# Studies on Dipeptidase

Mutumi MURAMATU*, and
Ryozo HIROHATA**.
*From Laboratory for Protein Chemistry,*
*(Director: Prof. R. Hirohata)*
*Yamaguchi Medical School, Ube*
(Received October 31, 1964)

Studies on dipeptidase which were initiated by Grassmann et al. and Linderström-Lang et al. disclosed the multiplicity of dipeptidase. Smith et al. isolated and purified leucine-amino-peptidase (LAPase) from hog kidney (*1*) and applied it to protein chemistry in order to study the amino acid sequence (*2*). Because their studies have not given all possible dipeptidases, in the present paper, the effects of metal ions on the hydrolytic activity of acetone powder of hog intestinal mucosa and hog pancreas to the synthesized substrates will be examined. Such a study may lead to the discovery of new types of dipeptidase and may also offer a scheme of the digestion of protein in the intestinal tract. Experiments were carried out in low substrate concentration of four series of dipeptides containing glycine at its N-terminal (Gly-X); and glycine (X-Gly), D, L-valine (X–Val) and D, L-isoleucine (X-Ileu) at its C-terminal respectively, and it was found that the hydrolytic activity of the acetone powder of hog intestinal mucosa is much stronger than that of the pancreas, and moreover that the hydrolysis of dipeptides Gly-X occurs easier than that of the dipeptides of other types. Furthermore, in the high substrate concentration, the mode of hydrolysis of dipeptides was studied, replacing X with various amino acids, and kinetic data were presented.

On the other hand, LAPase was isolated from hog kidney and partially purified according to Smith's method. The purified LAPase had the specific activity of $C_1=15$. It is found that the enzyme located in intestinal mucosa functioned especially to Gly–X, however it is quite different from LAPase. Replacing the X in dipeptides Gly-X, with glycine or threonine, the enzymatic activity of intestinal mucosa is far weaker than in case X in dipeptides is replaced by another amino acid. Since dipeptides Gly-X are hydrolyzed at almost the same rate with the exception of glycyl-glycine and glycyl-D, L-threonine, this enzyme found in hog intestinal mucosa seems to be one and the same enzyme. The enzyme will tentatively be called glycine amino peptidase (GAP

* Present adress: The Department of Enzyme Physiology, Institute for Enzyme Research, School of Medicine, Tokushima University, Tokushima.
** Present adress: Dept. of Biochem. Daiichi College of Pharmaceutical Sciences, Fukuoka.

ase) or glycyldipeptidase as it requires N-terminal glycine for its action. Considering the fact that the action of LAPase is interrupted at glycine residue of the polypeptide chain, the presence of GAPase is important as regards the digestion of protein. An attempt to purify GAPase failed to obtain any good results. It was concluded, however, that GAPase requires metal ion for its activity, because the complete inhibition by versen occured, however renewed activity was not attained by adding any one of $Zn^{++}$, $Mg^{++}$, $Ca^{++}$, $Mn^{++}$, and $Co^{++}$.

Though Smith described the fractionation of glycyl-leucine-dipeptidase from hog intestinal mucosa, no study has been made to examine the substrate specificity with various dipeptides.

Therefore, it remains uncertain whether GAPase is the same enzyme to glycyl-leucine-dipeptidase or not, however, the latter enzyme seems to be different from GAPase because of its behavior towards $Mn^{++}$, by which glycyl-leucine-dipeptidase is activated.

## EXPERIMENTALS

*1. Synthesis of the Substrate.* Among the dipeptidases, Gly-X, Gly-L-Phe, Gly-S-benzyl-L-CyS and Gly-ε-N-carbobenzoxy-L-Lys were synthesized by Fischer's chloroacetyl chloride method and the other dipeptides were obtained by Vaughan's mixed anhydride method (*3*) from carbobenzoxy glycine and amino acid esters.

In order to get dipeptides X-Gly, Pro, Ala, Met, Leu, Phe, Val and Ileu were converted to carbobenzoxy derivatives, Lys to N, N′-dicarbobenzoxy, CyS to S-benzyl-N-carbobenzoxy and Tyr to N-carbobenzoxy-O-benzylderivatives and thereafter these derivatives were coupled with glycine ethylester.

The dipeptides of two series, X-Val and X-Ileu were synthesized by coupling of X with D, L-Val or D, L-Ileu ethyl ester.

Carbobenzoxy derivaives of dipeptides containing S were reduced by means of metalic sodium in liquid ammonia, and those without S were reduced catalytically by using palladium black.

*2. Preparation of the Acetone Powder of Hog Intestinal Mucosa.*

All subsequent procedures were carried out at $0^{\circ}-4^{\circ}C$. Cutting off a piece of 60 cm length from the upper end of fresh hog duodenum, its mucosa was scratched and put into fifteen to twenty volumes of cold acetone under constant vigorous stirring. The precipitaties thus obtained were filtrated through a funnel and washed with purified acetone. During the filtration through the funnel the substance must be protected from the contact with humidity, this was obtained by passing dried air through the funnel. The airation of the substance with dried air must be continued for 30 minutes in order to remove the acetone quickly. The white powder was preserved on phosphorus pentoxide in vacuo

in the ice chamber without loss of activity for many months.

*3. Preparation of the Acetone Powder of Hog Pancreas.* Fresh hog pancreas was treated exactly as above.

*4. Preparation of LAPase.* LAPase of "Step 6" was prepared according to the method described by Smith et al (*1*). Its activity was found to be $C_1=15$.

*5. Enzymatic Action of Acetone Powder to Low Substrate Concentration.* Sixty mg of acetone powder was suspended in 10 ml of 1/30 M phosphate buffer solution (pH 7.6) and kept standing for 15 minutes at room temperature. After centrifugation at 3,000 r. p. m., the supernatant was used as enzyme solution. Reaction mixtures were composed of 0.5 ml of enzyme solution described above, 0.5 ml of substrate solution which contains 100 μmole of substrate in 10 ml of water, 1 ml of 1/30 M phosphate buffer solution (pH 7.6) and 0.5 ml of 1/200 M metal ion. The final concentration of substrate and metal ion was 2 mM and 1 mM respectively. After the reaction mixtures thus obtained were incubated at 37°C, 1 ml of the solution was heated in boiling water in order to stop the enzyme action, and then diluted to 20 ml. These diluted solutions were used to determine the amino acids splitted.

