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# The Utilization of Prolyl Peptides by *Escherichia coli*

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Peptides that have an *N*-terminal proline residue are taken up by *Escherichia coli* and are degraded by intracellular peptidases. A mutant that is unable to transport oligopeptides with *N*-terminal α-amino acids is also unable to transport the peptides with *N*-terminal proline. Dipeptides and oligopeptides can prevent the uptake of the corresponding prolyl peptides and the converse competitive interactions are also observed. Although the peptide α-amino group is essential to the process of peptide transport, the results with the prolyl peptides indicate that the dipeptide and oligopeptide permeases can handle peptides with either an α-amino or α-imino group.

In studies of the process of peptide transport in *Escherichia coli*, it has been found that dipeptides and oligopeptides employ distinct uptake systems (Payne & Gilvarg, 1971). Although more than one dipeptide transport system may exist (Payne & Gilvarg, 1971), all oligopeptides appear to use a single system (Payne, 1968). In the uptake of both di- and oligo-peptides the α-amino group on the peptide plays an essential role, as evidenced by the loss of transport caused by substitution of this group (Gilvarg & Katchalski, 1965; Losick & Gilvarg, 1966; Payne, 1971). In view of the importance of the α-amino group to the transport process it was decided to ascertain whether *N*-terminal prolyl peptides, in which an imino group replaces the amino group, make use of a general peptide transport system or whether a unique system has been evolved to ensure their uptake. Specific means are frequently required to handle proline, both in its free form, and when it occurs in peptide linkage. Thus, in bacteria a specific proline permease is found (Kessel & Lubin, 1962; Britten & McClure, 1962; Tristram & Neale, 1968), and a similar situation appears to apply to the uptake of proline into mammalian cells (Oxender & Christensen, 1963). Further, proteases and peptidases of broad specificity are generally unable to cleave peptide bonds involving proline, necessitating the occurrence of specific enzymes to split such linkages (Sarid, Berger & Katchalski, 1959, 1962; Yaron & Mlynar, 1968; Stone, 1953; Vogt, 1970). The studies described here indicate that dipeptides and oligopeptides in which proline forms the *N*-terminal residue share the transport systems used by other peptides.

## MATERIALS AND METHODS

*Peptides.* Lysine and ornithine homopeptides were synthesized as described by Payne (1971). All other peptides were purchased from the following commercial sources: Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; Miles–Seravac Ltd., Maidenhead, Berks., U.K.; and Cyclo Chemical Co. Ltd., through Baxter Laboratories, Thetford, Norfolk, U.K. The purity of each peptide was checked by high-voltage paper electrophoresis by using a Shandon flat-plate apparatus with acetic acid (8%, v/v) and formic acid (2%, v/v) buffer, pH 2.1. Peptides were stained with cadmium–ninhydrin reagent (Heilmann, Barrolier & Watzke, 1957), or with isatin (Smith, 1953), for *N*-terminal prolyl peptides.

*Bacterial strains.* Strain M-26-26 is a lysine auxotroph of *E. coli* W (A.T.C.C. 9637) (Davis, 1952; Dewey & Work, 1952). Strain M-26-26.R is a spontaneous non-auxotrophic revertant that corresponds to the wild-type strain. Strain M-26-26.TOR is unable to transport oligopeptides but retains the ability to transport dipeptides; the isolation of this mutant and its characterization were described by Payne (1968). Strain M-26-26.R.TOR is the corresponding non-auxotrophic revertant. Strain M-123 is a glycine–serine auxotroph derived from *E. coli* W strain M-22-93, originally provided by Dr W. K. Maas (Payne, 1968). The bacterial cultures were grown in the medium A of Davis & Mingioli (1950). Bacterial growth was followed by measuring the $E_{560}$ of the culture with a Bausch and Lomb Spectronic 20 instrument. Inocula (0.1–0.2 ml) were removed, either from an exponential-phase culture or from a stationary-phase culture growing in medium A supplemented with the required amino acid, and were added to the test solutions (6.0 ml) in culture tubes (20 mm diam.). The culture tubes were incubated at 37°C with shaking.

*E. coli* K12 strain EMG 29, pro$^-$, his$^-$, tryp$^-$, lac$^-$, F$^-$, Str$^r$, was kindly provided by Dr W. Hayes. This strain was grown in minimal medium M-56 (Weismeyer & Cohn,

1960) supplemented with 0.5% glucose, and the required amino acids were supplied at 20 $\mu$g/ml. The cultures were inoculated and growth was measured in a manner similar to that described for the strains of *E. coli* W.

