Purification and Properties of a Mouse Ascites Tumor Dipeptidase, a Metalloenzyme*

(Received for publication, July 13, 1970)

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SUMMARY
A dipeptidase that hydrolyzes L-Ala–Gly and a wide spectrum of other L-α-dipeptides has been purified 800-fold from the soluble fraction of Ehrlich-Lettre mouse ascites tumor cells. The highest specific activity (micromoles of dipeptide hydrolyzed at 40° per min per mg of protein) achieved was 2,600 with Ala–Gly, the substrate with which purification was followed. With the best substrate, Ala–Ile, this specific activity was equivalent to a molecular activity (moles of dipeptide hydrolyzed at maximum velocity at 40° per min per mole of enzyme) of 2 × 10⁶. Instability of the enzyme at this high activity prevented determination of homogeneity; a sample of molecular activity of 1 × 10⁶ (Ala–Gly specific activity, 1,360) was estimated to be 50% pure by acrylamide gel electrophoresis. Studies with metal chelators indicate that the dipeptidase is a metalloenzyme. For instance, o-phenanthroline completely inhibited the enzyme activity (50% at 0.1 mM), whereas m-phenanthroline had no effect. Although atomic absorption analyses showed a correlation of zinc content with enzyme activity in the final chromatographic step and a value of 0.9 ± 0.1 g atom of zinc per mole of enzyme of specific activity 2,600 (Ala–Gly), it has not been confirmed that the dipeptidase is a zinc metalloenzyme. The enzyme was separated from leucine aminopeptidase, prolidase, and tripeptidase by Sephadex G-150 filtration and, by comparison with standard proteins, was shown to have a molecular weight of 85,000 ± 5,000. The dipeptidase hydrolyzes only L-α-dipeptides with a free amino and carboxyl group. Kinetic studies of 15 dipeptides have shown the $K_m$ values to vary between 0.4 and 22 mM and the relative $V_{max}$ values (Ala–Gly standard, $V_{max}$ – 1) to vary from 5.3 (Ala–Ile) to 0.01 (Gly–Gly). Inhibition by high substrate concentrations (3 to 50 mM) was observed in cases where the $K_m$ was low. Peptides with small NH₂-terminal and bulky nonpolar COOH-terminal R groups were preferred. The hydrolyses of the relatively poor substrates, Pro–Gly and Gly–Gly, were activated by mM Mn²⁺ and Cu⁺⁺, respectively, whereas that of Ala–Gly was inhibited by both these metals as well as by 10 other metals. No evidence was obtained that these hydrolyses were carried out by separate enzymes: the enzyme peaks were congruent, the ratios of activities were constant throughout purification, and the activities toward the three substrates decayed at the same rate on exposure to 30° for periods up to 24 hours.

Although many proteolytic enzymes have been studied intensively during recent years, the dipeptidases have received little attention, probably because of their lability. This class of enzymes hydrolyzes only dipeptides with a free amino and carboxyl group (3, 4). It has long been known that dipeptidases occur in all cells studied; most notably, the enzymes that hydrolyze Ala–Gly are found in highest activity in rapidly growing and protein-synthesizing cells (5, 6). The Ala–Gly dipeptidase has been shown to increase in activity along with the active protoplasm (5), but to decrease in mature cells and cells that are breaking down (5–7). Simmonds (8), from her studies of peptidase activity in log and stationary phase Escherichia coli K-12 cells, has postulated that the intracellular crypticity of peptidase activity is controlled by metal ions. The function of the dipeptidases has been clarified by the work of Coffey and de Duve (9), who have shown that proteins are hydrolyzed to small peptides (mainly dipeptides) by liver lysosomal enzymes and that soluble dipeptidases split the dipeptides into amino acids which can then be used for protein synthesis.

Several stable dipeptidases have been highly purified from hog kidney (4, 10–15) and E. coli (16), but these occur in most cells in low activity compared to the unstable enzyme that hydrolyzes Ala–Gly (4, 17, 18). One renal particulate dipeptidase has been crystallized and is a zinc metalloenzyme (13). In addition, Cordonnier (19) has isolated a dipeptidase from yeast that hydrolyzes Ala–Gly as well as a variety of other dipeptides. The present paper describes progress in the purification (800-fold) of a relatively labile soluble dipeptidase from a rich mammalian source, a mouse ascites tumor. This enzyme hydrolyzes Ala–Gly and a variety of other dipeptides and has been shown to be a metalloenzyme.