*6. The Enzymatic Activity of Acetone Powder of Hog Intestinal Mucosa to Substrate of High Concentration.* The amount of 0.15 ml of supernatant solution described above, 0.5 ml of the substrate solution (1/8M) dissolved in 1/30 M of phosphate buffer (pH 7.6) or 1/10 M of Tris buffer (pH 9.0) and 0.6 ml of the same buffer solution were mixed and incubated at 40°C. The final concentration of the substrate was 1/20 M. Of the reaction mixture 0.1 ml was taken off and heated in boiling water, in order to stop the reaction, and then, by adding water diluted to 2.5 ml. One ml of the solution thus obtained was diluted to 20 ml and the percentage of hydrolysis was determined.

*7. Hydrolysis of Dipeptides by LAPase.* 1 ml of LAPase solution (Step 6) was mixed with 1.15 ml of 1/25 M Tris buffer (pH 8.0) containing 1/250 M of $MgCl_2$, and it was activated for 15 minutes at 40°C. 0.65 ml of 1/10 M Tris buffer (pH 9.0) containing 0.008 M $MgCl_2$, 0.5 ml of 1/8 M dipeptide solution and 0.1 ml of the activated LAPase solution were mixed and incubated at 40°C. The hydrolysis percentage of dipeptide was determined according to 6.

*8. Calculation of the Hydrolysis Percentage of Dipeptide.*

Each diluted solution of reaction mixture was heated with ninhydrin by Rosen's method (*4*) and the hydrolysis percentage was calculated according to Schwartz's method (*5*). As regards to the hydrolysis of Gly-L-Pro, proline, which is released by the enzymatic action, was determined by Chinard's method (*6*).

*9. Preparation of Calcium Phosphate Gel.* To the solution of 6.7 g of $CaCl_2.2H_2O$ in 800 ml of water, the solution of 11.4 g of $Na_3PO_4.12\ H_2O$ in 75 ml water was added under stirring. The pH of the mixture was adjusted to 7.4 with

acetic acid and the precipitate was washed ten times with water by decantation. The suspension containing 19.4 mg of dried weight of gel was usualy applied.

## RESULTS

*1. Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas to Low Substrate Concentration.* Dipeptidase activities of acetone powder of both hog intestinal mucosa and hog pancreas are indicated in Fig. 1 to Fig. 8.

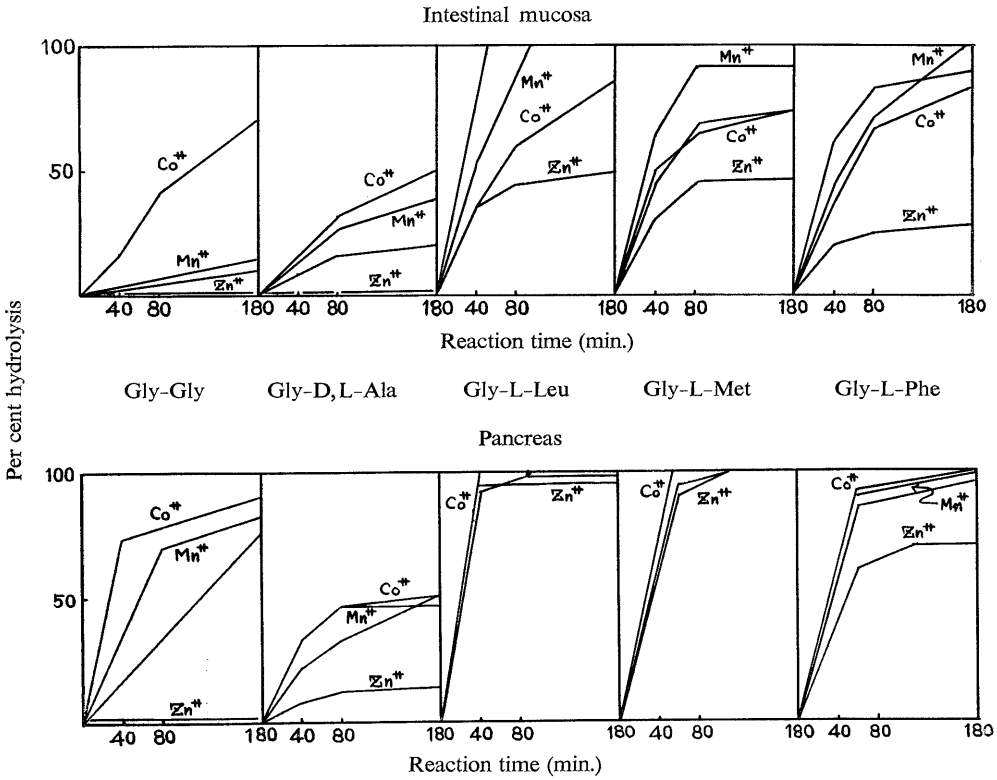



Fig. 1 Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas on Glycyl-X

Dipeptidase activities were examined under the following conditions.

Phosphate buffer (pH 7. 6, 1/30M)
Substrate concentration: 0. 002 M
Metal ion concentration: 0. 001 M
Enzyme concentration:
Kjeldahl N 0. 025 mg/ml (intestinal mucosa)
0. 093 mg/ml (pancreas)

Per cent hydrolysis was calculated as described in text.