*Peptidase assays.* The preparation of bacterial cell-free extracts and the conditions used for peptidase assays are described in the accompanying paper (Payne, 1971). Peptide cleavage was assayed by the procedure of Moore & Stein (1948).

## RESULTS

The uptake of a peptide by a bacterium may be conveniently measured from the ability of a peptide to support growth of an amino acid auxotroph. To employ this procedure successfully demands the absence of extracellular peptidase activity, and the presence of intracellular peptidases able to liberate the required amino acid at a rate greater than that which is growth limiting. These criteria seem to obtain in the bacterial utilization of most peptides (Gilvarg & Katchalski, 1965; Payne & Gilvarg, 1971). However, the utilization of prolyl peptides by proline auxotrophs of *E. coli* has sometimes proved to be an exception in that low activities of proline peptidases present in exponential-phase cells may lead to slow growth or prolonged lag periods (Stone, 1953; Fruton & Simmonds, 1950; Stone & Hoberman, 1953). For these reasons we initially studied not a proline auxotroph but a mutant in which the required amino acid was not linked directly to the *N*-terminal proline residue.

The utilization of prolylphenylalanyl-lysine by the lysine auxotroph M-26-26 is shown in Fig. 1. An identical growth response is obtained with the tripeptide as with lysine itself, which implies that prolylphenylalanyl-lysine can enter the cell and be cleaved to yield free lysine. However, this fact does not in itself indicate whether the prolyl peptide enters the cell by the same uptake system as is used by oligopeptides with *N*-terminal $\alpha$-amino groups or one specific for prolyl peptides. This problem was studied by using strain M-26-26.TOR. This mutant was isolated on the basis of its resistance to the toxic peptide triornithine; its resistance arises from its inability to concentrate triornithine, which reflects a general inability to transport oligopeptides (Payne, 1968). When prolylphenylalanyl-lysine was tested with strain M-26-26.TOR it was totally unable to support growth (Fig. 1). In earlier studies it was shown that all oligopeptides of lysine that could act as a source of the amino acid for strain M-26-26 were devoid of nutritional effect with strain M-26-26.TOR (Payne, 1968). Therefore, the analogous result with prolylphenylalanyl-lysine implies that the TOR mutation also results in loss of the transport facility for prolyl peptides, and indicates that peptides with an $\alpha$-imino group must at least share some component of the transport system used by other peptides. The growth responses of strains M-26-26 and M-26-26.TOR to lysine and to dipeptides of lysine are identical (Fig. 1) (Payne, 1968).

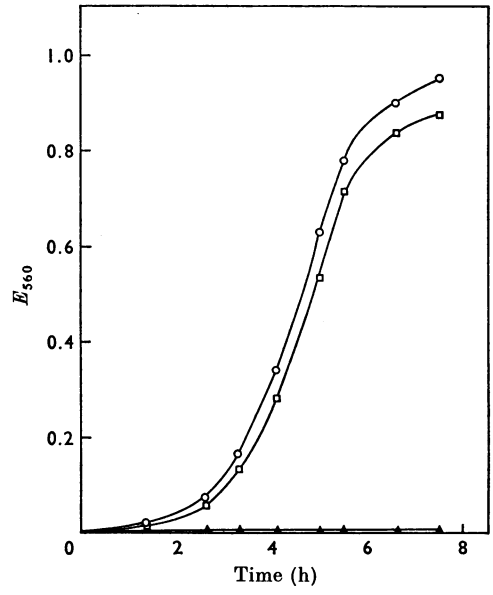


Fig. 1. Growth responses of the *E. coli* W lysine auxotroph M-26-26, and the oligopeptide-transport-deficient strain M-26-26.TOR, to prolylphenylalanyl-lysine. ○, Strain M-26-26 or M-26-26.TOR + lysine (0.15 $\mu$mol/ml), or strain M-26-26 + prolylphenylalanyl-lysine (0.25 $\mu$mol/ml); □, strain M-26-26 + prolylphenylalanyl-lysine (0.125 $\mu$mol/ml); ▲, strain M-26-26 or M-26-26.TOR without lysine, or strain M-26-26.TOR + prolylphenylalanyl-lysine (0.25 $\mu$mol/ml).

