The Characterization of Dipeptidases from *Escherichia coli*

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SUMMARY

The supernatant extracts from disrupted *Escherichia coli* had only low hydrolytic activity towards diglycine, divaline and prolylglycine, but latent peptidases were activated by Co^{2+} or Mn^{2+} . The cations K^+ , Mg^{2+} , Zn^{2+} , Fe^{2+} , Ni^{2+} , Cu^{2+} , Ca^{2+} and Cd^{2+} caused no increase in peptidase activity while Cd^{2+} , Zn^{2+} and Cu^{2+} antagonized the effects of Co^{2+} and Mn^{2+} . Measurements of optimum cation concentration, of pH optimum and of effects of various buffers led to the conclusion that the main peptidase activity towards these three peptides, and towards a broad spectrum of other dipeptides, resided in a single Co^{2+} activated enzyme. The activity of this peptidase was enhanced by phosphate and inhibited by veronal. Similar measurements with Mn^{2+} suggested the presence of several enzymes with lower activities and narrower substrate specificity than the Co^{2+} activated peptidase. Procedures were devised for the assay, under optimum conditions, of the main peptidase activities of a crude extract.

INTRODUCTION

Various amino acid auxotrophs of *Escherichia coli* can utilize peptides as sources of their required amino acids (Payne, 1971 *a, b*; Payne & Gilvarg, 1971; Sussman & Gilvarg, 1971). The transport and intracellular concentration of a peptide appears not to be a rate-limiting step in its utilization and, in general, the auxotrophic growth rate on a peptide equals that obtained with the free amino acid. However, low levels of intracellular peptidase activity can lead to negligible growth rates on certain peptide substrates (Stone, 1953; Payne, 1971 *b*). Furthermore, the ability of certain peptides to reach high internal concentrations as a consequence of low peptidase activity can lead to inhibition of growth and even death in certain cases (Simmonds, Tatum & Fruton, 1947; Simmonds, Harris & Fruton, 1951; Payne, 1968; Simmonds, 1970; Sussman & Gilvarg, 1970).

The effect of a peptide on a bacterium is related to the physiological condition of the organism, which in turn reflects an apparent variation in peptidase activity in response to changes in culture conditions and phase of growth (Kogoma & Nishi, 1965; Nishi & Hirose, 1966; Simmonds & Toye, 1967; Simmonds, 1970; Sussman & Gilvarg, 1971). This paper describes the general characteristics of the main peptidase activities found in extracts of *Escherichia coli*, towards three structurally dissimilar dipeptides; it forms the first part of a study of the effects of environmental conditions on bacterial peptidases.

METHODS

Chemicals: Di- and triglycine were purchased from British Drug Houses Ltd, Poole, Dorset. All other peptides were obtained from Cyclo Chemical Corporation, through Baxter Laboratories, Thetford, Norfolk. Trinitrobenzene sulphonate (TNBS) was purchased from Sigma (London) Chemical Co. Ltd, London, S.W. 6.

Table 1. *The effect of cations on the peptidase activity of crude extracts towards diglycine*

The incubation mixtures (1 ml) contained diglycine (7.5 mM) and either potassium phosphate pH 7.5 (20 mM) or tris-HCl pH 7.9 (20 mM); crude extract was added to give approximately 15 µg protein/ml with Co²⁺ (upper part of Table), 75 µg protein/ml with Mn²⁺ (lower part of Table), and amounts up to 1500 µg protein/ml with other cations. All salts were supplied as chlorides apart from ZnSO₄. Peptidase activity was assayed as described in the Methods. Activities of 100 % correspond to 4.8 and 0.85 µmol glycine released/min/mg protein for Co²⁺ (upper part of Table) and Mn²⁺ (lower part of Table) respectively. P, potassium phosphate; T, tris-HCl.

