The Kinetics of Low Temperature Acid Hydrolysis of Gramicidin and of some Related Dipeptides

By R. L. M. SYNGE, *The Lister Institute of Preventive Medicine, London*

(*Received* 12 *June* 1945)

The work described in this paper was undertaken in order to throw light on the course of acid hydrolysis of gramicidin, and has especial significance in connexion with the analytical data already reported for a partial acid hydrolysate of gramicidin (Synge, 1944). The time course of evolution, during low temperature acid hydrolysis, has now been studied for amino groups, 'hydroxyamino' groups (i.e. free ethanolamine—Synge, 1945) and free amino-acids. Interpretation of the mode of breakdown of gramicidin has been assisted by parallel studies of the hydrolysis, under the same conditions, of a number of dipeptides embodying peptide bonds representative of those possibly present in gramicidin.

Data in the literature on the kinetics of acid hydrolysis of peptides, etc. are fragmentary and scattered (e.g. Abderhalden & Bahn, 1932, 1935; Abderhalden & Brockmann, 1927; Abderhalden & Köppel, 1927; Abderhalden & Mahn, 1927, 1928; Abderhalden & Sickel, 1927; Steiger, 1934), whereas extensive data on the kinetics of alkaline hydrolysis have been published, especially by Levene and Abderhalden and their respective collaborators. The peptides employed in the present experiments were mostly the same as those employed in a study of alkaline hydrolysis kinetics by Levene, Steiger & Rothen (1932). Comparison has therefore been possible of the effects of some amino-acid side-chains on the rate of hydrolysis of adjacent peptide bonds by acid and alkali; in this connexion the present observations have general significance in protein chemistry.

EXPERIMENTAL

Materials

Synthetic dipeptides. Glycyl-*dl*-alanine was prepared according to Fischer (1904), *dl*-alanylglycine according to Axhausen (1905) and glycyl-*l*-tryptophan according to Abderhalden & Kempe (1907). Other dipeptides employed were the preparations referred to by Synge (1944).

Gramicidin. Preparation R 3 (Synge, 1944) was employed.

Methods

Hydrolysis of synthetic dipeptides. Unless otherwise stated, a 0·2 M solution of peptide in 10 N-HCl was diluted to 2 vol. with glacial acetic acid. The resulting mixture was kept in a glass-stoppered vessel in the incubator at 37° and 0·2 ml. portions were withdrawn at intervals for analysis.

The hydrolysis solutions remained colourless except for those containing tryptophan peptides. The mixture with *l*-leucyl-*l*-tryptophan rapidly darkened through purple to deep brown. Measured samples of the mixture with glycyl-*l*-tryptophan were kept in separate sealed evacuated tubes to prevent access of air; nevertheless, the solution progressively darkened to a dark green.

Determination of extent of hydrolysis of synthetic peptides. This was effected by the ninhydrin-CO_2 manometric microprocedure of Van Slyke, Dillon, MacFadyen & Hamilton (1941). The solution for analysis was evaporated to dryness in the reaction vessel over H_2SO_4 and soda-lime in a vacuum desiccator at room temperature overnight. The reaction was effected with 50 mg. ninhydrin and 50 mg. of the pH 4·7 citrate buffer in water at a volume of 2 ml.

'Blank' determinations on portions of the hydrolysis mixtures evaporated immediately after making up exceeded the reagent blank, and indicated that 3–10% hydrolysis occurred during the overnight evaporation. The results quoted below have been corrected by subtraction of the blank determined in this way for each peptide. This probably introduces an excessive correction of the readings in the later stages of the hydrolysis, but the effect is small.

Portions of each hydrolysis mixture were subjected to 'complete' hydrolysis by heating for 36 hr. in sealed evacuated tubes at 110–115° before the ninhydrin-CO_2 determination was done. The results lay within 5% of the theoretical for all the peptides except those containing tryptophan; glycyl-*l*-tryptophan gave 81% and *l*-leucyl-*l*-tryptophan 86% of the theoretical result for complete hydrolysis. However, in calculating the hydrolysis constants, the extent of hydrolysis of these peptides, as for the others, was expressed on the basis of the theoretical final value, since destruction of tryptophan residues is probably less serious at 37° than at 110–115°.