EXPERIMENTAL PROCEDURE

Materials and Methods

Chemicals—The peptides were purchased from Miam with the exception of Ala–Ile and Ala–Leu, which were obtained from Sigma. The dehydropeptides were a gift from Dr. Benedict
Campbell of the University of Missouri. Crystallized bovine serum albumin, five times crystallized egg albumin, and mouse albumin,
Fraction V, were all purchased from Pentex. Goat antibody to mouse serum was produced by Hyland Laboratories, Los Angeles, California. Cytochrome c (Kellin-Hartree) was purchased from Boehringer. Mannheim. Bromsulphalein was obtained from Hyson, Westcott, and Dunning, Inc., Baltimore, Maryland. Chemicals for disc electrophoresis were supplied by Canal Industrial Corporation, Bethesda, Maryland. Dansylbovine serum albumin (20) was synthesized by Judith Liebowitz of this Institute. The ammonium sulfate and sucrose, special enzyme grade, were manufactured by Mann, and analytical grade chemicals and glass-distilled water (greater than 106-ohm resistance) were used for all solutions. DEAE-Cellulose (microgranular DE 32) was obtained from the Whatman Company, Reeve Angel, Clifton, New Jersey, Distributors, and Sephadex G-150 and Ficoll from Pharmacia. p-Phenanthroline was obtained from Fisher and m-phenanthroline was a gift from Dr. Barbara Nevaldine, Syracuse University. Chelex 100 was from BioRad Laboratories, and Coomassie brilliant blue, R-250, from Colab Laboratories, Inc., Chicago Heights, Illinois.

General Conditions—The following precautions were taken to reduce metal ion contamination. All operations were carried out in a relatively dust-free room in glassware which had been chromic acid-cleaned and boiled in water of greater than 106-ohm resistance. The enzyme preparations were stored frozen in distilled water, 0.1 M and titrimetrically was run in the same experiment with each glycyl dipeptide. The ninhydrin method could not be used for NH2-terminal glycyl dipeptides, since there is too small a difference in ninhydrin color between the glycyl peptides and their constituent amino acids for practical use. For assay of the hydrolysis of these dipeptides (e.g. Gly-Pro, the substrate for prolidase) the ultramicrochemical titrimetric method of Lindenberg-Lang and Holter (5) was used. A control of Ala-Gly hydrolysis estimated titrimetrically was run in the same experiment with each glycyl peptide.

Enzyme Units—A unit of enzyme activity is defined as 1 pmole of substrate hydrolyzed per min at 40°. The substrate concentrations were 50 mM except in the kinetic experiments. Unless otherwise specified, enzymatic activity is the rate of hydrolysis (40°) of Ala-Gly (90 mM, pH 8.3). Enzyme concentrations are recorded as units per ml of solution. Specific activity is defined as enzyme units per mg of protein. Relative maximum velocity (V(max)) is defined as the ratio of the maximum velocity with a given substrate to that with Ala-Gly.

Enzyme Kinetics—For determination of Michaelis constants and maximum velocities, peptide hydrolysis was followed by monitoring the loss of ultraviolet absorbance due to the peptide bond with a modification of the method of Schmitt and Siebert (26). Although the absorption peak for the peptide bond is at 235 rnp (27), it was found that at 235 rmp molar absorbance differences (eAIA; eBIA, 56 rnp) for the millimolar range of substrate concentrations required, fell in a region measurable with accuracy in the Gilford spectrophotometer. The course of the reaction was recorded on a Honeywell strip chart recorder attached to the Gilford spectrophotometer. The reaction was carried out in silica microcells (2 x 10 mm) with a 155-ml volume of reaction mixture. The cell compartment was thermostated at 30°, a temperature which was chosen for enzyme stability in the absence of albumin. Buffers used were phosphate or phosphate-borate which had low absorbances at 235 rnp.

Protein Determination—The method of Nayyar and Glick (28) was modified for determination of soluble proteins. Bovine serum albumin, which is an acidic protein, as is the dipeptidase, was used as the reference standard (20). The NaOH step was omitted and the citrate-sulfobromophthalain reagent (0.2 to 0.4 mg of sulfobromophthalain per ml of 0.5 M citric acid) was adjusted accordingly. By variation of the volumes of reagents and samples so that the weight ratio of reagent to protein was in the range 1.5 to 6, protein concentrations from 0.1 to 5 mg per ml were determined in three overlapping ranges.

Metal Content—A Varian-Techtron atomic absorption spectro-

1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

2 S.-H. Hsiao, unpublished observations.
The effect of pH on dipeptidase activity. Ala-Gly, 0.10 M, was made up to the required pH with mixtures of 50 mM Na₂B₄O₇ with 0.1 M NaOH or 0.1 M KH₂PO₄. Seven microliters of an enzyme preparation (specific activity 164) diluted 5-fold with ovalbumin (1.5 mg per ml in 20 mM potassium phosphate buffer, pH 7.8) were incubated for 8 min at 40° with 7 μl of substrate. The pH values are given as measured at 25°. Relative activity refers to percentage of activity at the optimal pH.

### Table I

| Preparation       | Volume | Total Protein | Total Activity | Specific Activity | Recovery |
|-------------------|--------|---------------|----------------|-------------------|----------|
| Soluble fraction. | 414    | 10,500        | 36,750         | 3.5               | 100%     |
| (NH₄)₂SO₄ (50-75%) | 46     | 3,200         | 37,200         | 11.6              | 101%     |
| Sephadex G-150... | 76     | 360           | 33,000         | 91.7              | 90%      |
| DEAE-cellulose    |        |               |                |                   |          |
| Fractions 90-91...| 2.8    | 1.9           | 5,000          | 2,600             | 14%      |
| Fractions 89-94...| 8.8    | 8.0           | 14,300         | 1,800             | 39%      |

* The results in this table represent the best purification achieved. The figures in parentheses give the ranges obtained in all purifications.

