The Requirement for the Protonated α -Amino Group for the Transport of Peptides in *Escherichia coli*

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Many glycine peptides support growth of a glycine auxotroph of *Escherichia coli*. If the α -amino group of these peptides is methylated, the products are still utilized for growth, and also retain comparable ability with the unsubstituted peptides to compete with natural peptides for transport into the cell. In contrast, glycine peptides devoid of an α -amino group, or that have the α -amino group substituted by one of a number of acyl groups are not utilized, although *E. coli* possesses intracellular enzymic activity able to release glycine from such compounds; further, these derivatives do not compete with natural peptides for transport into the cell.

A number of studies have been carried out over the last few years into the process of peptide transport in bacteria (Payne & Gilvarg, 1971). In general, these studies have sought to establish which structural features of a peptide play a role in the transport process. The uptake of a peptide into a bacterium can be established from the ability of a peptide to support the growth of an appropriate amino acid auxotroph (Gilvarg & Katchalski, 1965). It was demonstrated that lysine and arginine homopeptides, for example, could support the growth of the respective *Escherichia coli* auxotrophs, but that nutritional effectiveness ceased abruptly beyond the tetrapeptides (Gilvarg & Katchalski, 1965). With other homologous oligopeptides, analogous observations were made that nutritional effectiveness was lost by higher oligomers (Gilvarg & Katchalski, 1965; Payne & Gilvarg, 1968a; Payne, 1968). The structural parameter responsible for the loss of nutritional efficiency was shown to be the overall volume of the peptide (Payne & Gilvarg, 1968b). Further studies have shown that separate transport systems exist for the uptake of dipeptides and of oligopeptides (Payne & Gilvarg, 1968a; Payne, 1968), which differ with respect to their terminal carboxyl-group requirements. Whereas a dipeptide must possess an unsubstituted terminal carboxyl group to be transported, oligopeptides with the terminal carboxyl group substituted or even completely absent are still readily taken up (Payne & Gilvarg, 1968a). In contrast, the presence of an unsubstituted α -amino group is important for the transport of both di- and oligo-peptides (Gilvarg & Katchalski, 1965; Losick & Gilvarg, 1966). This conclusion was based on the observation that

acetylation of the α -amino group of lysine and arginine homopeptides effectively destroyed their nutritional capacity. However, acetylation not only substitutes the α -amino group but also removes its positive charge. It appears of some importance therefore, before any mechanism of peptide transport can be proposed, to assess the importance or otherwise of this positive charge on the α -amino group.

In the present study, we describe the relative abilities of glycine oligopeptides and α -N-methylglycine oligopeptides (sarcosine peptides) to enter *E. coli*; N-methylation leaves intact the positive charge on the amino group. Further, to substantiate the generality of the earlier conclusions based on the α -N-acetylated lysine and arginine peptides we have prepared a variety of α -N-acylglycine peptides and have tested them for their ability to enter *E. coli*.

MATERIALS

Oligopeptides. Lysine and ornithine homo-oligopeptides were prepared by the controlled polymerization of ϵ -N-benzyloxycarbonyl-lysine-N-carboxylic anhydride (Bergman, Zervas & Ross, 1935) and δ -N-benzyloxycarbonylornithine-N-carboxylic anhydride in a manner analogous to that described by Payne & Gilvarg (1968a). The individual oligomers were selectively eluted from a column of CM-cellulose (Na⁺ form) with a gradient of exponentially increasing salt concentration by the procedure of Stewart & Stahmann (1962). Di- and tri-glycine were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. All other peptides were purchased from Cyclo Chemical Corp., through Baxter Laboratories, Thetford, Norfolk, U.K., or from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

Table 1. *Characterization of N-acyl derivatives*

Electrophoretic mobilities are relative to aspartic acid (1.0) at pH 6.5, and serine (1.0) at pH 2.1, measured from the neutral band. The net charge is that expected from the nature of each particular acyl substituent. Molecular weights are estimated on the basis of the electrophoretic mobility and net charge by the method of Offord (1966). Abbreviations are as follows: Suc, succinyl; Glt, glutaryl; Mal, maleyl; Cit, citraconyl; Ac, acetyl; Sarc, sarcosyl; n.d., not determined.