Cation supplement (mM)											Buffer	Activity (%)
Co ²⁺	Mg ²⁺	Zn ²⁺	Fe ²⁺	K ⁺	Ni ²⁺	Cu ²⁺	Cd ²⁺	Mn ²⁺	EDTA	Ca ²⁺		
0.5	—	—	—	—	—	—	—	—	—	—	P	100
1.0	—	—	—	—	—	—	—	—	—	—	P	100
0.5	1.0	—	—	—	—	—	—	—	—	—	P	94
0.5	0.5	—	—	—	—	—	—	—	—	—	P	100
—	1.0	—	—	—	—	—	—	—	—	—	P	0
—	1.0	—	—	—	—	—	—	—	0.25	—	P	0
—	1.0	—	—	—	—	—	—	—	—	—	T	2
—	1.0	—	—	—	—	—	—	—	0.25	—	T	2
1.0	—	0.01	—	—	—	—	—	—	—	—	P	100
1.0	—	0.1	—	—	—	—	—	—	—	—	P	58
1.0	—	—	—	—	—	—	—	—	—	—	T	19
1.0	—	0.5	—	—	—	—	—	—	—	—	T	6
—	—	1.0	—	—	—	—	—	—	—	—	P	0
1.0	—	—	1.0	—	—	—	—	—	—	—	P	100
—	—	—	1.0	—	—	—	—	—	—	—	P	0
1.0	—	—	—	10.0	—	—	—	—	—	—	P	100
1.0	—	—	—	20.0	—	—	—	—	—	—	P	86
—	—	—	—	3.0	—	—	—	—	—	—	P	4
—	—	—	—	3.0	—	—	—	—	0.25	—	P	3
—	—	—	—	3.0	—	—	—	—	—	—	T	0
1.0	—	—	—	—	—	—	—	—	0.25	—	P	108
1.0	—	—	—	—	1.0	—	—	—	—	—	P	100
—	—	—	—	—	3.0	—	—	—	—	—	P	4
—	—	—	—	—	3.0	—	—	—	0.25	—	P	3
—	—	—	—	—	3.0	—	—	—	—	—	T	0
1.0	—	—	—	—	—	1.0	—	—	—	—	P	41
1.0	—	—	—	—	—	10.0	—	—	—	—	P	0
—	—	—	—	—	—	1.0	—	—	—	—	P	0
—	—	—	—	—	—	20.0	—	—	—	—	P	0
—	—	—	—	—	—	20.0	—	—	0.25	—	P	0
1.0	—	—	—	—	—	—	0.1	—	—	—	P	7
1.0	—	—	—	—	—	—	0.01	—	—	—	P	27
1.0	—	—	—	—	—	—	0.005	—	—	—	P	37
1.0	—	—	—	—	—	—	1.0	—	—	—	P	0
0.5	—	—	—	—	—	—	—	0.5	—	—	P	103
0.5	—	—	—	—	—	—	—	1.0	—	—	P	89
1.0	—	—	—	—	—	—	—	1.0	—	—	P	114
1.0	—	—	—	—	—	—	—	3.0	—	—	P	53
1.0	—	—	—	—	—	—	—	10.0	—	—	P	31
1.0	—	—	—	—	—	—	—	—	—	1.0	P	100
—	—	—	—	—	—	—	—	—	—	1.0	P	3
1.0	—	—	—	—	—	—	—	—	—	1.0	T	22
—	—	—	—	—	—	—	—	1.0	—	—	P	100
—	—	—	—	—	—	—	—	1.0	0.25	—	P	122
0.5	—	—	—	—	—	—	—	0.5	—	—	P	53
—	—	—	—	10.0	—	—	—	1.0	—	—	P	89
—	—	0.01	—	—	—	—	—	1.0	—	—	P	20
—	—	0.1	—	—	—	—	—	1.0	—	—	P	0
—	—	0.5	—	—	—	—	—	1.0	—	—	T	2

Table 1 (cont.)

Cation supplement (mM)										Buffer	Activity (%)
Co ²⁺	Mg ²⁺	Zn ²⁺	Fe ²⁺	K ⁺	Ni ²⁺	Cu ²⁺	Cd ²⁺	Mn ²⁺	EDTA		
—	—	—	—	—	—	—	—	1.0	—	T	98
—	—	—	—	—	—	—	—	1.0	0.25	T	130
—	—	—	—	—	—	1.0	—	1.0	—	P	14
—	—	—	—	—	—	10.0	—	1.0	—	P	0
—	—	—	—	—	—	—	0.01	1.0	—	P	12
—	—	—	—	—	—	—	0.1	1.0	—	P	5
—	—	—	—	—	—	—	—	1.0	—	P	115
—	—	—	—	—	—	—	—	1.0	—	T	125

Growth of bacteria and preparation of crude extracts

For most studies here, *Escherichia coli* B (MRE strain 160) was grown in a chemostat under conditions of carbon limitation at a dilution rate of 0.18 h^{-1} , and harvested at $E_{540} = 10.3$ (dry wt 4.4 mg/ml). In one case batch culture was used. Both these procedures are described elsewhere (Payne, 1972). The strains employed and batch growth of *E. coli* W and K12 have been described previously (Payne, 1971a). Bacteria were disrupted, and crude extracts were prepared as described elsewhere (Payne, 1972). Protein concentrations were kindly determined by Dr H. E. Wade by an automated Folin-Ciocalteu procedure (Wade & Phillips, 1971).

Peptidase assay

The procedure of Binkley, Leibach & King (1968), employing trinitrobenzene sulphonate (TNBS) was used. In this method, the presence of Cu^{2+} in the reagent solution effectively prevents reaction of the TNBS with the peptide α -amino group while allowing its reaction with the α -amino groups of the amino acids liberated by peptidase hydrolysis. It therefore has the advantage over most assays employing ninhydrin that the peptide 'blank' values are low. In exploring the optimum conditions for peptidase action, the individual components of the incubation mixture were varied widely as shown in the Results section. However, a standard incubation mixture of 1 ml comprised: peptide, 7.5 mM; Co^{2+} or Mn^{2+} , 1 mM; EDTA, 0.25 mM; buffer, 20 mM; and crude extract containing, when diglycine was substrate, approximately $15 \mu\text{g}$ protein/ml with Co^{2+} or $75 \mu\text{g}$ protein/ml with Mn^{2+} , and approximately five times these amounts of protein when using divaline or prolylglycine. The reaction was started by the addition of protein to the test mixture previously equilibrated to 37°C and incubation was continued at this temperature. At zero time, and at three or four intervals thereafter up to 80 min, samples (0.1 ml) were removed and added to the TNBS- Cu^{2+} reagent solution (4.5 ml). These were incubated at 37°C for exactly 20 min when hydrochloric acid (11.6 M, 0.1 ml) was immediately added, the solutions were mixed and the E_{420} were recorded. Calibration curves for the amino acid constituents of the peptides were prepared by similar procedures. Proline does not react with TNBS under the above assay conditions, and when prolylglycine was used as substrate the total concentration of amino acid released was therefore taken as twice the value obtained for glycine.