I obtained exactly analogous results when I tested the relative growth responses to prolylglycylglycine of the glycine auxotrophs M-123 and M-123.TOR (Fig. 2). However, in this case I found that the use of exponential-phase cells as inocula gave a negligible growth response not only with prolylglycylglycine but also with prolylglycine (Fig. 2). A similar situation has been reported for growth of an *E. coli* proline auxotroph on proline peptides (Stone, 1953), and the explanation offered was that exponential-phase cells are deficient in proline peptidase activity. In agreement with these studies I found that when stationary-phase cells were used as inocula the prolyl peptides were well utilized. The stationary-phase cells were cultured in minimal media and growth ceased through acidification of the media rather than through nutrient depletion; when the cells reached the stationary phase they were incubated for a further 6–8 h before use as inocula. Nevertheless, even when stationary-phase cells were used as inocula strain

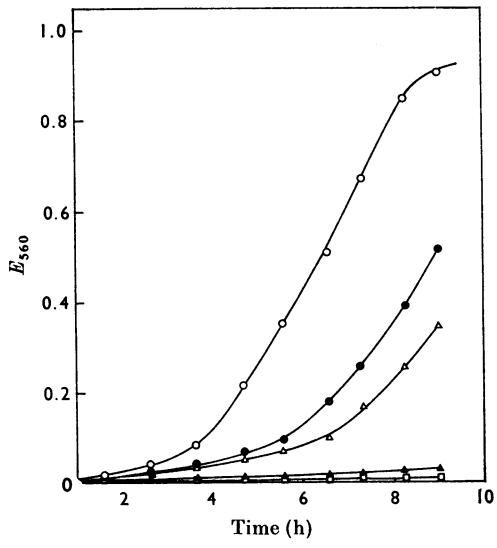


Fig. 2. Growth responses of the *E. coli* W glycine auxotroph M-123, and the oligopeptide-transport-deficient strain M-123.TOR, to prolylglycylglycine. Inocula were either stationary (stat) or exponential phase (expo) cells. ○, Strain M-123 or M-123.TOR (expo or stat) + glycine (1.5 $\mu$mol/ml); ●, strain M-123 (stat)+prolylglycylglycine (1.5 $\mu$mol/ml); △, strain M-123 or M-123.TOR (stat) + prolylglycine (1.5 $\mu$mol/ml); ▲, strain M-123 (expo) + prolylglycylglycine (1.5 $\mu$mol/ml); □, strain M-123.TOR (stat) + prolylglycylglycine (1.5 $\mu$mol/ml).

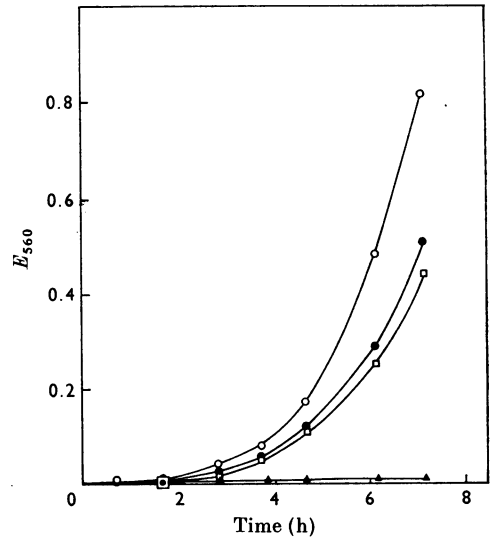


Fig. 3. Ability of prolylphenylalanyl-lysine to overcome the inhibitory effect of triornithine in wild-type *E. coli* W. Except where indicated, triornithine was present at a concentration of 0.04 $\mu$mol/ml. ○, No triornithine; ●, + prolylphenylalanyl-lysine (0.88 $\mu$mol/ml); □, + prolylphenylalanyl-lysine (0.22 $\mu$mol/ml); ▲, triornithine alone, or + proline + phenylalanine + lysine (1.0 $\mu$mol/ml of each).

M-123.TOR was unable to use prolylglycylglycine (Fig. 2). Previous studies indicated that this mutant has lost the normal ability to utilize a wide variety of glycine oligopeptides because of a deficiency in oligopeptide transport (Payne, 1968). The result substantiates the conclusion with the lysine auxotroph that oligopeptides with *N*-terminal proline employ a similar uptake system to other oligopeptides. Once again it should be noted that the growth response to dipeptides is identical in the parent and in the TOR derivative.

The lack of growth seen with the proline tripeptides and the TOR mutants does not reflect an inhibitory activity, for in controls I found that prolylphenylalanyl-lysine (2.1 $\mu$mol/ml) and prolylglycylglycine (2.5 $\mu$mol/ml) do not inhibit growth of strains M-26-26.TOR and M-123.TOR on media supplemented with the required amino acids.