Peptide	Net charge at pH 6.5	Electrophoretic mobility		Estimated molecular weight	Theoretical molecular weight
		pH 6.5	pH 2.1		
Suc-Gly	-2	1.49	n.d.	189	185
Suc-Gly ₂	-2	1.27	n.d.	240	242
Suc-Gly ₃	-2	1.15	n.d.	285	299
Glt-Gly	-2	1.40	n.d.	202	199
Glt-Gly ₂	-2	1.24	n.d.	250	256
Glt-Gly ₃	-2	1.12	n.d.	293	313
Mal-Gly ₂	-2	1.30	n.d.	232	258
Mal-Gly ₃	-2	1.17	n.d.	275	315
Cit-Gly ₂	-2	1.23	n.d.	254	272
Cit-Gly ₃	-2	1.11	n.d.	300	329
Ac-Gly ₂	-1	0.89	n.d.	160	174
Ac-Gly ₃	-1	0.74	n.d.	212	231
Sarc-Gly ₂	0	0.00	1.33	138	146
Sarc-Gly ₃	0	0.00	1.15	190	203
Sarc-Ala	0	0.00	1.24	160	160
Sarc-Ser	0	0.00	1.15	189	176
Gly-Sarc	0	0.00	1.27	153	146
Gly ₂	0	0.00	1.40	120	132
Gly ₃	0	0.00	1.20	176	189

N-Substituted glycine oligopeptides. Standard procedures are usually employed for the acylation of proteins by using several common acid anhydrides (Riordan & Vallee, 1967; Klotz, 1967). However, for the acylation of simple glycine peptides the lower pK_a of the α -amino group (about 7.2) in the glycine oligopeptides compared with that of an ϵ -amino group (about 10.3) allows the reaction to be readily carried out at neutral pH, thus decreasing the spontaneous hydrolysis of the acid anhydride (Klotz, 1967). The α -*N*-acyl derivatives of glycine oligopeptides were prepared as follows. The glycine substrate was treated with the appropriate anhydride at pH 7.1 by using a Radiometer Titrimeter (Copenhagen, Denmark) in its pH-stat mode. Glycine (160 mg, 2.13 mmol), diglycine (300 mg, 2.28 mmol) or triglycine (400 mg, 2.10 mmol) was dissolved in 1 M-NaHCO₃ (5.0 ml) in the titration cup of the titrimeter and the pH was adjusted to 7.1. The anhydride (2.5 mmol) was added in a single amount to the stirred solution at room temperature. The pH was maintained at 7.1 by the automatic addition of 10 M-NaOH. The reaction was rapid and in all cases was judged to be complete (addition of base ceased) after about 20 min. The pH of the final reaction mixture was adjusted to 7.5 before storage at -10°C. These solutions were diluted as required and were used directly for bacterial growth studies. The anhydrides used were acetic anhydride, maleic anhydride, 2-methylmaleic anhydride (citraconyl anhydride) obtained from British Drug Houses Ltd., and succinic anhydride and glutaric anhydride purchased from Ralph N. Emanuel Ltd., London S.E.1, U.K. Glutaric anhydride was recrystallized before use. The

N-acyl derivatives were characterized by high-voltage paper electrophoresis by using a Shandon flat-plate apparatus with a buffer (pH 6.5) containing pyridine (8%, v/v) and acetic acid (0.4%, v/v). At this pH value the glycine substrates are neutral, whereas the *N*-acyl derivatives each possesses an overall negative charge. Electrophoretograms were developed with cadmium-ninhydrin reagent (Heilmann, Barrolier & Watzke, 1957), followed by chlorine-tolidine (Von Aux & Neher, 1963). The initial colour reaction with cadmium-ninhydrin showed the complete absence from the neutral band of any of the glycine starting materials, except for the citraconyl derivatives, which contained a few per cent of the free peptide; the citraconyl group is the most acid-labile of the acyl derivatives and the free peptides probably arose through deacylation (Dixon & Perham, 1968). The acetyl, succinyl and glutaryl derivatives are all ninhydrin-negative, a fact that is used as a test for complete acylation (Klotz, 1967). The maleyl, and in particular the citraconyl, derivatives gave a yellow colour with cadmium-ninhydrin, characteristic of peptides with unsubstituted *N*-terminal glycine. However, we believe that this may be attributed to deacylation of these acid-labile substituents during running and subsequent drying of the electrophoretograms, for the ninhydrin colour response was considerably greater if heat was used. All the *N*-acyl derivatives were readily detected as single spots on the electrophoretograms by chlorine-tolidine. By the method of Offord (1966), it is possible to estimate a value for the molecular weight of each species from the observed mobilities of the *N*-acyl derivatives and from the net charge on each derivative.

The results of such calculations are shown in Table 1 and confirm that the observed products are indeed the expected *N*-acyl derivatives. Glycine and sarcosine peptides were characterized by high-voltage electrophoresis at pH 2.1 in acetic acid (8%, v/v) and formic acid (2%, v/v). The α -*N*-methyl compounds are ninhydrin-positive, but yield a purple colour which for full development takes somewhat longer at 105°C than for the unsubstituted glycine peptides, which stain yellow. Each gave a single spot with cadmium-ninhydrin with the expected electrophoretic mobility.