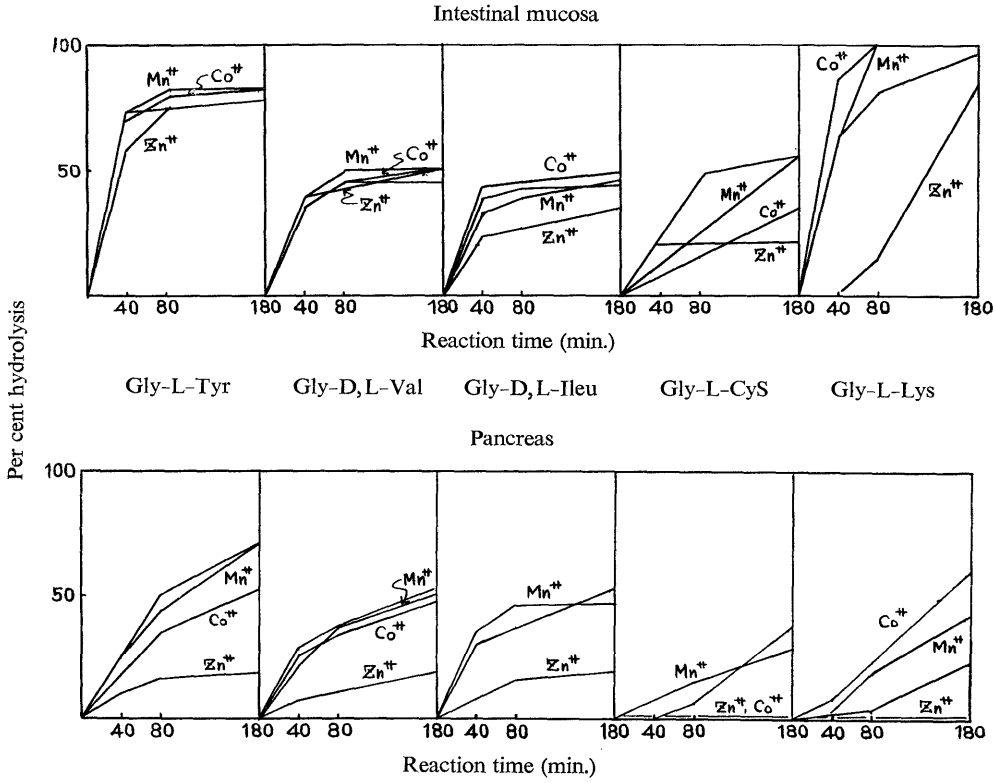



Fig. 2 Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas on Glycyl-X.

The experimental conditions were described in Fig. 1.

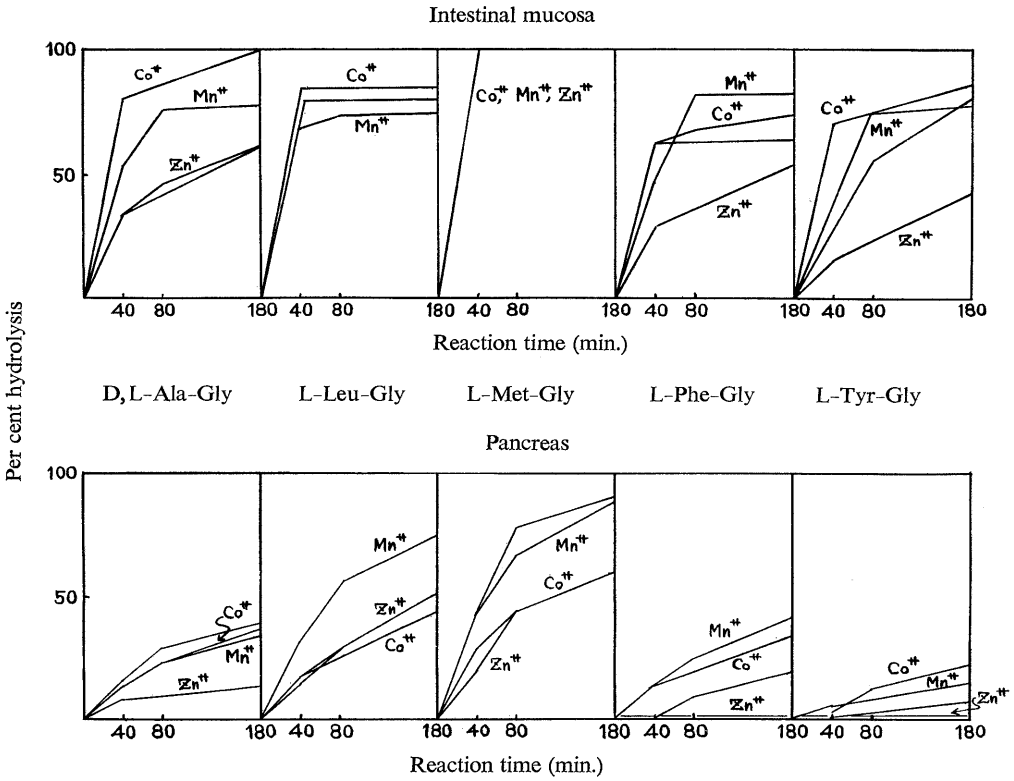



Fig. 3 Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas on X-Glycine

The experimental conditions were described in Fig. 1.

