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Studies on Rat-Liver Glycylglycine Dipeptidase

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The metal-ion-activated enzyme found in various mammalian tissues which catalyses the hydrolysis of glycylglycine (Smith, 1948*a*; Adams, McFadden & Smith, 1952; Robinson & Shaw, 1960; Rademaker & Soons, 1957; Uzman, Rumley & Van Den Noort, 1960) has been named 'glycylglycine dipeptidase' (Smith, 1948*a*). Analogous enzymic activity has also been demonstrated in micro-organisms (Crewther & Lennox, 1953; Nishi, 1958). The glycylglycine-splitting activity demonstrable in all preparations studied can be activated by Co^{2+} ions, and in some cases by Mn^{2+} ions (Smith, 1948*a*). In contrast with the marked lability of most of the preparations described, the extracts from mammalian uterus (Smith, 1948*a*) and rat liver (M. Fried & S. M. Cain, unpublished work) are relatively stable when frozen.

The purpose of the present paper is to study and compare the glycylglycine-hydrolysing activities of rat liver in the presence and absence of bivalent metal ions, as well as in the presence of some organic substances. Such a comparison provides evidence for at least two separate glycylglycine-hydrolysing activities.

MATERIALS AND METHODS

Assay procedure. The enzymic hydrolyses were carried out at 35° in a volume of 2 ml. The solutions were 50 mM with respect to substrate, 1 mM with respect to the bivalent metal ion, and 50 mM with respect to tris buffer, giving a final pH of 8.2–8.4 throughout the activity measurement. Solutions of other substances, added in various experiments, were adjusted to pH 7 and included in the assay mixture. The enzyme was added last after preincubation of the other constituents for 20 min. at 35°. Longer preincubation did not alter the reaction rate.

The extent of hydrolysis was determined by the method of Grassman & Heyde (1929); samples of the reaction

mixture were titrated at various time-intervals until hydrolysis was about 60% completed. As reported by Smith (1948*a*), the reaction was zero order to this point. The results were plotted and the rate of hydrolysis was determined graphically from the slope of the line drawn by the method of least mean squares (Brey, 1958).

Protein concentrations were determined by the biuret method (Gornall, Bardawill & David, 1949), with bovine serum albumin as the protein standard. Specific activities are expressed as μmoles of substrate hydrolysed/min./mg. of protein.

Three enzymic systems were studied: the 'residual system', assayed without added bivalent metal ions, the ' Co^{2+} ion system', assayed in the presence of Co^{2+} ions at an initial concentration of 1 mM, and the ' Mn^{2+} ion system', assayed in the presence of Mn^{2+} ions at an initial concentration of 1 mM.

Tris buffer was used because its buffer capacity is high in the pH ranges studied and because it forms metal complexes to an extent too small to affect these studies (Wehber, 1959).

Enzyme preparation. White rats (300–500 g. each) were killed by decapitation. After exsanguination, the livers were removed and homogenized for 2 min. with 3–5 vol. of 5 mM-phosphate buffer, pH 7.5, at 4°. This homogenate was then centrifuged for 20 min. at 7000g to remove cell debris. Crude homogenates prepared in this manner had specific activities ranging from 0.20 to 0.25 when measured in the Co^{2+} ion system.

Since serum contains some glycylglycine-dipeptidase activity (Smith, 1948*a*; Adams *et al.* 1952), most of the blood was removed by perfusing the liver with 0.9% sodium chloride. This was done by inserting a polyethylene tube into the portal vein of the freshly excised liver and forcing cold 0.9% sodium chloride through the tissue until it assumed an even blanched colour. After homogenization, differential centrifuging (Hogeboom, 1955) was employed to obtain nuclear, mitochondrial, microsomal and supernatant fractions. All activity was found in the supernatant, as has been reported by Rademaker & Soons (1957). The supernatant fraction, obtained after centrifuging for 3–4 hr. at 104 000g, represented a 2.5–3.0-fold increase in

specific activity over the crude homogenate and this preparation was used for the characterization, specificity and inhibition studies.

