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On the Mode of Action of Peptides as Growth Factors for *Leuconostoc mesenteroides*

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In a previous paper [1] we recorded results showing that *Leuconostoc mesenteroides* P-60, which requires glycine for an amino acid, is also able to use glycine peptides instead of glycine. In connection with the present study we were particularly interested in ascertaining whether the action of peptides was due to the cleavage of glycine from them caused by bacterial cells, hence, the growth-affecting factor being always free glycine, or whether bacteria may utilize glycine peptides without prior hydrolysis. The former explanation strikes at first more natural, but some observations reported in the literature seem to favour the latter one. Particular reference should be made in this connection to the findings of Simmonds and Fruton [2] of the exceptional activity of proline peptides which is greater than that of the equimolar free proline. In our investigations, however, no glycine peptide exceeded in activity the free glycine.

To elucidate the problem, we examined the hydrolysis of some glycine peptides by the action of *Leuconostoc mesenteroides* P-60. The results are reported here.

EXPERIMENTAL

The bacterial mass was grown in a nutrient solution containing 1 % glucose, 1 % sodium citrate, 0.5 % Bacto-tryptone, and 1.5 % Bacto-yeast extract. A stab culture of the strain was first inoculated into 10 – 20 ml of the above nutrient solution and allowed to grow for about 1 day at 37° C. Then it was further inoculated into 500 ml of the same nutrient solution and kept again for 1 day at 37° C. The bacterial mass was then aseptically centrifuged, washed once and suspended in 15 – 20 ml of 0.9 % NaCl-solution. Dry weight of bacteria was determined from the suspension after standing overnight at 103° C.

The examination included the following peptides: DL-alanylglycine (Schuchardt), DL-leucylglycine (University of California), glycylglycine (the same), glycyl-L-tyrosine

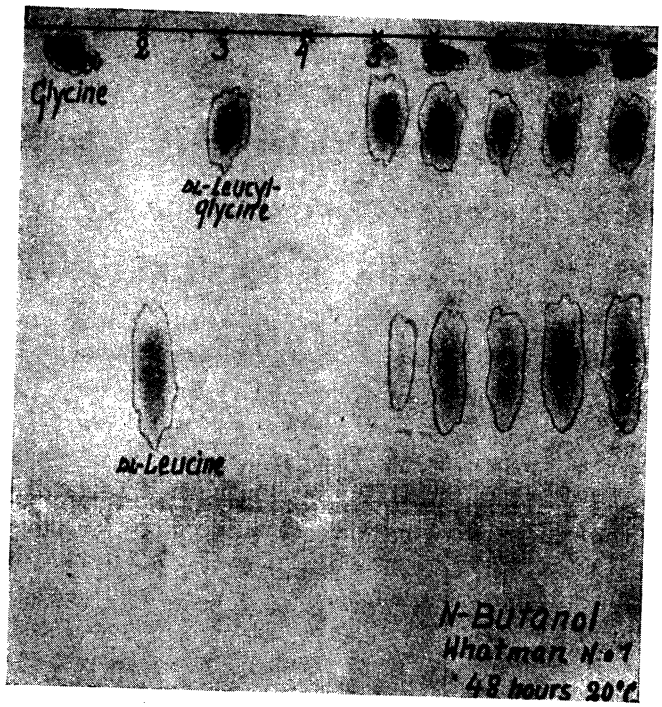

Fig. 1. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL*-leucyl-glycine with bacteria.*

1) Glycine alone
2) DL*-Leucine alone*
3) DL*-Leucylglycine alone*
4) Bacteria alone after 45 h
5—9) DL*-Leucylglycine with bacteria*
5) after 3 h
6) » 9 »
7) » 21 »
8) » 32 »
9) » 45 »

methylesterhydrochloride (Sir Ian Heilbron) and leucyl-DL-phenylalanine methylesterhydrochloride (the same). The peptide concentration of the three first mentioned ones was 10 mg/ml and that of the two last mentioned 15 mg/ml. The concentration of the respective amino acids was: glycine, alanine, and leucine 5 mg/ml, tyrosine and phenylalanine 10 mg/ml.