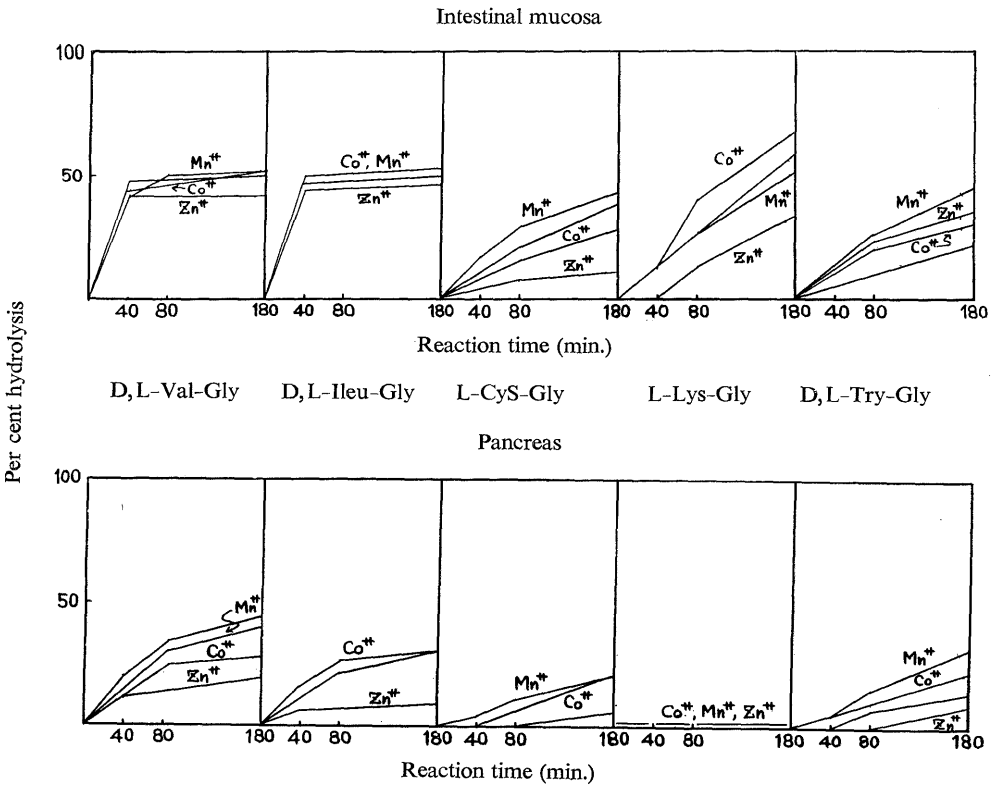



Fig. 4 Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas on X-Gly.

The experimental conditions were described in Fig. 1.

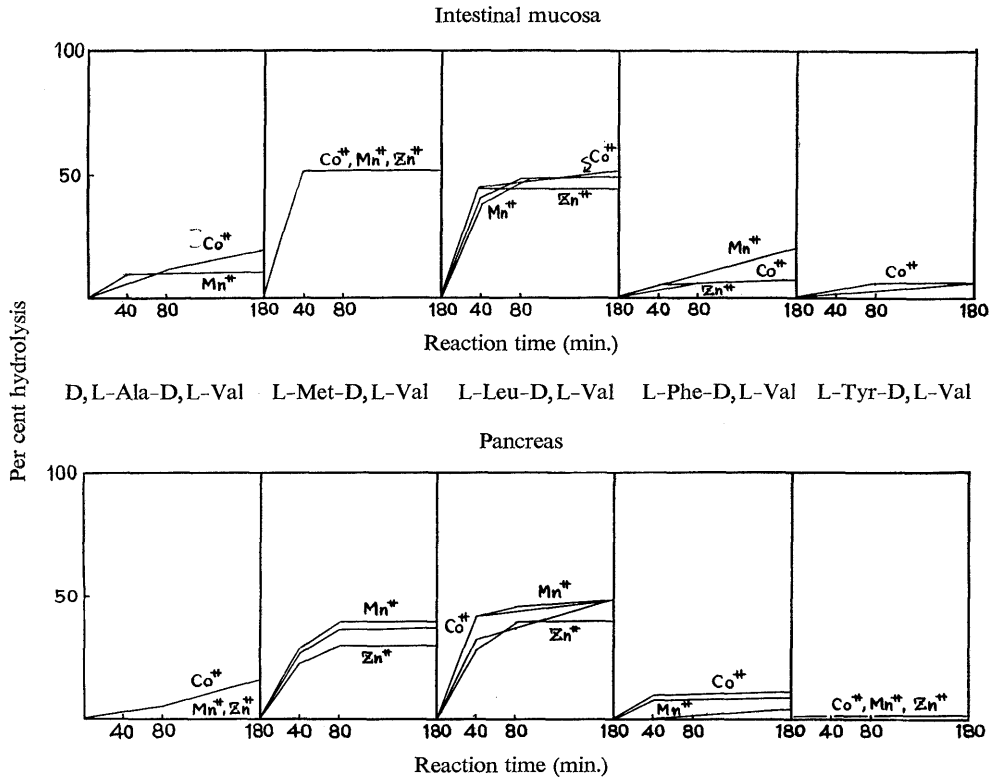



Fig. 5 Dipeptidase Activiiy of Acetone Powder of Hog Intestinal Mucosa and Pancreas on X-D,L-Val.

The experimental conditions were described in Fig. 1.

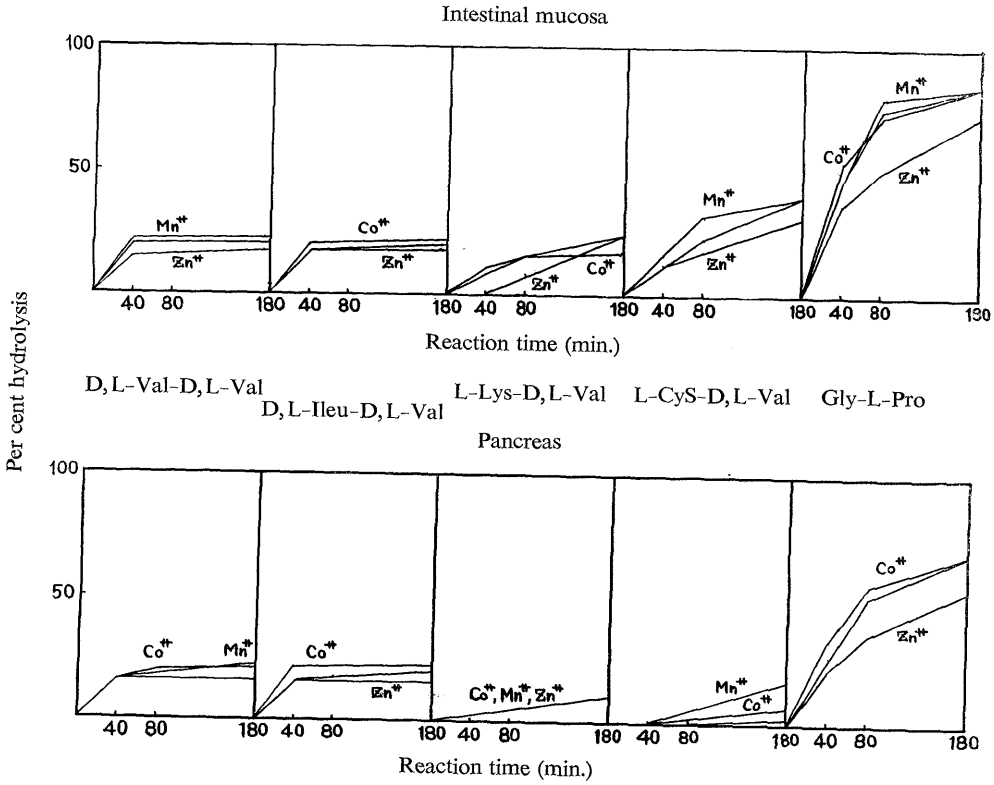



Fig. 6 Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas on X-D, L-Val and Gly-L-Pro.
The experimental conditions were described in Fig. 1.

