animals revealed that (a) equilibration times were closely similar, the equilibration of globulin (65–140 hr.) being generally slower than that of albumin (40–65 hr.). (b) Equilibration was followed by a decline in plasma specific activity which maintained a constant exponential rate during 3–4 weeks of observation. Biological half-lives of plasma proteins were closely similar when measured with ^{14}C and ^{131}I . In general, albumin (160–230 hr.) was eliminated more slowly than globulin (140–170 hr.) and both much more slowly than fibrinogen (52–85 hr.). (c) When equilibration was complete, the mass of protein in the extravascular compartment was about one and a half times that in the blood stream. Albumin had a lower extra-/intra-vascular distribution ratio than globulin, while that of fibrinogen was considerably less. The complex significance of these ratios is discussed.

3. The presence of denatured, rapidly eliminated components in the injected protein was associated with a high urinary output of ^{131}I in the first 72 hr. and with unusually low plasma specific activity values subsequently, the latter giving rise to incorrectly high estimates of pool size.

4. ^{131}I detached from plasma protein was rapidly and almost completely excreted in the urine and provided an accurate measure of the protein elimination rate. There was no detectable re-incorporation of ^{131}I into newly synthesized plasma protein. Retention of ^{131}I by the thyroid gland was negligible in animals receiving iodide in the drinking water.

5. It is concluded that rabbit plasma can be labelled with radioactive iodine in such a way as to provide data concerning the *in vivo* distribution and elimination of albumin and globulin closely similar to that obtained with the corresponding ^{14}C -labelled proteins.

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The Kinetics of Acid Hydrolysis of Dipeptides

By J. I. HARRIS,* R. D. COLE AND N. G. PON

Hormone Research Laboratory, University of California, Berkeley, California

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Hydrolysis by means of acid is the method most generally used for the degradation of proteins. Although all the peptide bonds in a protein molecule are susceptible to hydrolysis by acid, the rate of hydrolysis of a particular bond will depend in large measure upon its accessibility to the approach of hydrogen ions. Thus it is to be expected that the rate of hydrolysis of the different

bonds along a peptide chain will be greatly influenced by the electrostatic and steric properties of the chain, as well as by the nature of the amino acids forming each particular bond. The cumulative effects of these and other factors in a long polypeptide chain will vary as hydrolysis proceeds, but it seems reasonable to assume, at least as a first approximation, that the stability to hydrolysis of each bond will depend mainly on the nature of its constituent amino acids, in such a way that the

* Present address: Department of Biochemistry, University of Cambridge.

relative stabilities of the various bonds can be correlated with those of their corresponding dipeptides (Synge, 1945).

Numerous investigators (see Sanger, 1952) have studied the rates of hydrolysis of a limited number of simple dipeptides, but on the whole the available data are fragmentary, and difficult to correlate because of the diversity of hydrolytic conditions employed.

Synge (1945) investigated the rate of hydrolysis of a series of simple dipeptides in a mixture of equal volumes of 10N-HCl and glacial acetic acid at 37°; the results showed that, among the peptides studied, the most important factor influencing the rate of hydrolysis was the effective size of the amino acid side chains on either side of the peptide bond. Furthermore, this steric effect was found to depend not only on the actual size of the side chain but also upon its position relative to the bond in question. Thus dipeptides containing valine were found to be particularly stable, presumably because the bulky *isopropyl* group is close to the main peptide chain and effectively retards the approach of hydrogen ions over a wide angle. Similarly, peptides containing leucine, where the *isopropyl* grouping is further removed from the peptide bond, were found to be less stable than the corresponding valine peptides, while peptides containing alanine and glycine, where the steric factors are reduced to a minimum, were still more labile.