The amino acid and peptide solutions, 4 ml each, were sterilized in an autoclave at 112° C in glass ampoules for 5 min. After this 1 ml of bacterial suspension, corresponding to 15—18 mg dry bacteria, was pipetted into each ampoule. Respectively, 1 ml of distilled

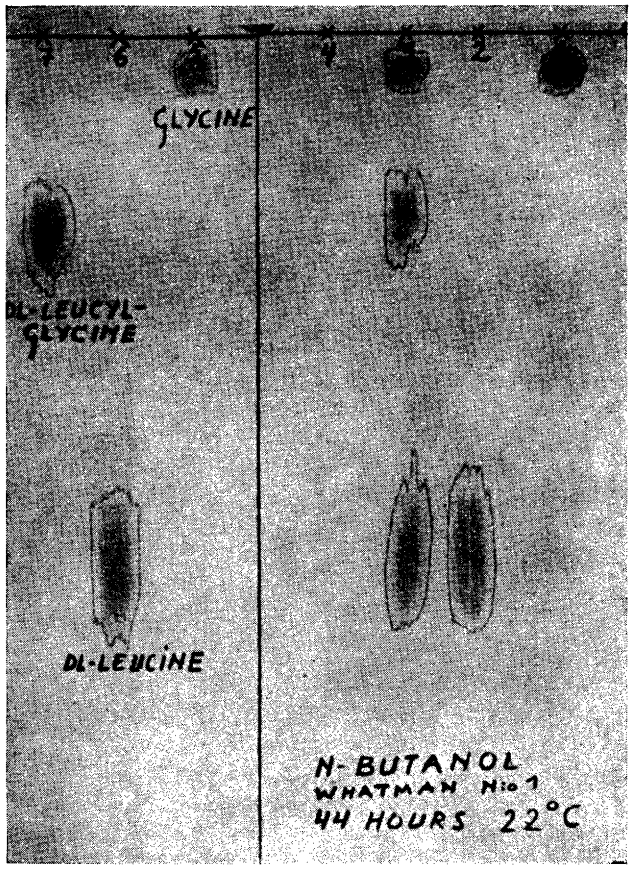

Fig. 2. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL-*leucylglycine with bacteria after 24 hours.*

1) Glycine with bacteria
2) DL-*Leucine with bacteria*
3) DL-*Leucylglycine with bacteria*
4) Bacteria alone
5) Glycine alone
6) DL-*Leucine alone*
7) DL-*Leucylglycine alone*

water was pipetted into the control solutions. The tubes were again sealed and warmed on a water-bath to 37° C.

After hydrolysis the bacterial mass was centrifuged and amino acids and peptides were determined in the clear solution by means of one-dimensional paperchromatography employing the technique introduced by Miettinen and Virtanen [3]. In some of the experi-