The lability of the activity was evident when conventional methods of purification were employed. The use of acetone or ammonium sulphate for concentrating the activity into narrow fractions resulted in considerable loss of the activity within short periods, even in the presence of concentrations of Co^{2+} or Mn^{2+} ions which stabilize similar enzymes (Smith, 1948a).

Materials. The glycylsarcosine and sarcosylglycine used in the present studies were prepared as described by Koltun, Fried & Gurd (1960). All other organic compounds used were obtained commercially. Inorganic compounds used were all of analytical reagent grade. All metal salts were the chlorides except the mercuric and lead salts, which were the nitrates.

RESULTS

Characteristics of the time-course of hydrolysis. The rates of hydrolysis were determined by the use of four or five experimental points. Preliminary work showed that the rate was linear with time up to the point of 60–70% completion. In plotting these results there was a suggestion of a slight lag period in the Co^{2+} ion system which did not seem to occur when assaying the other two systems. For this reason, all three systems were examined more critically at the early time periods. The results are shown in Fig. 1. All plots were obtained by the method of least mean squares, all experimental points being used. There is a lag period of about 7–10 min. in the Co^{2+} ion system, and of about 5 min. in the residual system, but no lag period in the Mn^{2+} ion system.

The lag period in the Co^{2+} ion system could be reduced to about 5 min. when the Co^{2+} ions and the enzyme preparation were preincubated for 10 min. This preincubation also resulted in the loss of 25% of the activity (Fig. 1). In an experiment in which the Co^{2+} ions were added after the enzyme and glycylglycine had been preincubated for 10 min., there was a lag period of about 7 min.

Effect of temperature. The rate of hydrolysis of glycylglycine was determined at 23°, 30°, 35° and 40° in each of the three systems. The heat of activation, ΔH , for each overall reaction was determined by plotting $1/T$ against $\log_{10}(k/T)$ from the relation (Lawrence & Moore, 1951):

$$k = \frac{kT}{h} \exp\left(\frac{-\Delta H}{RT}\right) \exp\left(\frac{\Delta S}{R}\right) \quad (1)$$

which, on taking logarithms, gives

$$\log_{10}\left(\frac{k}{T}\right) = C - \left(\frac{\Delta H}{R}\right)\left(\frac{1}{T}\right) \quad (2)$$

where ΔS is the entropy of activation, k is Boltzmann's constant, h is Planck's constant, R is the gas constant, T is the absolute temperature, k is the rate constant expressed as percentage hydro-

lysis/min. and C is a constant which includes all of the constants in eqn. (1). The least-mean-square lines obtained on plotting these data are shown in Fig. 2. From the slope of each line the following ΔH values were calculated: 6.9 kcal. for the residual activity, 16.3 kcal. for the Co^{2+} ion system and 12.9 kcal. for the Mn^{2+} ion system.

Fig. 1. Time-curves for the hydrolysis of glycylglycine by rat-liver glycylglycine-dipeptidase preparation. Experimental details are given in the text. $\circ$, Co^{2+} ion system; $\blacksquare$, enzyme and substrate separately preincubated with Co^{2+} ions (1.0 mM) for 10 min.; $\triangle$, enzyme preincubated with Co^{2+} ions, substrate not preincubated with the assay system; $\bullet$, Mn^{2+} ion system; $\blacktriangle$, residual system.

Fig. 2. Effect of temperature on the activity of rat-liver glycylglycine-dipeptidase preparation. Experimental conditions and definitions are given in the text. $\bullet$, Co^{2+} ion system; $\circ$, Mn^{2+} ion system; $\blacktriangle$, residual system.

Effect of pH. Preliminary experiments indicated a possibility of the presence of more than one activity in the rat-liver preparation, as had been suggested for rat intestine by Robinson & Shaw (1960). Studies were carried out to determine the effect of pH on the different systems. Fig. 3 shows the pH-activity curves of each of the three activities in tris buffer. The residual activity is relatively independent of pH over the range studied, i.e. 7-9, with the maximum activity at about 8.1-8.2, whereas the pH optimum in the Co^{2+} ion and Mn^{2+} ion systems is about 8.5. Both of the metal-ion-activated systems appear to be sensitive to pH over the range studied.