It was observed earlier by Levene, Steiger & Rothen (1932), in studies of the hydrolysis of dipeptides in dilute alkali, that a 'spreading' side chain confers the greatest stability when it is part of the residue which contributes its carboxyl group to the peptide bond. Synge (1945) found the same relative stabilities to obtain in the case of hydrolysis with acid; thus Val.Gly and Leu.Gly respectively were found to be more stable than Gly.Val and Gly.Leu. However, according to expectation, the most stable bonds have been found to be those formed between two 'stabilizing' residues, as exemplified by Val.Val (Christensen, 1943) and Leu.Leu (Synge, 1945).

In addition to the steric effects described above, the presence of charged groups in the neighbourhood of a peptide bond will also affect the approach of hydrogen ions. In strong acid solutions carboxyl groups are uncharged, but basic groups become positively charged and would thus be expected to oppose the approach of similarly charged hydrogen ions. α -Amino groups are in general closer to the peptide backbone than side-chain amino, imidazole, and guanidinol groups, so that in tripeptides and higher peptides the bonds which are adjacent to free α -NH₂ groups should be relatively more resistant to hydrolysis by acid than bonds that are further removed from the charged group. This enhanced stability of bonds involving *N*-terminal

residues leads to the accumulation of dipeptides in partial hydrolysates of proteins (Gordon, Martin & Synge, 1941; Stein, Moore & Bergmann, 1944). The effect of positively charged groups on the rate of hydrolysis of suitable model dipeptides has not been investigated, and in any case it would probably be difficult to show any marked effect, other than that conferred by the α -NH₂ group, which they would all possess in common.

Despite the general importance of electrostatic and steric effects in determining the course of hydrolysis of a protein molecule it is becoming increasingly apparent that these are by no means the only factors involved. In fact it has already been shown that the bonds in a protein that are split most rapidly by strong acid are those involving the amino groups of serine and threonine residues (Gordon *et al.* 1941; Desnuelle & Casal, 1948). In addition, the specific release of free aspartic acid from proteins by hydrolysis with dilute acid has been reported by Partridge & Davis (1950) and by Blackburn (1950).

Except for the work of Abderhalden & Bahn (1932), where the preferential splitting of bonds involving the amino group of serine was used to prepare seryl peptides by the selective hydrolysis of the corresponding diketopiperazines, and the observation by the same authors (1935) that *N*-hippurylserine could be selectively hydrolysed in acid to give hippuric acid and serine, no information about the rate of hydrolysis of model peptides containing serine is available; nor has there been any report of such investigations on peptides containing aspartic and glutamic acids. Hitherto, studies of this kind have been hampered by the unavailability of a representative selection of synthetic model peptides. The synthesis of a series of dipeptides containing *L*-serine (Harris & Fruton, 1951) has now made possible a study of the specific effect of *N*- and *C*-terminal serine residues on the rate of hydrolysis of a peptide bond which is free from the other less specific factors introduced when a similar bond is part of a larger peptide sequence. Dipeptides containing *C*-terminal aspartic acid and glutamic acid have also been studied, together with a miscellaneous selection of other dipeptides for purposes of comparison.

In all, the rates of hydrolysis of nineteen dipeptides in 2N-HCl at 100° have been investigated. Since many of the peptides used were available only in small amounts, a micro technique based on the ninhydrin procedure of Moore & Stein (1948) has been used for measuring the extent of hydrolysis. Furthermore, it should be emphasized that considerations of speed, simplicity and the conservation of test substances have to some extent taken precedence over the quest for highly precise kinetic data.

EXPERIMENTAL

Materials. The peptides used for this investigation were all tested for purity by elementary analysis (C, H, N) and by chromatography on paper in at least two different solvent systems before and after hydrolysis. The source of the peptides, together with their molar colour yields in the ninhydrin reaction and R_F values in *n*-butanol-acetic acid-water (4:1:5, by vol.) are recorded in Table 1.

The hydrochloric acid used for hydrolysis was prepared from glass-distilled constant-boiling HCl by dilution with distilled water.