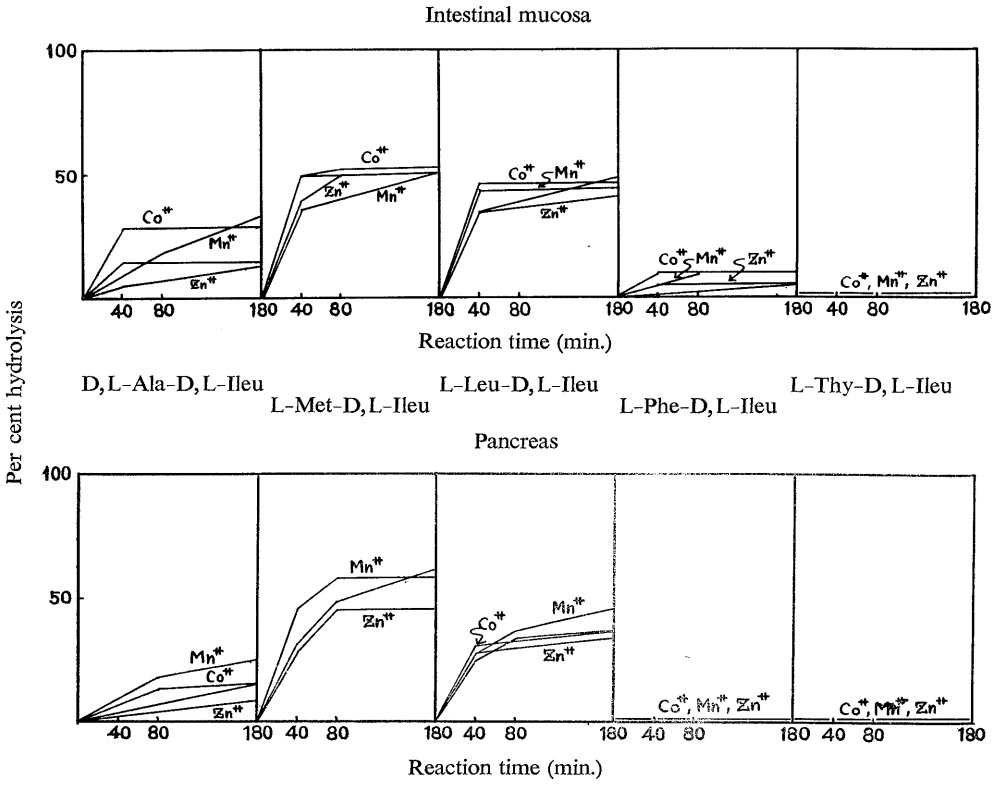



Fig. 7 Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas on X-D,L-Ileu.

The experimental conditions were described in Fig. 1.

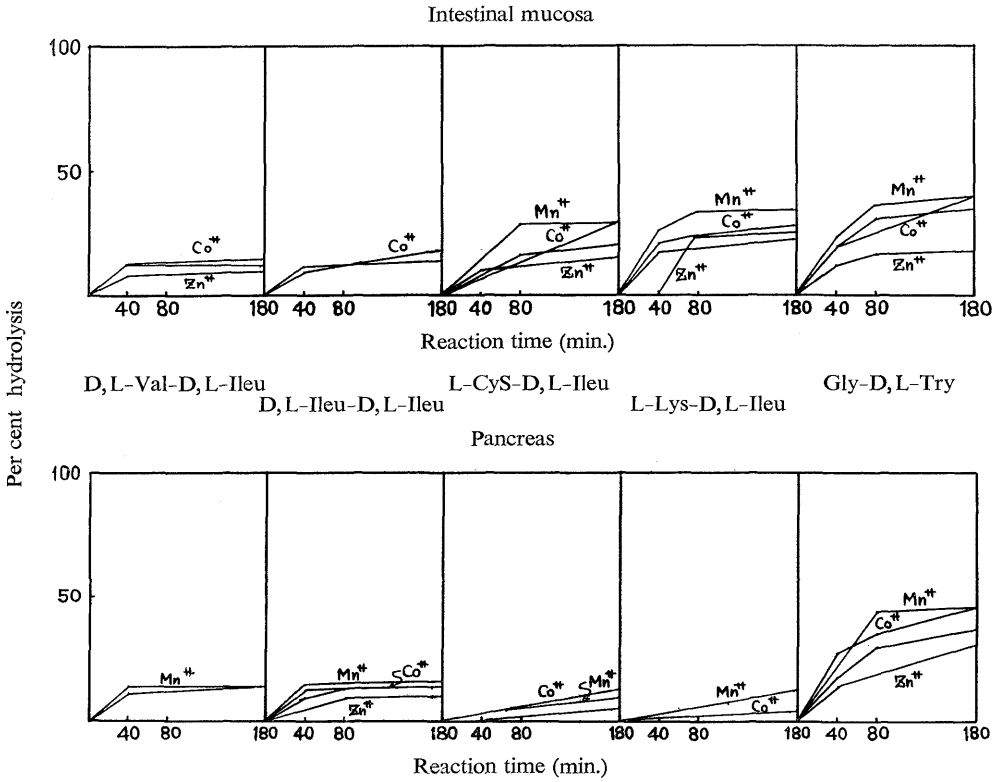



Fig. 8 Dipeptidase Activity of Acetone Powder of Hog Intestinal Mucosa and Pancreas on X-D, L-Ileu and Gly-D, L-Try.
The experimental conditions were described in Fig. 1.