RESULTS

The peptidase activity of crude extracts

The peptidase activities (expressed as μmol amino acid released/min/mg of protein) of crude extracts measured in incubation mixtures containing peptide (7.5 mM) and either

potassium phosphate pH 7.5 (20 mM) or tris-HCl pH 7.9 (20 mM) respectively were 0.012 and 0.018 with diglycine, 0.018 and 0.003 with divaline and 0.004 and 0.003 with prolylglycine. When dialysed extracts were used, the activities were unchanged.

The effect of cations on the peptidase activity of crude extracts

Bacterial peptidases frequently require metal ions for activity (Sarid, Berger & Katchalski, 1959, 1962; Simmonds & Toye, 1967; Yaron & Mlynar, 1968; Simmonds, 1970; Sussman & Gilvarg, 1970; Vogt, 1970). Using diglycine as substrate, a potentiating effect was also observed here when crude extracts were supplemented with certain cations (Table 1). Of the divalent cations tested, Co^{2+} and Mn^{2+} specifically activated the cleavage of diglycine approximately 500 and 60 times respectively. With the present assay procedure cation stimulation was immediate, and there appears to be no advantage in prolonged activation procedures as are required, for example, with leucine aminopeptidase (Smith, 1951). The cations Mg^{2+} , Zn^{2+} , Fe^{2+} , Ni^{2+} , Cu^{2+} , Cd^{2+} , and K^{+} were all without significant potentiating action, while Cd^{2+} and Zn^{2+} , and to a lesser extent Cu^{2+} , all antagonized the activation by Co^{2+} and Mn^{2+} . These observations on the inhibitory properties of Zn^{2+} and Cd^{2+} extend the findings of others (Sarid *et al.* 1962; Simmonds, 1970; Vogt, 1970). The studies were extended to divaline and to prolylglycine and the effects of Co^{2+} and Mn^{2+} concentration on the cleavage of all three peptides is shown in Fig. 1 and 2. For Co^{2+} activation (Fig. 1) in phosphate buffer, optimum cation concentration was within a narrow range around 1 mM for all three substrates; in tris buffer the optimum Co^{2+} concentration for the cleavage of diglycine was somewhat higher, about 5 mM. In all cases, higher concentrations of Co^{2+} led to marked inhibition of activity. With Mn^{2+} as activator (Fig. 2), the variation of activity (towards different substrates) as a function of concentration was greater than was observed with Co^{2+} . For example, in phosphate buffer, diglycine and divaline both showed optimal cleavage around 1 mM- Mn^{2+} , whereas maximum cleavage of prolylglycine required about 7 mM- Mn^{2+} . In contrast to Co^{2+} activated cleavage, the Mn^{2+} activated hydrolysis of diglycine showed the same rate and concentration dependence in tris as in phosphate buffer. Although a high ionic strength is required by certain carboxypeptidases (Smith, 1951), the results with added K^{+} ions indicated that with neither Co^{2+} nor Mn^{2+} did high ionic strength stimulate hydrolytic activity, in fact the opposite was observed (Table 1); this result therefore argues against the routine inclusion of K^{+} or Na^{+} in assay mixtures.

Effect of EDTA on peptidase activity

When tested in phosphate buffer pH 7.5, the addition of EDTA (0.05 to 0.20 mM) slightly stimulated the Co^{2+} (1 mM) activated hydrolysis of diglycine and stimulated the Mn^{2+} (1 mM) activated cleavage more (Table 1). On further increasing the EDTA concentration to 0.5 mM the rate of both Mn^{2+} (1 mM) and Co^{2+} activated cleavage decreased.

Effect of cysteine on peptidase activity

Cysteine activates certain proteases (Smith, 1951), and peptidases (Stone, 1953) but inhibits others, e.g. leucine aminopeptidase (Smith, 1951). Using the extracts here (Table 2), cysteine alone (5 mM) did not stimulate hydrolysis of diglycine, or divaline or prolylglycine; on the contrary it slightly inhibited the Mn^{2+} activated cleavage of these dipeptides and markedly inhibited the Co^{2+} activated hydrolysis. The inhibition with Co^{2+} presumably arises from reduction of the free cation concentration as a result of cysteine- Co^{2+} -complex formation (Michaelis & Schubert, 1930).

Fig. 1

Fig. 2

Fig. 1. The effect of Co^{2+} concentration on the cleavage of divaline (a), prolylglycine (b), and diglycine (c, d) by crude extracts. Incubation mixtures (1 ml) contained peptide (7.5 mM) and either potassium phosphate pH 7.5 (20 mM) or tris-HCl pH 7.9 (20 mM), and indicated amounts of Co^{2+} ; crude extract was added to give, with divaline, prolylglycine and diglycine, approximately 300, 150 and 20 μg protein/ml respectively. Peptidase assays were performed as described in Methods. $\circ$, Potassium phosphate; $\triangle$, tris-HCl.

