PEPTIDES AND BACTERIAL GROWTH

II. L-ALANINE PEPTIDES AND GROWTH OF LACTOBACILLUS CASEI

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Lactobacillus casei grows in the absence of vitamin B₆, provided that D-alanine and an unidentified peptide are added to a medium complete in other nutrients (1, 2). A partial hydrolysate of protein contained several chromatographically distinct fractions that possessed this "peptide factor" activity (3). It appeared likely that a single amino acid linked in a variety of combinations was responsible for these growth effects. An active, purified fraction was identified tentatively as a mixture of alanyl and tyrosyl dipeptides of valine, leucine, and isoleucine (3). An expeditious solution of the problem thus appeared possible by testing synthetic peptides containing these amino acids. Such studies, described below, revealed that dipeptides containing L-alanine in either the carboxyl or amino position possessed growth-promoting activity; L-alanine was inactive under the same conditions, as were peptides of tyrosine.

Examination of the reasons for this enhanced activity of L-alanine peptides revealed that D-alanine, which is itself essential for growth in the absence of vitamin B₆, strongly inhibited utilization of L-alanine, but did not inhibit utilization of peptides of this amino acid. Despite this fact, resting cells of L. casei hydrolyzed each of the L-alanine peptides utilized for growth. Certain implications of these and related findings are discussed briefly.

EXPERIMENTAL

Assay Methods—The assay procedures were those described previously (3), except that culture volumes were increased to 4.0 ml. to permit turbidity readings without dilution. This increased volume made incubation under carbon dioxide unnecessary.

Growth Promotion by Synthetic Peptides—The comparative effects of vitamin B₆ or of various pure peptides on growth of L. casei are shown in Fig. 1. Vitamin B₆ permits growth in shorter incubation times than do active peptides; with longer incubation times, however, certain of the peptides are as effective as vitamin B₆ in permitting high levels of growth. Such peptides, like partial protein digests, have no growth-promoting activity in the absence of D-alanine; vitamin B₆ continues to permit growth under...
the latter conditions (1–3). The rate of growth with various L-alanine peptides differs considerably, possibly reflecting the different rates at which the peptides are hydrolyzed (see below).

In accordance with expectation from a consideration of fractionation experiments (3), L-alanyl-L-leucine and L-alanyl-L-valine have high activity; however, all peptides of L-alanine examined promote growth under these conditions (Table I). This fact undoubtedly explains the previous observation (3) that each of the ninhydrin-reactive bands separated from a partial hydrolysate of protein by paper chromatography possessed growth-promoting activity. Peptides of D-alanine are without activity, as are other peptides that do not contain L-alanine as one component. L-Alanine itself is inactive at all levels under the conditions of this experiment; growth with it did not occur even after 42 hours of incubation.

Hydrolysis of Active Peptides by L. casei—In considering possible explanations for the high activity of peptides of L-alanine, it was important to know whether such peptides could be hydrolyzed by the test organism. This was tested in a manner similar to that described by Virtanen and Nurmikko (4). L. casei was grown for 24 hours in the inoculum medium (3), centrifuged, washed, and resuspended in 0.01 M phosphate buffer, pH 6.25. A 0.1 ml. aliquot of this suspension, equivalent to 0.24 mg. of dry cells, was mixed with 200 γ of peptide in 0.1 ml. of the phosphate buffer. Cells alone and peptides alone were incubated simultaneously with buffer as controls. After incubation at 37° for 24 hours, the cells were centri-

![Figure 1](http://www.jbc.org/) The comparative growth responses of L. casei to peptides of L-alanine and to vitamin B₆. Curves A, B, C, and D are the responses to pyridoxine, L-leucyl-L-alanine, L-alanyl-L-histidine, and L-alanylglycine, respectively.
fuged out. The supernatant solutions were spotted on filter paper and chromatographed with water-saturated phenol as the developing solvent.