METHODS

Bacterial strains. The following strains of *E. coli* W (A.T.C.C. 9637) were used. Strain M-26-26 is a lysine auxotroph that lacks the decarboxylase that converts *meso*-diaminopimelic acid into L-lysine (Davis, 1952; Dewey & Work, 1952). Strain M-26-26.R is a spontaneous non-auxotrophic revertant of strain M-26-26 corresponding to the wild-type strain. Strain M-123 is a glycine-serine auxotroph derived from strain M-22-93, originally obtained from Dr W. 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} in a Bausch and Lomb Spectronic 20 instrument. Inocula (0.1–0.2 ml) were removed from a culture growing exponentially on minimal medium and 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 AS013, a lysine auxotroph derived from strain 2001c, F⁻, Thr⁻, Leu⁻, thiamin⁻, was kindly provided by Dr C. Gilvarg. This strain was grown in minimal medium M-56 (Weismeyer & Cohn, 1960) supplemented with 0.5% glucose; the required amino acids were provided at 50 mg/litre and thiamin at 5 mg/litre.

Peptidase assay. To prepare cell-free extracts, bacteria were grown in minimal medium, harvested in exponential phase, washed once with minimal medium A and then disrupted for 10 min in a Braun cell disintegrator. The extract was separated from cell debris by centrifugation at 15000g for 50 min. Protein concentration was determined by the method of Warburg & Christian (1941). Peptide cleavage was measured in an incubation mixture containing 50 mM-potassium phosphate buffer, pH 7.1, crude extract corresponding to 0.5–1.0 mg of protein, and peptide or peptides in a total volume of 1.2 ml. When required, Co²⁺, Zn²⁺, Mg²⁺ and Mn²⁺ were each added to give a final concentration of 0.5 mM. Reaction mixtures were incubated at 37°C, samples were removed periodically, and the peptidase action was stopped by delivering into HCl with lysine peptides, or by cooling in ice-water, with other peptides. Cleavage of lysine peptides was followed by the ninhydrin method of Shimura & Vogel (1966), which is specific for lysine and gives no colour with lysine peptides. Cleavage of glycine peptides was assayed by the liberation of ninhydrin-positive material (α -amino groups) by using the procedure of Moore & Stein (1948). By this procedure, the absorption at zero time was identical for the *N*-acyl derivatives tested and for the control containing protein alone, confirming that these *N*-acyl peptides are not contaminated with the unsubstituted parent peptides.

We occasionally made use of the decrease in E_{220} that is

a consequence of the cleavage of the peptide bond, as a preliminary procedure to assess rapidly the peptidase activity in any incubation mixture. Extinction changes were automatically recorded with a Gilford model 2000 recording spectrophotometer. This assay is extremely simple, and with appropriate controls, lends itself readily to determination of the usual parameters of an enzymic reaction. Samples of this same incubation mixture may subsequently be used for standard colorimetric assays. In confirmation of peptidase action the products of these reactions were characterized by high-voltage electrophoresis as described above.

RESULTS

The *N*-acyl derivatives of glycine were synthesized to assess the importance of the α -amino group of a peptide to the bacterial utilization of peptides. Some of these compounds can be considered to be either *N*-acyl peptide derivatives or peptides that lack an α -amino group. Thus, an α -acetyl substituted amino acid or peptide may equally well be regarded as a glycyl peptide devoid of its α -amino group. Similarly, α -propionyl, α -succinyl and α -glutaryl derivatives respectively may be considered as alanyl, aspartyl and glutamyl peptides devoid of α -amino groups. In interpreting the results of the bacterial utilization of these *N*-acyl compounds it is occasionally helpful to consider that some *N*-acyl derivatives are peptides without their α -amino groups.

Growth response of glycine auxotroph to free and N-substituted glycine peptides

In earlier studies it was shown that the homo-oligopeptides up to and including hexaglycine could act as a source of glycine for the glycine auxotroph M-123 (Payne, 1968). However, in the present study when the *N*-acyl compounds were tested at concentrations up to 3.0 μ mol/ml, that is about six times the concentration at which glycine oligopeptides can sustain exponential growth, no growth was observed. These *N*-acyl derivatives do not inhibit growth, for at 3.0 μ mol/ml they are without effect on the growth of the wild-type strain or on the growth of auxotroph M-123 on media supplemented with glycine. Slow linear growth was observed with the citraconyl derivatives, but this was compatible with that expected from their content of deacylated contaminants. However, α -glutamylglycine and α -aspartylglycine each at 0.8 μ mol/ml supported exponential growth of strain M-123, whereas neither of the corresponding peptides that lack the α -amino group, that is, *N*-glutaryl- and *N*-succinyl-glycine, was utilized. Similarly, α -*N*-acetyldiglycine and α -*N*-acetyltri-glycine did not support growth although they are the analogues of tri- and tetra-glycine respectively,

both of which allow exponential growth (Payne, 1968).

