The inhibitory action of D-leucine, D-valine, and DL-valine upon the growth of *Lactobacillus arabinosus* has been demonstrated experimentally (1, 2). Such studies are of interest in cellular chemistry. The specificities observed are related theoretically to those found in the substrates of proteases (3, 4). The information obtained is also pertinent to experiments on the retardation of growth of laboratory animals by nutritional amino acid mixtures (5) and to knowledge of the factors influencing microbiological assay (6). Data of this type may furthermore help to form a picture of the essential structures and modes of action of such antibiotics as gramicidin, tyrocidine, and penicillin (1, 2, 7).

The study of bacterial inhibition by D-amino acids has now been extended to other species in order to determine whether or not the effect may be a special or general one. The effects on *Escherichia coli* reported here for D-valine and D-leucine are similar to those found with *Lactobacillus arabinosus*. A pattern of inhibition of the same sort has been observed for *Staphylococcus aureus* also. In the case of *Staphylococcus aureus*, however, the inhibitions were not consistently observed, although the majority of tests showed inhibition. The nature of this variation is receiving further investigation.

The influences of amino acids on *Escherichia coli* have corresponded closely to the specificity of amino acid residues in substrates of proteases (3). These results differ qualitatively from those observed on *Lactobacillus arabinosus* in the case of D-alanine only. Attempts to clarify the knowledge of the particular function of D-alanine in these organisms were therefore made; data on the competitive behavior of D-alanine are presented below. The effect of D-alanine which has been studied concerns its ability to counteract the inhibition of bacterial growth by glycine. This relationship had previously been observed in *Streptococcus lactis* by Snell and Guirard (8).
E. coli GROWTH INHIBITION

Materials and Methods

Amino Acids—The preparation of the six optical forms of alanine, valine, and leucine has been described (1, 2). The purification of benzoylalanine employed in the resolution of alanine was facilitated by washing out the contaminating benzoic acid with hot carbon tetrachloride, as for hippuric acid (9), instead of with ligroin (10). The glycine used was Merck’s aminoacetic acid.

Growth of Microorganisms—The growth of Lactobacillus arabinosus 17-5 was estimated by titration of acid produced (1).

The growth of Escherichia coli was measured turbidimetrically. Stock cultures of Escherichia coli were maintained in the refrigerator by monthly transfer to nutrient agar slants (11). The identity of the organism was checked after completion of the experiments (12). Transfers for inhibition tests were made to tubes of nutrient broth (13) and incubated for 24 hours at 37°, centrifuged, washed with physiological saline solution, and resuspended in 10 ml. of saline solution.

The amino acids were included in tubes in 0.50 ml. of aqueous solution, and 0.50 ml. of nutrient broth was added to each tube. These tubes were sterilized for 15 minutes at 15 pounds steam pressure, allowed to cool, and inoculated with 1 drop of the shaken suspension. They were then incubated for 24 hours at 37°, treated with 9.0 ml. of water each, shaken thoroughly, and read in a Coleman model 11 spectrophotometer with Filter PC-4 at 450 mu.

Standard dilution curves were determined for each experiment in order to evaluate variations in the rate of growth of the control. These standard curves were prepared by dilution of the incubated control tubes with appropriate amounts of nutrient broth solution which had been diluted to one-twentieth of its original concentration. The spectrophotometer scale was set at 100 per cent transmission with this latter solution.

For the experimental tubes, the galvanometer readings were converted to a calculated value, growth ratio, in order to furnish a basis of comparison between sets of experiments. The growth ratio was determined by reading from the corresponding standard dilution curve the per cent of maximum growth (in absence of inhibitors) to which the observed turbidity corresponded.

**EXPERIMENTAL**

In Fig. 1 is presented a typical standard curve for the relationship of galvanometer readings and successive dilutions of a control culture of Escherichia coli.

In Fig. 2 is presented a typical series of curves illustrating the growth ratios of Escherichia coli in the presence of glycine, D-alanine, D-valine, and
Fig. 1. Graph of galvanometer readings and successive dilutions of a control culture of *Escherichia coli*.

**Fig. 2.** Effects of varying amounts of D-leucine, D-valine, D-alanine, and glycine on the growth of *Escherichia coli*. The growth ratio was determined by reading from the corresponding standard dilution curve the per cent of maximum growth (in absence of inhibitors) to which the observed turbidity corresponded.

D-leucine. In all cases the inhibition by D-amino acids on an equimolar basis was in the order D-leucine > D-valine > D-alanine. In no case were
the L forms inhibitory in the range studied. Glycine was more inhibitory than D-alanine.

