Ever since peptidases were shown to be present in serum, various theories have been proposed to explain the origin and the mode of release of these enzymes (1–4). A thorough study of the properties of various serum peptidases has, however, not been made; instead, most investigators have tacitly assumed that the serum enzymes behave in a manner analogous to that of the known peptidases isolated from animal tissues. This lack of information has prompted us to investigate in greater detail the hydrolytic action of human serum on a number of peptides. Our results indicate that serum contains a multiplicity of peptidases, some of which have apparently not been found in tissues or cells. Similar investigations on the cellular elements of human blood, which will be described at a later time, have shown less complicated patterns. These findings, together with the results of our previous study (5) that some serum peptidases but not others may be altered in pathologic conditions, suggest the possibility of tracing certain peptidases to their tissues of origin.

In this paper our experimental findings on the hydrolysis of glycylglycine and glycyl-L-leucine by normal serum will be described.

Methods

The serum used in this study was obtained from venous blood of four healthy individuals whose peptidase activities were comparable to one another. 0.5 ml. of serum, freed from its bicarbonate content with 0.03 volume of N HCl, was mixed with 0.2 ml. of acid or alkali to obtain the required pH, and with 1.25 ml. of one of the following buffers, Sörensen's phosphate, Michaelis' Verona1 or dimethylglycine, Gomori's 2-amino-2-methyl-1,3-propanediol (6) (used as 0.1 m). The activating ion was added in 0.05 ml. of water. These mixtures were preincubated at 38° for 2 to 4 hours, if desired. 0.5 ml. of M/40 glycylglycine or glycyl-L-leucine, adjusted to the required pH, was then added and a 0.5 ml. sample was withdrawn immediately. The remainder was incubated at 38° for the time
indicated in each experiment. Finally the pH was checked with a glass electrode. All concentrations refer to this final mixture.

The samples were precipitated with 2 ml. of 1 per cent picric acid and centrifuged. They were analyzed in triplicate by the colorimetric ninhydrin method of Moore and Stein (7). A known solution of L-leucine served as the standard. It was shown by Moore and Stein that Beer's law does not hold in this test for optical densities above 0.5. Because the degree of peptide hydrolysis is determined from the difference of two color

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Corrections to be added to observed optical densities in the colorimetric ninhydrin method of Moore and Stein (7). Coleman junior spectrophotometer, model 6A; wave-length, 570 mμ; cuvettes, thick walled test-tubes with 18.35 ± 0.10 mm. outer diameter.

values, it appeared that a greater accuracy could be obtained by utilizing the entire colorimeter range. The necessary corrections are plotted in Fig. 1; for added convenience, a table was constructed from this curve. The corrected densities were converted to the fractions $p$ of initial substrate hydrolyzed during the time of observation; the proportionality factors were obtained as described by Schwartz and Engel (8) and corrected according to the color value of the leucine standard.

It has become rather common practice to treat most enzyme reactions as if they followed first order (monomolecular) kinetics. Recently, the author pointed out (9) that a closer fit of rate curves can be obtained if the integrated Michaelis-Menten equation in the form
is applied. Of the two parameters, \( r' \) is understood to contain the concentration and the Michaelis constant of substrate, as well as concentrations and dissociation constants of all inhibitors which may be present initially or formed during the reaction, while \( m \) is the initial rate per minute in a plot of \( p \) versus \( t \), and as such is a measure of enzyme activity. For the different optimal conditions of peptide hydrolysis, the parameters \( r' \) were

\[
t = \frac{1}{m(r' + 1)} \left( \ln \frac{1}{1 - p} + r'p \right)
\]
determined from time reactions whenever possible; these values were assumed to be constant for all sera and were used to calculate the enzyme activities of various sera from single determinations. It must be emphasized that all values are given for a serum dilution 1:5 and for a peptide concentration of $5 \times 10^{-3}$ M. Conversion to activities at different substrate concentrations cannot be made from these parameters alone, unless the absence of inhibitors is known.

Fig. 3. Hydrolysis of glycylglycine by normal serum in the presence of Mn$^{++}$. a, effect of pH; ◦, Veronal buffer; X, no buffer; ○, phosphate buffer; all with $2 \times 10^{-3}$ M MnCl$_2$; time 3 hours. b, effect of varying concentrations of Mn$^{++}$. Veronal buffer, pH 7.4; time 3 hours.
Results

Glycylglycine-Splitting Activity—In the presence of Co\(^{++}\) the hydrolysis of glycylglycine shows three distinct optima. Varying somewhat with the buffer used, these are located at pH 6.2 to 6.3, 6.8, and 8.0 to 8.3 (Fig. 2, a). The cobalt concentration curves (Fig. 2, c) indicate that the activities are practically nil in the absence of Co\(^{++}\), that the best concentration of Co\(^{++}\) is not the same for the three pH optima, and that high concentrations cause inhibition or inactivation. At pH 8.0 the curve was obtained in Veronal buffer, since higher concentrations of Co\(^{++}\) cannot be reached in phosphate buffer. The adverse effect of excess Co\(^{++}\) on the pH 6.8-activity
appears to be due to inactivation, for preincubation of serum with Co\textsuperscript{++}, but not in its absence, has been shown repeatedly to reduce the activity. This is illustrated in Fig. 2, b and may also be seen from the time curve (Fig. 4, a). The activity with optimum at pH 6.2 is in some part affected