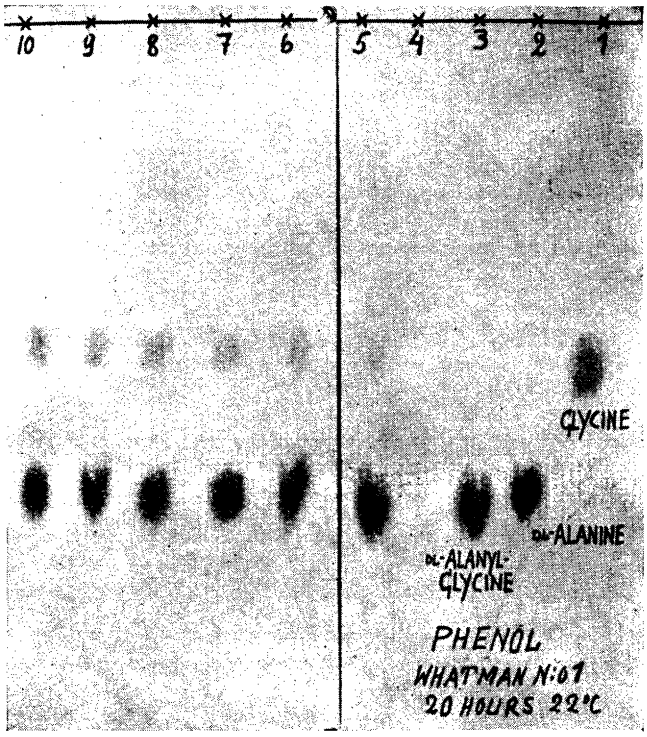

Fig. 3. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL-*alanylglycine with bacteria.*

1) Glycine alone
2) DL-*Alanine alone*
3) DL-*Alanylglycine alone*
4) Bacteria alone after 24 h
5—10) DL-*Alanylglycine with bacteria*
5) after 3 hours
6) » 6 »
7) » 9 »
8) » 12 »
9) » 18 »
10) » 24 »

ment series, besides, 1 % ninhydrin solution (1 : 1) was used for following the hydrolysis of peptides according to Abderhalden [4]. The intensity of the red-blue colour, obtained with ninhydrin in 2 h at 21° C in experiments with glycyl-L-tyrosine and glycyl-DL-phenylalanine, was determined with a Klett-Summerson photoelectric colorimeter using filter S 52.

In order to find out how the leucine component of leucylglycine acts in the growth experiments of *Leuconostoc mesenteroides* an experiment was carried out, using the same

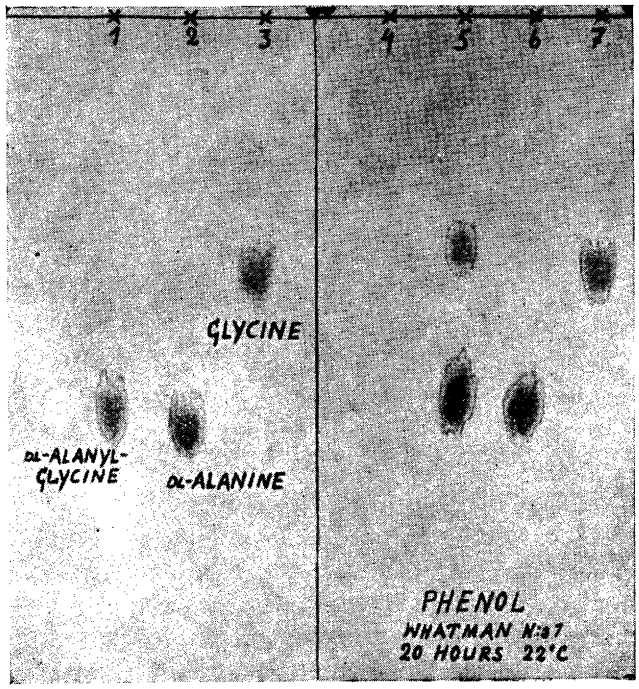

Fig. 4. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL-*alanyl-glycine with bacteria after 24 hours.*

1) Alanylglycine alone
2) DL-*Alanine alone*
3) Glycine alone
4) Bacteria alone
5) DL-*Alanylglycine with bacteria*
6) DL-*Alanine with bacteria*
7) Glycine with bacteria

method as earlier [1], in a leucine-free basal medium, prepared according to Henderson and Snell [5].