An evaluation of the variation in extent of inhibition may be obtained from the results presented in Table I, at a common value of 0.050 M of the added D-valine or D-leucine. These results were obtained in a graphically sensitive region of concentration; at 0.100 M all D-valine and D-leucine tubes were visually clear. The set of four experiments in Table I was preceded by three experiments which showed the same trends but which lacked sufficient points in the curves for interpolation to 0.050 M.

The effect of glycine upon the growth of Lactobacillus arabinosus, in combination with other substances, is illustrated in the data in Table II. Table II shows that the inhibition caused by glycine is reversed by either D-alanine or L-alanine, or by pyridoxine. It may be noted that D- and L-alanine were equally effective. The inhibition caused by D-leucine is, however, not reversed by either L-alanine or pyridoxine. The same lack of reversibility is found for the inhibition by D-valine.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Growth ratio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-Valine</td>
<td>D-Leucine</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>37</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table I**

Inhibition of Escherichia coli by D-Valine and D-Leucine at 0.050 M Concentration

**Table II**

Effect of Pyridoxine and Alanine on Inhibition of Lactobacillus arabinosus Caused by Glycine, D-Valine, and D-Leucine

<table>
<thead>
<tr>
<th>Addition to basal medium (2.50 ml.) in tube</th>
<th>Average, 0.100 N acid produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.39 ± 0.02</td>
</tr>
<tr>
<td>15 mg. glycine</td>
<td>2.18 ± 0.01</td>
</tr>
<tr>
<td>30 &quot; &quot;</td>
<td>1.48 ± 0.06</td>
</tr>
<tr>
<td>30 &quot; &quot; + 30 mg. L-alanine</td>
<td>2.56 ± 0.05</td>
</tr>
<tr>
<td>30 &quot; &quot; + 30 &quot; D-alanine</td>
<td>2.69 ± 0.04</td>
</tr>
<tr>
<td>30 &quot; &quot; + 0.5 mg. pyridoxine</td>
<td>2.35 ± 0.03</td>
</tr>
<tr>
<td>30 &quot; D-leucine</td>
<td>1.17 ± 0.07</td>
</tr>
<tr>
<td>30 &quot; &quot; + 30 mg. L-alanine</td>
<td>1.25 ± 0.02</td>
</tr>
<tr>
<td>30 &quot; &quot; + 0.5 mg. pyridoxine</td>
<td>1.40 ± 0.01</td>
</tr>
<tr>
<td>30 &quot; D-valine + 30 mg. L-alanine</td>
<td>1.30 ± 0.10</td>
</tr>
</tbody>
</table>
In Table III are presented the effects of some of these same substances on Escherichia coli. For this species D-alanine is inhibitory, and the inhibition by glycine is not reversed by L-alanine, D-alanine, or pyridoxine.

### Table III

*Effect of Pyridoxine and Alanine on Inhibition of Escherichia coli Caused by Glycine*

All results were observed in duplicate.

<table>
<thead>
<tr>
<th>Addition to basal medium in tube</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Normal visible growth</td>
</tr>
<tr>
<td>25 mg. glycine</td>
<td>No visible growth</td>
</tr>
<tr>
<td>25 “ D-alanine</td>
<td>“ “ “</td>
</tr>
<tr>
<td>25 “ L-alanine</td>
<td>Normal visible growth</td>
</tr>
<tr>
<td>25 “ glycine + 25 mg. D-alanine</td>
<td>No visible growth</td>
</tr>
<tr>
<td>25 “ “ + 0.5 mg. pyridoxine</td>
<td>“ “ “</td>
</tr>
</tbody>
</table>

### Discussion

The inhibitory action of some of the D-amino acids upon bacterial growth is an effect which is not limited to the first species studied, *i.e.*, *Lactobacillus arabinosus*. At least two other species of bacteria are so affected, and preliminary evidence exists for retardation of growth of laboratory animals by high levels of D-amino acids. The concentrations of D-amino acids which are necessary for inhibiting bacterial growth are higher than those usually employed for practical use of other antibacterials. On the other hand the relatively low mammalian toxicity of some D-amino acids (14, 15) does not warrant preclusion of investigation of such properties. The conceivable long term detrimental effects which might result from certain n-amino acids, when present in solutions employed in large amounts clinically, require further study. Of related interest is an investigation of the effects of D-amino acids and derivatives upon neoplasms.