From these figures it can be seen that dipeptidase activity of intestinal mucosa is much higher than that of pancreas.

As to the effect of metal ion, $Zn^{++}$ shows to be inhibitor, but in other cases, particularly in the case of glycylglycine, it was found to be completely inhibitory. Concerning $Co^{++}$ and $Mn^{++}$, a general tendency could not be found.

Comparing each series of synthetic substrate Gly-X, X-Gly, X-Val and X-Ileu the acetone powder of intestinal mucosa showed a high enzymatic activity to Gly-X or X-Gly, particularly towards Gly-X.

The peptides containing cystine seemed not to be affected by intestinal mucosa or pancreas. In case of dipeptides containing aromatic or basic group at its N-

terminal residue, such as L-Tyr-Gly, L-Tyr-D, L-Val, L-Tyr-D, L-Ileu, L-Phe-Gly, L-Phe-D, L-Val, L-Phe-D, L-Ileu, L-Lys-Gly, L-Lys-D, L-Val and L-Lys-D, L-Ileu, intestinal mucosa or pancreas showed very low activity; no activity at all in case of L-Thr-D, L-Val, L-Tyr-D, L-Ileu, L-Phe-D, L-Val and L-Phe-D, L-Ileu.

2 *Dipeptidase Activity of Acetone Powder of Intestinal Mucosa to the Substrate of High Concentration.* It was found that the acetone powder of intestinal mucosa showed high dipeptidase activity towards the Gly-X or X-Gly series in low concentration, therefore, in order to examine this fact more exactly, dipeptidase of intestinal mucosa was investigated in the concentration of 0.05 M; the results thereof were indicated in Fig. 9 and Fig. 10.

Fig. 9 shows that the dipeptides Gly-L-Phe, Gly-L-Met and Gly-L-Leu are hydrolyzed approximately at the same rate. In comparison to these three dipeptides, Gly-D, L-Ala, Gly-D, L-Val and Gly-D, L-Ileu are hydrolyzed at half this rate. These effects must perhaps be attributed to the fact that D, L-compounds were used. Therefore, if the L-isomers would be used instead of D, L-compounds, these dipeptides should be hydrolysed at approximately the same rate as Gly-L-Leu. Gly-D, L-Thr and Gly-Gly are hydrolyzed at lower rate, though they belong to the Gly-X series. The Gly-X series is hydrolyzed at almost the same rate, except in case of Gly-Gly and Gly-D, L-Thr. The kinetic data of hydrolytic reaction towards Gly-L-Leu are shown in Tab. I.

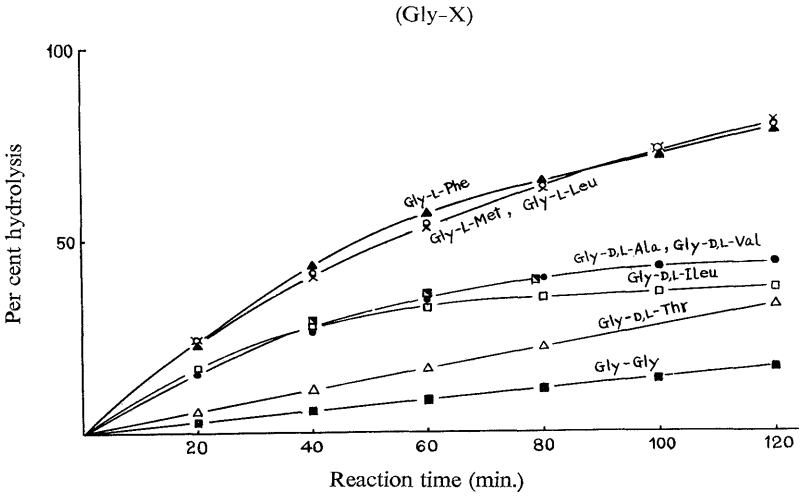



Fig. 9 Hydrolysis of Dipeptides by Hog Intestinal Mucosa,
Hydrolysis was examined under the following conditions.
Phosphate buffer (pH 7. 6, 1/30 M)
Substrate concentration: 0. 05 M
Enzyme concentration: Kjeldahl N 0. 025 mg/ml
Per cent hydrolysis was calculated as described in text.

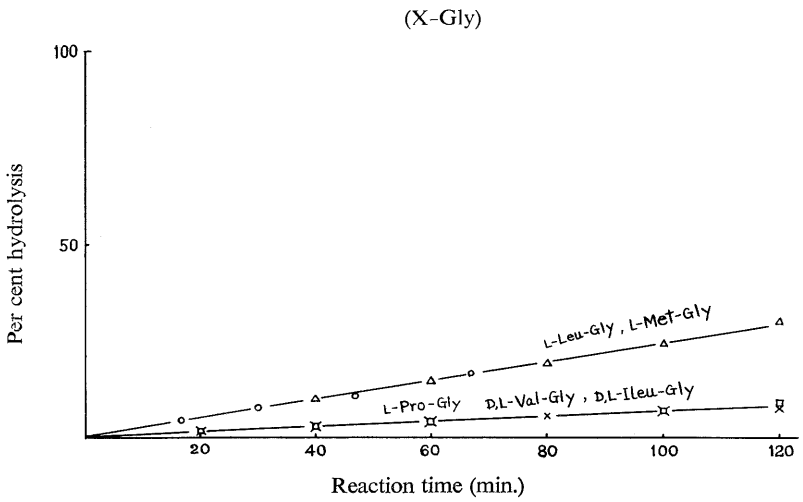



Fig. 10 Hydrolysis of Dipeptides by Hog Intestinal Mucosa
The experimental conditions were as described in Fig. 9.