RESULTS

The results of our hydrolysis experiments are presented in Figs. 1—5 and in Table 1. The photographs were taken from the paperchromatograms, which were obtained from DL-leucylglycine, DL-alanylglycine and glycylglycine before and after the hydrolysis with bacterial mass and from the respective amino acids using one-dimensional run in butanol or in phenol. As appears from the figures, all these peptides are hydrolyzed by the bacterial mass. The longer

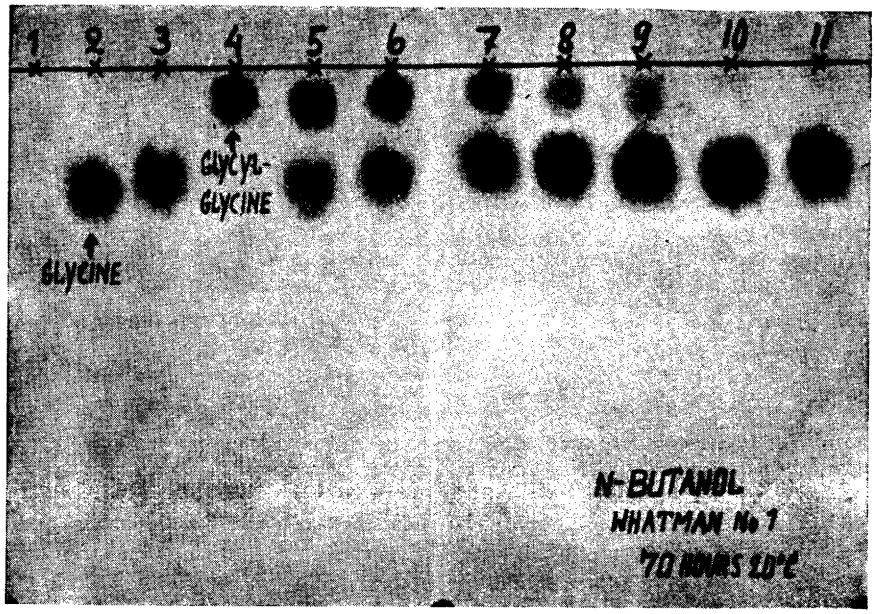

Fig. 5. Photograph of one-dimensional paperchromatogram of the hydrolysis of glycylglycine with bacteria.

1) Bacteria alone after 24 h
2) Glycine alone
3) Glycine with bacteria
4) Glycylglycine alone
5—11) Glycylglycine with bacteria
5) after 1 ½ h
6) » 3 »
7) » 6 »
8) » 12 »
9) » 19 »
10—11) » 24 »

the time of hydrolysis, the higher the hydrolysis degree. DL-leucylglycine and DL-alanylglycine are slightly hydrolyzed already within 3 hours and glycylglycine within 1 ½ h. Within 24 hours the hydrolysis of glycylglycine was quantitative.

Bacterial mass alone did not produce compounds detectable in paper chromatograms.

Hydrolyzation of DL-leucylglycine and DL-alanylglycine was also noted by colour reaction with 1 % ninhydrin solution at room temperature. The inten-

Table 1. Hydrolysis of glycyl-L-tyrosine methylesterhydrochloride and glycyl-DL-phenylalanine methylesterhydrochloride with bacteria after 24 hours at 37° C. 1 % ninhydrin solution added to the solution under examination in ratio 1 : 1 according to R. Abderhalden.

	Optical density		Optical density
Glycine	0.346	Glycine	0.336
Tyrosine	0.028	Phenylalanine	over 1.800
Glycyl-L-tyrosine methylester-hydrochloride	0.210	Glycyl-DL-phenylalanine methylesterhydrochloride	0.190
Bacteria alone	0.028	Bacteria alone	0.036
Glycyltyrosine methylesterhydrochloride with bacteria	0.432	Glycyl-DL-phenylalanine methylesterhydrochloride with bacteria	0.334

Both experiments indicate that hydrolysis has taken place. Provided that the results are quantitatively comparable, the hydrolysis of glycyl-L-tyrosine methylesterhydrochloride is almost complete, whereas that of glycyl-DL-phenylalanine methylester is insignificant. Phenylalanine gives with ninhydrin so intense a colour that a hydrolysis of phenylalanine, proceeded to a high extent, raises the colour intensity to manyfold. However, it must be taken into account that tyrosine and phenylalanine have been free in the controls, whereas they are liberated as methylesters in the hydrolysis. This affects considerably the colour intensity.

sities of colour were then consistent with the results obtained by paperchromatography.