**Table I**

*Comparative Activities of Synthetic Peptides in Supporting Growth of L. casei*

<table>
<thead>
<tr>
<th>Compound†</th>
<th>Amount permitting half maximum growth*</th>
<th>Compound†</th>
<th>Amount permitting half maximum growth*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>Inactive</td>
<td>L-Alanyl-L-histidine</td>
<td>0.208</td>
</tr>
<tr>
<td>L-Alaninamide</td>
<td>0.803</td>
<td>DL-Alanyl-L-histidine</td>
<td>0.161</td>
</tr>
<tr>
<td>L-Alanyl-L-leucine</td>
<td>0.094</td>
<td>L-Alanyl-L-histidine</td>
<td>0.112</td>
</tr>
<tr>
<td>L-Alanyl-L-leucinamide</td>
<td>0.089</td>
<td>Glycyl-L-alanine</td>
<td>0.075</td>
</tr>
<tr>
<td>L-Leucyl-L-alanine</td>
<td>0.074</td>
<td>L-Alanyl-L-histidine</td>
<td>0.208</td>
</tr>
<tr>
<td>L-Leucyl-d-alanine</td>
<td>Inactive</td>
<td>D-Alanyl-L-histidine</td>
<td>Inactive</td>
</tr>
<tr>
<td>L-Alanyl-L-valine</td>
<td>0.088</td>
<td>Glycyl-L-tyrosine</td>
<td>“</td>
</tr>
</tbody>
</table>

* Incubation time, 42 hours. “Maximum” growth was taken as that obtained in this time with an excess of pyridoxine.

† Additional peptides tested, all of which were inactive, included glycyl-L-histidine, L-α-amino-n-butyryl-L-histidine, β-L-aspartyl-L-histidine, L-leucylglycine, glycyl-L-tryptophan, L-glutamyl-L-leucine, L-glutamyl-L-phenylalanine, glycylglycine and glycylphenylalanine. We are indebted to the following individuals for samples of peptides: Dr. K. Folkers, Dr. K. P. Link, Dr. W. G. McCullough, Dr. W. S. McNutt, Dr. E. L. Smith, Dr. W. H. Stein, and Dr. V. du Vigneaud.

**Fig. 2.** The hydrolysis of peptides of L-alanine by resting cells of *L. casei*. Water-saturated phenol was the developing solvent.

The results (Fig. 2) showed that each of three active peptides tested was hydrolyzed by the resting cells. No ninhydrin-positive zones resulted from incubating cells with phosphate buffer in the absence of peptide.
Separate trials, in which the time of incubation of cells with peptide was varied, showed that L-leucyl-L-alanine was hydrolyzed more rapidly than L-alanylglycine (Fig. 3). In this case, therefore, the most rapidly hydrolyzed peptide is the most active in promoting growth during short incubation periods (Fig. 1).

Inhibition of Response to L-Alanine by D-Alanine—The hydrolysis of active peptides by L. casei was somewhat surprising in view of the inactivity of free L-alanine in supporting growth. The basal medium used in these experiments contains hydrolyzed casein as the source of amino acids, with added cystine, tryptophan, and glycine (3). Glycine is known to inhibit growth of Streptococcus faecalis, and alanine to counteract this inhibition (1). When the supplementary glycine was omitted, L-alanine did permit growth, provided that the inoculum was increased 4-fold. Under these conditions, the relation of various concentrations of D- and L-alanine to growth of L. casei is shown in Fig. 4. With low concentrations (10 γ per ml.; Curve A) of D-alanine, no added L-alanine is required for growth, since the hydrolyzed casein of the basal medium supplies approximately 185 γ
per ml. of this amino acid. An additional 300 γ of L-alanine per ml. are required to permit maximum growth at this level of d-alanine. As the concentration of d-alanine is increased, the amount of L-alanine required for growth is greatly increased; it is evident that d-alanine is effectively inhibiting the utilization of L-alanine. The amount of L-alanine required at these higher levels of d-alanine greatly surpasses the total yield of cells obtained in the medium; it is thus evident that only a fraction of the L-alanine present actually is utilized. The ratio of L- to d-alanine required for maximum growth at 10, 20, 50, and 100 γ levels of d-alanine is 48, 47, 24, and 22, respectively. The absence of a strictly competitive relationship is not surprising, since (a) a variable fraction of the d- and L-alanine is being utilized for growth (particularly at low concentrations of d-alanine, therefore, the amount acting solely as an inhibitor is less than the amount added); (b) amino acids other than d-alanine may interfere with utilization of L-alanine (their effects would be most noticeable at low concentrations of d-alanine); and (c) L-alanine itself inhibits growth at very high levels for reasons yet unknown. This latter inhibition, like that by d-alanine, is not apparent in media that contain sufficient vitamin B₆.

