METABOLISM OF PHENYLALANINE-CONTAINING PEPTIDE AMIDES IN ESCHERICHIA COLI

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Received for publication July 20, 1961

ABSTRACT

SIMMONDS, SOFIA (Yale University, New Haven, Conn.) AND DAVID D. GRIFFITH. Metabolism of phenylalanine-containing peptide amides in Escherichia coli. J. Bacteriol. 83:256–263. 1962.—A study was made of the hydrolysis, by fresh and lyophilized cells of a phenylalanine auxotroph of Escherichia coli, of L-phenylalanylglycinamide and glycyl-L-phenylalaninamide. The former dipeptide amide is hydrolyzed to yield phenylalanine and glycine, the latter to yield glycine and phenylalaninamide. The slow degradation of phenylalaninamide yields the free amino acid.

In spite of the presence of intracellular activity toward the two dipeptide amides, neither permits as much growth as that observed with an equimolar concentration of free phenylalanine. The relatively small extent of growth on the dipeptide amides, and also on L-phenylalaninamide, appears to result from the inability of the cells in a growing culture to take up all of the exogenous amide added to the growth medium. This, in turn, probably reflects the presence in the medium of the amides as a mixture of charged and uncharged forms, of which only the latter are readily taken into the cells.

Although considerable information is available concerning the ability of peptides to replace amino acids as growth factors for bacteria, relatively little is known about the growth-promoting action of amino acid or peptide amides. Some years ago it was observed that the amides of L-phenylalanine, L-tyrosine (Simmonds, Tatum, and Fruton, 1947a), L-leucine (Simmonds, Tatum, and Fruton, 1947b; Simmonds and Fruton, 1949), L-proline, L-glutamic acid (Simmonds and Fruton, 1948), and L-methionine (Taylor, Simmonds, and Fruton, 1950) can support the growth of specific amino acid auxotrophs of Escherichia coli strain K-12, but all the amides, except L-glutamine, are very much less active than the corresponding amino acids.

The only peptide amides that have been tested as growth factors are derivatives of phenylalanine and tyrosine, and, like the amino acid amides, they are relatively poor growth factors (Simmonds et al., 1947a). The reason for the poor growth on the amides has remained unclear, and we have recently renewed the study of these compounds in an attempt to provide an explanation for the phenomenon. The results obtained with the phenylalanine auxotroph (strain 58-278) of E. coli form the substance of the present report.

MATERIALS AND METHODS

Growth experiments. All growth tests were carried out by methods similar to those of Simmonds, Dowling, and Stone (1954), in which the growth of cultures (consisting of 7 ml of medium inoculated with about 10⁶ cells and incubated on a shaker at 30 C) was followed as a function of time by periodic absorbancy measurements in an Evelyn colorimeter (Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.) (filter no. 540). The three types of basal media used (Table 1) differ mainly in their buffering capacity, initial pH, and source of nitrogen. The GlyPhe,³ PheGly, GlyPhe-NH₂ acetate, PheGly-NH₂ ace-

¹ This study was supported in part by a research grant (G-7586) from the National Science Foundation. Some of the data presented are taken from a dissertation submitted by David D. Griffith to the Yale University School of Medicine in candidacy for the degree of Doctor of Medicine.

² Recipient of research stipends awarded for the summers of 1959 and 1960 to the Yale University School of Medicine from a National Science Foundation grant.

³ The following abbreviations are used: GlyPhe and PheGly for glycyl-L-phenylalanine and L-phenylalanylglycine; GlyPhe-NH₂ and PheGly-NH₂ for the corresponding dipeptide amides; and Phe-NH₂ and Gly-NH₂ for the amides of L-phenylalanine and glycine, respectively.
TABLE 1. Composition of basal media

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<thead>
<tr>
<th>Constituent*</th>
<th>Medium</th>
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<tbody>
<tr>
<td></td>
<td>A†</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Na₂SO₄</td>
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<tr>
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<td>7.0</td>
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<tr>
<td>pH, after sterilization</td>
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</tr>
</tbody>
</table>

* The composition is given in terms of the mM concentration of the final media. In addition to the compounds listed, all media contained L-asparagine (11.4 mM), D-glucose (11.1 mM), MgSO₄ (0.4 mM), and also 1 μg of biotin and 1 ml of a trace-element solution (Horowitz and Beadle, 1943) per liter of medium.