Fig. 2. The effect of Mn^{2+} concentration on the cleavage of divaline (a), prolylglycine (b), and diglycine (c) by crude extracts. Incubation mixtures contained peptide (7.5 mM) and either potassium phosphate pH 7.5 (20 mM) or tris-HCl pH 7.9 (20 mM), and indicated amounts of Mn^{2+} ; crude extract was added to give with divaline, prolylglycine and diglycine approximately 1500, 750, and 75 μg protein/ml respectively. Peptidase assays were performed as described in Methods. $\circ$, Potassium phosphate; $\triangle$, tris-HCl.

Effect of pH and buffer on Co^{2+} activated peptidase

The effects of pH on the Co^{2+} activated cleavage of diglycine, divaline and prolylglycine are shown in Fig. 3. The diglycine-splitting enzyme was active over a wide pH range, with a broad maximum around pH 7.8 (Fig. 3c). However, although the buffers used provided overlapping pH profiles, for a given pH the absolute activities varied greatly depending on the nature of the buffer used. Certain aspects of the influence of buffers on peptidase activity were studied further and the results are shown in Table 3. For example, with diglycine as substrate, the activity in phosphate was about five times greater than that measured

Table 2. *The effect of cysteine on the cleavage of diglycine, prolylglycine, and divaline*

The incubation mixtures (1 ml) contained peptide (7.5 mM), potassium phosphate pH 7.5 (20 mM), and supplements as indicated. Crude extracts were added to give approximately 15 μ g protein with Co^{2+} and diglycine, approximately 50 μ g with Co^{2+} and other peptides and with Mn^{2+} and diglycine, and approximately 300 μ g protein with other mixtures. Peptidase activity was assayed as described in Methods, and is expressed as μ mol amino acid released/min/mg protein.

Peptide	Supplements to crude extracts Concn (mM)			Activity
	Co^{2+}	Mn^{2+}	Cysteine	
Diglycine	—	—	—	0.01
Diglycine	1	—	—	5.25
Diglycine	1	—	5	0.72
Diglycine	—	1	—	1.00
Diglycine	—	1	5	0.82
Diglycine	—	—	5	0.02
Prolylglycine	—	—	—	0.01
Prolylglycine	1	—	—	0.54
Prolylglycine	1	—	5	0.19
Prolylglycine	—	1	—	0.08
Prolylglycine	—	1	5	0.08
Prolylglycine	—	—	5	0.01
Divaline	—	—	—	0.02
Divaline	1	—	—	0.38
Divaline	1	—	5	0.06
Divaline	—	1	—	0.04
Divaline	—	1	5	0.02
Divaline	—	—	5	0.02

in tris-HCl, and this difference was preserved over a range of pH values (Fig. 3) and at various concentrations of Co^{2+} (Fig. 1). The cleavage of divaline and prolylglycine was also maximal in phosphate buffer although the enhancement was not so marked as with diglycine. The reason for the stimulatory effect of phosphate is not known but did not arise from its ability to reverse possible inhibition by contaminating Ca^{2+} ions, as has been reported for other peptidases (Smith, 1951; Kenny, WongLeung & Ingram, 1968), for the addition of Ca^{2+} ions was without effect on activity (Table 1). With tris, decreasing its concentration did not lead to greater activity, neither did increasing its concentration further reduce the rate of diglycine hydrolysis, suggesting that tris *per se* was not inhibitory (Table 3). In fact, increasing the concentration of tris from 20 to 80 mM increased the hydrolysis of diglycine, whereas a similar increase in the concentration of phosphate buffer decreased cleavage (Table 3). On the other hand, the addition of phosphate ions to the tris buffer markedly stimulated the diglycine splitting activity. Veronal buffer inhibited the Co^{2+} activated cleavage at all pH values tested (Fig. 3), and in this case activity could not be restored by addition of phosphate ions to the incubation mixture.

Effect of pH and buffer on Mn^{2+} activated peptidase

The Mn^{2+} activated cleavage of diglycine, divaline and prolylglycine (Fig. 4) occurs over a wide pH range with an optimum in each case at about pH 8. In contrast to the variable activities found with Co^{2+} , when Mn^{2+} was used essentially equal activities were observed for a given pH irrespective of the buffer used. It is especially noteworthy that the activity was in no way inhibited by veronal. Increasing the concentration of tris in the incubation mixture

Fig. 3

Fig. 4

Fig. 3. The effect of pH on the Co^{2+} activated hydrolysis of divalinal (a), prolyl-glycine (b) and diglycine (c). Incubation mixtures (1 ml) contained peptide (7.5 mM), EDTA (0.25 mM), Co^{2+} (1 mM), buffer (20 mM) and crude extract (with divalinal and prolyl-glycine approximately 100 μg protein and with diglycine approximately 20 μg protein). Peptidase assays were performed as described in Methods. The buffers used were: ○, potassium phosphate; Δ, tris-HCl; ●, carbonate; ×, veronal.

