The attachment of 4-[N-(2-mercaptoethyl)aminopyridine-2,6-dicarboxylic acid] to AlaAlaCys through a disulfide bond to the cysteine residue has been described (Boehm, J. C., Kingsbury, W. D., Perry, D., and Gilvarg, C. (1983) J. Biol. Chem. 258, 14850–14855). The peptide disulfide showed enhanced growth inhibitory properties in Escherichia coli compared to the free sulfhydryl compound. Genetic evidence was presented to show that this side chain-modified peptide utilizes the oligopeptide transport system to gain entry to the cell. Following transport of the peptide, MEPDA is liberated by disulfide exchange reactions with sulfhydryl-containing components of the cell pool. In this paper, we examine in more detail the metabolism of this peptide. Using gel filtration chromatography to examine filtrates from cell suspensions incubated with the peptide, it was shown that loss of the peptide from the medium is accompanied by a corresponding increase in a component having the properties of MEPDA. The release of sulfhydryl groups from the peptide by cell suspensions could be monitored by Ellman’s reagent and was found to be dependent upon peptide transport. Following cleavage of the disulfide bond, MEPDA is able to cross the cytoplasmic membrane and exit from the cell as a relatively lipophilic uncharged metal chelate.

The di- and oligopeptide transport systems of Escherichia coli have been shown to exhibit a lack of side chain specificity (reviewed by Payne and Gilvarg, 1978). Part of the evidence for this conclusion was the demonstration of the ability of the oligopeptide transport system to effect the passage of normally impermeant amino acid analogues across the cytoplasmic membrane when they were incorporated into a peptide (Fickel and Gilvarg, 1973; Ames et al., 1973). Since then, numerous natural and synthetic examples of both di- and tripeptides which elaborate this phenomenon (for which several terms have been suggested, the one which we shall use being “portage transport” (Gilvarg, 1981)) have been reported.

We are interested in extending the variety of molecules which can be transported via peptide uptake systems, and in the previous paper (Boehm et al., 1983), we describe the first example of portage transport involving the attachment of compounds of interest to an amino acid side chain of a di- or tripeptide. A convenient method for the attachment of sulfhydryl-containing compounds to a cysteine residue of a peptide by a disulfide bond was described. It was suggested that such peptides are transported across the cytoplasmic membrane by the appropriate transport system, after which the sulfhydryl compound is released from the cysteine residue by disulfide exchange reactions with the high concentration of free sulfhydryl compounds normally present in the cell pool. 4-[N-(2-Mercaptoethyl)aminopyridine-2,6-dicarboxylic acid was selected as a candidate for portage transport by this method. This compound was a very weak inhibitor of the growth of E. coli, but its mixed disulfide cysteinylation peptide derivatives were much more efficient growth inhibitors. The experiments described in this paper examine the metabolism of one of these toxic peptides in detail and provide an explanation for its enhanced toxicity.

EXPERIMENTAL PROCEDURES

Materials—1-alamyl-1-alamyl-β-l-alamyl-2-pyridyl disulfide was a generous gift from Dr. W. D. Kingsbury of Smith, Kline and French Laboratories. AlaAlaCys-MEPDA was synthesized from these reagents as described (Boehm et al., 1983) except that the initial purification by Sephadex G-10 (Pharmacia) chromatography used acetate buffer (10 mM, pH 4) containing 1 mM EDTA. Salts and EDTA were removed by gel filtration on the same column using water as eluent. Carbonyl cyanide m-chlorophenylhydrazone, Ala, Ala, Gly, and Gly2 were from Sigma. DTNB was from Pierce Chemical Company. Dipicolinic acid was from K & K Laboratories, Inc. and n-octanol was from Fisher. Inorganic salts were reagent grade from Fisher.

Bacterial Strains and Growth Conditions—E. coli K12 strains CB64 recA/F'123, CB64TOR recA/F'123, and CB64TOR recA/F'123TOR and growth media were as described (Boehm et al., 1983). Seedled agar plates were made as described (Boehm et al., 1980) except that in some instances citrate was omitted from the medium.

Preparation of Cell Extracts and Culture Filtrates from Cell Suspensions Incubated with AlaAlaCys-MEPDA—CB64 recA/F'123 or CB64TOR recA/F'123TOR were grown in A medium (100 to 200 ml) to late log phase, centrifuged (4000 X g, 10 min), and washed twice by resuspension in potassium phosphate buffer (equal volume, 10 mM, pH 7.6) and centrifugation. The cells were resuspended in this buffer (20 to 60 ml) and the absorbance of the suspension was measured at 660 nm. Typical suspensions had absorbances of 3 to 5, corresponding to approximately 4 X 10^10 cells/ml.