Effect of dialysis. The residual activity could be ascribed to the presence of either Co^{2+} or Mn^{2+} ions or both in the rat-liver homogenate. The effect of dialysis on the three systems was investigated to ascertain whether there was sufficient endogenous free metal ion in the homogenate to account for the residual activity.

The rat-liver homogenate was dialysed against running distilled de-ionized water at 4° (approx. 50 ml./hr. in a total volume of 500 ml.) for various times and the activity was measured under the conditions specified for the three assay systems, i.e. after the addition of the standard amounts of Co^{2+} or of Mn^{2+} ions, or in the absence of metal ions. Fig. 4 shows the results of such an experiment which indicate that dialysis lowers the activity in all three systems and that the ratio of activities does not remain constant. The residual activity is inactivated considerably more slowly than are the two

Fig. 3. Effect of pH on the activity of rat-liver glycylglycine-dipeptidase preparation. Experimental conditions are given in the text. All reactions were carried out in 0.05M-tris buffer. ●, Co^{2+} ion system; ○, Mn^{2+} ion system; ▲, residual system.

metal-ion-activated systems, which are inactivated at essentially the same rate.

Stabilization of the enzyme(s) by metal ions, which has been shown for carnosinase (Rosenberg, 1960), may also contribute significantly to the interpretation of these observations and was tested in an experiment in which the residual activity was assayed after dialysis against $10\mu\text{M-Mn}^{2+}$ ion or against $10\mu\text{M-Co}^{2+}$ ion for 1 hr. Dialysis against distilled water was used as a control. All three were then compared with the undialysed preparation. A 25% loss of activity was found both in the control and in the preparation dialysed against Mn^{2+} ions, whereas the preparation dialysed against Co^{2+} ions showed only a 10% loss.

Fig. 4. Effect of dialysis on the activity of rat-liver glycylglycine-dipeptidase preparation. Experimental conditions are given in the text. ●, Co^{2+} ion system; ○, Mn^{2+} ion system; ▲, residual system.

The residual activity was inhibited completely by 1 mM-EDTA at pH 7, although 0.1 mM-EDTA had no effect. Sodium citrate, another metal-ion chelator, inhibited the residual activity by about 12% at a concentration of 50 mM.

Metal-ion specificity. Table 1 shows the effect of several bivalent cations on the rate of glycylglycine hydrolysis by the rat-liver preparation. The significant activity demonstrable without the addition of bivalent metal ions, the residual activity, is represented as 100% in Table 1. The Table shows that only Co^{2+} and Mn^{2+} ions are activators, causing five- and four-fold increases in the rate of hydrolysis respectively.

Table 2 shows the effect of various metal ions on the Co^{2+} ion system. Inhibition by the added metal ion is observed in all cases; the general order of the degree of inhibition is similar to that in the residual system except for the reversal of the order of Zn^{2+} and Cu^{2+} ions.

Table 1. *Effect of bivalent metal ions on the glycylglycine-dipeptidase activity of rat liver*

Experimental details are given in the text. The activity of the metal-ion-free (residual) system is set at 100.

Metal ion present	Activity (%)
None	100
Co^{2+} (1.0 mM)	544
Co^{2+} (0.1 mM)	400
Mn^{2+} (1.0 mM)	414
Mn^{2+} (0.1 mM)	280
Ni^{2+} (1.0 mM)	80
Ni^{2+} (0.1 mM)	116
Pb^{2+} (1.0 mM)	39
Pb^{2+} (0.1 mM)	71
Mg^{2+} (1.0 mM)	94
Fe^{2+} (0.1 mM)	100
Zn^{2+} (0.1 mM)	50
Cu^{2+} (0.1 mM)	12
Cd^{2+} (0.1 mM)	12
Hg^{2+} (0.1 mM)	0

Table 2. *Effect of bivalent metal ions on the activation by cobaltous ions of the glycylglycine-dipeptidase activity of rat liver*

The concentration of Co^{2+} ion was 1.0 mM throughout, and that of the other metal ions 0.1 mM. Experimental details are given in the text. The activity in the presence of 1.0 mM- Co^{2+} ion alone is set at 100.