Fig. 4. The effect of pH on the Mn^{2+} activated cleavage of divalinal (a), prolyl-glycine (b), and diglycine (c). Incubation mixtures (1 ml) contained peptide (7.5 mM), EDTA (0.25 mM), Mn^{2+} (1 mM), buffer (20 mM) and crude extract (approximately 200 μg protein). Peptidase assays were performed as described in Methods. The buffers used were: ○, potassium phosphate; Δ, tris-HCl; ●, carbonate; ×, veronal.

slightly increased the activity at all pH values tested, whereas a decrease in hydrolysis resulted from comparable increases in the concentration of phosphate buffer (Table 3).

The effect of substrate concentration on Co^{2+} peptidase activity

The variation in the rates of cleavage of diglycine, prolyl-glycine and divalinal as a function of dipeptide concentration is shown in Fig. 5. In each case, over an appropriate concentration range the relationship between the extent of hydrolysis and the substrate concentration was linear in short-duration experiments. The enzymes appear to be saturated at substrate concentrations of approximately 2.5, 3, and 6 mM for divalinal, prolyl-glycine and diglycine respectively. In most standard incubation mixtures, the dipeptides were used at concentra-

Table 3. *The influence of buffer concentration and phosphate ions on the cleavage of diglycine by crude extracts*

Standard incubations and peptidase assays were performed as described in Methods. Peptidase activities are expressed as μ mole glycine released/min/mg of protein.

Buffer			Supplement		Cation	Activity
Type	pH	Concn (mM)	Type	Concn (mM)		
Tris	7.47	10	—	—	Co ²⁺	0.91
Tris	7.47	20	—	—	Co ²⁺	0.91
Tris	7.47	75	—	—	Co ²⁺	1.62
Tris	7.90	10	—	—	Co ²⁺	0.92
Tris	7.90	20	—	—	Co ²⁺	0.92
Tris	7.90	75	—	—	Co ²⁺	1.45
Phosphate	7.50	4	—	—	Co ²⁺	4.60
Phosphate	7.50	10	—	—	Co ²⁺	4.84
Phosphate	7.50	20	—	—	Co ²⁺	4.84
Phosphate	7.50	40	—	—	Co ²⁺	4.00
Phosphate	7.50	75	—	—	Co ²⁺	2.32
Phosphate	8.10	20	—	—	Co ²⁺	4.90
Phosphate	8.10	75	—	—	Co ²⁺	2.74
Tris	7.47	20	K ₂ HPO ₄	20	Co ²⁺	4.15
Tris	7.90	20	KH ₂ PO ₄	2	Co ²⁺	1.25
Tris	7.90	20	KH ₂ PO ₄	20	Co ²⁺	2.34
Tris	7.90	20	K ₂ HPO ₄	2	Co ²⁺	1.00
Tris	7.90	20	K ₂ HPO ₄	20	Co ²⁺	2.61
Tris	8.51	20	—	—	Co ²⁺	0.75
Tris	8.51	20	K ₂ HPO ₄	20	Co ²⁺	2.68
Veronal	7.80	10	—	—	Co ²⁺	0.00
Veronal	7.80	10	KH ₂ PO ₄	2	Co ²⁺	0.00
Veronal	7.80	10	KH ₂ PO ₄	20	Co ²⁺	0.07
Veronal	7.80	10	K ₂ HPO ₄	2	Co ²⁺	0.00
Veronal	7.80	10	K ₂ HPO ₄	20	Co ²⁺	0.09
Tris	7.47	20	—	—	Mn ²⁺	0.58
Tris	7.47	80	—	—	Mn ²⁺	0.93
Tris	7.90	20	—	—	Mn ²⁺	0.84
Tris	7.90	80	—	—	Mn ²⁺	1.14
Phosphate	7.50	20	—	—	Mn ²⁺	0.85
Phosphate	7.50	80	—	—	Mn ²⁺	0.27
Phosphate	8.10	20	—	—	Mn ²⁺	0.81
Phosphate	8.10	80	—	—	Mn ²⁺	0.16

tions of 7.5 mM. Lineweaver–Burk plots, for the hydrolysis of the three dipeptides, are also shown in Fig. 5. The apparent K_m values for divalinaline, prolylglycine and diglycine are 6.5×10^{-3} M, 2.5×10^{-3} M, and 2.0×10^{-3} M respectively.

Effect of substrate concentration on Mn²⁺ peptidase activity

This was studied using diglycine as substrate (Fig. 6). In short-duration experiments, a linear relationship again exists between the amount of glycine released and the substrate concentration within an appropriate concentration range, with the enzyme apparently saturated at substrate concentrations in excess of 6 mM. A Lineweaver–Burk plot of the data indicates an apparent K_m of 9.1×10^{-3} M.

Effect of protein concentration on Co²⁺ activated cleavage of diglycine

For short incubation periods (20 min), the relationship between the amount of glycine liberated and the amount of protein present in the reaction mixture was linear up to about 0.1 mg of protein/ml.