Table I Hydrolysis of Glycyl-L-leucine by Hog Intestinal Mucosa

| Reaction time (min) | Percentage of hydrolysis | $K_1=\frac{1}{t}\log\frac{100}{100-\%}$ | $C_1=\frac{K_1}{E}$ |
|---|---|---|---|
| 20 | 23.5 | 0.00586 | 0.234 |
| 40 | 40.5 | 0.00563 | 0.225 |
| 60 | 52.8 | 0.00544 | 0.218 |
| 80 | 62.8 | 0.00589 | 0.236 |
| 100 | 71.0 | 0.00538 | 0.215 |
| 120 | 77.3 | 0.00537 | 0.215 |
| mean | mean | 0.00559 | 0.225 |

The experimental conditions were as described in Fig. 9.

From these results it was found that the dipeptidase action of hog intestinal mucosa takes place according to the first order reaction; its velocity constant and proteolytic coefficient is 0.00559, and 0.225 respectively. These figures show that the acetone powder of intestinal mucosa has a high dipeptidase content, which may possibly be raised by further purification. In Fig. 10, it is demonstrated that intestinal mucosa has only low activity to dipeptides X-Gly contrary to Gly-X.

*3 Hydrolysis of Gly-X and X-Gly by LAPase.* Fig. 11 shows the hydrolysis of Gly-X and X-Gly by LAPase "Step 6". Though Smith et al. (7) found that LAPase has only low activity for the hydrolysis of glycine amide or dipeptides Gly-X, the experimental data of present investigations on the activity of the

enzyme towards dipeptides are shown in Fig. 11, which had up to now never been studied.

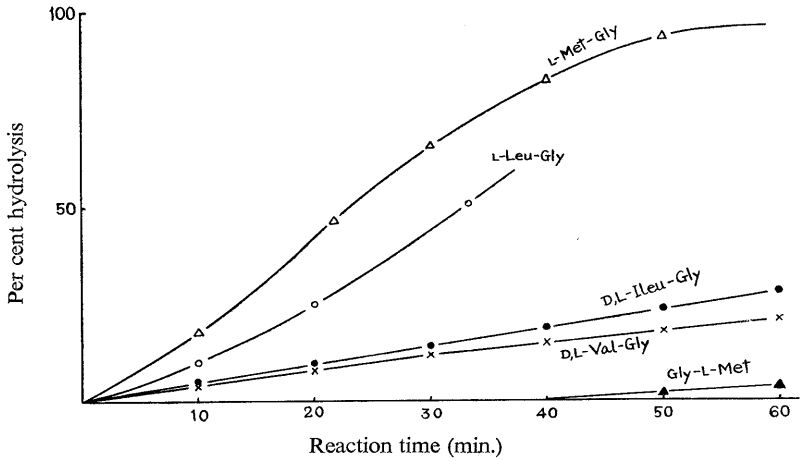



Fig. 11 Hydrolysis of Dipeptides by Leucine-amino-peptidase.
Various dipeptides were hydrolyzed by $Mg^{++}$-activated LAPase obtained from hog kidney. Details were described in text.
Per cent hydrolysis was calculated as described in text.

This experiment also makes clear that, as the results obtained in Fig. 9 and 10 indicate the enzyme located in intestinal mucosa, and acting specifically on dipeptide Gly-X, is different from LAPase. From Fig. 11 it is seen that the activity of LAPase is higher towards dipeptides X-Gly than towards dipeptides Gly-X, however, the activity varies towards other peptides. From the results of Fig. 9, 10 and 11, we confirm the existence of an enzyme which participates specifically in the hydrolysis of dipeptides Gly-X but which is different from LAPase. As it was found that an enzyme contained in intestinal mucosa and acting specifically towards dipeptides Gly-X, requires glycine at N-terminal in dipeptides Gly-X, it seems to be logical to call this enzyme glycine-amino-peptidase (GAPase) or Glycyl-dipeptidase. In this report the former name will be adopted for this enzyme.

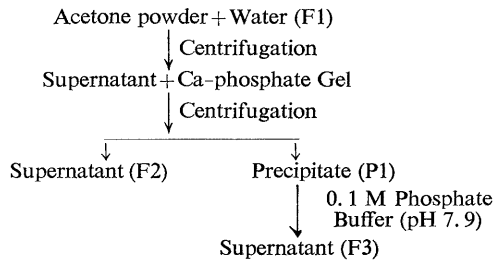
*4 Fractionation of GAPase by Calcium Phosphate Gel.* The purification of GAPase in intestinal mucosa was undertaken. The enzymatic activity was investigated using Gly-L-Leu as a substrate for each fraction.

Though an enzymatic activity was recognized in the fraction precipitated with ammonium sulfate of the saturation between 0.4 and 0.8, the activity was so low that further purification could not be attained. Therefore, the fractionation

of GAPase from intestinal mucosa with ammonium sulfate was given up, and the purification with calcium phosphate gel was applied. All subsequent procedures were carried out at 4°C.

600 mg of acetone powder of hog intestinal mucosa was suspended in 50 ml of water and stirred for 15 minutes, thereafter centrifugated at the velocity of 1,800×g for 5 minutes. The precipitates were discarded. The supernatant is designated F1. To F1 15ml of calcium phosphate gel (19.4 mg dry weight per ml of gel suspension) was added and stirred for 15 minutes, followed by centrifugation. This supernatant is designated F2. The protein fraction adsorbed on the gel was eluted with 15ml of 1/10 M phosphate buffer (pH 7.9), stirred for 15 minutes and then centrifugated. The elution was repeated with the same buffer once more, as described above. The combined supernatant is designated F 3. The enzymatic activities of F 1, F 2 and F 3 are shown in Tab. II.

Table II Fractionation of GAPase by Calcium Phosphate Gel




Hydrolysis of Glycyl-L-leucine by Various Fractions

| Fraction | Total N mg | Percentage of hydrolysis | $C_1$ |
|---|---|---|---|
| F1 | 49.0 | 43.2 | 0.046 |
| F2 | 32.6 | 0 | |
| F3 | 16.6 | 46.4 | 0.050 |

The experimental conditions were as described in text.