When sarcosylglycine and sarcosyldiglycine were tested at concentrations up to $5.0\mu\text{mol/ml}$ very slow growth of auxotroph M-123 was observed, with a minimum generation time of 120 min; the rate of growth was not faster with higher concentrations of these peptides. With sarcosylalanine and glycylsarcosine no growth was observed after incubation for 8 h. None of these compounds was inhibitory at this concentration to the growth of the wild type or to the growth of auxotroph M-123 in media supplemented with glycine. Sarcosine itself ($1.5\mu\text{mol/ml}$) is unable to support growth of the glycine auxotroph; it is also without inhibitory effect, although certain other α -N-methyl-amino acids are inhibitory to *E. coli* (Fowden, Lewis & Tristram, 1967). However, sarcosylserine was found to support exponential growth of auxotroph M-123, from which it must be concluded that *E. coli* possesses the means to concentrate and to cleave α -N-methyl-peptides.

The results of the above growth tests indicate that peptides that either lack an α -amino group, or that have this group substituted in certain ways cannot be utilized by *E. coli*. The question that remains is whether this arises because these compounds are immune to intracellular peptidase action, or because they are unable to enter the cell via the peptide transport systems. To decide between these alternatives we have carried out the following tests.

Enzymic cleavage of glycine peptides and N-substituted glycine peptides

Cell extracts were prepared and peptidase assays were carried out by using glycine and N-substituted glycine peptides as described in the Methods

section. Crude extracts contain enzymic activity able to cleave all peptides tested, with the exception of glycylsarcosine, for which we could detect no cleavage (Table 2). Thus, since the cell contains enzymes able to release glycine from the non-utilizable N-acyl peptides as easily as from the utilizable unsubstituted peptides, it implies that the N-acyl derivatives are unable to enter the cell. On the other hand, the very slow growth obtained with sarcosylglycine and sarcosyldiglycine may stem from their lower rates of cleavage (about one-third that of the unsubstituted peptides) which may supply glycine at a rate less than the critical rate required to sustain exponential growth. At first sight the fact that sarcosylserine is also cleaved at a slow rate but nevertheless supports exponential growth of auxotroph M-123 appears to invalidate the above argument, but it should be remembered that auxotroph M-123 requires either glycine or serine for growth, and serine appears to be nutritionally superior; indeed the rate of growth on glycylserine is considerably faster than that of glycylglycine. Thus, the slow cleavage of sarcosylserine may nevertheless provide a superior nutrient at a rate adequate for exponential growth. In view of the importance of cations to the activity of a number of peptidases (Simmonds, 1970; Sussman & Gilvarg, 1971), the assays were repeated in the presence of various cations (Table 2). Of those tested, Co^{2+} had the greatest effect, producing about a tenfold stimulation of cleavage of the unsubstituted and the sarcosyl peptides but no stimulation with glycylsarcosine or the N-acyl derivatives.

Competitive effects of N-substituted peptides

Competition that overcomes growth inhibition. We tested the ability of the various peptide derivatives

Table 2. *Cleavage of glycine peptides by crude extracts and by cation-supplemented extracts of E. coli W*

Cell extracts were prepared, and peptidase assays were carried out as described in the Methods section. Results are expressed as μmol of α -amino groups (glycine) released/min per mg of protein per ml. For abbreviations see Table 1.

Peptide	Cleavage of peptide				
	Crude extract alone	Crude extract supplemented with			
		Co^{2+}	Mn^{2+}	Zn^{2+}	Mg^{2+}
Gly ₂	0.021	0.121	0.008	0.003	0.026
Gly ₃	0.015	0.171	0.044	0.016	0.017
Sarc-Gly	0.006	0.134	0.004	0.003	0.005
Sarc-Gly ₂	0.008	0.115	0.010	0.010	0.004
Sarc-Ser	0.005	0.200	0.009	n.d.	n.d.
Gly-Sarc	0.000	0.000	0.000	n.d.	n.d.
Ac-Gly ₂	0.021	0.014	n.d.	n.d.	n.d.
Ac-Gly ₃	0.018	0.012	n.d.	n.d.	n.d.
Glt-Gly ₃	0.021	0.013	n.d.	n.d.	n.d.
Suc-Gly ₃	0.014	n.d.	n.d.	n.d.	n.d.