Metal ion(s) present	Activity (%)
Co^{2+}	100
$\text{Co}^{2+} + \text{Mn}^{2+}$	68
$\text{Co}^{2+} + \text{Ni}^{2+}$	85
$\text{Co}^{2+} + \text{Pb}^{2+}$	82
$\text{Co}^{2+} + \text{Zn}^{2+}$	32
$\text{Co}^{2+} + \text{Cu}^{2+}$	64
$\text{Co}^{2+} + \text{Cd}^{2+}$	4
$\text{Co}^{2+} + \text{Hg}^{2+}$	0

Binding constants for the metal ion in the two metal-ion-activated systems were determined by the method described by Jones (1960). Experimental plots are shown in Fig. 5. The apparent K_m values determined for Co^{2+} ion and Mn^{2+} ion systems are $26 \mu\text{M}$ and $23 \mu\text{M}$ respectively. These values were obtained without considering the metal ion-substrate binding. The constant for Co^{2+} ions obtained here is close to the value of $28 \mu\text{M}$ reported by Smith (1948a). Taking the Co^{2+} ion-glycylglycine binding into consideration, Rabin & Crook (1956) have determined a value of $0.11 \mu\text{M}$ for the Co^{2+} ion-enzyme binding constant, which is in good agreement with the value of $0.097 \mu\text{M}$ which they recalculated from Smith's data.

A similar treatment of the present data gives a value of $0.63 \mu\text{M}$ for the Co^{2+} ion-enzyme binding constant and $3.4 \mu\text{M}$ for the Mn^{2+} ion-enzyme binding constant. These were calculated by determining the free metal-ion concentration from the value of K_1 , 1.2 mM/sec., for Co^{2+} ion-glycylglycine binding (Datta & Rabin, 1956) and from that of K_1 , 14 mM/sec., for Mn^{2+} ion-glycylglycine binding (Monk, 1951), which were then substituted into the relationship (Warburg & Christian, 1942):

$$K = \left(\frac{V_{\max} - v}{v} \right) [\text{M}^{2+}] \quad (3)$$

where $V_{\max}$ and v are substituted for $A_{\max}$ and a , and $[\text{M}^{2+}]$ refers to the metal-ion concentration calculated as described above. The $V_{\max}$ values were determined from the plots in Fig. 5.

Since both Co^{2+} and Mn^{2+} ions were strong activators, the effect of the presence of both metal ions in the assay system was investigated at various

Fig. 5. Lineweaver-Burk curves of rat-liver glycylglycine-dipeptidase activity at different metal-ion concentrations. The assay conditions are those described in the text except for the variation of the metal-ion concentration. The velocity, v , is expressed as μmoles of glycylglycine hydrolysed/min. and $[\text{M}^{2+}]$ refers to the initial metal-ion concentration. ●, Mn^{2+} ion binding; ○, Co^{2+} ion binding.

Table 3. *Specific activities of rat-liver glycylglycine-dipeptidase preparation in the presence of both cobaltous and manganous ions*

Experimental details are given in the text.

Concn. of Co ²⁺ (mM)	...	Sp. activity (μ moles of substrate hydrolysed/min./mg. of protein)			
		0	0.1	0.2	1.0
Concn. of Mn ²⁺ (mM)					
0		0.196	0.562	0.644	0.644
0.1		0.330	0.378	0.366	0.412
0.2		0.430	0.372	0.379	0.412
1.0		0.455	0.443	0.443	0.345

Table 4. *Hydrolysis of glycine derivatives by rat-liver glycylglycine-dipeptidase preparation*

Each derivative was added to give an initial concentration of 50 mM. Experimental details are given in the text. The activity in the absence of added metal ions is set at 100.

	Activity (%)		
	1.0 mM-Co ²⁺ present	No metal ion present	1.0 mM-Mn ²⁺ present
Glycylglycine	522	100	410
Sarcosylglycine*	36	8	32
Glycylsarcosine*	11	4	14
N-Acetyl glycine	0	0	—
Benzoxycarbonylglycine	0	0	—
Glycyl-L-proline*	35	26	35
Glycyl-L-leucine	473†	1180†	—
Glycyl-DL-phenylalanine	489†	763†	—

* Because of lower rates hydrolysis was followed for 4 hr. with continued linearity.