† Medium A is essentially that of Gray and Tatum (1944), which was used in earlier work with the auxotroph (Simmonds et al., 1947a).

‡ Phosphate was provided as an appropriate mixture of K₂HPO₄ and KH₂PO₄.

tate, Phe-NH₂ acetate, and Gly-NH₂ acetate were gifts of J. S. Fruton. Paper chromatography (in the solvent system described below) of 50-μg samples of the dipeptide amides and of 15-μg samples of the other compounds showed each to contain only one ninhydrin-positive component. Each compound was tested for its growth-factor activity at concentrations of 0.005 to 2 mM.

The growth curves of the auxotroph in media containing suboptimal concentrations of L-phenylalanine are diphasic (Simmonds, 1950), and show an initial rapid rise to a "primary maximum" (dependent on the initial concentration of phenylalanine) followed by a "plateau," whose duration depends both on the phenylalanine concentration and on the pH and buffering power of the medium (Simmonds et al., 1954), and a second slow rise. The "secondary growth" results from the utilization of phenylpyruvic acid formed by the spontaneous decomposition of prephenic acid accumulated in the medium during the earlier phases of the growth cycle.

In practice, it is sometimes difficult to estimate the primary maxima accurately (e.g., if primary growth occurs at a slow rate or if the duration of the plateau period is short). Under even the most favorable conditions (medium C), primary growth to absorbancies above about 0.30 is followed by a plateau period of only 2 to 3 hr, although nonlimiting concentrations of growth factors permit growth to an absorbancy of 0.70 to 0.80. Consequently, to compare the relative growth-factor activities of various test compounds, the concentration of each compound required to produce a culture absorbancy of 0.15 was estimated from a concentration curve that was prepared by plotting the concentrations tested against the culture absorbancies after incubation for 20 hr. At that time the growth curves for most of the cultures show clearly defined primary maxima, i.e., the cultures are in the plateau period.

Hydrolysis experiments. After growth to a culture absorbancy of about 0.75, the cells in 100 ml of medium containing an excess of L-phenylalanine (0.4 mM) were harvested by centrifugation and washed twice with 0.9% saline. The washed cells were either suspended in 15 ml of 25 mM phosphate buffer (pH 6.8) and used immediately (fresh cells) or were frozen and lyophilized.

Complete reaction mixtures contained, per 1 ml of 25 mM phosphate buffer (pH 6.8), 10 μmoles of a dipeptide amide and an amount of cells providing 0.05 to 0.15 mg of bacterial nitrogen (determined by micro-Kjeldahl analysis). Control mixtures lacked either the dipeptide amides or the cell preparation. In each experiment, a given cell preparation was tested with GlyPhe-NH₂ and with PheGly-NH₂. All mixtures were shaken at 30 C for 24 hr and, at intervals during that period, 0.01-ml samples were removed from each mixture for analysis by paper chromatography, with n-butanol–pyridine–water (2:1:1) as the developing solvent. To each sheet of Whatman no. 1 chromatography paper were applied, with heat, one complete series of samples from a given reaction mixture and also a series of standard solutions containing a mixture of equimolar amounts (12.5, 25, 37.5, 50, 75, or 100 mM) of per 0.01 ml of the phosphate buffer) of the dipeptide amide substrate and its hydrolysis products. To effect satisfactory separation of the hydrolysis products of GlyPhe-NH₂, the chromatograms were developed by the ascending method for 18 to 26 hr; the approximate Rₚ values were: Phe-NH₂, 0.78; GlyPhe-NH₂, 0.56; phenylalanine, 0.48; GlyPhe, 0.31; and glycine, 0.12.
Because ascending chromatography failed to separate PheGly-NH$_2$ from phenylalanine, for this substrate the hydrolysis mixtures were analyzed by the descending method; the approximate $R_f$ values were: PheGly-NH$_2$, 0.53; phenylalanine, 0.47; PheGly, 0.36; Gly-NH$_2$, 0.21; and glycine, 0.11. After it had been found in preliminary experiments that GlyPhe and PheGly are not formed during the bacterial hydrolysis of the corresponding dipeptide amides, the dipeptides were omitted from the standard solutions.