Fig. 5. The effect of substrate concentration on the rate of Co^{2+} activated hydrolysis of divaline (a), prolylglycine (b), and diglycine (c). The incubation mixtures contained potassium phosphate pH 7.5 (20 mM), EDTA (0.25 mM), Co^{2+} (1 mM), and the indicated amounts of dipeptide substrates. Crude extracts were added to give approximately 15 μg protein with diglycine as substrate and approximately 75 μg protein with divaline and prolylglycine. Peptidase assays were performed as described in Methods. Inserts are Lineweaver-Burk plots of the data; $1/v$, $\mu\text{mol amino acid released per min}$; $1/s$, peptide concn (mM^{-1}).

Fig. 6. The effect of substrate concentration on the rate of Mn^{2+} activated hydrolysis of diglycine. The incubation mixtures (1 ml) contained potassium phosphate buffer pH 7.5 (20 mM), EDTA (0.25 mM), Mn^{2+} (1 mM), and crude extract (75 μg protein), together with the indicated amounts of diglycine. Peptidase assays were performed as described in Methods. Inset is Lineweaver-Burk plot of the data; $1/v$, $\mu\text{mol amino acid released per min}$; $1/s$, peptide concn (mM^{-1}).

Table 4. *The substrate specificities of Co²⁺ and Mn²⁺ activated peptidases*

The incubation mixtures (1 ml) contained peptide (5 mM), EDTA (0.25 mM), potassium phosphate pH 7.5 (20 mM), crude extract was added to give approximately 750 µg protein/ml with Mn²⁺ (1 mM) and with the unsupplemented mixture, and 150 µg protein/ml with Co²⁺ (1 mM). Peptidase activity was assayed as described in Methods. Peptidase activities are expressed as µmol amino acid released/min/mg of protein, and are calculated using the appropriate extinction coefficients for each amino acid.

Peptide	Activity of crude extract supplemented with		
	No ion	Mn ²⁺	Co ²⁺
Pro-Gly	0.004	0.082	0.540
Gly-Pro	0.042	0.028	0.127
Pro-Val	0.009	0.023	0.042
Pro-Tyr	0.000	0.025	0.042
Pro-Phe	0.009	0.036	0.085
Gly-Lys	0.055	0.049	0.860
Lys-Gly	0.036	0.079	0.615
Lys-Lys	0.066	0.051	0.382
Leu-Phe	0.032	0.042	0.466
Leu-Tyr	0.032	0.045	0.312
Val-Val	0.018	0.041	0.381
Val-Gly	0.007	0.012	0.498
Gly-Val	0.002	0.045	0.360
Gly-Gly	0.012	1.000	5.250
Gly-Leu	0.004	0.021	0.191
Gly-Phe	0.032	0.085	0.222
Gly-His	0.042	0.072	0.106
Gly-Tyr	0.070	0.094	0.816
α-Glu-Gly	0.000	0.015	0.202
γ-Glu-Gly	0.000	0.003	0.011
α-Asp-Ala	0.002	0.038	0.032
β-Asp-Ala	0.006	0.009	0.005

The substrate specificities of the Co²⁺ and Mn²⁺ activated peptidases

One reason for selecting diglycine, divaline and polyglycine for study was the assumption that their cleavage by crude extracts was likely to be effected by separate peptidases of limited specificities. However, the results presented so far suggest that the Co²⁺ (and perhaps also the Mn²⁺) activities may well reside in only one enzyme, unless several peptidases of remarkably similar properties are present. Isolating and purifying the individual peptidases was outside the present objectives of assessing the main peptidase activities of the bacteria, but to study this aspect further we measured the effects of Co²⁺ and Mn²⁺ on the cleavage of a wider selection of dipeptides. Several general conclusions emerge from the data shown in Table 4. First, the crude extracts displayed uniformly low activity towards a broad spectrum of dipeptides; secondly, Co²⁺ dramatically stimulated the hydrolysis of all α-linked peptides tested but to extents that vary with the nature of the peptide substrate; thirdly, Mn²⁺ stimulates the cleavage of only a few of the peptides tested and with these to a lesser extent than found with Co²⁺. The effect of Mn²⁺ upon the cleavage of peptides containing basic amino acids seems to be minimal; the greatest stimulation occurred with peptides that possess neutral residues only. Neither Co²⁺ nor Mn²⁺ stimulated cleavage of the β- or γ-linked peptides tested.

DISCUSSION

While studying peptide transport in *Escherichia coli* we became interested in the growth of amino acid auxotrophs on peptides (Payne, 1971*a, b*; Payne & Gilvarg, 1971), and in the

general role of peptidases in the bacterial utilization of peptides. The subject of the transport and utilization of peptides in bacteria has recently been reviewed (Sussman & Gilvarg, 1971).

