
<th>Canavanine sulfate</th>
<th>Arginine hydrochloride</th>
<th>Canavanine to arginine ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei</td>
<td>0/5</td>
<td>0/5</td>
<td>15:1</td>
</tr>
<tr>
<td>&quot; arabinosus</td>
<td>0</td>
<td>8</td>
<td>3:1</td>
</tr>
<tr>
<td>&quot; delbrueckii</td>
<td>0</td>
<td>10</td>
<td>0.5:1</td>
</tr>
<tr>
<td>1000</td>
<td>200†</td>
<td>5:1†</td>
<td></td>
</tr>
</tbody>
</table>

* The growth levels selected for comparison are the same with any one organism, but vary from one organism to another from about one-fourth to one-half of maximum growth (24 hours).
† This experiment was conducted in parallel with the one immediately above, but a 3-fold heavier inoculum was used.
tirely similar data were obtained with Escherichia coli Y117, which requires one of these three compounds for growth. For this latter organism, DL-citrulline and DL-ornithine hydrochloride were 0.78 and 0.83 as active, respectively, as L-arginine hydrochloride in promoting growth. In alleviating canavanine inhibition, however, each was less than one-eighth as effective as arginine.

The effectiveness of canavanine in inhibiting arginine utilization suggested that canaline might show a similar relationship to ornithine utilization. This amino acid was consequently tested (a) for its ability to replace ornithine in promoting growth of Escherichia coli Y117 and Lactobacillus fermenti, and (b) for its ability to inhibit growth of E. coli Y109

| Table III |
|-----------------|-----------------|-----------------|
| Comparative Effectiveness of Arginine, Citrulline, and Ornithine in Overcoming Inhibition of Escherichia coli Y109 by Canavanine |
|                | L-Arginine hydrochloride | DL-Citrulline | DL-Ornithine hydrochloride |
| No canavanine present | γ per 2 cc. | γ per 2 cc. | γ per 2 cc. |
| 0 | 55 | 55 | 55 |
| 200 | 55 | 55 | 0 | 55 |
| 0 | 95 | 95 | 95 | 95 |
| 3 | 93 | 91 | 20 | 88 |
| 10 | 82 | 85 | 100 | 78 |
| 20 | 71 | 81 | 200 | 75 |
| 50 | 61 | 68 | 400 | 67 |
| 100 | 55 | | 1000 | 62 |
| 200 | 56 | | 100 | 62 |

* Arbitrary scale; distilled water = 100, uninoculated medium = 95.

and to prevent the response of E. coli Y117 and Lactobacillus fermenti to minimum levels (10 γ per 2 cc.) of ornithine. It showed no activity in either capacity in amounts up to 1 mg. per 2 cc. Another analogue of ornithine, α-amino-β-hydroxyvaleric acid, was synthesized as described by Sørensen (8), and similarly tested. It showed neither growth-promoting nor growth-inhibiting properties in amounts up to 4 mg. per 2 cc.

**DISCUSSION**

That Neurospora synthesizes arginine via reactions of Krebs' ornithine cycle has been shown by the careful investigations of Srb and Horowitz

*Because of possible lability of this hydroxylamine derivative to autoclaving with the medium, it was filtered and added aseptically to the previously sterilized medium for these tests.
It is interesting that the same enzymatic deficiencies induced in *Neurospora* by artificial production of mutants are found to occur naturally in various lactic acid bacteria. Thus representatives of these organisms have been found (Table I) whose requirement for arginine can be met by ornithine, citrulline, or arginine; by citrulline and arginine, but not ornithine; and by arginine only. Apparently arginine synthesis in the lactic acid bacteria, too, proceeds via this same series of reactions, illustrating anew the essential similarity of the synthetic mechanisms in different forms of life.