### *Competitive interaction of* N-*prolyl peptides*

*Ability of* N-*prolyl peptides to inhibit peptide uptake.* The conclusion from the study of the TOR mutants, that prolyl oligopeptides share the uptake system that is used by other oligopeptides, leads to the expectation that the two groups should compete for entry to the cell. Such tests should also provide the means to check whether a single system is used for the transport of prolyl dipeptides and other dipeptides.

The absence from exponentially growing cells of *E. coli* of peptidase activity able to cleave triornithine leads to an intracellular accumulation of the tripeptide, which can reach toxic concentrations (Payne, 1968; Sussman & Gilvarg, 1970). The inhibitory effect of triornithine can therefore be alleviated if its entry into the cell can be limited. For this reason, it has been demonstrated that the addition to the growth media of other oligopeptides can overcome triornithine inhibition (Payne, 1968, 1971). We have therefore used this system to measure the competitive ability of prolyl oligopeptides. Triornithine (0.04 $\mu$mol/ml) completely inhibits growth of strain M-26-26.R; however, the addition of prolylglycylglycine (0.15 $\mu$mol/ml) (not shown) or prolylphenylalanyl-lysine (0.22 $\mu$mol/ml) very effectively overcomes this inhibition (Fig. 3) and a family of growth curves may be obtained by varying the concentration of the prolyl peptides.

### *Inhibition of prolyl peptide uptake as a result of competition from other peptides*

In carrying out these tests I used the proline auxotroph EMG 29 of *E. coli* K12. With this mutant, an exponential rate of growth, equal to that

obtained with proline itself (0.3 $\mu$mol/ml), was obtained with the following peptides supplied at 0.3 $\mu$mol/ml: prolylglycine, prolylphenylalanine, prolyltyrosine and prolylglycylglycine. Prolylvaline (0.8 $\mu$mol/ml) completely inhibited growth in media supplemented with proline (0.3 $\mu$mol/ml), as expected from the known toxicity of valine to *E. coli* K12 (Leavitt & Umbarger, 1962). We found that the growth responses to the prolyl peptides were identical when either exponential- or stationary-phase cells were used to inoculate the cultures.

To explore the possibility that the uptake of prolyl peptides can be curtailed by other peptides in the growth media, I studied the effects of lysine homopeptides on the growth response of strain EMG 29 to prolylglycine and prolylglycylglycine. The results in Fig. 4 indicate that dilysine and trilysine can completely inhibit growth on prolyl di- and tri-peptides respectively. These competitive inhibitions are specific, for at these concentrations, dilysine does not affect growth on prolylglycylglycine and neither is growth on prolylglycine affected by trilysine; similarly, neither lysine peptide affects the growth on proline.

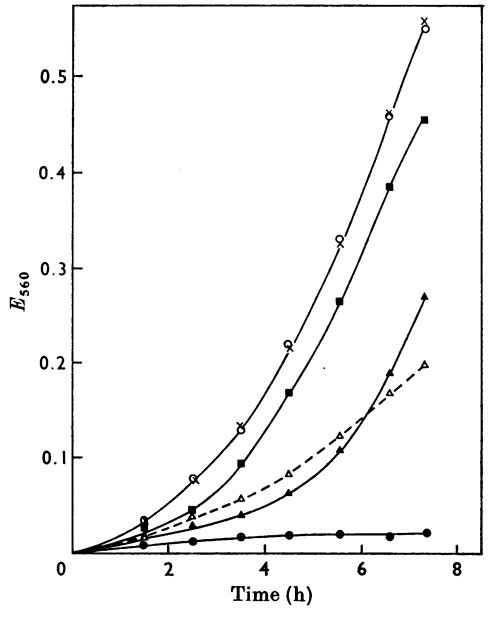


Fig. 4. Ability of lysine peptides to inhibit growth of *E. coli* K12 proline auxotroph EMG 29 on prolyl peptides. Except for the case ○, of prolylglycine (0.20 $\mu$mol/ml) plus trilysine (0.05 $\mu$mol/ml), trilysine was studied in competition with prolylglycylglycine (0.13 $\mu$mol/ml) only, and dilysine was studied in competition with prolylglycine (0.20 $\mu$mol/ml) only. X, Prolylglycine (0.20 $\mu$mol/ml) alone or prolylglycylglycine (0.13 $\mu$mol/ml) alone; ■, + dilysine (0.002 $\mu$mol/ml); ▲, + dilysine (0.01 $\mu$mol/ml); △, + trilysine (0.01 $\mu$mol/ml); ●, + dilysine (0.1 $\mu$mol/ml) or + trilysine (0.05 $\mu$mol/ml).