The antipodal specificity found for the amino acids in the present studies makes it desirable to consider that some of the bacterial inhibitions which have been reported for DL-amino acids (16, 17) may have been due to the D isomer. On the other hand, in some cases the activity of the racemate is not the average of the activities of the optical isomers. DL-Valine, for instance, had almost the same activity against *Lactobacillus arabinosus* as had D-valine (2). Analogous behavior has been reported for substrates of proteases (18, 19), but more recent results (20) are at variance with one of these reports (19). In the other case (18), chymotrypsin failed to hydrolyze benzoyl-DL-tyrosylglycylamide, although it hydrolyzed the L isomer. The explanation which has been offered is that the racemate in solution repre-
sents a third substance with distinct properties. The distinct nature of such properties is well illustrated in the solubilities of the various forms of amino acids (21) and in the values for the heats of combustion of DL-leucine as compared to that of either D-leucine or L-leucine (22). With the added fact that L-amino acids possess inhibitory activity in some experiments (23), it appears that conclusions in bacterial experiments can be drawn most safely in comparisons between L and D forms rather than between L and DL molecules.

A similarity in type of inhibition of growth of bacteria by D-amino acids to their effects as substrate residues for proteases has been cited for the belief that D-amino acids may interfere quite directly with the action of “proteosynthetic” enzymes (2). This analogy was based upon the non-hydrolyzability of peptide substrates on one hand (3) and on effects of added unsubstituted D-amino acids (2) on the other. It is therefore of interest that Abderhalden and Abderhalden (4) found that added D-leucine itself greatly decreases the hydrolytic activity of peptidases from a variety of sources. If one accepts the concept that a proteolytic enzyme catalyzes the same reaction in both directions, the accumulated observations substantiate the interpretation that the D-amino acids studied hinder bacterial growth by interfering with proteolytic (= proteosynthetic) enzymes.

The correspondence of the bacterial effects and the substrate effects was close enough to suggest originally such a conclusion (2) for Lactobacillus arabinosus. This correspondence held for both isomers of valine, both isomers of leucine, and for L-alanine, but not for D-alanine. In the case of Escherichia coli, the correspondence holds for all six forms, since D-alanine is inhibitory to this species. On such a basis, the effect of D-alanine on L. arabinosus is anomalous. A clue to such behavior is found in the ability of alanine, especially D-alanine, to reverse the inhibition by glycine. The effect was observed in Streptococcus faecalis R by Snell (24) and such behavior has also been found here to hold for L. arabinosus. Snell suggested that D-alanine functioned as an intermediate in an inefficient conversion to pyridoxine. Perhaps the important points in the present study are that E. coli, which is inhibited by D-alanine, cannot use it in the same way as L. arabinosus does (Tables II and III), and that the pattern of inhibition by monoaminomonocarboxylic acids for E. coli bears such a close analogy to the effects with peptidase substrates.

Besides the parallelism in the enzymic and bacterial effects of alanine, valine, and leucine, analogous behavior is also seen with glycine. Glycine is found to be inhibitory for the two species of organisms reported here and for others reported previously (8, 25). The glycine residue also exhibits the property of interference with hydrolysis by enzymes (3, 26) as exemplified by “sluggish” peptidolysis of glycylglycine.
The relationship of the \(\alpha\)-amino acids to antibiotic \(\alpha\)-amino acid derivatives is receiving further attention. The \(\alpha\)-amino acid residue has been shown to be one of a number of critical structural features for penicillin through comparison of the activity with the \(L\) isomer (27). The corresponding investigation for gramicidin, requiring the preparation of a gramicidin type molecule containing only \(L\) residues is not readily feasible by present synthetic methods. A number of molecules in which the \(\alpha\)-amino acid residue is repeated do not, however, have high antibacterial activity. If the \(\alpha\)-amino acid residue is generally critical for antibiotics of the penicillin and gramicidin classes, other structural features must be concurrently critical.

**SUMMARY**

The growth of *Escherichia coli*, like that of *Lactobacillus arabinosus*, is inhibited by added \(\alpha\)-amino acids, at levels at which the \(L\) forms do not exhibit such an effect. \(\alpha\)-Alanine, which is not inhibitory for *Lactobacillus arabinosus*, slows the growth of *E. coli* to a lesser extent than do \(\alpha\)-valine or \(\alpha\)-leucine. These effects of amino acids on *E. coli* correspond closely to those observed by others for the hydrolyzability of peptides, constructed from these same amino acids, when subjected to the action of peptidases.

High levels of added glycine inhibit both *Lactobacillus arabinosus* and *Escherichia coli*. In the case of the former species, this inhibition is counteracted by added \(\alpha\)-alanine or pyridoxine, whereas such addition to the culture medium of *Escherichia coli* fails to counteract these effects.

The theoretical implications of the results reported are discussed.

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E. coli growth inhibition

ANTIPODAL SPECIFICITY IN THE INHIBITION OF GROWTH OF ESCHERICHIA COLI BY AMINO ACIDS
Yutaka Kobayashi, Marguerite Fling and Sidney W. Fox


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