Procedure. Approx. 2 mg. of each peptide (weighed to $\pm 1 \mu\text{g}$.) were dissolved in hydrochloric acid (1.0 ml.; 2*N*, except where otherwise indicated); samples of the peptide solution (0.100 ml.) were immediately withdrawn by means of a Carlsberg constriction micropipette and sealed in individual Pyrex tubes (10 $\times$ 45 mm.). The tubes were then immersed in a boiling-water bath (99°). At appropriate intervals tubes were withdrawn and immediately transferred to a Dewar flask containing acetone-solid CO₂ to prevent further hydrolysis. When the last tube had been removed from the bath the tubes were opened, the contents were neutralized with 2*N*-NaOH (0.10 ml.) and then diluted with 1.0 ml. of water. The extent of hydrolysis was measured by analysis of 0.100 ml. samples of the neutralized reaction solution according to the ninhydrin procedure of Moore & Stein (1948); optical-density measurements were carried out with a Coleman spectrophotometer (model 6A) at wavelength 570 $m\mu$. Portions of the neutralized hydrolysis solutions were also chromatographed on paper as a check

on the identity and chromatographic purity of the peptides under investigation, as noted above.

RESULTS

The hydrolysis of a dipeptide in a solution of strong acid can be represented as:

On this basis, one H₃O⁺ would be consumed for each peptide bond that is hydrolysed. In the presence of a large excess of acid the rate of hydrolysis of a dipeptide would then be expected to follow the kinetics of a first-order reaction. The rate of such a reaction is described by the equation

$$\ln \frac{a_0}{a_0 - a} = k_{\text{obs}} t, \quad (1)$$

where a_0 represents the initial concentration of dipeptide and a the concentration of dipeptide which has been hydrolysed after time t .

Following Schwartz & Engel (1950), who used the photometric ninhydrin procedure (Moore & Stein, 1948) for the determination of the degree of enzymic hydrolysis of synthetic peptides,

$$D_0 = m_0 a_0, \quad D_\infty = (m_1 + m_2) a_0,$$

$$\text{and} \quad D_x = m_0(a_0 - a) + (m_1 + m_2)a,$$

Table 1. R_F values and quantitative data on the hydrolysis of various dipeptides by acid

The peptides were hydrolysed by heating in 2*N*-HCl at 99°. In determining the relative colour yield, that given by alanine was taken as 1.00. R_F values were obtained by descending chromatography on Whatman no. 1 paper, using as solvent a mixture of butanol-acetic acid-water (4:1:5, by vol.). The average deviation in the k_2 values was $\pm 0.2 \times 10^{-3}$.

Peptide	Source*	Relative ninhydrin colour yield	R_F	$10^3 k_2$ (mole ⁻¹ min ⁻¹)
Alanylalanine†	1	1.99	0.39	—
DL-Alanylglycine	1	1.48	0.23	2.4
Alanylleucine (A)†	1	1.45	0.60	1.1
Glycyl-L-alanine	2	0.87	0.25	1.3
Glycyl-D-alanine	2	0.87	0.25	1.4
Glycylglycine‡	4	1.11	0.16	3.5
Glycyl-L-leucine	3	1.05	0.55	1.2
Glycyl-L-serine	3	0.93	0.12	6.4
Glycyl-L-tyrosine	4	1.09	0.37	1.5
Glycyl-L-aspartic acid	1	1.11	0.13	6.8
L-Leucyl-L-aspartic acid	1	1.29	0.49	3.0
L-Leucyl-L-glutamic acid	1	1.29	0.55	0.8
L-Leucylglycine	1	0.83	0.52	0.7
L-Leucyl-D-leucine	1	0.83	0.88	0.2
L-Prolyl-L-phenylalanine	1	0.02	0.62	1.0
L-Seryl-L-alanine	3	1.22	0.28	2.6
L-Serylglycine	3	0.76	0.19	1.4
L-Seryl-L-serine	3	0.87	0.15	1.4
L-Alanyl-L-serine	3	1.76	0.19	> 4.0§

* Sources of peptides:

1. Emil Fischer peptide collection. We are indebted to Professor H. O. L. Fischer for samples of these peptides.
2. Obtained through the courtesy of Dr J. P. Greenstein, National Cancer Institute, Bethesda, Md.
3. J. I. Harris. Synthesized in part during the tenure of a James Hudson Brown Memorial Fellowship in Professor J. S. Fruton's laboratory at Yale University Medical School.
4. Hoffman-La Roche, Inc., Nutley, N.J.