**Photometer**—fitted with an Aztec log expander and a Sola constant voltage transformer, was used for determination of the metal content of the enzyme preparations. Calibration of the instrument was made with appropriate dilutions of Fisher certified atomic absorption standards. The buffer blanks contained less than 0.1 μM zinc, which was the noise level of the instrument.

**Other Determinations**—Phosphatase was analyzed by the procedure of Kolb, Weidner, and Tomnies (30) scaled down for 5-μl aliquots. Chloride was measured by the method of Schales and Schales (31) modified for use with 10 to 20-μl samples. Mouse serum albumin was estimated by a micromodification by Weiler, Melletz, and Breuninger-Peck (32) of the Ouchterlony gel diffusion method. Mouse serum albumin (0.1 mg per ml) served as the standard and goat antibody to mouse serum as the antibody. Acrylamide disc electrophoresis (33) was carried out for 1 to 6 hours at 3 mA per tube in Tris glycine buffer at pH 8. Amido black or Coomassie brilliant blue was used for staining. The densitometry of the resulting bands was performed with a linear scanning attachment to a Gilford spectrophotometer and the area under the curves was measured with a planimeter.

**Biological Material**—The Ehrlich-Lettre hyperdiploid mouse ascites carcinoma was maintained by serial intraperitoneal transplantation of 0.2 to 0.3 ml of ascites into adult (24- to 3 month old) female mice of the ICR albino strain. After 7 to 8 days of growth, the peak of peptidase activity in the cells (34), tumor samples were aspirated by sterile syringe and collected in tubes in an ice bath. All subsequent operations were carried out at 2-5°. The ascites serum, which contains a dipeptidase inhibitor (34), was removed from the cells by centrifugation and subsequent washing with buffered 0.9% NaCl-KCl (sodium to potassium ratio, 10:1; 0.01 M NaHCO₃). The pelleted, washed ascites cells were then subjected to a 2-min hypotonic shock treatment (35) with water, in order to cytolyze any contaminating red cells. After centrifugation and repetition of this treatment, the cell pellets were cream-colored.

**Homogenization of Ascites Cells**—The packed, washed cells, suspended in about one-sixth their volume of 20 mM potassium phosphate, pH 8.2, were transferred to an iced Servall Omi-
mixer and homogenized at full speed (14,000 rpm) for 5 min. After addition of sufficient 2 M sucrose in 20 mM potassium phosphate (pH 7.6) to give 0.25 M sucrose, final homogenization was carried out for 2 min.

Preparation of Soluble Fraction—The homogenate was centrifuged at 17,500 rpm (20,000 × g) in a Spinco model L centrifuge for 20 min in order to sediment the remaining whole cells, nuclei, and mitochondria. The supernatant fluid was then centrifuged at 40,000 rpm (105,000 × g) for 2 hours. After removal of the top lipid layer, the clear soluble fraction (pH 6.9 to 7.0) was pipetted off, leaving behind the micromolar pellet and the fluffy layer. The dipeptidase is localized entirely in the soluble fraction of the ascites cell (36).

RESULTS

Dipeptidase Purification

Buffer and pH Optimum—As seen from Fig. 1, the pH optimum of the enzyme is pH 8.3. Since the highest enzyme activities were obtained with preparations in phosphate (see "Effect of Added Metal Ions on Dipeptidase Activity"), this buffer (20 mM, pH 8.2) was used throughout the purification.

Ammonium Sulfate Fractionation—Table I summarizes the purification of the enzyme. Approximately 400 ml of ascites-soluble fraction from 350 mice were fractionated between 50 and 75% saturated ammonium sulfate. The resultant precipitate was dissolved in the minimal volume of buffer and dialyzed against the same buffer overnight. Yields of enzyme in the ammonium sulfate step usually exceeded 100%, probably because of removal of an inhibitor.

Sephadex G-150 Filtration—A portion of the ammonium sulfate preparation, a volume of 13 to 15 ml, was filtered through a G-150 column. The elution pattern (Fig. 2) shows the separation of leucine aminopeptidase (substrate Leu- NH2), prolidase (substrate Gly-Pro; arrow points to peak), and tripeptidase (substrate Ala-Gly-Gly) from the dipeptidase that hydrolyzes Ala-Gly. The latter is also freed from hemoglobin which is at the position of the 250 μL peak to the right of the tripeptidase. The hydrolyses of Pro-Gly (Mn2+-activated) and of Gly-Gly (Co2+-activated and not shown in Figure 2) parallel that of Ala-Gly.