Fig. 1. Ability of glycine peptides to overcome the inhibitory effect of triornithine on the growth of wild-type *E. coli* W. Except where indicated, triornithine was present at a concentration of $0.04\mu\text{mol/ml}$. Glycine, diglycine and *N*-methyldiglycine were tested at concentrations up to $0.5\mu\text{mol/ml}$. *N*-Succinyl- and *N*-glutaryl-glycine, *N*-acetyl-, *N*-succinyl- and *N*-glutaryl-diglycine, *N*-acetyl-, *N*-succinyl-, *N*-glutaryl- and *N*-maleyl-triglycine were tested at concentrations up to $1.4\mu\text{mol/ml}$. $\circ$, No triornithine; $\bullet$, + triglycine ($0.033\mu\text{mol/ml}$); $\blacksquare$, + *N*-methyltriglycine ($0.033\mu\text{mol/ml}$); $\blacktriangle$, + *N*-methyltriglycine ($0.016\mu\text{mol/ml}$); $\triangle$, + triornithine alone or + *N*-acylglycine peptides, *N*-methyldiglycine, diglycine or glycine.

to use the peptide transport systems by measuring their competitive efficiency in limiting the entry of various unsubstituted peptides. Triornithine has been shown to be extremely toxic to *E. coli* (Payne, 1968). The exact mechanism of its toxicity is still unclear, but it results from the build-up of a high intracellular concentration caused by the absence of peptidases able to cleave the tripeptide (Payne, 1968; Sussman & Gilvarg, 1970). Thus, if triornithine can be prevented from entering the cell, growth may be achieved.

Fig. 1 shows that an approximately equimolar concentration of triglycine can virtually overcome the inhibitory effect of triornithine, and a family of growth curves may be obtained by using graded concentrations of triglycine, $0.15\mu\text{mol/ml}$ completely reversing the inhibition caused by $0.04\mu\text{mol}$ of triornithine/ml. *N*-Methyltriglycine also overcomes triornithine inhibition (Fig. 1), suggesting that the transport system does not distinguish between a peptide with a free α -amino group and one with a methylated α -amino group. The reversal of inhibition is not attributable to the glycine moiety.

At comparable concentrations neither the free amino acid nor diglycine are effective, a result to be expected from the distinct modes of uptake employed by amino acids, dipeptides and oligopeptides (Payne & Gilvarg, 1971; Payne, 1968). In further studies it was shown that the *N*-acyl peptides afforded no protection against inhibition even at a concentration 100 times that at which *N*-methyltriglycine was effective.

Norleucine and norvaline both inhibit the growth of *E. coli* and peptides containing these amino acids are inhibitory if they can enter the cell and be cleaved. The peptides norleucylnorvaline, glycyl-norvaline, alanyl-norvaline, glycyl-norleucine, glycyl-glycyl-norleucine and norvalylglycylglycine were all inhibitory to various extents. In confirmation of the fact that these oligopeptides containing unnatural amino acids use the peptide transport system to enter the cell we found that a mutant unable to transport oligopeptides (Payne, 1968), is not inhibited by these tripeptides. It was therefore possible, in a manner analogous to the triornithine experiments, to test whether the *N*-substituted glycine peptides are able to use the transport system by measuring their ability to overcome the inhibitory effect of the norvaline and norleucine peptides. The results (Fig. 2) were analogous to those observed with triornithine. Triglycine and *N*-methyltriglycine are almost equally efficient in restoring growth whereas the *N*-acyl derivatives, even at much higher concentrations, afford no protection. Similarly, glycine is devoid of competitive ability. A noteworthy case of competition of a type distinct from that concerned with transport is possible in this system, for it should be recalled that norleucine toxicity arises from its interference with the functions of methionine (Fowden *et al.* 1967), and that its toxic effect can be overcome by the addition of methionine to the growth medium. We confirmed that the inhibitory effect of glycyl-norleucine and glycyl-glycyl-norleucine (each at $1.0\mu\text{mol/ml}$) can be reversed by adding methionine ($1.5\mu\text{mol/ml}$) to the media. Further, the inhibitory effect of norleucine itself can be reversed non-specifically by leucine, presumably because the two amino acids share a common permease and leucine can therefore competitively prevent the uptake of norleucine. However, with glycyl-norleucine or glycylglycyl-norleucine (each at $1.0\mu\text{mol/ml}$), leucine ($4.0\mu\text{mol/ml}$) lacked any ability to reverse inhibition, in accord with the distinct transport systems used by amino acids and peptides.