![Graph showing the effect of varying pH on the hydrolysis of glycyl-L-leucine by normal serum.](image)

**Fig. 5.** Hydrolysis of glycyl-L-leucine by normal serum. a, effect of varying pH. ○, Veronal buffer; □, 2-amino-2-methyl-1,3-propanediol buffer; both with 10\textsuperscript{-4} M MnCl\textsubscript{2}; ●, Veronal buffer; X, phosphate buffer; ■, 2-amino-2-methyl-1,3-propanediol buffer; the last three with 10\textsuperscript{-3} M CoCl\textsubscript{2}; time 2 hours. b, effect of varying concentrations of Mn\textsuperscript{++}. ○, Veronal buffer, pH 9.1; □, 2-amino-2-methyl-1,3-propanediol buffer, pH 9.2; time, 2 hours. c, effect of varying concentrations of Co\textsuperscript{++}. ●, Veronal buffer, pH 9.0; ■, 2-amino-2-methyl-1,3-propanediol buffer, pH 9.4; time 4 hours.
by the same sensitivity to Co++, since the ranges of the two activities overlap. Without preincubation with Co++, the time curves at pH 6.2 and 6.8 do not follow, therefore, Michaelis-Menten kinetics. This situation is best recognized by plotting $p/t$ versus $(1/t) \ln (1/(1-p))$, similarly to the method of Walker and Schmidt (10); instead of the usual straight line, a curve is obtained. The reaction at pH 8.0 is best described by first order kinetics; no difference was seen whether or not serum was preincubated with Co++.

In the presence of Mn++, the hydrolysis of glycylglycine shows only a single peak at pH 7.4 (Fig. 3, a). The optimal concentration of Mn++ is $2 \times 10^{-3}$ M (Fig. 3, b), as determined in Veronal buffer; the activity is much lower in phosphate buffer because of the precipitation of manganese phosphate. Under optimal conditions Mn++ is usually a better activator than Co++, if comparisons are made in 3 hour experiments. The activation by Mn++ does not require time (Fig. 4, b). The enzymatic reaction in the presence of Mn++ fails to follow Michaelis-Menten kinetics for reasons not as yet understood; since preincubation with Mn++ does not affect the activity, it appears unlikely that Mn++ is directly responsible for the rapidly declining rate.

While the effect of Zn++ alone on the hydrolysis of glycylglycine was not investigated, the simultaneous presence of equimolar amounts of this ion and optimal amounts of Co++ (at the three maxima of pH) or of Mn++ caused almost complete loss of activity.

**Glycyl-L-leucine-Splitting Activity**—The hydrolysis of glycyl-L-leucine by serum is distinguished by its requirement for a high pH and by its relatively great activity (Fig. 5). With the optimal concentration of Mn++, the maximum is observed at pH 9.4 in Veronal or dimethylglycine buffer. In the presence of Co++, the optimum is at a somewhat lower pH, and the activity is considerably less. Whether Mn++ and Co++ activate the same enzyme cannot be decided as yet. From a study of reaction rates, the parameters $r'$ were calculated to be 1.7 and 1.1, respectively, for Mn++ and Co++. Preincubation with the ions is not required.

Because Mn++ is autoxidizable at pH 9.4, we have also investigated the enzymatic hydrolysis of glycyl-L-leucine in the absence of oxygen. The result was identical with that of a second reaction, which was allowed to proceed in the usual way under air, indicating that the formation of MnO(OH) is of no consequence for the enzymatic reaction.

Zn++ is not only ineffective as an activator ($2 \times 10^{-4}$ M) but, when present together with Mn++, neutralizes the activating effect of the latter.

**Distribution of Activities in Normal Serum**—With the optimal conditions for the glycylglycine and glycyl-L-leucine-splitting activities established, their distribution was investigated in twenty-five normal sera. The time
of reaction was chosen to be 3 hours in the case of glycylglycine and 2 hours for glycyl-L-leucine. Phosphate buffer was used below, Verona1 buffer above pH 7.0. The normal subjects were nine men and sixteen women, ranging in age from 18 to 40 years. No correlation was noted with age; as to sex, it appeared that men have slightly higher values than women; however, more data are needed before such difference can be proved.

Table I summarizes our results in condensed form without any sex differ-

Table I
Peptidases in Human Serum
Hydrolysis of glycylglycine (GG) and glycyl-L-leucine (GL) by normal serum under optimal conditions of pH and ion concentration.