† Optical configuration undetermined.

‡ Hydrolysed also in *N*-HCl.

§ Approximate value.

where D_0 represents the initial optical density of the unhydrolysed peptide, D_∞ the final optical density after complete hydrolysis and D_x the optical density of a sample drawn from the hydrolysis reaction after a time x ; m_0 represents the molar colour yield of the unhydrolysed peptide, and m_1 and m_2 are the molar colour yields of its constituent amino acids. Thus equation (1) becomes

$$\ln \frac{D_\infty - D_0}{D_\infty - D_x} = k_{\text{obs.}} t. \quad (2)$$

The observed rate constants ($k_{\text{obs.}}$), shown in Table 1, may be derived in either of two ways. A direct calculation may be carried out with equation (2) (see Table 2 for a typical calculation).

Alternatively, $\ln \frac{D_\infty - D_0}{D_\infty - D_x}$ may be plotted against time (see Fig. 1), to give a line whose slope equals the observed rate constant. The method gives the best results when $m_1 + m_2 \simeq 2m_0$. As m_0 approaches ($m_1 + m_2$), D_0 approaches D_∞ , and the method loses

Table 2. *Typical experiment for a pseudo-unimolecular reaction, the hydrolysis of glycyl-D-alanine with 2.026 N-HCl at 99°*

For significance of symbols see text.

Time (min.)	D	$\frac{D_\infty - D_0}{D_\infty - D}$	$\ln \left[\frac{D_\infty - D_0}{D_\infty - D} \right]$	$10^3 k_{\text{obs.}}$ (min. ⁻¹)	$10^3 k_2^*$ (l. mole ⁻¹ min. ⁻¹)
0	0.493	1.00	0.000	—	—
50	0.620	1.17	0.157	3.14	1.54
85	0.683	1.27	0.239	2.82	1.39
133	0.765	1.44	0.365	2.75	1.36
190	0.865	1.72	0.542	2.86	1.41
250	0.943	2.02	0.703	2.81	1.39
330	1.070	2.84	1.044	3.16	1.55
450	1.147	3.77	1.327	2.95	1.46
605	1.222	5.53	1.710	2.83	1.40
∞	1.383	—	—	—	Av. 1.44 $\delta = 0.06$ (4%)†

* $k_2 = k_{\text{obs.}}/2.026$.

† The overall average deviation for all the experiments reported was found to be $\pm 8\%$.

Table 3. *Effect of acid concentration on the hydrolysis of glycylglycine*

Acid concn. (N)	$10^3 k_{\text{obs.}}$ (min. ⁻¹)	$10^3 k_2^*$ (l. mole ⁻¹ min. ⁻¹)
2.047	7.15	3.49
	7.50	3.66
1.023	3.78	3.69
	3.46	3.42

* $k_2 = k_{\text{obs.}}/(\text{acid concentration})$.

Fig. 1. Kinetics of hydrolysis of representative dipeptides in 2N-HCl at 99° plotted according to the first-order law. O, Glycyl-L-serine; ▲, glycylglycine; □, L-seryl-L-alanine; ●, L-serylglycine; ×, L-leucylglycine.

precision. As shown in Table 1, the molecular extinction coefficients of dipeptides vary appreciably with the nature of the component amino acids. Alanine peptides tend to give unusually high molar colour yields; in the case of Ala. Ala, m_0 is approximately $2m_{\text{Ala}}$, and consequently it was not found possible to follow the hydrolysis by this procedure. For similar reasons, the results obtained with Ala.Ser were also difficult to interpret, and consequently the rate constant recorded in Table 1 is only an approximate value.