DEAE-cellulose Chromatography—The peak fractions from several Sephadex runs were pooled and chromatographed on DEAE-cellulose (Fig. 3). A 20-fold purification (Table I) of the enzyme resulted, largely because of a lack of adsorption of most of the inert protein. It is apparent, however, from the lack of superposition of the enzyme and protein peaks, that the entire enzyme peak is not homogeneous. The contaminating protein on the right side of the enzyme peak (fractions at 47- and 55 ml elution volume) was identified as mouse serum albumin by use of the Ouchterlony (32) immunological technique. Attempts to remove this protein by precipitation with the γ-globulin fraction of antiserum to mouse serum were unsuccessful. The highest specific activity (2600, Ala-Gly, 50 mM substrate) was found at the trough of the protein curve (elution volume 53 to 37 ml) and may represent pure dipeptidase. The 3A280:4A560 ratio of these preparations varied from 0.26 to 0.50.

An artifact of several Sephadex G-150 runs has been the occurrence of a rapid marked shrinkage of the column during the period of sample addition. This phenomenon has rendered the use of either upward flow elution or of flow adaptors impossible, but does not appear to impair the resolution unduly. It is probably due to the osmotic effect described by Edmond et al. (37).
log molecular weight of standard proteins and dipeptidase. Three milligrams of blue dextran, 40 mg of rabbit γ-globulin, 10 mg of cytochrome c, and 40 mg of ovalbumin were dissolved in 0.5 ml of 20 mM potassium phosphate, pH 8.2, and the flow rate was 6 to 8 ml per hour. Blue dextran was determined by means of its absorbance at 625 μm and cytochrome c by its absorbance at 416 μm. The elution positions of ovalbumin and γ-globulin were determined by using the peaks of 280 μm absorbance which were appropriate for the molecular weights of these proteins. Dansyl-bovine serum albumin was estimated by its fluorescence which was activated at 330 nm and emitted at 450 nm. The elution volume was 2.9 ml. The column measured 2.5 X 91 cm, the buffer volume was 2.9 ml. The column was considered the total volume that emerged from the column from the start of sample addition. The molecular weights of the standard proteins were taken from Reference 39. The position of the dipeptidase is indicated by a straight line.

Fig. 4. Plot of elution volume from Sephadex G-150 against log molecular weight of standard proteins and dipeptidase. Three milligrams of blue dextran, 40 mg of rabbit γ-globulin, 10 mg of cytochrome c, and 40 mg of ovalbumin were dissolved in 0.5 ml of a dansyl-bovine serum albumin solution (containing 3.4 mg of protein) plus 1.0 ml of ammonium sulfate-fractionated peptidase (containing 40 mg of protein and 270 units of activity). The final volume was 2.9 ml. The column measured 2.5 X 91 cm, the buffer was 20 mm potassium phosphate, pH 8.2, and the flow rate was 6 to 8 ml per hour. Blue dextran was determined by means of its absorbance at 625 μm and cytochrome c by its absorbance at 416 μm. The elution positions of ovalbumin and γ-globulin were determined by using the peaks of 280 μm absorbance which were appropriate for the molecular weights of these proteins. Dansyl-bovine serum albumin was estimated by its fluorescence which was activated at 330 nm and emitted at 450 nm. The elution volume was considered the total volume that emerged from the column from the start of sample addition. The molecular weights of the standard proteins were taken from Reference 39. The position of the dipeptidase is indicated by a straight line.

The zinc values are discussed below.

Acrylamide Gel Electrophoresis.—For an estimate of the purity of the enzyme, a preparation of a specific activity of 1360 was subjected to acrylamide gel electrophoresis (33), which resulted in one major and three minor (faint) bands. Densitometric analysis gave a value of 50% for the major band. Electrophoresis of samples of higher purity revealed more bands, which may have been due to structural changes in the enzyme as a result of repeated freezing and thawing. Therefore no estimate of enzyme purity was attempted at a specific activity above 1360.

Stability.—The stability of the dipeptidase is described in Table II. Except for the requirement of added sucrose for stability to freezing, the enzyme is completely stable until the Sephadex step, when dilution in albumin is compulsory for stability during the assay at 40°. After DEAE-cellulose chromatography, a further degree of instability is evidenced by intolerance to dialysis, to freezing, and to further chromatographic steps, such as rechromatography on DEAE-cellulose or Sephadex. No increase in stability was achieved by addition of MgCl₂ or tRNA (which was removed in the DEAE-cellulose step), or replacement of phosphate buffer by triethanolamine.

Properties

Molecular Weight.—An estimate of the molecular weight of the dipeptidase was made by gel filtration in Sephadex G-150 of a small aliquot of the ammonium sulfate-precipitated enzyme, in conjunction with several standard proteins and blue dextran (38) (Fig. 4). The resolution was excellent and it was notable that the dipeptidase peak was eluted just prior to, and overlapping that of, dansyl-bovine serum albumin. With the assumption that the dipeptidase is a globular protein, a molecular weight of 85,000 ± 5,000 was calculated from the mean of four experiments. When more highly purified enzyme (after DEAE-cellulose chromatography which removes tRNA) was used, the elution profile was similar and the molecular weight obtained was 87,000.