† Determined as a zero-order rate by using the value obtained after 30 min., since the hydrolysis was not linear over the entire period.

concentrations (Table 3). The results indicate that when the concentration of either of the metals was held constant and the concentration of the other increased from 0 to 1 mM the specific activity fell.

The possibility that the increased total concentration of metal ion might account for such inhibition was investigated. Smith (1948a) reported that activation of glycylglycine dipeptidase by Co²⁺ ions was maximal at a concentration of about 1 mM. With the rat-liver preparation and 10 mM-Co²⁺ ion or 10 mM-Mn²⁺ ion, activities were respectively 43 and 51 % of the rate obtained with a concentration of 1 mM of each of the same metal ions. With either metal ion at a concentration of 2 mM, there was no discernible difference from the rates at a concentration of 1 mM.

Substrate specificity. Various substrates were tested to ascertain the specificity of the glycylglycine-hydrolysing activities in the rat-liver preparation (Table 4). Although several dipeptidase activities were present in the enzyme preparation

used, the effect of metal ions on each particular activity served to differentiate them.

The results show that the hydrolysis of the close glycylglycine analogues, glycylsarcosine and sarcosylglycine, is activated by Co²⁺ and Mn²⁺ ions, whereas Co²⁺ ions inhibit the hydrolysis of glycyl-DL-phenylalanine and glycyl-L-leucine. The Co²⁺ and Mn²⁺ ions have essentially equal activating effects on the rate of hydrolysis of glycyl-L-proline.

Effect of amino acid and glycine derivatives. The linearity of the rate of hydrolysis of glycylglycine indicates that the product, glycine, is not inhibitory at concentrations up to 60 mM (60 % hydrolysis of glycylglycine). This was also shown for higher concentrations by the addition of glycine to the initial assay mixture. The effect of glycine and several other amino acids and glycine derivatives on the Co²⁺ ion and residual systems is shown in Table 5.

The hydrolysis of glycylglycine is appreciably inhibited by L-leucine and L-isoleucine in the Co²⁺ ion system but less so in the residual system. L-Lysine is slightly inhibitory only in the residual system.

The effect of L-leucine on all three of the glycylglycine systems is shown in the Lineweaver & Burk (1934) plots in Fig. 6. The K_m values for glycylglycine and K_i values for L-leucine, determined by assuming competitive inhibition, are listed in Table 6.

DISCUSSION

The degree of purification of the preparation used in the present study is comparable with others studied from mammalian sources (Smith, 1948a), although glycylglycine-hydrolysing activities from micro-organisms have been more highly purified (Crewther & Lennox, 1953; Nishi, 1958).

Metal-ion-activated peptidases frequently exhibit dual metal-ion specificity; however, none has been reported that is activated only by Mn²⁺ and Co²⁺ ions, as are the glycylglycine-hydrolysing systems studied here. These two ions may be expected to exhibit similar activating effects because

Table 5. *Effect of amino acids and glycine analogues on the hydrolysis of glycyglycine by the rat-liver glycyglycine-dipeptidase preparation*

Experimental details are given in the text. The two columns are treated separately; 100 in each column is the relative rate for that system with only glycyglycine present.

Substrate and amino acid or glycine analogue present	Activity (%)	
	1.0 mM-Co ²⁺ present	No metal ion present
Glycyglycine (50 mM)	100	100
Glycyglycine (50 mM) + sarcosylglycine (50 mM)	102	—
Glycyglycine (50 mM) + <i>N</i> -acetylglucine (70 mM)	104	—
Glycyglycine (50 mM) + sarcosine (50 mM)	88	—
Glycyglycine (50 mM) + glycine (25 mM)	88	100
Glycyglycine (50 mM) + L-leucine (6.0 mM)	12	65
Glycyglycine (50 mM) + L-isoleucine (6.0 mM)	42	59
Glycyglycine (50 mM) + L-lysine (6.0 mM)	98	84

Table 6. *Kinetic constants of rat-liver glycyglycine-dipeptidase preparation*

Experimental details are given in the text. K_m (glycyglycine), the Michaelis constant for glycyglycine determined without added L-leucine; K_i (L-leucine), the inhibition constant obtained by assuming competitive inhibition by L-leucine.