Since hydrolysis of glycyl-L-tyrosine methylester and glycyl-DL-phenylalanine methylester could not be proved for certain by paperchromatography, 1 % ninhydrin was used for the purpose (Table 1). Glycyl-L-tyrosine methylester gave with the bacterial mass in 24 h a distinctly more intense red-blue colour than peptide alone. The greater intensity must be due to the partial hydrolysis of peptide since glycine produces a comparatively strong colour reaction. Likewise, glycyl-DL-phenylalanine gave with bacterial mass in 24 h a more intense red-blue colour than peptide alone. Hydrolysis must therefore have taken place. Phenylalanine gave a very intense colour, whereas tyrosine gave practically none at all.

In comparing the results of the hydrolysis experiments with those obtained in the growth experiments with peptides [1], it can be ascertained that the active normal peptides, which are composed of amino acids, also are regularly hydrolyzed. This suggests that glycine, set free on the cleavage of peptides, is responsible for the action of glycine peptides. This concept is also supported by the observation made in this work that leucylglycine is approximately as active both as glycine and leucine.

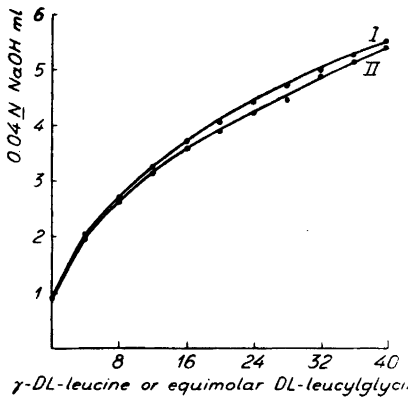

Fig. 6. DL-*leucine activity of* DL-*leucylglycine in growth experiment with Leuconostoc mesenteroides P-60. (Growth 72 h at 37° C, basal medium according to Henderson & Snell.)*

I = DL-*leucine 100 % activity.*
II = DL-*leucylglycine 97 % activity.*

On the contrary, benzoylglycine, which is glycine-active in the growth experiments with *L. mesenteroides*, was not hydrolyzed, at least not in detectable amounts. It would therefore seem likely that benzoylglycine as such would replace glycine. Its non-amino acid component (benzoic acid) would thus change the mode of action of peptide. It is possible that benzoylglycine joins the enzyme on the surface of the bacterial cells and is not hydrolyzed until in the cell during the metabolism. This concept is, however, quite hypothetical.

There is still reason to emphasize the fact that our hydrolysis experiments were carried out with greater amounts of bacteria than our growth experiments. Thus the hydrolysis velocity is not directly comparable with the glycine activity of peptides in the growth experiments. With such amounts of bacteria, which come into question in the growth experiments, hydrolysis of peptides is not detectable by the technique employed by us. Therefore our experiments do not suffice to solve convincingly the problem whether the hydrolysis of peptides is so rapid that the liberation of glycine from normal peptides would explain the mode of action of active glycine-peptides in the growth experiments with *Leuconostoc mesenteroides*. The results show at any rate for certain that this organism hydrolyzes the normal glycyl and leucine peptides which can replace the said amino acids in the growth experiments.

SUMMARY

Leuconostoc mesenteroides P-60 hydrolyzes leucylglycine, alanylglycine and glycylglycine, which replace glycine in growth experiments with this organism. Similarly, hydrolysis has been noted with glycyl-L-tyrosine methylester and

glycyl-DL-phenylalanine methylester, which also show activity with the same bacteria. The results suggest that the activity of glycine peptides depends on the hydrolysis of these peptides to free amino acids. DL-Leucylglycine replaces as well leucine as glycine in the growth experiments with *L. mesenteroides*, which is to be expected if the amino acids set free by hydrolysis are the active factors in the growth experiments.

Benzoylglycine is also glycine-active in the growth experiments with *L. mesenteroides*. Its hydrolyzation could not be noted with this organism.

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