Aliquots (10 to 40 ml) of cell suspension were placed in conical flasks (125-m1 capacity) and glucose (20%, w/v) was added to give a final concentration of 0.5%, w/v. The flasks were incubated at 37 °C in a rotatory shaking water bath (200 rpm, 5 min) after which AlaAlaCys-MEPDA was added to 50 or 200 μM. (Control suspensions were treated identically except that no peptide was added.) Portions of the suspensions (10 ml) were filtered immediately or at 20-min intervals through glass fiber filter discs (Whatman GF/C, 2.4-cm diameter) placed on top of damp Millipore filters (type HA, 0.45 μ) on a Millipore filtration manifold. Filtration time was 1 to 2 min. The filtrates were collected and lyophilized.

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The abbreviations used are: MEPDA, 4-[N-(2-mercaptoethyl)-aminopyridine-2,6-dicarboxylic acid (AlaAlaCys-MEPDA denotes attachment of MEPDA to the peptide through a disulfide bond); TOR, triornithine resistant; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
The filters plus cells were then transferred to screw-cap tubes (20 × 125 mm) and a solution of dithiothreitol was added (5 ml, 20 mM), followed by heating in a boiling water bath (10 min) to release cell contents. The extracts were centrifuged and filtered as above, the boiled filters being washed with a further aliquot (2 ml) of dithiothreitol solution and also filtered. The filtrates were lyophilized and stored under desiccator conditions until required.

**Gel Filtration Chromatography of Lyophilized Culture Filtrates—**
The lyophilized culture filtrates were dissolved in a solution of EDTA (0.2 ml, 10 mM) and applied to a column of Sephadex G-10 (2 × 22 cm) in potassium phosphate buffer (50 mM) containing EDTA (10 mM). Using this buffer as eluent, fractions of 0.8 ml were collected and their absorbance at 300 nm was measured. The elution positions of MEPDA, oxidized MEPDA, and AlaAlaCys-MEPDA were determined under the same conditions.

**Determination of MEPDA in cell extracts by High Performance Liquid Chromatography—**Lyophilized cell extracts were dissolved in water (50 μl) and diluted with acid phosphate buffer (10 mM, pH 2.5, 100 μl). Aliquots (20 μl) were analyzed on an Altex column (4 × 250 mm) packed with Lichrosorb RP-18 using a 5 to 10% methanol gradient in the same acid phosphate buffer (1 ml/min) and detection at 313 nm. Under these conditions, control cell extracts exhibited several peaks, but co-injection of pure MEPDA (100 ng) showed that it was well separated from them, having a retention time of 12.86 min.

**Determination of Sulfhydryl Release from AlaAlaCys-MEPDA by E. coli Cell Suspensions Using DTNB—**Suspensions of E. coli strains in phosphate buffer, pH 7.6, were prepared as described above but at 60 μM and 2 ml to 20 ml of culture and resuspending to an A600 of 0.7 to 0.8 (100-115 Klett units). The cell suspension was dispersed into cuvettes (10-mm path length) and glucose (to 0.5%, w/v, from a 20% w/v stock) and DTNB (usually 0.5 mM from a 100 mM solution in 0.3 M dipotassium hydrogen phosphate) was added as appropriate to give a final volume of 1.0 ml. Readings of absorbance at 412 nm were usually taken against a blank of cells plus glucose. After addition of AlaAlaCys-MEPDA (1 to 40 μl, 37.5 mM), the suspension was mixed by agitation with a bent sealed capillary tube and readings were taken every 1 to 2 min with similar agitation before every reading. Linear rates of increase were observed during the first 15 to 20 min, and these were converted to values of nanomoles/min/A600 unit using the molar absorption coefficient for DTNB reaction of 13,600 M⁻¹ cm⁻¹.

**Extraction of MEPDA and Dipicolinic Acid into n-Octanol—**n-Octanol (200 ml) was shaken with a solution of dithiothreitol (10 mM) in Tris-HCl buffer (10 mM, pH 8.0). The aqueous phase was then used to make solutions (approximately 1 mM) of MEPDA and dipicolinic acid. Aliquots (0.1 ml, in duplicate) of these solutions were diluted 10-fold with a solution of EDTA (5 mM) and their absorbance was measured at 300 (MEPDA) or 270 nm (dipicolinic acid) against a blank prepared by similar dilution of the buffer. A portion of each solution (0.2 ml) was mixed with 10-fold volume of buffer-equilibrated n-octanol and vortexed vigorously for 30 s, followed by centrifugation (4000 × g, 10 min). The aqueous phase was then separated and diluted for absorbance readings as above. A portion of the remaining aqueous phase was then re-extracted and diluted for absorbance measurements two more times. The average percentage loss in absorbance following extraction was calculated.