Hydrolysis of gramicidin. To 2·5 ml. portions of an accurately made up 2% (w/v) solution of gramicidin in glacial acetic acid were added 2·5 ml. 10 N-HCl and the resulting clear solutions were kept at 37° in sealed evacuated tubes. The appearance of the mixture as hydrolysis proceeds has been described by Synge (1944). At intervals, tubes were removed for analysis; their contents were transferred quantitatively with glacial acetic acid to a flask having a ground-glass joint connexion to the evacuation apparatus and evaporated to dryness *in vacuo* below 40°. (When water is employed for transference, the mixture froths uncontrollably, and precipitation occurs.) During the evaporation of the earlier hydrolysates there was separation of insoluble material, which sometimes took the form of a skin and impeded the evaporation; this decreased markedly with increasing time of hydrolysis, and after 20 days' hydrolysis no such phase separation occurred on evaporation. The evaporation residues were made to 2·5 ml. with glacial acetic acid, in which they were all completely soluble.

Determination of amino-N of gramicidin hydrolysates. This was done in duplicate on 0·25 ml. samples of the above acetic acid solutions. The solution was washed into the apparatus with a further 0·75 ml. glacial acetic acid (added in 2 portions) and then with 5 ml. water, after which the standard 4 min. Van Slyke manometric amino-N procedure was followed. The earlier hydrolysates gave precipitation when the water was added, and further precipitation and frothing on addition of the $NaNO_2$, but this did not occur with 20-day (or longer) hydrolysates. In all cases the reaction mixture turned yellow when the $NaNO_2$ was added.

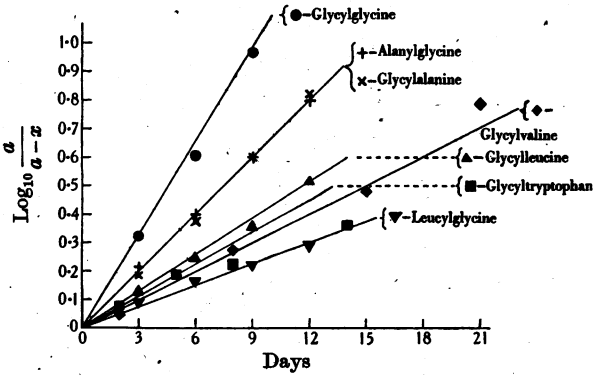


Fig. 1. Data on kinetics of hydrolysis of dipeptides. (a = initial quantity of peptide; x = quantity of peptide (same units) hydrolyzed after given time.)

Determination of 'hydroxyamino'-N of gramicidin hydrolysates. This was effected in Conway units by the adaptation of the periodate-NH_3 method of Van Slyke, Hiller & MacFadyen (1941) employed throughout for the work with the hydroxyamino constituent of gramicidin (Synge, 1945). Two 0·5 ml. samples of the acetic acid solution of evaporated hydrolysate were placed in the outer chamber of Conway dishes whose rims had been smeared with vaseline, and were allowed to evaporate overnight in a vacuum desiccator containing H_2SO_4 and soda-lime. One dish was employed for the NH_3 determination, and the other for the (NH_3 + 'hydroxyamino')-N determination. No free NH_3 was found at any stage of the hydrolysis. Since, on addition of the reagents to the dishes, much material, particularly in the early stages of hydrolysis, did not dissolve or was precipitated, it appeared that low values might be resulting from failure of reagents or NH_3 to diffuse through the lumps of insoluble material. Accordingly, the dishes were titrated as usual after 2 hr. equilibration, and were then re-closed for a further 22 hr., after which any further NH_3 which had reached the centre chamber was titrated. The results obtained after 2 and 24 hr. are recorded separately; the 2 hr. values have been corrected for the incomplete equilibrium absorption of NH_3 (95%), whereas the 24 hr. values (sum of 2 and 24 hr. titrations) are naturally not so corrected. All results are further corrected, a 90% yield of NH_3 from ethanolamine being assumed (cf. Synge, 1945).