For none of the bacteria studied is canavanine able effectively to replace arginine for growth. Its structural similarity to arginine is reflected, however, in the fact that it inhibits utilization of arginine by many of these organisms. The competitive nature of the inhibition further indicates that inhibition results from the combination of canavanine with cellular constituents normally involved in arginine metabolism. Perhaps in organisms such as *Streptococcus faecalis* which are extremely resistant to inhibition by canavanine the structural features required for such combination are not fully met by canavanine. It is known, for example, that canavanine is a much weaker base than arginine (9). Perhaps, as suggested by Horowitz and Srb (1), such resistant organisms are able to detoxify the inhibitor. In this latter connection, the presence of arginine dihydrolase in *Streptococcus faecalis* and other streptococci of Group D (10), and its possible occurrence in other canavanine-resistant organisms, suggests a mechanism by which the inhibitor might be decomposed. The action of this enzyme on canavanine is not known; the latter is, however, split by a liver enzyme (probably arginase) to yield canaline (11). That such cleavage would in fact detoxify canavanine is shown by the observed inactivity of canaline as an inhibitor.

In contrast to many antimetabolites, such as those of pantothenic acid (7, 12), which are effective inhibitors only for organisms which do not synthesize the corresponding metabolite, canavanine inhibits organisms which synthesize their own arginine as well as those which require preformed arginine. It resembles in this respect the sulfonamides and certain other amino acid inhibitors, such as β-2-thienylalanine (13) and hydroxyaspartic acid (14). It is not necessary, however, to assume a fundamentally different mode of action for these two classes of inhibitors. According to present concepts, the antimetabolite and its corresponding metabolite compete for an enzyme involved in the further transformation of the metabolite. To permit such competition the metabolite must be present in a free and diffusible form. Where an antimetabolite functions against cells which synthesize the metabolite, it may be assumed that the metabolite normally appears in free and diffusible form in the cell preliminary to utilization in the process which the inhibitor affects. In those cases in
which the antimetabolite is ineffective against cells which synthesize the metabolite, this apparently is not true.

Horowitz and Srb (1) found that lysine was also effective in alleviating canavanine inhibition of Neurospora. With the organisms used here, lysine was ineffective; mixtures of lysine and arginine were no more effective than would be predicted from their arginine content.

It was pointed out above that, at equivalent levels of growth, Escherichia coli Y109 (which synthesizes its own arginine) was much more readily inhibited by canavanine than was Escherichia coli Y117, for growth of which arginine (or a precursor) must be supplied. However, the two organisms showed similar sensitivity to canavanine when supplied with equal amounts of arginine. Reasons for such behavior were discussed above. It should be expected, on this basis, that arginine precursors, such as citrulline and ornithine, would be much less effective than arginine in preventing canavanine inhibition, and this was observed in all instances tested. These observations are thus consistent with previous conclusions (e.g. (14)) that precursors of a metabolite are generally less effective than the metabolite itself in preventing inhibition of growth by an antimetabolite. All of our data support the conclusion that inhibition of growth by canavanine results from its interference in the utilization of arginine for various synthetic reactions, presumably the synthesis of cell proteins.

SUMMARY

The specificity of the arginine requirement of a number of lactic acid bacteria was examined. Some grew only when arginine was supplied, others with either arginine or citrulline; still others could utilize ornithine, citrulline, or arginine. None of the bacteria tested could effectively use canavanine in place of arginine. Similarly, canalinine did not replace ornithine for those organisms which utilize the latter amino acid.

For some, but not all, of the bacteria tested canavanine was an effective growth inhibitor. This was true both for organisms which synthesize arginine, and for those which require this amino acid preformed. For both types of organisms, inhibition by canavanine was competitively alleviated by arginine. In many instances, the ratio of canavanine to arginine at which a given level of growth was observed was not constant, but decreased as the concentration of canavanine was increased. The magnitude of this ratio was markedly dependent upon the size of the inoculum. For organisms which utilized them for growth, citrulline and ornithine showed limited effectiveness in counteracting canavanine inhibition; they were, however, much less effective than arginine. Lysine was ineffective.

Canalinine and α-amino-δ-hydroxyvaleric acid did not inhibit utilization of ornithine by the organisms tested.

The significance of these various results is discussed briefly.
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