**RESULTS AND DISCUSSION**

**Metabolism of AlaAlaCys-MEPDA by Suspensions of E. coli—**MEPDA has a UV spectrum showing a broad maximum at 300 nm with an extinction coefficient of 11,000 M⁻¹ cm⁻¹. We used this fact to determine the level to which MEPDA was accumulated in the intracellular contents of suspensions of CB64recA/F'123 which had been incubated for various times with AlaAlaCys-MEPDA (50 μM), by subjecting cell extracts to high performance liquid chromatography analysis using a 313-nm detection system as described under "Experimental Procedures." Only low levels of MEPDA were found, so low as to be beyond exact quantitation by this method because of the variability in blank values due to binding of MEPDA or AlaAlaCys-MEPDA and retention of filtrate by the filters used for isolation of the cells. The failure to find any appreciable accumulation of 313-nm-absorbing material raised the possibilities of either extensive metabolism of MEPDA or its loss from the cells after uptake. When the absorption of the culture filtrates at 300 nm was measured at various times, no change in absorption was found within experimental error. We therefore examined the culture filtrates from such experiments by gel filtration, by which means AlaAlaCys-MEPDA and MEPDA can be easily distinguished.

Fig. 1 shows the results of gel filtration chromatography of lyophilized filtrates of suspensions of CB64recA/F'123 which had been incubated for various times with AlaAlaCys-MEPDA (200 μM). The absorbance of the fractions was measured at the wavelength of maximum absorbance of MEPDA and control filtrates from suspensions without addition of peptide showed no absorption at this wavelength (data not shown). The absorption of fractions at the position corresponding to AlaAlaCys-MEPDA diminished with continuing incubation using CB64recA/F'123 and a new peak appeared at a position similar to that found for oxidized MEPDA and whose UV spectrum corresponded to that of MEPDA. (The incubation conditions used in this experiment (vigorous aeration, 37 °C, pH 7.6) would lead to rapid oxidation of any free MEPDA to its disulfide. Under the moderate salt conditions used for elution (50 mM phosphate buffer, pH 7.6, 10 mM EDTA), MEPDA adsors to the Sephadex and consequently elutes past the included volume of the column.) The conversion of peptide-associated MEPDA to free MEPDA was greatly reduced when suspensions of the oligopeptide transport-deficient strain CB64TORrecA/F'123TOR were used. As shown in Fig. 1, even after 60 min incubation with this strain, very little conversion of AlaAlaCys-MEPDA to MEPDA had occurred, and simple disulfide exchange reactions with released sulfhydryl compounds could account in part for this low level of conversion.

In a study of the effect of peptide concentration on the rate of peptide disappearance in filtrates from suspensions of CB64recA/F'123 which had been incubated with 50 μM peptide, the rate of loss of the peptide peak was 30% slower than that found using 200 μM peptide (data not shown).

It appears that, following transport of AlaAlaCys-MEPDA by the oligopeptide transport system and the liberation of cysteine, MEPA or its loss from the cells after uptake. When the absorption of the culture filtrates at 300 nm was measured at various times, no change in absorption was found within experimental error. We therefore examined the culture filtrates from such experiments by gel filtration, by which means AlaAlaCys-MEPDA and MEPDA can be easily distinguished.

**FIG. 1.** Gel filtration chromatography of lyophilized culture filtrates from cell suspensions incubated with AlaAlaCys-MEPDA. Suspensions of CB64recA/F'123 or CB64TORrecA/F'123TOR were incubated with AlaAlaCys-MEPDA (200 μM) and filtered at various times as described under "Experimental Procedures." Lyophilized filtrates were dissolved, applied to a column of Sephadex G-10, and eluted with phosphate buffer/EDTA as described. After the passage of 15 ml of eluent, fractions (0.8 ml, 10 mM) and applied to a column of Sephadex G-10, and eluted with phosphate buffer/EDTA as described. After the passage of 15 ml of eluent, fractions (0.8 ml, 10 mM) and applied to a column of Sephadex G-10, and eluted with phosphate buffer/EDTA as described.
Pseudomonas fluorescens could be treated with the reagent, presence of DTNB, the sulfhydryl compounds excreted by this showed that the addition of 0.2 mM DTNB had no effect washed free of it, and returned to logarithmic growth without cultures that we could find in the literature was that of Laakso and zoic acid (as much as 10000000-fold excess of Gly, and Ala, but not Ala, and was completely inhibited by the protonophore CCCP (25 \mu M).