Effect of Metal Chelators.—Fig. 5 shows the inhibitory effect of o-phenanthroline, contrasted with the lack of inhibition by similar concentrations of m-phenanthroline, on the hydrolysis of Ala-Gly by the purified dipeptidase. It was determined that a
contained no magnesium. The absorbance of a ZnCl₂ (0.1 mM)-o-phenanthroline (Zn-OP) (0.1 mM) solution was read with a blank of o-phenanthroline (0.2 mM), ——-O. The absorbance of a solution of dipeptidase (specific activity 2200)-o-phenanthroline (Dipeptidase-OP) (0.37 mM) mixture was read with a blank of o-phenanthroline (0.2 mM), ——-O. Similarly, the absorbance of a MgCl₂ (0.01 mM)-o-phenanthroline (Mg-OP) (0.2 mM) solution was read with a blank of o-phenanthroline (0.03 mM), ——-O. The absorbance of a MgCl₂ (0.1 mM)-o-phenanthroline (Mg-OP) (0.2 mM) solution was read with a blank of o-phenanthroline (0.1 mM), A-A. The absorbance of a ZnCl₂ (0.1 mM)-o-phenanthroline (Zn-OP) (0.1 mM) solution was read with a blank of o-phenanthroline (0.2 mM), ——-O. The absorbance of a MgCl₂ (10 mM)-o-phenanthroline (Mg-OP) (0.2 mM) solution was read with a blank of o-phenanthroline (0.37 mM) and the absorbance of the dipeptidase solution read against water subtracted from the absorbance of the Mg-OP mixture. EDTA gave an inhibition curve similar to that of o-phenanthroline, but the concentration for 50% inhibition was 10⁻³ times higher. The factor of 10⁻³ difference between the two chelators was not caused by differences in ionic strength, inasmuch as the enzyme activity was not affected by 0.15 M NaCl or KCl. Histidine (50 mM) caused an 88% inhibition of the hydrolysis of Ala-Gly (12.5 μM) by the dipeptidase and (μM) dithiothreitol caused an 85% inhibition of the hydrolysis of Ala-Gly by the dipeptidase, whereas mercaptoethanol (5 μM) inhibited only 38%. The rough correlation of degree of inhibition by the thios with chelating ability (40), rather than reducing power, leads to the conclusion that inhibition is due to metal chelation.

The technique of differential spectrophotometry (41) was used in order to detect whether a metal ion in the enzyme was chelated by o-phenanthroline. Fig. 6 shows the difference absorption curve of Zn⁺⁺ o-phenanthroline as compared with o-phenanthroline, Mg⁺⁺-o-phenanthroline as compared with o-phenanthroline, and dipeptidase (specific activity 2500, 0.66 μM)-o-phenanthroline corrected for absorbance of enzyme and o-phenanthroline. The fact that the dipeptidase-o-phenanthroline curve resembles the metal-o-phenanthroline curves, with a major peak in absorbance in the 290 μM region and minor peaks at 327 and 343 μM, strongly suggests that the dipeptidase is a metalloenzyme. Similarly, the absorbance of a MgCl₂ (0.1 mM)-o-phenanthroline (Mg-OP) (0.2 mM) solution was read with a blank of o-phenanthroline (0.13 mM), ——-O. The control rate is that obtained for each substrate without added metal ions.

15-min prior incubation (23°) with o-phenanthroline gave full inhibition. EDTA gave an inhibition curve similar to that of o-phenanthroline, but the concentration for 50% inhibition was 0.13 μM, in contrast to the 0.13 mM concentration of o-phenanthroline. This factor of 10⁻⁶ difference between the two chelators was not caused by differences in ionic strength, inasmuch as the enzyme activity was not affected by 0.15 M NaCl or KCl. Histidine (50 mM) caused an 88% inhibition of the hydrolysis of Ala-Gly (12.5 μM) by the dipeptidase and (μM) dithiothreitol caused an 85% inhibition of the hydrolysis of Ala-Gly by the dipeptidase, whereas mercaptoethanol (5 μM) inhibited only 38%. The rough correlation of degree of inhibition by the thios with chelating ability (40), rather than reducing power, leads to the conclusion that inhibition is due to metal chelation.

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phenanthroline curve can only be considered as suggestive of a zinc metalloenzyme.

Prolonged (24 hours) dialysis at 5° of stable enzyme preparations (specific activity 12) against several changes of mm o-phenanthroline, followed by dialysis (6 hours) against changes of phosphate buffer until dialysates contained no o-phenanthroline, resulted in reduction of enzyme activity to 18% of a control preparation dialyzed for similar times. The activity could not be restored by addition of metal ions.