The air-dried chromatograms were sprayed with a 2% solution of ninhydrin in 95% ethanol, the colors were allowed to develop in the dark at 30°C for several hours, and the colored areas were cut out and eluted in 7 ml of 50% ethanol. The absorbancy of the eluates was measured in an Evelyn colorimeter (filter no. 580) set for 100% transmission with eluates of ninhydrin-negative areas of the chromatograms. The amount of each compound in a hydrolysis mixture was estimated from a standard curve prepared from the appropriate component of the standard mixture run on the same sheet of paper. This procedure permits detection of 5 to 100 mM moles of compound, with a maximal error of about 10%.

**RESULTS**

*Growth experiments.* In agreement with the results of earlier growth tests carried out under somewhat different conditions (Simmonds et al., 1947a), the amides are relatively poor growth factors compared to free phenylalanine or to simple dipeptides (Table 2). In all the media used here, PheGly-NH$_2$ is the most effective of the amides, and the isomeric GlyPhe-NH$_2$ is even less effective than Phe-NH$_2$.

Changes in the composition of the basal medium have relatively little effect on the growth response to phenylalanine. No significant variations were observed either in the concentrations required to produce a given culture absorbancy (Table 2) or in the growth rates (generation time, 90 to 100 min in media A and B, and 80 to 90 min in medium C for all phenylalanine concentrations used). Changes in the basal medium do, however, have a profound effect on the growth responses to the amides, and media of high buffering power (medium B) and high initial pH (medium C) permit more efficient utilization of all the amides. This enhanced utilization is especially marked for Phe-NH$_2$ and GlyPhe-NH$_2$, whose relative growth-promoting activities are greatly increased in media B and C compared to their activities in medium A.

Another noteworthy aspect of the growth response to Phe-NH$_2$ is the fact that the rate of visible growth on this compound in each medium increases as the initial concentration of the amide is raised. For example, in medium C (where the growth rate was measured most precisely) the generation times for 0.04, 0.10, 0.20, and 0.40 mM Phe-NH$_2$ were 170, 150, 105, and 85 min, respectively. Thus, in medium C the

<table>
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<th>Compound</th>
<th>Growth factor activity*</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Concentration</td>
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<tr>
<td>L-Phenylalanine</td>
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</tr>
<tr>
<td>GlyPhe†</td>
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</tr>
<tr>
<td>PheGly†</td>
<td>0.015 mM</td>
</tr>
<tr>
<td>PheGly-NH$_2$‡</td>
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</tr>
<tr>
<td>Phe-NH$_2$</td>
<td>1.1 mM</td>
</tr>
<tr>
<td>GlyPhe-NH$_2$</td>
<td>1.55 mM</td>
</tr>
</tbody>
</table>

* The activities are estimated from the mM concentrations required to produce culture growth to an absorbancy of 0.15 in 20 hr.
† The dipeptides have not been tested in media B and C. Their activities in medium A are approximately equal to that of phenylalanine throughout the pH range 6.5 to 7.9.
‡ The growth rate increases as the initial concentration in the medium is raised, as described in the text.
growth rate on Phe-NH₂ resembles that on phenylalanine only when the initial concentration of the amide is about 0.40 mM or higher. In medium B the growth rate characteristic of phenylalanine is attained only when the amide concentration is raised to about 1 mM; in the more poorly buffered medium A this optimal growth rate requires about 2 mM Phe-NH₂.

A similar marked dependence of growth rate on amide concentration was not observed with either of the dipeptide amides. In each medium, the growth rates on the dipeptide amides are approximately the same as the rate on phenylalanine.

As expected, changes in the basal medium influenced the duration of the plateau period that follows growth on any exogenous source of phenylalanine. For example, with 0.01 mM phenylalanine, the duration was about 7 hr in medium A, 14 hr in medium B, and 30 hr in medium C; at the 0.02 mM level, the times were 2 to 3, 6, and 18 hr, respectively.

Hydrolysis experiments. Preliminary tests, in which the course of the hydrolysis of PheGly-NH₂ and GlyPhe-NH₂ by fresh cells was followed qualitatively, showed that each dipeptide amide is cleaved at the peptide bond and yields the NH₄-terminal amino acid and an amino acid amide. Free dipeptides were not detected on the paper chromatograms of any hydrolysis mixture. These results were confirmed in the quantitative experiments described below.