D-Alanine does not interfere with utilization of peptides of L-alanine, for, in contrast to the results with L-alanine, the same concentration of L-alanylglycine (about 100 γ per ml.) was required for maximum growth in the presence of 20, 50, or 100 γ of d-alanine per ml. The greatly en-
hanced activity of these peptides, as compared to L-alanine, in supporting
growth under conditions in which D-alanine is an essential nutrient (i.e.,
in the absence of added vitamin B₆) is thus readily understandable.

_Inhibition of Response to D-Alanine by Glycine—_That high concentra-
tions of L-alanine failed to permit growth when the basal medium contained
extra glycine was pointed out above. This suggested that glycine was
antagonistic to alanine for _L. casei_, as it is for _S. faecalis_ (1, 6) and several
other lactic acid bacteria (6). In cases so far examined, this antagonistic
action of glycine has been exerted primarily against D-alanine, and only
to a lesser extent against the L isomer (6). In accordance with these
results, data of Fig. 5 show that, as the concentration of D-alanine in the
medium is increased, the tolerance to glycine is greatly increased. It is
certain, therefore, that the inhibitory action of glycine is exerted to a large
extent against D-alanine.

The possibility that glycine simultaneously inhibited utilization of
L-alanine was tested by supplying a high concentration of D-alanine (100
γ per ml.), together with sufficient glycyl-L-alanine (15 γ per ml.) to permit
suboptimum growth. Under these conditions, the same concentration of
L-alanine (1.0 mg. per ml.) was required to permit maximum growth with
0, 100, 500, and 1000 γ per ml. of added glycine. At these concentrations,
therefore, no inhibition of utilization of L-alanine by glycine could be
demonstrated.
DISCUSSION

The complex nutritional interrelationships revealed by these investigations are perhaps best understood in terms of the diagram presented in Fig. 6. Growth of any organism involves absorption of the required nutrients from the medium, followed by synthesis of essential cellular components, e.g. proteins, within the cell.\(^1\) In principle, inhibitory metabolites may exert their effects either upon the absorption process (i.e., at the cell wall) or upon the essential synthetic processes within the cell; in few, if any, cases is the exact locale of their action known.

In the present instance glycine, D-alanine, and L-alanine must be supplied to permit growth in the absence of vitamin B\(_6\), because the vitamin B\(_6\) enzymes required for their synthesis are not present (cf. (1, 7-10)). Under these circumstances, they must be absorbed from the medium (Reactions a to c, Fig. 6). Under these conditions, glycine interferes with utilization of D-alanine, either at Reaction b or f. Similarly, D-alanine interferes strongly with the utilization of the structurally similar L-alanine, but not the structurally dissimilar peptides of L-alanine. If the latter are hydrolyzed by the growing cells, as they are by resting cells (Reaction h), then utilization of the L-alanine formed from them within the cell (Reaction i)

\(^1\) Investigations in progress show that most of the D-alanine present in lactic acid bacteria occurs in the protein fraction.
is not inhibited by d-alanine, and by extension, Reaction $g$ should not be that prevented by d-alanine. Thus the evidence is most simply interpreted by assuming that Reaction $c$, the absorption of L-alanine through the cell wall, is that inhibited by d-alanine, whereas Reaction $d$ proceeds unhindered. This evidence is consistent with the finding that neither glycine nor d-alanine, at the levels used here, is inhibitory if an excess of vitamin $B_6$ is supplied. Under these conditions, glycine, L-alanine, and d-alanine can be synthesized within the cell (Reactions $j$ to $l$), since they need not be supplied in the medium to permit growth. The utilization (Reactions $m$ and $n$) of the d- and L-alanine formed in this way is not susceptible to inhibition by glycine or d-alanine, respectively, as it is when their absorption from the medium is required.\footnote{In some experiments, the maximum growth obtained with vitamin $B_6$ is decreased slightly when glycine is added, but the effect is small compared to that in the absence of vitamin $B_6$, and does not change the argument presented.}