*Peptidase activities*

It is important to establish for the above growth tests with auxotrophs (Fig. 4) that the observed effects are not a consequence of competition between the different peptides for cleavage by intracellular peptidases, that is, lysine peptides do not exert their inhibitory effect through competitively preventing breakdown of the proline peptides. I studied this feature by using cell extracts from both exponential- and stationary-phase cultures. In general, the extracts of stationary-phase cells possessed higher peptidase activity than those from exponential-phase cells; $Co^{2+}$, but not $Mn^{2+}$, stimulated the activity of both the crude extracts towards several substrates (Table 1). Because of the low activities observed with crude extracts, peptide mixtures were assayed in the presence of $Co^{2+}$. With exponential-cell extracts, cleavage of proline peptides was not inhibited by the addition of lysine peptides to the incubation mixture, the observed cleavage being the sum of that observed when the peptides were incubated separately. Similarly, with stationary-phase extracts, dilysine was without effect on the cleavage of prolylglycine. When trilysine and prolylglycylglycine were assayed together, the cleavage was about 15% less than that observed when the two peptides were assayed individually; a similar effect was observed when trilysine and prolylglycine were incubated together.

The significance of the general lack of competition in the above peptidase assays is emphasized by two examples in which marked inhibition is observed. In growth studies with *E. coli* W it is observed that the toxic effect of glycylnorleucine is reversed by adding prolylphenylalanine to the growth media; the finding that prolylphenylalanine completely inhibits cleavage of glycylnorleucine by cell extracts is in itself sufficient to explain the growth studies. Similarly, prolylphenylalanyl-lysine specifically inhibits growth of a glycine auxotroph on triglycine, which may be a consequence of its ability to inhibit cleavage of the glycine peptide (Table 1). In both the above cases, paper electrophoresis of the initial products of peptidase action indicated that the glycyl peptides remained intact.

## DISCUSSION

A number of workers have studied the utilization of peptides of proline by *E. coli* (Simmonds & Fruton, 1948; Fruton & Simmonds, 1950; Stone,

Table 1. *Peptide cleavage by cell extracts of* E. coli

Results are expressed as μmol of amino groups released/min per mg of protein. Cell extracts of *E. coli* K12 strain EMG 29 were used for the assays involving lysine peptide competition (1–7); extracts of *E. coli* W were used for the remaining assays (8–13). Stationary-phase cells were a culture in which exponential growth ceased after acidification of the medium, and incubation was continued for a further 10 h before the cells were broken. $Co^{2+}$ or $Mn^{2+}$ was added at 0.5 mM. n.d., Not detectable; —, not measured; Nle, norleucine.

| Assay no. | Peptide added (μmol/ml) | Cleavage by crude extracts from: Stationary-phase cells, Alone | Stationary-phase cells, $+Co^{2+}$ | Stationary-phase cells, $+Mn^{2+}$ | Exponential-phase cells, Alone | Exponential-phase cells, $+Co^{2+}$ | Exponential-phase cells, $+Mn^{2+}$ |
|---|---|---|---|---|---|---|---|
| 1 | Pro-Gly (2.8) | 0.009 | 0.076 | 0.006 | n.d. | 0.012 | n.d. |
| 2 | Pro-Gly-Gly (2.0) | 0.012 | 0.075 | 0.008 | n.d. | 0.011 | n.d. |
| 3 | Lys-Lys (1.6) | n.d. | 0.007 | — | — | 0.004 | — |
| 4 | Lys-Lys-Lys (1.5) | 0.006 | 0.017 | — | — | 0.016 | — |
| 5 | Pro-Gly (1.6)+Lys-Lys (1.6) | — | 0.088 | — | — | 0.014 | — |
| 6 | Pro-Gly (1.6)+Lys-Lys-Lys (1.2) | — | 0.071 | — | — | 0.030 | — |
| 7 | Pro-Gly-Gly (1.6)+Lys-Lys-Lys (1.2) | — | 0.078 | — | — | 0.030 | — |
| 8 | Gly-Nle (0.8) | — | 0.065 | — | — | — | — |
| 9 | Pro-Phe (3.8) | — | 0.026 | — | — | — | — |
| 10 | Gly-Nle (0.8)+Pro-Phe (3.8) | — | 0.026 | — | — | — | — |
| 11 | Gly-Gly-Gly (2.5) | — | 0.136 | — | — | — | — |
| 12 | Pro-Phe-Lys (2.0) | — | 0.040 | — | — | — | — |
| 13 | Gly-Gly-Gly (2.5)+Pro-Phe-Lys (2.0) | — | 0.042 | — | — | — | — |