The dependence of the rate of production of absorbance on the concentration of AlaAlaCys-MEPDA for a suspension of CB64recA/F'123 is shown in Fig. 3. It shows saturable kinetics which when displayed on a Lineweaver-Burk plot (Fig. 3) gives a value of apparent \( K_a = 310 \mu M \) and \( V_{max} = 6.1 \text{ mmol min}^{-1} A_{660}^{-1} \). A number of different processes in the metabolism of this peptide might be expected to influence the \( K_a \) found by this method apart from its affinity for the transport system. The cleavage of the disulfide bond is obviously a prerequisite for the excretion of MEPDA and cysteine, and its rate will depend on the concentration of free sulfhydryl groups (principally glutathione) in the cell pool available for disulfide exchange. During active transport of the disulfide, this concentration could be limited by the ability of glutathione disulfide reductase to recycle oxidized glutathione (the end product of a series of disulfide exchanges). After cleavage of the disulfide bond, the exit of cysteine will be controlled by the factors normally responsible for the regulation of its intracellular level. The exit of MEPDA will be governed by its ability to partition in and out of the cytoplasmic membrane, and data on this are presented in the next section.

Although the calculated \( K_a \) may not reflect simply the affinity of the peptide for the transport system, the fact that the rate of loss of peptide from cell suspensions, as determined directly by Sephadex G-10 chromatography of lyophilized culture filtrates was 30% lower at an initial concentration of 50 \mu M compared to that at 200 \mu M (see above) agrees well with a \( K_a \) of 310 \mu M.

**Metal Chelation by MEPDA and Its Effect on Growth Inhibition by AlaAlaCys-MEPDA** —It might be expected that MEPDA would not be capable of easy exit from the cell as it will exist predominantly as negatively charged species in the alkaline cell pool, its carboxyl group \( pK_a \) values presumably being very similar to those of dipicolinic acid (2.16 and 4.76, Tichane and Bennett, 1957). A consideration of its metal-chelating properties, however, suggests a way in which such exit could be facilitated.

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**Fig. 2.** Release of sulfhydryl compounds from AlaAlaCys-MEPDA by suspensions of E. coli as measured by DTNB. AlaAlaCys-MEPDA (750 \mu M) was added to cell suspensions (\( A_{660} = 0.78 \)) in phosphate buffer containing glucose and DTNB as described under "Experimental Procedures." The release of sulfhydryl compounds was monitored by following the increase in absorbance at 412 nm. \( \bullet \), CB64recA/F'123; \( \odot \), CB64TORrecA/F'123; \( \xmark \), CB64TORrecA/F'123TOR.

**Fig. 3.** Concentration dependence of rate of production of sulfhydryl compounds from AlaAlaCys-MEPDA by CB64recA/F'123. The rates of production of sulfhydryl compounds from varying concentrations, \( S \), of AlaAlaCys-MEPDA by buffered suspensions of CB64recA/F'123 in the presence of glucose and DTNB were determined as described under "Experimental Procedures." Rates, \( V \), are expressed as nanomoles of sulfhydryl produced/min, and are normalized to a cell density of \( A_{660} = 1.0 \). The solid curve is theoretical for \( K_a = 310 \mu M, V_{max} = 6.1 \text{ mmol min}^{-1} \); points are experimental. Inset, reciprocal plot of data.
Dipicolinic acid is known to be a good chelator of metal ions, forming 1:1 complexes with stability constants at room temperature and ionic strength of 0.1 of between approximately $10^5$ (Mg$^{2+}$) and $10^{15}$ (Fe$^{3+}$) (tabulated by Martell and Smith, 1974). MEPDA presumably binds metals in a similar fashion, and such binding is shown by a blue shift in the UV spectrum from 300 to 285 nm upon the addition of molar equivalent of various metal salts (e.g. MnCl$_2$, ZnCl$_2$, CuSO$_4$; data not shown). The neutral chelate of doubly negatively charged MEPDA and a divalent metal ion will be much more lipophilic than the free charged MEPDA, and we propose that passage of MEPDA across the cytoplasmic membrane occurs as such uncharged metal chelates. The rapid loss of dipicolinic acid from germinating spores of the bacilli (Powell and Strange, 1953; Powell, 1953) may occur by just such a mechanism, i.e. diffusion of the calcium chelate across the cytoplasmic membrane (no evidence for facilitated or active transport of dipicolinic acid out of the cell has been found). A comparison of the relative lipophilicity of dipicolinic acid and MEPDA both as free compounds and as manganese chelates was performed by measuring the loss of UV absorbance from a buffered solution following extraction with 10-fold volumes of buffer-equilibrated n-octanol as described earlier as "Experimental Procedures." (Manganese was chosen for this experiment because it binds moderately well, and its chelate is more soluble than those formed by some of the other ions. It is also present at a concentration of some 15 $\mu$M in the cell water of E. coli (Silver et al., 1970).) The average of three such extractions showed that in the presence of EDTA there was no detectable loss of either substance from the aqueous phase as would be expected, but extraction in the presence of manganese removed some 20% of MEPDA at each extraction and some 2% of dipicolinic acid. The manganese chelate of MEPDA is therefore significantly more lipophilic than that of dipicolinic acid and both are vastly more lipophilic than the free compounds. It is probable that such chelation provides the basis for MEPDA's rapid exit from the cell following its liberation from the peptide.