The peptidases of *Escherichia coli* appear to be truly intracellular and are neither released by osmotic shock (Van Lenten & Simmonds, 1967) nor by sphaeroplast formation (Matheson & Murayama, 1966; Simmonds & Toye, 1966; Van Lenten & Simmonds, 1967). Upon disruption of the organism these intracellular peptidases may be characterized further either as ribosomal (Dick, Matheson & Wang, 1970), or soluble (Stone, 1953; Sarid *et al.* 1959, 1962; Simmonds & Toye, 1966, 1967; Haley, 1968; Yaron & Mlynar, 1968; Simmonds, 1970; Vogt, 1970; Sussman & Gilvarg, 1970, 1971). In this paper we have investigated only the soluble peptidases that are found in the supernatant extract after disruption of the bacteria; previous studies indicate this to be the major source of cellular peptidase activity (Simmonds, 1970). Although earlier studies have provided evidence for a number of peptidases of varied substrate specificities, and several of these enzymes have been purified and characterized, our aim here was to study the peptidase complement of the extract with a view to assessing the most active peptidases towards several dipeptides used as nutrients for amino acid auxotrophs (Payne, 1971*a, b*). It is presumed that the enzymes that display the greatest peptidase activity *in vitro* towards certain peptides are likely to be the peptidases that play the most important role in the utilization of these same peptides by cultures of auxotrophic bacteria. Three dipeptides were selected as being structurally rather dissimilar and likely therefore to be mainly cleaved by dipeptidases of differing substrate specificities. The characterization of such peptidases forms a necessary preliminary to studies of the variation of peptidase activity with growth conditions (Payne, 1972). The results suggest that the predominant hydrolytic activity towards all three dipeptides probably resides in a single Co^{2+} activated peptidase, although confirmation of this would require fractionation of the crude extract. The observations with the three dipeptides that suggest a low substrate specificity are reinforced by the further studies (Table 4) with the selection of dipeptides that contain a broad spectrum of amino acid types; in all cases Co^{2+} significantly stimulated hydrolysis. Several facts indicate that the Mn^{2+} activated hydrolysis derives from a distinct Mn^{2+} activated enzyme(s) rather than from an inferior activation by Mn^{2+} of the Co^{2+} enzyme(s); the most important of these are the following: first, the activity towards diglycine, when Co^{2+} (1 mM) and Mn^{2+} (1 mM) are present together, essentially equals the sum of the individual activities when they are tested separately (Table 1); second, veronal buffer inhibits the Co^{2+} activated cleavage while permitting normal Mn^{2+} activated hydrolysis; and third, although phosphate enhances the Co^{2+} cleavage it is without specific effect on Mn^{2+} cleavage.

Comparison of the results here with those of other workers indicates that the Co^{2+} enzyme, which in our hands appears to be the most active peptidase in *Escherichia coli*, has yet to be purified and characterized. Sussman & Gilvarg (1970) described a Co^{2+} activated dilysine peptidase from *E. coli* but this was only partially purified and its activity towards other substrates was not tested. Simmonds & Toye (1967) showed that a peptidase present in the soluble portion of an extract of *E. coli* K12 was activated by Co^{2+} , but they did not make detailed studies of this activity and concentrated instead upon the Mn^{2+} activated peptidases (Simmonds & Toye, 1966, 1967; Van Lenten & Simmonds, 1967; Simmonds, 1970). Simmonds and her co-workers were studying a different strain of *E. coli*, although the peptidase complement of different strains of *E. coli* does appear to be very similar (see later and Sussman & Gilvarg, 1970).

A number of Mn^{2+} activated peptidases have been reported in *Escherichia coli* extracts and several have been isolated (Stone, 1953; Sarid *et al.* 1962; Simmonds & Toye, 1966,

1967; Van Lenten & Simmonds, 1967; Yaron & Mlynar, 1968; Simmonds, 1970; Vogt, 1970). It is likely that the Mn^{2+} activated hydrolysis described here resides in one or more of these enzymes; certainly, the results of the Mn^{2+} concentration studies (Fig. 2) and the substrate specificity results (Table 4) are consistent with the presence of several Mn^{2+} enzymes.

Further studies with veronal suggest that its inhibitory effect is not an artifact of the assay procedure, neither does it significantly sequester Co^{2+} ions, and it seems more likely to be affecting the enzyme itself. Veronal has often been used for assays of *Escherichia coli* peptidases (Sarid *et al.* 1959, 1962; Nishi & Hirose, 1966; Yaron & Mlynar, 1968). In further studies therefore we compared the activities of both sonic extracts and toluene-treated organisms from batch-grown strains of *E. coli* B, K12 and W towards diglycine, divalinal and prolylglycine. In all cases, Co^{2+} greatly enhanced cleavage of the dipeptides, the activities were greatest in phosphate buffer, and low activities (about 10 %) were observed in veronal. In general therefore, veronal appears to be an unsuitable buffer, although it could be useful for assaying other peptidases, e.g. isolation of a specific proline iminopeptidase was probably facilitated by using veronal and therefore inhibiting hydrolysis of the substrate prolylglycine by the more active Co^{2+} peptidase (Sarid *et al.* 1959).

These results therefore provide an assessment of the effects of incubation conditions on the measurement of *Escherichia coli* peptidase activity, and indicate procedures for optimal expression of the major peptidase activities that are found in a crude extract. The fact that the Co^{2+} and Mn^{2+} activated enzymes retain about 90 % of their activities towards all three dipeptides after storage at $-20^{\circ}C$ for three months in phosphate pH 7 (10 mM), should encourage further work on the isolation of the individual activities.