After GlyPhe-NH₂ is hydrolyzed by fresh cells, the Phe-NH₂ formed is further metabolized to yield phenylalanine; the sum of the Phe-NH₂ and phenylalanine is approximately equal to the amount of the dipeptide amide that is used up (Fig. 1A). The glycine liberated does not accumulate in amounts equivalent to the amount of GlyPhe-NH₂ that disappears. Even less glycine was detected in an experiment with another fresh-cell preparation. In the latter experiment, the maximal amount of glycine observed was 0.8 μmole and was found in a 4-hr sample which also contained 4.1 μmoles of Phe-NH₂, 0.5 μmole of phenylalanine, and an amount of GlyPhe-NH₂ corresponding to the hydrolysis of 4.1 μmole of substrate. This destruction of glycine liberated from the dipeptide amide may be attributed to the action of the glycine deaminase known to be present in the cells (King and Simmonds, unpublished data).

With PheGly-NH₂ as the substrate (Fig. 1B), the hydrolysis is accompanied by the quantitative formation of phenylalanine. However, the amount of Gly-NH₂ detected is always less than would be expected on the basis of the amount of phenylalanine formed and PheGly-NH₂ hydrolyzed. Although no free glycine appears, it is probable that the Gly-NH₂ formed from PheGly-NH₂ is hydrolyzed to yield glycine, which is immediately deaminated. The production of ammonia from Gly-NH₂ (as well as from glycine) has been observed in independent studies on fresh cells of a number of mutant strains of E. coli (King and Simmonds, unpublished data). With fresh cells of a serine-glycine auxotroph, strain S/G of Simmonds and Miller (1957), whose activity has been examined in detail, the production of ammonia from Gly-NH₂ is not accompanied by the formation of significant amounts of glycine.
However, cell-free extracts devoid of glycine deaminase activity are still capable of forming ammonia from Gly-NH₂ and so contain glycine amidase activity.

GlyPhe-NH₂ is hydrolyzed at a faster rate (Fig. 1) than PheGly-NH₂ (e.g., 50% of the GlyPhe-NH₂ is lost within 2.5 hr; a similar loss of PheGly-NH₂ requires about 5 hr). This rate difference has been found also with preparations of lyophilized cells (Fig. 2) and with fresh cells from a culture grown in medium A. The data obtained with the latter preparation of fresh cells are essentially similar to those shown in Fig. 1.

Like fresh cells, preparations of lyophilized cells split each dipeptide amide only at the peptide bond to liberate the NH₂-terminal amino acid. However, lyophilization produces a cell preparation that has lost much of the glycine deaminase activity of fresh cells (compare Fig. 1A and 2A). There also may be some loss of the ability to hydrolyze Phe-NH₂, since the rapid disappearance of GlyPhe-NH₂ and rapid accumulation of Phe-NH₂ are not accompanied by a correspondingly rapid appearance of phenylalanine (Fig. 2A). The loss of "phenylalaninamidase" activity was more pronounced with two other preparations of lyophilized cells, where no more than 0.5 to 1.0 μmole of phenylalanine was found after 24 hr; at 12 hr practically all the GlyPhe-NH₂ had been converted to glycine and Phe-NH₂, and the amount of the latter did not decrease significantly in the interval between 12 and 24 hr.

Lyophilization also causes some loss in the ability to metabolize the Gly-NH₂ formed from PheGly-NH₂ (compare Fig. 1B and 2B). Independent experiments on the ability of various E. coli preparations to form ammonia from Phe-NH₂ and from Gly-NH₂ indicate that the amidase activity toward both amino acid amides is relatively unstable to lyophilization (King and Simmonds, unpublished data). It is evident that peptidase activity toward PheGly-NH₂ is much less stable to lyophilization than peptidase activity toward GlyPhe-NH₂. This result has been obtained with two other preparations of lyophilized cells, both of which had only a trace of activity toward PheGly-NH₂ but were still highly active toward the other dipeptide amide.

The data shown in Fig. 2 indicate that lyophilized cells may have some ability to destroy phenylalanine. Thus, at the end of the experiment, the disappearance of 8.9 μmoles of GlyPhe-NH₂ is accompanied by the appearance of only 2.7 μmoles of Phe-NH₂ plus 3.4 μmoles of phenylalanine, and only 6.4 μmoles of phenylalanine appear during the hydrolysis of 8.4 μmoles of PheGly-NH₂ and the formation of 9.0 μmoles of glycine. This experiment is the only one in which evidence suggesting destruction of phenylalanine was obtained; the ability of the cells to deaminate or decarboxylate phenylalanine has not been tested directly.