Table III Fractional Elution by Phosphate Buffer of GAPase Adsorbed in Calcium Phosphate Gel

| Concentration of the buffer | Kjeldahl N mg/ml | Percentage of Hydrolysis | $C_1$ |
|---|---|---|---|
| 1/200M | 0.340 | 0 | |
| 1/150 | 0.343 | 0 | |
| 1/100 | 0.348 | 0 | |
| 1/50 | 0.351 | 0 | |
| 1/10 | 0.484 | 25 | 0.054 |

GAPase adsorbed on calcium phosphate gel was eluted by various concentrations of phosphate buffer (pH 7.9) as described in text.

The enzyme activity was determined as described in text.

As can be seen, the active fraction is adsorbed on calcium phosphate gel and easily eluted from the gel with 1/10 M phosphate buffer (pH 7.9). Based on these observations, fractional elutions from P1 with 15 ml of phosphate buffer (pH 7.9) of various concentrations were carried out; the results being presented in Tab. III.

No elution was attained below 1/50 M phosphate buffer. However, no increase of the specific activity is found after the treatment with calcium phosphate gel. It is often observed that inactivation of the enzyme occurs during the purification process. One reason of this phenomenon may be the loss of the co-factor. Generally, there are many proteinases which require the divalent metal ion. Therefore, the following experiments were executed in order to investigate the co-factor of GAPase.

*5 Necessity of Metal Ion in GAPase.* The hydrolytic activity of intestinal mucosa to Gly-L-Leu was completely inhibited, when the acetone powder of hog intestinal mucosa was suspended for 20 minutes in 1/10 M Tris buffer (pH 8.0) containing 1/1000 M versen. Therefore, GAPase requires metal ion as a co-factor for its action.

To the extract, treated with versen described above, $MnSO_4$, $MgCl_2$ $CoCl_2$, $CaCl_2$, and $ZnCl_2$ were added in final concentration of 1/1,000 M and 1/100 M, and incubated at 40°C from 0 to 120 minutes. However, the activation of GAPase by these metal ions failed to reverse its activity. Also, the fraction obtained with calcium phosphate gel was inhibited by the metal ions mentioned above. Therefore, at present, no co-factor or activator is found yet.

## DISCUSSION

Since the studies on dipeptidases by Grassmann, Linderström-Lang, the multiplicity of dipeptidase has been discussed, and the dipeptidases have been named according to the substrates acted upon. However, by studies examined in Smith's Laboratory, it had deen clarified that LAPase, which was purified from hog kidney, is an amino-exopeptidase hydrolyzing not only leucine amide, but also peptides containing various amino acids at N-terminal. In addition to LAPase, the existence of dipeptidase, glycyl-glycine-dipeptidase, glycyl-leucine-dipeptidase, prolidase and prolinase was presumed, even though their purification had never been attained. Moreover, the fact that an enzyme GAPase, as shown in Fig. 9 and 10, hydrolyzing dipeptides such as Gly-X except Gly-Gly at the same rate, exists in hog intestinal mucosa, might produce some interesting information on the digestion and absorption of protein into intestinal tract, and also some knowledge in regard to the multiplicity of dipeptidase.

Because the substrate specificity of glycyl-leucine-peptidase (GLPase) in case

leucine resldue was replaced with another amino acid, has never been investigated or described, a comparison between GAPase and GLPase is not possible. However, since GLPase is activated by Mn ion (*8*) whilst GAPase is inhibited, the latter is assumed to be different from the former. As the report of Sato et al. (*9*), the effect of $Mn^{++}$ on GLPase is different, according to the extraction method applied, therefore GAPase should not be distinguished from GLPase only on ground of the $Mn^{++}$ dependency. The difference between both enzymes could be stated only when purification of GAPase would be attained successfully. The fact that GAPase shows low activity towards glycyl-glycine might be an indication that the hydrolysis of this dipeptide is attributable to another enzyme, i. e. glycyl-glycine dipeptidase. The activity of GAPase being very low towards Gly-D, L-Thr can be compared to the low activity of LAPase towards peptides containing hydrophylic group at N-terminal amino acid residue (*7*).

When LAPase acts on peptides, it removes N-terminal amino acid residue successively, but at glycine residue in peptide chain its activity will weeken. Supposed it might be possible that such a delayed hydrolytic reaction can be recovery, if the glycine residue is cleaved by GAPase, the interest in the problem, presented in this paper, might be greatly increased.

## SUMMARY

The hydrolytic activity of the acetone powder of hog intestinal mucosa and hog pancreas on four series of dipeptides, i. e. Gly-X, X-Gly, X-D, L-Val and X-D, L-Ileu, was examined at both low and high substrate concentrations. The hydrolytic activity of hog intestinal mucosa was much more active than that of pancreas. In particular, Gly-X was the most actively hydrolyzed dipeptide by acetone powder of intestinal mucosa, except Gly-Gly. Further, this enzyme was named glycine-amino-peptidase (GAPase). Although the purification of GAPase was tried with calcium phosphate gel, no remarkable results were obtained. GAPase requires a metal ion as co-factor, however, neither the definite metal ion nor an activation method have been found as yet.

## REFERENCES

1) D. H. Spackman, E. L. Smith and D. M. Brown: *J. Biol. Chem.*, **212**,: 255, 1955
2) R. H. Hill and E. L. Smith: *J. Biol. Chem.*, **228**,: 577, 1957
3) J. R. Vaughan, and R. L. Osato: *J. Amer. Chem. Soc.*, **73**,: 5553, 1951
4) H. Rosen: *Arch. Biochem. Biophys.*, **67**,: 10, 1957
5) T. B. Schwartz and F. L. Engel: *J. Biol. Chem.*, **184**,: 197, 1950
6) F. B. Chinard: *J. Biol. Chem.*, **199**,: 91, 1952
7) E. L. Smith and D. H. Spackman: *J. Biol. Chem.*, **212**,: 271, 1955
8) E. L. Smith: *J. Biol. Chem.*, **176**,: 9, 1948
9) M. Sato, T. Akatsuka and Y. Awaya: *J. Agr. Chem. Soc. Japan*, **29**,: 894, 1955