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REFERENCES

- BINKLEY, F., LEIBACH, F. & KING, N. (1968). A new method of peptidase assay and the separation of three leucylglycinases of renal tissues. *Archives of Biochemistry and Biophysics* **128**, 397–405.
- DICK, A. J., MATHESON, A. T. & WANG, J. H. (1970). A ribosomal bound amino peptidase in *Escherichia coli* B: purification and properties. *Canadian Journal of Biochemistry* **48**, 1181–1188.
- HALEY, E. E. (1968). Purification and properties of a β -aspartyl peptidase from *Escherichia coli*. *Journal of Biological Chemistry* **243**, 5748–5752.
- KENNY, A. J., WONGLEUNG, Y. L. & INGRAM, J. (1968). Comparison of enzymes in rat kidney that hydrolyse L-leucinamide, L-leucyl-*p*-nitroanilide and iodinated insulin B chain. *Biochemical Journal* **110**, 6p.
- KOGOMA, T. & NISHI, A. (1965). Rhythmic variations in proteolytic activities during the cell cycle of *Escherichia coli*. *Journal of General and Applied Microbiology* **11**, 321–329.
- MATHESON, A. T. & MURAYAMA, T. (1966). Limited release of ribosomal peptidase during formation of *Escherichia coli* spheroplasts. *Canadian Journal of Biochemistry* **44**, 1407–1415.
- MICHAELIS, L. & SCHUBERT, M. P. (1930). Cobalt complexes of thioglycollic acid. *Journal of the American Chemical Society* **52**, 4418–4426.
- NISHI, A. & HIROSE, S. (1966). Further observations on the rhythmic variation of peptidase activity during the cell cycle of various strains of *Escherichia coli*. *Journal of General and Applied Microbiology* **12**, 293–297.
- PAYNE, J. W. (1968). Oligopeptide transport in *Escherichia coli*: specificity with respect to side chain and distinction from dipeptide transport. *Journal of Biological Chemistry* **243**, 3395–3403.
- PAYNE, J. W. (1971*a*). The requirement of the protonated α -amino group for the transport of peptides in *Escherichia coli*. *Biochemical Journal* **123**, 245–254.
- PAYNE, J. W. (1971*b*). The utilization of prolyl peptides by *Escherichia coli*. *Biochemical Journal* **123**, 255–260.
- PAYNE, J. W. (1972). Variations in the peptidase activities of *Escherichia coli* in response to environmental changes. *Journal of General Microbiology* **71**, 281–291.
- PAYNE, J. W. & GILVARG, C. (1971). Peptide transport. *Advances in Enzymology* **35**, 187–244.

- SARID, S., BERGER, A. & KATCHALSKI, E. (1959). Proline iminopeptidase 1. *Journal of Biological Chemistry* **234**, 1740–1746.
- SARID, S., BERGER, A. & KATCHALSKI, E. (1962). Proline iminopeptidase 2. *Journal of Biological Chemistry* **237**, 2207–2212.
- SIMMONDS, S. (1970). Peptidase activity and peptide metabolism in *Escherichia coli* K12. *Biochemistry* **9**, 1–9.
- SIMMONDS, S., HARRIS, J. I. & FRUTON, J. S. (1951). Inhibition of bacterial growth by leucine peptides. *Journal of Biological Chemistry* **188**, 251–262.
- SIMMONDS, S., TATUM, E. L. & FRUTON, J. S. (1947). The utilization of leucine derivatives by a mutant strain of *Escherichia coli*. *Journal of Biological Chemistry* **170**, 483–489.
- SIMMONDS, S. & TOYE, N. O. (1966). Peptidases in spheroplasts of *Escherichia coli* K12. *Journal of Biological Chemistry*, **241**, 3852–3860.
- SIMMONDS, S. & TOYE, N. O. (1967). The role of metal ions in the peptidase activity of *Escherichia coli* K12. *Journal of Biological Chemistry* **242**, 2086–2093.
- SMITH, E. L. (1951). The specificity of certain peptidases. *Advances in Enzymology* **12**, 191–257.
- STONE, D. (1953). Some aspects of the hydrolysis of proline peptides by a prolineless mutant of *Escherichia coli*. *Journal of Biological Chemistry* **202**, 821–827.
- SUSSMAN, A. J. & GILVARG, C. (1970). Peptidases in *Escherichia coli* K12 capable of cleaving lysine homopeptides. *Journal of Biological Chemistry* **245**, 6518–6524.
- SUSSMAN, A. J. & GILVARG, C. (1971). Peptide transport and metabolism in bacteria. *Annual Reviews of Biochemistry* **40**, 397–408.
- VAN LENTEN, E. J. & SIMMONDS, S. (1967). Dipeptidases in spheroplasts and osmotically shocked cells prepared from *Escherichia coli* K12. *Journal of Biological Chemistry* **242**, 1439–1444.
- VOGT, V. M. (1970). Purification and properties of an aminopeptidase from *Escherichia coli*. *Journal of Biological Chemistry* **245**, 4760–4769.
- WADE, H. E. & PHILLIPS, B. W. (1971). Automated determination of bacterial asparaginase and glutaminase. *Analytical Biochemistry* **44**, 189–199.
- YARON, A. & MLYNAR, D. (1968). Aminopeptidase P. *Biochemical and Biophysical Research Communications* **32**, 658–663.