Experiments with strain S/G. In growth tests carried out with medium B, GlyPhe-NH₂ served as a fairly good growth factor for the serine-glycine auxotroph mentioned earlier. The growth rate on low concentrations (below 10 mM) of the dipeptide amide is slower than that on glycine and increases as the amide concentration in the medium is raised. Comparisons of growth factor activity (made from concentration curves plotted for the maxima of the growth cycles) show the amounts required to produce cultures of absorbancy 0.15 are about 0.3 mM for glycine, for PheGly, and for GlyPhe, and about 0.6 mM for GlyPhe-NH₂. However, neither PheGly-NH₂ nor Gly-NH₂ (tested at concentrations of 1 to 10 mM) supported growth. Although incubation of this
auxotroph for 5 days in growth medium containing 10 mM PheGly-NH₂ did not produce visible growth, the culture filtrate was found (both by microbiological tests with the phenylalanine auxotroph and by paper chromatography) to have significant amounts of free phenylalanine (Simmonds, unpublished data).

**Discussion**

From the data accumulated in several laboratories, it has become clear that the utilization of simple peptides as sources of amino acids for bacterial growth usually involves the preliminary hydrolysis of the peptide to its constituent amino acids. This appears to be true both for amino acid auxotrophs of *E. coli* (Simmonds and Miller, 1957; Levine and Simmonds, 1960) and for the lactic acid bacteria (Kihara and Snell, 1960; Leach and Snell, 1960). It is reasonable to assume that the amides of amino acids and of peptides also undergo such hydrolysis. Consequently, if the bacteria can take up and hydrolyze an amide and if there is no destruction of the essential amino acid formed, the extent of growth in a medium containing an amino acid or dipeptide amide should equal the extent of growth in a medium supplying an equivalent molar concentration of the required amino acid in the free state or as a simple dipeptide. The rate of growth on the amide may differ from that on the unamidated compound, since growth rate will depend on the rate at which the required amino acid is made available within the cell. The latter process is determined both by the rate of uptake of an amide from the medium and by the rate of hydrolysis, either of which may be too slow to support growth at the rate characteristic of growth on the free amino acid.

In the present study, it has been shown that preparations of the phenylalanine auxotroph of *E. coli* can form free phenylalanine from both PheGly-NH₂ and GlyPhe-NH₂. The initial cleavage of the dipeptide amides, which liberates the NH₂-terminal amino acid and an amino acid amide, appears to be catalyzed by different peptidases, one of which is less stable to lyophilization than the other. The amino acid amides formed by this initial cleavage undergo further hydrolysis. Free glycine is destroyed rapidly, but free phenylalanine is not. Thus, the initial hydrolysis of PheGly-NH₂ provides phenylalanine for growth, but the production of phenylalanine from GlyPhe-NH₂ involves two successive hydrolytic reactions. All cell preparations split the peptide bond of PheGly-NH₂ at a slower rate than they split GlyPhe-NH₂, but the two-step degradation of the latter results in a relatively slower liberation of phenylalanine from GlyPhe-NH₂ than from PheGly-NH₂. Nonetheless, the rates of growth on the two isomeric dipeptide amides do not significantly differ. Nor do they differ detectably from the rate of growth on free phenylalanine. Hence, the over-all process of uptake and hydrolysis occurs at a rate at least as fast as the rate at which exogenous phenylalanine is made available for growth.

Although the data from the hydrolysis experiments indicate that each dipeptide amide can be converted quantitatively to phenylalanine, the extent of growth on GlyPhe-NH₂, PheGly-NH₂, and Phe-NH₂ is not equal to that observed with an equimolar amount of free phenylalanine, even under the most favorable conditions. It must be noted, however, that the growth-factor activities were compared at relatively low concentrations of the various test compounds in the growth media (i.e., concentrations permitting growth to an absorbancy of 0.15 under conditions where cultures provided with an excess of any of these growth factors grow to an absorbancy of 0.7 to 0.8). One may assume, therefore, that not all the amide added to the medium is available for degradation to phenylalanine by the cells of a growing culture. Such would be the case if only a part of the total exogenous amide is in a form that can be taken up by the cells. An explanation for this may be found in the fact that the pH of the media (pH 6.8 to 7.5) is near the pH' of the amides (about pH 7.8). Hence, approximately one-tenth to one-third of the total exogenous amide exists in the uncharged form (NH₂-ChRR) and the rest is present as the charged form ("NH₂-ChRR").