Determination of Metals in Enzyme Preparations by Atomic Absorption—Two metals, zinc and magnesium, have been measured in enzyme preparations at all stages of purification. Although enrichment of magnesium with respect to protein was observed, the fact that a net value of zero magnesium was obtained for one purified sample (specific activity 1,160) suggests that magnesium is not an essential constituent of the enzyme. On the other hand, there was a decrease in the ratio of moles of zinc to enzyme units during purification, but there was a correlation of zinc concentration with highly purified enzyme (Fig. 3).

The zinc values follow the enzyme curve on the left side but deviate on the right side, when mouse serum albumin is present. The control values were those obtained at zero time.

The Effect of Added Metal Ions on Dipeptidase Activity—As seen from Table III, the majority of metal ions commonly found in biological materials are inhibitory to the hydrolysis of Ala-Gly at concentrations less than millimolar. No significant activation was found at lower metal ion concentrations. The sensitivity of the dipeptidase to metal ions was seen in experiments in which compounds which act as better buffers at pH 8.2 were substituted for phosphate. Tris, N-ethylmorpholine, HEPES, sodium 5,5'-diethyl barbiturate and triethanolamine (all at 0.02 M) were inhibitory to varying extents. Less inhibition was observed with highly purified buffers. This result suggested that the inhibition was due to trace metal contamination in the buffers.

When the effect of millimolar concentrations of selected divalent metal ions was studied with highly purified enzyme preparations and various substrates, a complex pattern of inhibition and activation emerged. As seen from Table IV, although the hydrolysis of Ala-Gly was inhibited by Co²⁺, Mn²⁺, and Cd²⁺, the hydrolyses of Gly-Thr, Gly-Ser, and especially Gly-Gly were activated by Co²⁺, and those of Pro-Gly and Hyp-Gly by Mn²⁺. Since Gly Gly dipeptidase is known to be activated by Co²⁺ (42) and prolase (minidipeptidase) by Mn²⁺ (43), it seemed that at least three dipeptidases might be present in the enzyme preparations. Nevertheless, these activities could not be separated by Sephadex G-150 gel filtration (before or after the DEAE-cellulose step) or by DEAE-cellulose chromatography with a very slow phosphate gradient. Activities toward the three substrates, when normalized for their differences in hydrolysis velocity, were completely superimposable around the peaks. Moreover, the ratios of activities throughout purification were identical. The optimum pH for hydrolysis of Gly-Gly, Co²⁺-activated, was 8.2, similar to that of Ala-Gly. In addition, as seen in Fig. 7, when an enzyme preparation (specific activity 600) was held at 30° for 24 hours, the rates of loss in activity toward Ala-Gly (H₂O), Gly-Gly (Co²⁺), and Pro-Gly (Mn²⁺) were approximately the same for 24 hours. Therefore, no evidence has been obtained thus far for separate enzymes.

Specificity Studies and Kinetics—The enzyme is a true dipeptidase (3, 4) in that it hydrolyzes only N-α-dipeptides with a free amino and carboxyl group. Compounds which are not hydrolyzed are Gly-n-Leu, β-Ala-Gly, γ-phenylalanineamide, γ-leu-

### Table V

| Substrate | Vₘₐₓ | Kₘ |
|-----------|------|----|
| Ala-Ile   | 5.3  | 1.2 |
| Gly-Ile   | 3.6  | 7  |
| Gly-Val   | 2.9  | 4  |
| Gly-Leu   | 2.3  | 2.5|
| Ala-Ala   | 2.2  | 2  |
| Ala-Val   | 1.9  | 1.1|
| Leu-Ala   | 1.7  | 1.2|
| Gly-Met   | 1.6  | 2.5|
| Gly-Ala   | 1.1  | 22 |
| Val-Gly   | 0.92 | 1.4|
| Ala-Leu   | 0.88 | 0.4|
| Gly-Ser   | 0.29 | 12.5|
| Gly-Lys   | 0.15 | 5.5|
| Gly-Gly   | 0.01 | 15 |
| Gly-Asp   | <0.01|    |

### Kinetic data and relative substrate specificity

Substrate (150 μl) was equilibrated in a microcell to 30°, 5 μl of enzyme (specific activity 750) were added, and the reaction was followed at 233 μm with recording. Substrates were made up in 20 mM potassium phosphate-sodium borate buffer (borate to phosphate, 3:7), 250 mM sucrose, pH 8.4. The enzyme was diluted with 2 μg per ml albumin in 20 mM potassium phosphate buffer, pH 8.1, and was stored on ice. The enzyme concentration varied with the substrate but was of the order of a few units per ml. The rate of hydrolysis of each substrate was compared in the same experiment with the rate of hydrolysis of 12.5 mM Ala-Gly. Kₘ and Vₘₐₓ were determined from Lineweaver-Burk plots. Vₘₐₓ, the relative maximum velocity, is defined as the ratio of the maximum velocity with a given substrate to that with Ala-Gly. The maximum velocities for each substrate were calculated by the use of a factor based on the difference in extinction of the respective peptides and their constituent amino acids.
cinnamamide, Gly-Phe-amide acetate, benzyl oxycarbonyl-Gly-\text{L-Leu}, and \text{l-Ala-Gly-Gly}. The dehydropeptides, Gly-dehydro-Ala (0.24 mM) and Gly-dehydro-L-Phe (50 μM), are split 0.03% and 0.02% as fast as Ala-Gly (50 μM), respectively.