	Concn. of L-leucine (mM)	K_m (glycyglycine) (mM)	K_i (L-leucine) (mM)
Residual system (no metal ion present)	0	55	—
	4.0	—	65
	3.0	—	15
Co ²⁺ ion system (0.2 mM-Co ²⁺ ion present)	0	37	—
	4.0	—	0.85
Co ²⁺ ion system (0.1 mM-Co ²⁺ ion present)	0	29	—
	4.0	—	0.21
Mn ²⁺ ion system (0.2 mM-Mn ²⁺ ion present)	0	27	—
	3.0	—	0.21

of their similarity in size and electron affinity (Malmström & Rosenberg, 1959). The fact that Mn²⁺ ions are a less effective activator would be predicted if complex stability were accepted as one of the major contributing factors to enzyme activation.

It is more difficult to explain the effect observed when both Co²⁺ and Mn²⁺ ions are present (Table 3). Inhibition studies of this type have been reported by Malmström (1961) for enolase. He concluded that the inhibition was competitive, i.e. that the activating metal ions bind to a single site. The results obtained in the present study do not indicate competitive inhibition but might be explained if it is assumed that more than one enzyme site is responsible for the observed hydrolysis, with binding of metal ions to one site in some way rendering the other inactive. Where the existence of two separate enzymes is postulated, the presence of one metal ion might activate one and inhibit the other activity. A possible factor contributing to the inhibition, which was considered by Rosenberg (1960) for carnosinase, is the increased metal-ion concentration present in the mixed system. Such an effect does not seem operative here either, since doubling the concentration of either metal ion alone did not result in a diminished rate.

Results reported for the inhibition of the activity when both activating metal ions were added suggest the possibility of more than one glycyglycine-hydrolysing activity. This possibility is also supported by the results from the dialysis experiment (Fig. 4), which shows the residual activity to be inactivated by dialysis at a rate proportionately slower than either of the two metal-ion systems.

Although the inhibition of the residual system by EDTA and citrate is evidence for the presence of a metal-ion activator, a comparison of the heats of activation calculated for the overall reactions in the three systems shows that the residual system has ΔH about half of that of the Co²⁺ ion and Mn²⁺ ion systems. This would indicate that the residual activity cannot be the same as the others, unless a tightly bound metal ion is involved which is completely different or bound in a different way from those in the metal-ion systems.

The slight but perhaps significant difference between the pH optimum of the residual activity and that of the two metal-ion systems is also evidence for the existence of different enzymes. The greater relative stability of the residual activity may be accounted for by the effect of pH on the metal ion-substrate binding. Previous pH-activity curves for activation by Co²⁺ ions of various pre-

parations studied indicate pH optima ranging from 7.6 to 8.0, whereas that obtained in this study is about 8.5. This higher pH may be due to some property of the buffer (tris) used or may well be a valid characteristic of the rat-liver glycylglycine

Fig. 6. Effect of L-leucine on the hydrolysis of glycylglycine by rat-liver glycylglycine-dipeptidase preparation. Experimental conditions are given in the text. The velocity, v , is expressed as μ moles of glycylglycine hydrolysed/min. (a) Residual system: $\bullet$, 4.0 mM-L-leucine present; $\circ$, 3.0 mM-L-leucine present; $\blacktriangle$, no added L-leucine. (b) Mn^{2+} ion system (0.2 mM- Mn^{2+} ion present): $\bullet$, 4.0 mM-L-leucine present; $\circ$, no added L-leucine. (c) Co^{2+} ion system (0.2 mM- Co^{2+} ion present): $\bullet$, 4.0 mM-L-leucine present; $\circ$, no added L-leucine. (d) Co^{2+} ion system (0.1 mM- Co^{2+} ion present): $\bullet$, 4.0 mM-L-leucine present; $\circ$, no added L-leucine.

dipeptidase. The variation of pH can affect the metal-ion-activated rate in three ways. It may change (a) the enzyme molecule itself, (b) the binding of the metal ion to the enzyme or substrate or both, or (c) the availability of OH^- ions for the nucleophilic attack on the peptide bond (Klotz, 1954). These several factors make a comparison of pH optima, as a criterion for activity separation, unreliable.