1953; Stone & Hoberman, 1953; Sarid *et al.* 1959), but in none of these studies was attention focused upon the transport of these compounds. With the observation that the peptide α-amino group plays an essential role in the transport process (Gilvarg & Katchalski, 1965; Losick & Gilvarg, 1966; Payne, 1971), I thought it important to see if the unique character of peptides in which proline forms the *N*-terminus affects their ability to use the general peptide-transport systems. Because of the difficulties in obtaining a diverse selection of radioactively labelled peptides with which to measure peptide transport directly, other means were used to explore the uptake process.

The transport-deficient TOR mutants used here were selected on the basis of their resistance to the toxic peptide triornithine. The present observation that several TOR auxotrophs are unable to utilize prolyl peptides for growth implies that these strains are unable to transport these peptides. That a single mutation can affect the uptake of prolyl oligopeptides and other oligopeptides implies that all these peptides share a common transport system. However, until this mutation is characterized it remains a possibility that the defect may be to some component that is common to several oligopeptide-transport systems, e.g. the energy coupling process, and that they may possess distinct components for handling structurally dissimilar oligopeptides. Such a hypothetical situation finds analogy in the transport of carbohydrates by bacteria (Roseman, 1969; Egan & Morse, 1965).

The demonstration that different peptides can compete with one another for entry to the cell provides further evidence that, in fact, a single transport system is used by various peptides. The present results indicate that prolyl di- and oligo-peptides can interfere with the uptake of peptides that possess other amino acids as their *N*-termini (Fig. 3), and that other peptides can competitively prevent the uptake of prolyl di- and oligo-peptides (Fig. 4). It should be emphasized that the competitive growth effects observed (Fig. 4) are specific, i.e. dipeptides only interfere with dipeptide uptake and oligopeptides only inhibit oligopeptide uptake. These results are in accord with the nature of the TOR mutation, which distinguishes between di- and oligo-peptides, and all previous studies, which suggest that separate systems are operative for the uptake of the two peptide classes (Payne & Gilvarg, 1971). The conclusion from these studies, that oligopeptides in which proline constitutes the *N*-terminal residue use the same uptake systems as other peptides, is in agreement with other studies indicating that peptides in which the primary α-amino group has been converted into a secondary amino group by methylation possess identical transport characteristics to the unsubstituted peptides (Payne, 1971).

The peptidase assays (Table 1) exclude the

possibility that the effects of lysine peptides on the growth of the proline auxotroph on prolyl peptides is caused by inhibition of cleavage of the prolyl peptides. We observed no inhibition of breakdown of prolylglycine by dilysine even at relative concentrations 100 times that at which growth is inhibited, although at a similar relative concentration trilysine does cause slight inhibition of cleavage of prolylglycylglycine. However, it also inhibits cleavage of prolylglycine, although no comparable effect can be observed in growth tests. Therefore, competition at the level of intracellular peptidases does not offer an adequate explanation for the competitive inhibitions seen in the growth studies.

Certain studies on the utilization of peptides of proline by *E. coli* have shown that exponentially growing cells lack peptidase activity able to split such peptides at a rate sufficient to support growth of a proline auxotroph, although stationary-phase cells are able to cleave these peptides at an adequate rate (Stone, 1953). Our findings with the K12 proline auxotroph EMG 29 do not endorse these findings; although stationary-phase cells do possess a higher peptidase activity (Table 1), an equally good growth response is observed with either exponential- or stationary-phase inocula. However, I did observe an analogous situation with a strain of *E. coli* W; thus, with exponential-phase inocula, the lysine auxotroph grew well on prolyl-phenylalanyl-lysine (Fig. 1) but the glycine auxotroph failed to grow on prolylglycylglycine (Fig. 2), although in the latter case growth could be achieved when stationary-phase inocula were used. With neither peptide is proline peptidase action apparently essential to release the required amino acid and it is probable that peptidases of different specificities are involved. In this case, the differential growth responses may be caused by the greater requirement of the cell for glycine than for lysine.

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