Chelation and exchange of chelated ions can be envisaged in several ways. The medium used for growth contains approximately 0.4 mM magnesium and micromolar concentrations of trace metals such as zinc, manganese, and iron. These ions may therefore bind to AlaAlaCys-MEPDA and be co-transported into the cell. Once inside the cell, metal ions such as magnesium with poor stability constants could exchange for those with higher stability constants, e.g. zinc and iron, which will then exit from the cell as the MEPDA chelates. In addition, metal-free peptide may be transported into the cell, where MEPDA could then chelate a metal ion and exit. Such deprivation of metal ions in the cell pool could explain in part why AlaAlaCys-MEPDA is a much more efficient growth inhibitor than MEPDA itself (Boehm et al., 1983). It was of interest, therefore, to examine the effect of various metal salts on the growth inhibition produced by AlaAlaCys-MEPDA in seeded agar plates (lacking citrate) of CB64recA/F$^{123}$ and CB64TORrecA/F$^{123}$. Copper sulfate alone produced small zones of inhibition against both strains but these were almost eliminated by the addition of 1 molar equivalent of MEPDA. Of these two strains, AlaAlaCys-MEPDA produces a zone of inhibition against CB64recA/F$^{123}$ only, and the addition of 1 molar equivalent of copper sulfate resulted in enhanced toxicity to this strain. Although copper complexes may produce nonspecific inhibitory effects merely by facilitating diffusion of the copper across the cytoplasmic membrane (e.g. GlyAla and methionine, Counter et al., 1960; Ala$_3$, this laboratory, data not shown) such effects can be discounted here because they would be manifested against the oligopeptide transport-deficient strain as well. The adduct-copper complex is only inhibitory when accumulated by active transport through the olligopeptide transport system, and thus differs from the known examples of antimicrobial chelates which rely on simple diffusion to cross the cytoplasmic membrane (reviewed by Foye, 1977). The most intensively studied example of such chelates is that elucidated by Albert and co-workers for 8-hydroxyquinoline complexes of iron and copper (Albert, 1979).

The ability of portage transport through microbial peptide transport systems to provide antimicrobial agents with novel modes of action and new agents for the investigation of microbial physiology is apparent from this first study utilizing peptide side chain attachment.

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| Zone size | CB64recA/F$^{123}$ | CB64TORrecA/F$^{123}$ TOR |
|-----------|------------------|--------------------------|
| 200 nmol MEPDA | 0 | 0 |
| 200 nmol CuSO$_4$ | 9 | 11 |
| 200 nmol each, MEPDA and CuSO$_4$ | 7.5 | 7.5 |
| 200 nmol AlaAlaCys-MEPDA | 14, hazy zone | 0 |
| 200 nmol each, AlaAlaCys-MEPDA and CuSO$_4$ | 16, clear zone | 0 |

Table I

The effect of copper on zones of inhibition produced by AlaAlaCys-MEPDA in seeded agar plates

Agar plates (A medium minus citrate) seeded with E. coli strains were prepared as described under "Experimental Procedures." Test solutions (1-10 ml) were applied to paper discs (7-mm diameter) and placed on the agar. After incubation at 37°C for 24 h, zone of inhibition diameters were measured.
Portage Transport of a Metal-chelating Agent

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