Reports of lag periods for enzymic activity have been criticized (Rosenberg, 1960) because the apparent lag period has been found to be diminished with increasing purity of the enzyme preparation. This possibility also exists here. The quantitative interpretation of the plots for lag time is difficult, although with the Co^{2+} ion system there is a definite lag period of 7–10 min. A preincubation for 10 min. of Co^{2+} ions and enzyme diminishes this period somewhat to a new lag period of the same length as that found in the residual system.

The preparation used in this study contains several peptidase activities. Table 4 shows that the hydrolysis of glycyl-L-leucine is inhibited by Co^{2+} ions as is the hydrolysis of glycyl-DL-phenylalanine. The hydrolysis rates do not appear to be zero order at the concentrations employed, but both peptides are hydrolysed considerably more rapidly than is glycylglycine. Stereospecificity of the enzyme responsible was shown with glycyl-DL-phenylalanine, for the maximum amount hydrolysed approached one-half of the total quantity of peptide. The use of glycyl-L-proline as a possible substrate showed that it was hydrolysed slowly and the hydrolysis was activated by both Co^{2+} and Mn^{2+} ions equally. The hydrolysis of glycyl-L-leucine can be ascribed to glycyl-L-leucine dipeptidase (Smith, 1948*b*) and that of glycyl-L-proline to prolidase (Bergmann & Fruton, 1937).

The hydrolysis of glycylsarcosine and of sarcosylglycine is activated by both Co^{2+} and Mn^{2+} ions, in a manner similar to that of glycylglycine, but the rates of hydrolysis are much slower. This similarity in metal-ion specificity would suggest that the same enzyme may be responsible for the hydrolysis of all three dipeptides. The stabilities of Co^{2+} ion complexes of sarcosylglycine are only slightly lower than those of glycylglycine (Datta & Rabin, 1956), so that hydrolysis is not affected by differences in the extent of metal-ion binding.

Nishi (1960), using a preparation of glycylglycine dipeptidase from yeast, has reported the effect of various amino acids on its activity. The results are qualitatively similar to those reported here, i.e. the inhibition is greatest with L-leucine, that with L-isoleucine is somewhat less, and glycine has very little effect even at relatively high concentrations. The similarity in molecular size and shape of L-leucine and glycylglycine is the probable basis for

the competitive inhibition. The binding constant, pK_1K_2 , of Co^{2+} ions and L-leucine (8.26) (Maley & Mellor, 1950) is similar to that for glycylglycine and Co^{2+} ions (8.27) (Datta & Rabin, 1956). The plots in Fig. 6 show a competitive inhibition in the Co^{2+} ion system; perhaps a slight deviation from full competitive inhibition would result from a reduction in free metal-ion concentration in such a system. The deviation from full competitive inhibition in the Mn^{2+} ion system might be accounted for by the fact that Mn^{2+} ions form more stable complexes with L-leucine than with glycylglycine (Datta & Rabin, 1956; Maley & Mellor, 1950).

The present results indicate that the rat-liver preparation contains at least two glycylglycine-dipeptidase activities, one requiring for activity either Co^{2+} or Mn^{2+} ions and the other being active without the addition of a bivalent metal ion.

SUMMARY

1. The glycylglycine-hydrolysing activities present in a high-speed supernatant from homogenized rat liver have been studied. Both Co^{2+} and Mn^{2+} ions activate the hydrolysis but other bivalent cations are either without effect or inhibitory. The presence of one cation inhibits activation by others.

2. There is significant hydrolytic activity in the absence of metal ions. This activity is inhibited by bivalent cations and by complexing agents such as EDTA and citrate.

3. Studies of the effects of pH, reaction temperature, dialysis and time on these activities suggest the presence of at least two enzymes. Differences in substrate requirements were also observed.

4. L-Leucine and L-isoleucine inhibit the Co^{2+} ion-activated enzyme, L-leucine competitively, but were less effective inhibitors of the enzyme that did not require metal ions. L-Lysine inhibited the metal-ion-free system more than the Co^{2+} ion system.

5. The K_m and K_i values and heats of activation were calculated from the results and these also indicate the non-identity of the metal-ion-requiring and the metal-ion-free activities.

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