<table>
<thead>
<tr>
<th>Subject</th>
<th>GG</th>
<th>GL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Co(^{++}) pH 6.2</td>
<td>Co(^{++}) pH 6.8</td>
</tr>
<tr>
<td>1. Fasting</td>
<td>0.24</td>
<td>0.31</td>
</tr>
<tr>
<td>1. ½ hr. after meal</td>
<td>0.30</td>
<td>0.32</td>
</tr>
<tr>
<td>1. 1 &quot; &quot; &quot;</td>
<td>0.29</td>
<td>0.33</td>
</tr>
<tr>
<td>1. 2 hrs. &quot; &quot;</td>
<td>0.28</td>
<td>0.33</td>
</tr>
<tr>
<td>2</td>
<td>0.23</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>0.34</td>
<td>0.41</td>
</tr>
<tr>
<td>4</td>
<td>0.37</td>
<td>0.38</td>
</tr>
<tr>
<td>5</td>
<td>0.52</td>
<td>0.55</td>
</tr>
<tr>
<td>6</td>
<td>0.47</td>
<td>0.52</td>
</tr>
<tr>
<td>7</td>
<td>0.59</td>
<td>0.66</td>
</tr>
<tr>
<td>25 men and women; range (for GG) or mean and s.d. (for GL)</td>
<td>0.19</td>
<td>0.24</td>
</tr>
</tbody>
</table>

\* Fraction of substrate hydrolyzed in 3 hours.
† Activity per minute for serum dilution 1:5.

entiation. Included are data on the peptidase activities before and after a substantial meal; the total intake was about 2000 calories, of which 59 per cent was contributed by fat and 17 per cent by protein. It appeared that during a 2 hour period after ingestion of food there was no significant change of serum peptidase activities. In Table I the results on a few sera are listed in order to compare their individual activities. Especially the Co\(^{++}\)-activated glycylglycine hydrolysis at pH 8.0 may be seen to vary in a manner different from the other activities. From these observations it is concluded that Co\(^{++}\) at pH 8.0 and Mn\(^{++}\) at pH 7.4 probably do not
activate the same enzyme. Other conclusions do not seem to be warranted at this time.

DISCUSSION

The hydrolysis of glycylglycine by human serum, assumed to be due to a specific cobalt-activated dipeptidase similar to the enzyme in animal tissues (11, 12), has been tested previously at pH 7.3 (3) and pH 7.8 (4) in the presence of $10^{-3}$ M Co++. The activities reported under these conditions were low. The results of our experiments indicate that there are three regions of high activity with peaks near pH 6.2, 6.8, and 8.0. The three activities differ with respect to their sensitivity to Co++. In contrast to the glycylglycine dipeptidase from rat muscle (11), high concentrations of Co++ affect the serum activities adversely. At pH 6.8 this effect appears to be due to inactivation, and the abnormal kinetics of the reactions at pH 6.2 and 6.8 can thus be explained. The hydrolysis at pH 8.0, which was found to follow first order kinetics, may be somewhat affected by the formation of a complex between glycylglycine, Co++, and oxygen (13). In contrast to the glycylglycine dipeptidase from animal tissues which has been shown to be rather specific for Co++, serum contains also a manganese-activatable enzyme (3). Under the conditions of our test, the activating effect of Mn++ at pH 7.4 was even greater than that of Co++ at any of the three optima. Judging from the distribution of activities in normal sera, different enzymes appear to be responsible for the hydrolysis of glycylglycine in the presence of Mn++ at pH 7.4 and of Co++ at pH 8.0.

Glycyl-L-leucine has not been used commonly as a substrate for serum peptidases. Enzymes specific for this substrate have been obtained from animal tissues with pH optima between 7.8 and 8.0, some activated by Mn++, others by Zn++ (12, 14); phosphate has been said to be essential for maximal activity (14). The results of our studies indicate that there is very little reaction between serum and glycyl-L-leucine at pH 8.0. Instead, a rather marked activity was observed in the more alkaline region, with an optimum at pH 9.4 in the presence of Mn++. This enzyme shows greater activity than any other serum peptidase investigated so far. With Co++ the activation is considerably less, and may be due to the same enzyme.

SUMMARY

The hydrolytic action of normal human serum on glycylglycine and glycyl-L-leucine under a variety of conditions has been studied. With glycylglycine and Co++ as the activating ion, three apparently independent activities were observed, with optima near pH 6.2, 6.8, and 8.1; these differed in their susceptibility to the activating and inactivating effect of Co++ and in their distribution in the sera from twenty-five normal persons.
In the presence of Mn$$^{++}$$, on the other hand, the hydrolysis of glycylglycine seemed to be catalyzed by only a single peptidase. With glycyl-L-leucine as the substrate, the optimal pH was 9.0 to 9.6, depending on the buffer and activating ion used. Under the best conditions Mn$$^{++}$$ was about twice as active as Co$$^{++}$$, while Zn$$^{++}$$ showed only an inhibitory effect.

**BIBLIOGRAPHY**

PEPTIDASES IN HUMAN BLOOD: I.
THE HYDROLYSIS OF
GLYCYLGLYCINE AND
GLYCYL-L-LEUCINE BY NORMAL
SERUM
Gerard A. Fleisher and With the technical assistance of Norha Goplerud


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