Many studies have been made of the availability of peptides as amino acid sources for bacterial growth. In most instances, the peptides have been somewhat less or equally as active as the amino acid in supporting growth. In such instances, hydrolysis of the peptide prior to utilization of the amino acid has been suggested as the mechanism of utilization (4, 11–17). In a smaller number of studies several pure peptides have proved more active than the corresponding amino acids in promoting growth (18–20). Direct utilization of the peptide without prior hydrolysis has been postulated as a possible means of explaining this enhanced activity (18–20). The observations described in the experimental portion of this paper demonstrate, however, that these peptides also may be hydrolyzed prior to utilization.

Hills (21) observed an antagonistic relationship between d- and L-alanine in their effects on germination of spores of Bacillus anthracis reminiscent of the relationship described herein. Peptides of L-alanine were not tested as reversing agents. There is no reason to believe that this relationship of d-alanine to utilization of L-alanine and its peptides is unique, and since many cases of the inhibition of utilization of one amino acid by a related amino acid (1, 22, 23) or by synthetic analogues (24, 25) are known, we may expect to find such enhanced activity of peptides quite frequently.

It has been emphasized elsewhere (26, 27) that one essential metabolite may inhibit synthesis of another essential metabolite by a given organism. In such cases, the latter metabolite becomes a nutritional essential, even though the cell retains the ability to synthesize it and may do so (and

\footnote{An alternative but less simple hypothesis is that L-alanine formed by Reactions $h$ and $j$ differs (e.g., in the state of its chemical combination with enzyme surfaces) from that present at Reaction $g$ in such a way that d-alanine can interfere only with utilization of the latter.}
hence grow without it) under appropriately modified conditions. The data
of this paper show that apparent nutritional requirements may also arise
because one essential metabolite (D-alanine) may inhibit *utilization* of an-
other (L-alanine) when supplied in one form, but not when supplied in a
modified form (e.g., peptides of L-alanine). In such a case, the modified
form of the essential metabolite, rather than the free metabolite, will ap-
pear as the essential nutrient.

Finally, the possible bearing of these results on the use of antimetabolites
in tracing metabolic pathways should be mentioned. It is usually con-
sidered that the product of an enzymatic conversion blocked by a given
antimetabolite should counteract the inhibitory effects of the antime-
tabolite more effectively than does the substrate of the reaction, and
this in turn more effectively than does a precursor of the substrate (28).
In the present instance, these relationships appear not to hold, since
L-alanine peptides, which are hydrolyzed by the cells and hence act as
precursors of L-alanine, far surpass L-alanine itself in their ability to over-
come the inhibitory effects of D-alanine.

A second mechanism whereby peptides may show growth activity greater
than that of their component amino acids was suggested by Gale (29).
This is discussed in connection with an experimental demonstration of its
validity in an accompanying paper (30).

**SUMMARY**

*Lactobacillus casei* grows in the absence of vitamin B₆, provided that
D-alanine and certain peptides are supplied in an otherwise complete
medium. An active peptide fraction obtained from a partial hydrolysate
of protein contained alanyl and tyrosyl peptides of leucine, isoleucine, and
valine (3). Tests on synthetic peptides revealed that, while tyrosine-cont-
taining peptides were inactive, all peptides that contained L-alanine in
either the carboxyl or amino position were active. L-Alanine itself was
inactive under the same conditions. Despite this fact, resting cells of
*L. casei* hydrolyzed the active peptides to their component amino acids.

When the glycine content of the medium was lowered, L-alanine showed
growth-promoting activity of a much lower order than that shown by the
active peptides. Under these conditions, D-alanine was shown to an-
tagonize very strongly the utilization of L-alanine, but not that of L-alanine
peptides. The latter function as essential growth factors by supplying
L-alanine in an available form. High levels of glycine inhibit utilization
of D-alanine, but not that of L-alanine.

Many instances in which peptides show greater growth-promoting ac-
tivity than their component amino acids may have a similar explanation;
i.e., utilization of the free amino acid, but not that of its peptides, may be
inhibited by related amino acids present in the culture medium. A possible mechanism for this behavior is discussed.

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