It is noteworthy that the inhibition observed with glycylglycyl-norleucine at high concentrations (above $0.25\mu\text{mol/ml}$) cannot be completely reversed by increased concentrations of peptide competitors. The result accords with an earlier finding

Fig. 2. Ability of glycine peptides to overcome the inhibitory effect of glycylglycylnorleucine in auxotroph M-26-26. Except where indicated, glycylglycylnorleucine was present at a concentration of $0.082 \mu\text{mol/ml}$. Glycine, diglycine and *N*-methyldiglycine were tested at concentrations up to $2.0 \mu\text{mol/ml}$. *N*-Succinyl- and *N*-glutaryl-glycine, *N*-acetyl-, *N*-succinyl- and *N*-glutaryl-diglycine, and *N*-acetyl-, *N*-succinyl- and *N*-maleyl-triglycine were tested at concentrations up to $3.0 \mu\text{mol/ml}$. $\circ$, No glycylglycylnorleucine; $\blacksquare$, + triglycine ($0.82 \mu\text{mol/ml}$); $\square$, + *N*-methyltriglycine ($0.82 \mu\text{mol/ml}$); $\triangle$, + *N*-methyltriglycine ($0.33 \mu\text{mol/ml}$); $\bullet$, glycylglycylnorleucine alone or + *N*-acetyl-glycine peptides, *N*-methyldiglycine, diglycine or glycine.

with peptide-transport-deficient mutants, that in addition to entry via a specific peptide-transport system, neutral peptides can enter *E. coli* by a concentration-dependent mode that is insensitive to competition and is perhaps a diffusive process. Presumably, at high concentrations glycylglycylnorleucine can enter the cell by this secondary process at a rate sufficient to provide an inhibitory internal concentration of norleucine.

Diglycine did afford slight protection at very high molar ratios relative to glycylglycylnorleucine, in agreement with the previous findings that dipeptides possess limited ability to use the oligopeptide transport system (Payne & Gilvarg, 1971). We attempted to use this test system to measure the ability of glycine dipeptides to overcome the inhibition caused by glycylglycylnorleucine and by norleucyl-norvaline, but in neither case could we demonstrate any effect at competitor/inhibitor molar ratios up to 10:1. However, this may simply reflect a more complicated process of dipeptide uptake that may involve several systems.

Fig. 3. Ability of glycine peptides to reverse the inhibitory effect of valine peptides on *E. coli* K12 strain AS013. To preserve clarity, tests with the *N*-acyl peptides are not shown, but these were performed as follows: *N*-succinyl- and *N*-glutaryl-glycine, and *N*-acetyl-, *N*-succinyl- and *N*-glutaryl-diglycine were used at concentrations up to $20 \mu\text{mol/ml}$ in the presence of glycylvaline. *N*-Acetyl-, *N*-succinyl-, *N*-glutaryl- and *N*-maleyl-triglycine were tested at concentrations up to $20 \mu\text{mol/ml}$ in the presence of valyldiglycine. The results in each case paralleled those obtained with the valine peptides alone. $\circ$, No glycylvaline or valyldiglycine; $\blacksquare$, valyldiglycine ($0.4 \mu\text{mol/ml}$) + triglycine ($20 \mu\text{mol/ml}$) or + *N*-methyltriglycine ($20 \mu\text{mol/ml}$); $\bullet$, + glycylvaline ($0.4 \mu\text{mol/ml}$), + diglycine ($10 \mu\text{mol/ml}$) or + *N*-methyldiglycine ($10 \mu\text{mol/ml}$); $\blacktriangle$, + glycylvaline ($0.4 \mu\text{mol/ml}$) or valyldiglycine ($0.4 \mu\text{mol/ml}$).

In an effort to obtain further evidence for the importance of the positively charged α -amino group another strain of *E. coli* was examined. *E. coli* K12 possesses an unusual sensitivity to valine (Leavitt & Umbarger, 1962) and to valyl peptides (A. J. Sussman & C. Gilvarg, personal communication). The following valyl compounds completely inhibited growth of strain AS013 in our standard test culture, valine ($0.4 \mu\text{mol/ml}$), glycylvaline ($0.6 \mu\text{mol/ml}$), valylglycine ($0.4 \mu\text{mol/ml}$), trivaline ($0.1 \mu\text{mol/ml}$), valylglycylglycine ($0.5 \mu\text{mol/ml}$). The effects of peptides on the inhibition of growth by valine peptides were therefore examined. Diglycine and sarcosylglycine are indistinguishable in their ability to overcome glycylvaline inhibition, and similarly triglycine and sarcosyldiglycine are equally efficient in reversing valylglycylglycine

inhibition (Fig. 3). Further, the observations are specific for these combinations, for at these same concentrations, neither diglycine nor sarcosylglycine affect inhibition by valylglycylglycine, and triglycine and sarcosyldiglycine do not affect the inhibition by glycylvaline. The competitors do not relieve the inhibition by valine itself. In accord with all other studies we found the *N*-acyl peptides to be devoid of competitive activity in this test system.