In order to establish first-order dependence of the rate of hydrolysis on dipeptide concentration under the conditions of hydrolysis employed throughout the course of the investigation, apparent rate constants between successive points of observation for the hydrolysis of Gly. Ala in 2.020N-HCl were calculated from appropriate optical-density measurements. As shown in Table 2 the results conform to the kinetics of a pseudo first-order reaction at constant acid concentration.

First-order dependence of the rate of reaction on acid concentration was demonstrated when the rate of hydrolysis of Gly.Gly was measured at two different concentrations of acid (2.047 and 1.023N-HCl respectively); as shown in Table 3, the observed rate constants are directly proportional to acid concentration.

DISCUSSION

The stabilizing effect of a positively charged group on the rate of hydrolysis of a neighbouring peptide bond has been demonstrated by Lawrence & Moore (1951) in a study of the rates of hydrolysis of Gly.Gly.Gly and Gly.Gly under identical experimental conditions. The rate constant for the hydrolysis of the first bond to be split in the tripeptide was found to be about 8 times greater than the rate constant for the hydrolysis of the same bond in the dipeptide molecule. This ratio of 8 agrees with unpublished work by the present authors.

These results support the view that a peptide bond involving the $-CO-$ group of an *N*-terminal residue is more stable to acid hydrolysis than is the same bond when present in the interior of a peptide chain, which accounts for the selective accumulation of dipeptides in partial acid hydrolysates of proteins (Gordon *et al.* 1941; Stein *et al.* 1944).

In addition, our results (Table 4) indicate that steric effects, which are known to influence the rate of hydrolysis of peptide bonds in concentrated acid at 37°, also play an important role in determining the course of hydrolysis of similar bonds in more dilute acid (2*N*) at 99° (Table 1); furthermore,

residues, on the other hand, appear to have a stabilizing influence on the adjacent peptide bond. Thus, whereas Gly.Ser is five times more labile than Gly.Ala, Ser.Gly is twice as stable as Ala.Gly. In the case of Ser.Ser the labilizing effect of the *C*-terminal serine residue appears to be completely nullified by its *N*-terminal counterpart and Ser.Ser is in fact found to be more stable than Ser.Ala.

The relative stability to hydrolysis of Ser.Ser is of particular interest since it may mean that a peptide bond formed between two adjacent serine residues in a long peptide chain will not exhibit the specific lability which has been shown to be characteristic of other bonds involving the amino group of serine. In this connexion, it is interesting to speculate that the postulated shift of the peptide chain from the amino to the hydroxyl group of serine residues in proteins may not occur in Ser.Ser linkages; this could in part explain why only 60–70% of the bonds involving the amino groups of serine residues in silk fibroin undergo rearrangement in the presence of concentrated sulphuric acid (Elliott, 1952).

Although Gly.Ser is hydrolysed five times as fast as Gly.Ala in 2*N*-HCl at 99°, it may be expected that the relative difference would be even greater in cold concentrated acid, since the specificity of hydrolysis of such bonds in polypeptides and proteins is known to be much greater in cold concentrated acid than in hot dilute acid (Desnuelle & Casal, 1948).

Peptide bonds involving *C*-terminal aspartic acid residues have also been shown to be particularly susceptible to hydrolysis in 2*N*-HCl at 100°. Thus Gly.Asp is hydrolysed five to six times as fast as Gly.Ala, and Leu.Asp four times as fast as either Leu.Gly or Leu.Glu. The labilizing effects of β -carboxyl groups on neighbouring peptide bonds is consistent with the finding that aspartic acid is released specifically from proteins during hydrolysis at 100° with either weak organic acids or dilute hydrochloric acid (0.01*N*). To account for this phenomenon it was postulated (Partridge & Davis, 1950) that carboxyl groups may become negatively charged in sufficiently dilute acid, so that they attract hydrogen ions and act as proton donors to facilitate the hydrolysis of adjacent peptide bonds. In the case of Leu.Glu the side-chain carboxyl group is too far removed from the peptide chain to exert any appreciable labilizing effect. However, it is difficult to see how such an explanation can be applied to our results, since side-chain carboxyl groups would not be expected to carry a significantly negative charge in 2*N*-HCl.