The availability of a spectrophotometric assay permitted the determination of the kinetic parameters of the enzyme for a variety of dipeptides. The values obtained are listed in Table V in the order of decreasing $V_{\text{max}}$. Significant enzyme inhibition was observed with many substrates in the 3 to 50 mM range, the concentration at which inhibition occurred being lower, the lower the $K_m$.

For instance (Fig. 8), the hydrolysis of Ala-Gly ($K_m$ 2.5 mM) is inhibited by substrate concentrations over 25 mM, whereas no inhibition is observed at 50 mM Gly-Ala ($K_m$ 22 mM).

A molecular activity (micromoles of Ala-Ile hydrolyzed at 40° per min per g of enzyme) of 2 × 10⁸ can be calculated for the best substrate found thus far, Ala-Ile. This value is based on the $V_{\text{max}}$, the specific activity of 2,600 with Ala-Gly as substrate and a molecular weight of 85,000. Since the assumptions used include that of a pure enzyme, the molecular activity would be even higher if the dipeptidase was contaminated with extraneous protein.

## DISCUSSION

The choice of Ehrlich-Lettre mouse ascites carcinoma cells as the enzyme source was dictated by the reproducibility of a constant single cell type (avoidance of isoenzymes), the high specific activity (3.5), and the restriction of the dipeptidase to the soluble cell fraction (36). In material commonly used for dipeptidase purification, hog kidney (10, 13) and E. coli (44), the enzymes are distributed between the particulate and soluble fractions.

Estimation of the purity of the ascites dipeptidase is difficult because of the liability of the most highly purified preparations. By the criterion of congruity of enzyme and protein peaks (after the last step, DEAE-cellulose chromatography), the dipeptidase is obviously not homogeneous over the peak. A stable preparation of a specific activity of 1,900 was estimated to be 50% pure, with the assumption that the strongest band after acrylamide gel electrophoresis was the enzyme. If this assumption was valid, enzyme of specific activity 2,600 should be homogeneous, but preparations originally of this specific activity showed multiple bands, possibly due to denaturation. A preparation of this specific activity has a molecular activity of 2 × 10⁶ with the best substrate, Ala-Ile. This value is very high compared to other purified proteolytic enzymes; for instance, the 1,200 times purified prolidase (11) had a turnover number of 3.5 × 10⁵ moles of Gly-Pro hydrolyzed per min per 10⁶ g of enzyme of molecular weight 150,000. Therefore it was felt that studies of the properties of the ascites dipeptidase of specific activity values of 2,000 to 2,600 were worthwhile. For kinetic studies, a more stable enzyme of specific activity 130 was used. Since the relative rates at 50 mM substrate determined spectrophotometrically compared favorably with those determined titrimetrically with enzyme of 2,600 specific activity, the use of the former enzyme preparations seemed acceptable.

Comparison of the relative substrate specificity of the ascites dipeptidase with Ala-Gly-hydrolyzing enzymes from different sources can be made only on the basis of the high substrate concentration (17 or 50 mM) at which the hydrolyses were

### Table VI

Comparison of relative substrate specificities of ascites, hog kidney particulate, and yeast dipeptidases

| Substrate          | Metal   | Dipeptidases<sup>a</sup> |
|--------------------|---------|--------------------------|
|                    | Ascites<sup>b</sup> | Hog kidney particulate<sup>c</sup> | Hog kidney particulate<sup>c</sup> | Yeast<sup>d</sup> autolysate<sup>e</sup> |
| L-Ala-Gly          | %       | %                        | %                        | %                        |
| Gly-L-Leu          | 100     | 100                      | 100                      | 100                      |
| Gly-L-Leu          | 72      | 117                      | 20                       |
| Gly-L-Phe          | 184     | 3                        |
| Gly-L-Ile          | 139     | 173                      | 81                       | 300                      |
| Gly-L-Val          | 0       | 102                      | 53                       |
| Gly-L-Ala          | 125     | 155                      | 88                       |
| Gly-L-Ser          | 117     | 15                       |
| Gly-L-Lys          | 101     | 85                       | 70                       |
| Gly-L-Tyr          | 138     | 136                      |
| Gly-L-Pro          | 50      | 73                       | 44                       |
| Gly-L-Phe          | 80      | 196                      | 132                      |
| Gly-L-Asp          | 34      | 61                       | 19                       |
| Gly-dehydro-Ala    | 33      | 147                      |
| Gly-dehydro-Phe    | 14      | 41                       | 16                       | 14                       |
|                    | 57      | 116                      | 74                       |

<sup>a</sup> The following specific activities refer to Ala Gly hydrolysis and were calculated as micromoles per min per mg of protein:

<sup>b</sup> 2000, pH 8.3, 40°, 50 mM substrates except dehydropeptides which were assayed (13) at concentrations of 0.24 mM Gly-dehydro-Ala and 50 μM Gly-dehydro-Phe.