Competition that leads to growth inhibition. In contrast with the above situation in which competition between oligopeptides for uptake can curtail the entry of toxic peptides and thus enhance growth, it should be possible by competitively inhibiting the transport of a peptide containing a required amino acid to prevent growth of an auxotrophic strain.

The growth of the lysine auxotroph on trylisine can be competitively inhibited by the addition of triglycine or *N*-methyltriglycine to the growth media (Fig. 4). However, trylisine/triglycine molar ratios of about 1:50 are required before this inhibition is detectable. In contrast, no specific inhibition of growth on trylisine was observed with any of the *N*-acyl compounds when tested at molar ratios of up to 300:1 relative to trylisine. Further, as expected, neither glycine itself nor dipeptides of glycine inhibited growth on trylisine.

Absence of competitive peptidase activity. A possible objection to the interpretation of the above competition studies is that certain of the peptides may compete with one another for cleavage by intracellular peptidases. The negative results with the *N*-acyl peptides may simply reflect the absence of a competitive effect on peptidases unlike the unsubstituted and the *N*-methylated peptides. However, to accommodate this explanation, the varied nature of the above tests requires that the active competitors be capable of a variety of effects on intracellular peptidases. Thus, to overcome triornithine inhibition, triglycine and *N*-methyltriglycine must activate or induce peptidase activity to cleave triornithine. There is considerable evidence to indicate that peptidases are constitutive and are not induced by growth in the presence of peptides (Sussman & Gilvarg, 1970, 1971; Vogt, 1970), and further, it has been shown that the peptidase(s) activity responsible for cleaving triornithine is distinct from that which cleaves glycine peptides (Payne, 1968; Sussman & Gilvarg, 1970). In addition, triglycine neither activates nor inhibits the peptidase activity that cleaves triornithine in a crude extract of *E. coli*.

In contrast with the situation with triornithine, the effects observed with the peptides containing norleucine or valine (Figs. 2 and 3) require that the peptide competitors inhibit the cleavage of the toxic peptides, for in each case it is the free amino acid

Fig. 4. Ability of glycine peptides to inhibit growth of lysine auxotroph M-26-26 on trylisine. Except where indicated trylisine was present at a concentration of $0.04 \mu\text{mol/ml}$. Glycine, diglycine and *N*-methylglycine were tested at concentrations up to $8.5 \mu\text{mol/ml}$. *N*-Acetyl-, *N*-succinyl-, *N*-glutaryl- and *N*-maleyl derivatives of diglycine and triglycine were tested at concentrations up to $14 \mu\text{mol/ml}$. $\circ$, Trylisine alone, or + glycine, diglycine, *N*-methylglycine, or *N*-acyl peptides; $\square$, + *N*-methyltriglycine ($1.6 \mu\text{mol/ml}$); $\blacksquare$, + *N*-methyltriglycine ($3.2 \mu\text{mol/ml}$); $\blacktriangle$, + triglycine ($3.2 \mu\text{mol/ml}$); $\bullet$, no trylisine.

that is the toxic moiety. Similarly, tests in which peptide-dependent auxotrophic growth is inhibited (Fig. 4) demand that the competitors inhibit cleavage of trylisine. Earlier studies indicated that the cleavage of trylisine by crude extracts of *E. coli* is unaffected by relative concentrations of triglycine ten times that which inhibits growth on trylisine (Payne, 1968). We have now extended these studies, and by using the Shimura & Vogel (1966) assay for lysine, find that the cleavage of trylisine by crude extracts is unaffected by either triglycine or by *N*-methyltriglycine at molar ratios that cause inhibition of growth on trylisine. Cleavage of dilysine is similarly unaffected by the presence of diglycine or *N*-methylglycine in the incubation mixture.