Evidence suggesting that only one of these forms is readily taken up by the cells is provided by the effect of the initial pH and buffering power of the growth medium on the apparent growth-factor activities of all the amides. Thus, at pH 6.8 the extent of growth on a given concentration of amide is greater when the buffering power is high, and even more growth results when the initial pH of the highly buffered medium is raised to pH 7.5. This effect of pH on the utilization of amides also has been found with L-leucinamide, whose growth-
factor activity for a leucine auxotroph, strain 679-680 of Simmonds and Fruton (1949), rises markedly as the pH of the medium (medium B) is raised from 5.9 to 7.9 (Simmonds, unpublished data). The degradation of Gly-NH$_2$ to ammonia by fresh cells of the serine-glycine auxotroph is likewise sensitive to pH (King and Simmonds, unpublished data). Here, ammonia production is negligible at pH 6 but occurs fairly rapidly at pH 7, and the rate is almost doubled if the pH is raised from 7 to 8.

It is suggested, therefore, that bacterial cells normally take up amides of monoamino, monocarboxylic acids in the uncharged form. A greater proportion of the total amide will be present in the uncharged form in growth media of pH 7.5 than in a medium of pH 6.8. In the latter medium, a higher buffering capacity prevents the drop in pH that occurs during growth in a poorly buffered medium, and this, in turn, will favor the existence of the uncharged amide throughout the growth cycle. If, under even the most favorable conditions (medium C), no more than about one-third of the total exogenous amide is available in the uncharged form for uptake into the cells, extensive growth on the amides tested can not be expected when the initial amide concentration is low.

The rate, as well as the extent, of growth on Phe-NH$_2$ is influenced by the pH and buffering power of the growth medium. A similar result was found with leucinamide. For both these amino acid amides, too, there is an increase in the growth rate as the initial concentration of the exogenous amide is raised (for leucinamide, see Fig. 6 in Simmonds and Fruton, 1949). Since, at very high concentrations, growth on the amino acid amides is as fast as growth on the free amino acids, the amount of the uncharged amide present in the medium can be maintained at a level high enough to permit entrance of the amide into the cells, and hydrolysis therein at a rate sufficient to support optimal growth.

That the growth rates of GlyPhe-NH$_2$ and PheGly-NH$_2$ are not significantly altered by variation in the pH or the concentration of the exogenous dipeptide amide is somewhat unexpected. The pK' of the amino groups of the dipeptide amides probably does not differ greatly from that of Phe-NH$_2$. It must be assumed, therefore, that even at relatively low concentrations of dipeptide amides there is present a sufficient amount of

The uncharged form to permit rapid uptake and intracellular hydrolysis. The difference in growth response to Phe-NH$_2$ and the dipeptide amides may reflect the fact that the two classes of compounds are handled by independent uptake mechanisms. This is known to be true of certain dipeptides and amino acids (Cohen and Rickenberg, 1956; Levine and Simmonds, 1960; Leach and Snell, 1960).

None of the considerations discussed above explains why the efficacy of the phenylalanine-containing amides vary, i.e., why PheGly-NH$_2$ permits more growth than Phe-NH$_2$ and why the latter, in turn, is used better than GlyPhe-NH$_2$. It is possible either that the Phe-NH$_2$ and GlyPhe-NH$_2$ entering the cells of a growing culture are not completely degraded to phenylalanine or that, if complete degradation occurs, some of the free amino acid may be destroyed before it can be used for growth.

The difference in the growth-factor activities of GlyPhe-NH$_2$ and PheGly-NH$_2$ has been found to be even more marked when the two compounds were tested as sources of glycine for the serine-glycine auxotroph. The inability of PheGly-NH$_2$ to support growth, coupled with the fact that free phenylalanine can be formed from this dipeptide amide, suggests that PheGly-NH$_2$ (like Gly-NH$_2$) can be taken into the cells and hydrolyzed. It is probable, therefore, that the slow liberation of free glycine within the cells is followed immediately by its deamination, and, in the absence of cell multiplication, some unused phenylalanine is excreted into the medium.

LITERATURE CITED