While the present investigation was in progress Desnuelle & Bonjour (1952) described the influence of α -OH, and of α -, β - and γ -carboxyl groups, on the

Table 4. *Relative rates of hydrolysis of various peptides under different conditions*

Peptide	Relative rate of hydrolysis (Gly.Gly = 1.0)	
	2 <i>N</i> acid, 100°*	10 <i>N</i> acid, 37°†
Gly.Gly	1.00	1.00
Gly.Leu	0.40	0.35
Leu.Gly	0.23	0.20
Leu.Leu	0.05	0.06
Gly.Asp	1.94	—
Leu.Asp	0.86	—

* Present investigation. † Synge (1945).

steric effects are shown to depend on the position of the amino acid side chain relative to the bond as well as on its effective size. As shown in Table 4, our results are in close agreement with those obtained by Synge (1945) for the hydrolysis of the same peptides in concentrated acid at 37°.

As had been expected from earlier observations on proteins and long polypeptides (Gordon *et al.* 1941; Desnuelle & Casal, 1948), peptide bonds involving the amino groups of serine residues (i.e. in the present work dipeptides containing *C*-terminal serine) have been found to be significantly more labile than the corresponding dipeptides containing *C*-terminal glycine and alanine. *N*-Terminal serine

rate of hydrolysis of the *N*-acetyl derivatives of a series of model compounds in 0.25M oxalic acid at 105°. *N*-Acetylated molecules containing free CO₂H groups were found to be more labile than non-carboxylic *N*-acetylated molecules, and the labilizing efficiency of the various carboxyl groups was found to decrease in the order β-CO₂H, α-CO₂H, γ-CO₂H, a result which appears to confirm the findings of Partridge & Davis (1950). Furthermore, an α-hydroxyl group was found to exert a greater labilizing effect than any of the individual carboxyl groups.

The effect of an α-hydroxyl group, however, in strong acid hydrolysis is limited to the hydrolysis of bonds involving the amino groups of hydroxy-amino acid residues; thus, although peptides containing *N*-terminal serine and threonine are undoubtedly released during the early stages of hydrolysis of a protein with dilute oxalic, acetic or hydrochloric acids, no free serine or threonine is formed. With β-carboxyl groups, on the other hand, the initial effect, in which bonds involving the α-carboxyl groups of aspartic acid residues in peptide chains are split, is followed by the subsequent labilization of the adjacent bonds involving the amino groups of the *C*-terminal aspartic acid residues formed in the first stage of hydrolysis; in this way free aspartic acid is released specifically. It is unlikely, however, that all the aspartic acid residues in a protein can be released in this way, since the ease of hydrolysis will also depend upon the nature of the other amino acids forming the potentially susceptible bonds. For example, in the case of the hexapeptide Ala. Gly. Val. Asp. Ala. Ala, released in the ovalbumin → plakalbumin transformation (Ottesen & Villee, 1951), the -Asp. Ala-bond can be preferentially hydrolysed in 0.1N-HCl at 100° (Ottesen & Wollenberger, 1953); in this way Ala. Ala is released but no free aspartic acid is formed under these conditions, presumably because the bond involving its amino group is stabilized by being combined with the adjacent valine residue.

SUMMARY

1. The velocity constants for the hydrolysis of a series of synthetic dipeptides in aqueous 2N-HCl at 99° have been determined.

2. Dipeptides containing *C*-terminal serine and aspartic acid are found to be especially labile, whereas peptides containing leucine are observed to be particularly resistant to hydrolysis.

3. The significance of these results in relation to the more general problems of acid hydrolysis of proteins is discussed.

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