DISCUSSION

The experiments reported above were designed to investigate the function of the α -amino group in the process of peptide transport in *E. coli*. Previous studies showed that α -acetylation of lysine and arginine oligopeptides rendered them nutritionally

ineffective for lysine and arginine auxotrophs (Gilvarg & Katchalski, 1965; Losick & Gilvarg, 1966). In this case, the auxotrophs possessed peptidases able to cleave the acetylated peptides and the discrimination against these compounds was considered to arise from their inability to enter the cell. Simmonds and co-workers showed that the α -benzyloxycarbonyl derivatives of glycyl-phenylalanine, glycyltyrosine and prolylproline were much inferior to the free peptides in supporting the growth of phenylalanine, tyrosine (Simmonds, Tatum & Fruton, 1947), and proline auxotrophs (Simmonds & Fruton, 1948) respectively. The proline mutant was also unable to utilize α -N-acetylphenylalanylproline for growth (Simmonds & Fruton, 1948). Dunn & Dittmer (1951) reported that a number of peptides containing β -2-thienylalanine possessed inhibitory properties for *E. coli* equal to the free amino acid, but that the corresponding α -N-benzyloxycarbonyl peptides were about 1000 times less inhibitory. Unfortunately, in most of the above studies it was not demonstrated whether the cell could cleave the α -N-acyl peptides with intracellular peptidases and it is impossible to decide whether the distinction arises at the level of uptake or cleavage.

In an attempt to gain further insight into the function of the α -amino group we prepared a series of substituted peptides with different amino substituents. Thus, α -N-acetyl-, α -N-succinyl- and α -N-glutaryl-glycine peptides may be regarded as N-glycyl, N-aspartyl and N-glutamyl peptides devoid of α -amino groups; free glycine peptides and N-methylglycine peptides both possess a positively charged α -amino group; α -N-acetylglycine peptides have no positive charge, and in α -N-maleyl-, α -N-citraconyl-, α -N-succinyl- and α -N-glutaryl-glycine peptides the substituted α -amino group has acquired an overall negative charge.

Since sarcosylserine supports exponential growth of a glycine-serine auxotroph, and cells lack extracellular peptidase activity (Sussman & Gilvarg, 1971) an α -N-methylated peptide can evidently be accumulated and broken down by *E. coli*. The other N-methyl derivatives may be unable to support growth because *E. coli* lacks peptidase activity able to split these compounds at a sufficient rate. As crude extracts readily split the N-acyl derivatives, the nutritional discrimination against these compounds may result from their limited uptake. The effect of cations on peptidase activity was noteworthy. Co^{2+} activated cleavage of the free peptides and the N-methyl compounds by peptidases; no other cations tested enhanced peptidase action, and in certain cases inhibition was observed, although optimum conditions of pH and cation concentration were not explored. Therefore, if the Co^{2+} -sensitive peptidase(s) are fully active in the

growing bacterium the results could explain the lack of growth of the N-acyl derivatives (although presenting difficulties of interpretation for the N-methyl derivatives). However, exponential- and stationary-phase cells frequently differ in peptidase activity and it is not possible to decide from measurements of the peptidase activity of broken cells whether or not a peptide might be expected to support growth (Simmonds, 1970). Certainly, the simple observation that a cell extract contains activity able to cleave a peptide does not mean that the same peptide will support growth of an appropriate auxotroph.

Co^{2+} caused no stimulation in the cleavage of the N-acyl derivatives or of glycylsarcosine, and electrophoretic studies on the initial cleavage products of sarcosyl diglycine in the presence of Co^{2+} revealed glycine but not diglycine, suggesting that the enzyme is unlikely to be an aminopeptidase. Other Co^{2+} -activated peptidases have been reported (Sussman & Gilvarg, 1970, 1971).

In spite of the lack of growth in certain cases, there can be little doubt that N-methyl peptides can use the peptide transport system, for in every competitive test they were comparable in activity with the corresponding unsubstituted peptide. The diverse nature of the growth-competition experiments, and the direct tests that indicated the absence of any effects on peptidase activity, leave inhibition of peptide uptake as the most probable explanation for the results of the competitive tests. In contrast with peptides, the N-methylation of certain amino acids prevents their uptake by bacteria (Britten & McClure, 1962), although several of the amino acid transport systems in mammalian cells can tolerate α -N-methylation (Christensen & Handlogten, 1968). The fact that none of the N-acyl derivatives permits growth of the glycine auxotroph although the cells possess the enzymic machinery able to release glycine from these compounds suggests that they cannot enter the cell. The complete absence of competitive action with these compounds accords with this conclusion. It therefore appears that the peptide transport systems of *E. coli* can tolerate substitution of a peptide α -amino group provided that this can be achieved with retention of the positive charge. For this interpretation, it is necessarily assumed that neither steric effects nor overall negative charge are responsible for the inactivity of the N-acyl derivatives studied here but further studies are needed to substantiate this point, the most pertinent perhaps, being the utilizability of peptides with various α -N-alkyl substituents.

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