<sup>c</sup> 500, pH 8.0, 27°, 50 mM substrates.

<sup>d</sup> 50, pH 8.0, 30°, 17 mM substrates except dehydropeptides.

<sup>e</sup> 300, pH 8.0, 40°, 50 mM substrates.

*The following specific activities refer to Ala Gly hydrolysis and were calculated as micromoles per min per mg of protein:*
measured. Although the relative data for a given enzyme may be misleading, because of our finding that aspartic dipeptidase is inhibited at these substrate levels, the comparison (Table VI) may shed some light on the plurality of dipeptidases. In addition to some of the dipeptides listed in Table V, Table VI includes dipeptides with aromatic residues. It should be emphasized that the aspartic enzyme has the highest specific activity toward Ala-Gly of the four enzymes and, on a relative substrate specificity basis, most closely resembles the enzyme purified from yeast autolysates by Cordonnier (19). Although the hog kidney particulate dipeptidase of Campbell et al. (13) has been crystallized, the specific activity of 69 is comparatively low. This enzyme and that from the same source purified in 1953 by Robinson, Birnbaum, and Greenstein (10) differ from the aspartic enzyme in their relatively rapid hydrolysis of dehydropeptides and cleavage of peptides with COOH-terminal d-amino acids, and therefore clearly represent a separate class of enzymes.

The question of multiplicity of enzymes within a given dipeptidase preparation has repeatedly arisen because Co** and Mn** specifically activate hydrolyses of some substrates and inhibit those of others. As seen from Table VI, the hydrolysis of Ala-Gly by both the aspartic and yeast enzymes is inhibited by Co**, whereas the cleavage of Gly-Gly and Gly-Ser are strongly activated by this metal ion. As in the case of the aspartic enzyme, Cordonnier (19) was unable to separate these activities in the yeast preparations. Similarly, Capobianco and Vecsia (14), who purified a dipeptidase 6000-fold from hog kidney, believe that they have a single enzyme, although Co** enhances the activity toward Gly-Gly and inhibits the hydrolysis of Gly-Leu and Leu-Gly. A precedent for the dependence of enzymatic specificity upon specific metals exists in the case of carboxypeptidase (45) in which, for example, replacement of Zn** by Co** abolishes peptidase activity while enhancing esterase activity. Since there is no evidence of separation of Ala-Gly, Gly-Gly, and Pro-Gly activities in the aspartic enzyme preparations either by chromatography or by inactivation, it is tentatively concluded that all three activities are functions of a single enzyme molecule. Purification of this enzyme to homogeneity would clarify this question.

Since carboxypeptidase (46) and leucine aminopeptidase (47, 48) are both zinc metalloenzymes and hydrolyze peptide bonds with adjacent free carboxyl and amino groups, respectively, it is not surprising that the aspartic dipeptidase is a metalloenzyme. Positive identification of the metal ion in the enzyme cannot be 48 without evidence of separation of Ala-Gly, Gly-Gly, and Pro-Gly activities in the aspartic enzyme preparations either by chromatography or by inactivation. The question of multiplicity of enzymes within a given dipeptidase preparation has repeatedly arisen because Co** and Mn** specifically activate hydrolyses of some substrates and inhibit those of others. As seen from Table VI, the hydrolysis of Ala-Gly by both the aspartic and yeast enzymes is inhibited by Co**. Although these activities are functions of a single enzyme molecule, purification of this enzyme to homogeneity would clarify this question.

Tentative generalizations can be drawn from the kinetic data with different dipeptides (Table V). The substrates that are hydrolyzed most rapidly are those with bulky COOH-terminal R groups, especially those branching at the β carbon, isoleucine and valine. Most noteworthy are the low or zero values for the peptides with COOH-terminal polar groups and Gly-Gly. The enzyme appears to prefer small NH2-terminal R groups. In general, NH2-terminal alanyl substrates have lower K** values than the corresponding NH2-terminal glycyl peptides. Results with such peptides as Leu-Ala and Val-Gly indicate that even greater bulk in the NH2-terminal amino acid causes a further lowering of the K**. Although both Ala-Gly and Gly-Ala have the same V**, the markedly higher K** for Gly-Ala indicates that this substrate interacts less well with the active site of the enzyme.

Acknowledgments—Dr. Shu-Hsi Hsiao collaborated in the early portions of this work and we wish to thank her especially for her contribution to the purification of the dipeptidase (1). The excellent technical assistance of Augusta Keppel (1), Diana P. Steinmuller (2), and Josefa Gatmaitan is gratefully acknowledged. We wish to thank Evelyn Bremminger-Peck for teaching us the Ouchterlony technique. We are especially indebted to Dr. Melvin J. Bosma for discussions on the interpretation of our immunological experiments, and finally, we are grateful to Dr. Irwin Rose and Dr. Albert Mildvan for their critical review of the manuscript.

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