A determination was also carried out on intact gramicidin which had been evaporated from acetic acid solution in the dishes. The gramicidin dried to a clear skin, which, on addition of the reagents, broke away from the dish and floated as flakes. No NH_3 or 'hydroxyamino'-N resulted.

Determination of free amino-acids of gramicidin hydrolysate. Duplicate 0·25 ml. portions of the acetic acid solution of gramicidin hydrolysate were each mixed with 50 mg. of the pH 4·7 citrate buffer, evaporated in the reaction vessel in a desiccator overnight, and subjected to the ninhydrin-CO_2 procedure.

RESULTS

Hydrolysis of synthetic peptides. As expected, hydrolysis was found to follow the unimolecular law. Figs. 1 and 2 give the data, together with the

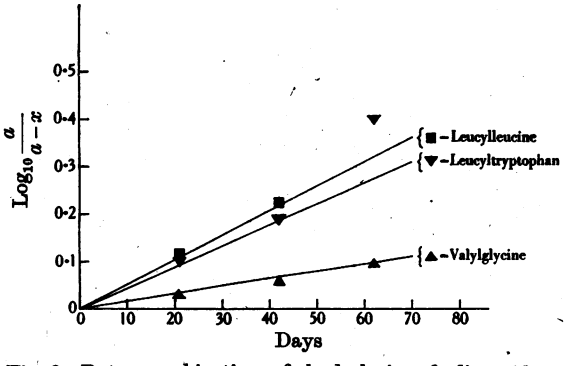


Fig. 2. Data on kinetics of hydrolysis of dipeptides. (a = initial quantity of peptide; x = quantity of peptide (same units) hydrolyzed after given time.)

best-fitting straight lines, from which the half-lives given in Table 1 have been deduced. It has seemed better, in view of the progressive decomposition of the tryptophan peptides, mentioned above, to draw their lines in agreement with the hydrolysis data from the earlier samples taken.

Table 1. *Hydrolysis of dipeptides by acid and alkali*

Peptide	Half-life period (days) in a mixture of equal volumes of 10 N-HCl and glacial acetic acid at 37°	Relative velocity of hydrolysis (glycylglycine = 1)	
		In acid (present study)	In alkali (Levene *et al.* 1932)
Glycylglycine	2·8	1	1
Glycyl-*dl*-alanine	4·5	0·62	0·24
dl-Alanylglycine	4·5	0·62	0·21
Glycyl-*dl*-leucine	7·0	0·40	0·061
Glycyl-*l*-tryptophan	8·0	0·35	—
Glycyl-*dl*-valine	9·0	0·31	0·049
dl-Leucylglycine	12	0·23	0·048
l-Alanyl-*l*-alanine	—	—	0·043
dl-Leucyl-*dl*-leucine 'A'	58	0·048	0*
l-Leucyl-*l*-tryptophan	68	0·041	—
dl-Valylglycine	190	0·015	0
Glycyl-α-amino-*iso*-butyric acid	—	—	0

* *d*-Leucyl-*d*-leucine.

With glycyl-*dl*-alanine, the unimolecular mode of hydrolysis was further checked (i) by employing an initial concentration three times as great as usual, and (ii) by adding equimolar quantities of the hydrolysis products, glycine and *dl*-alanine, to the initial hydrolysis mixture. In both these experiments hydrolysis was unimolecular and the constant was the same as before.

Hydrolysis of gramicidin. In Table 2 the data on the hydrolysate of gramicidin are tabulated (*a*) as N in percentage of total N of the gramicidin and (*b*) as percentage of the theoretical figure for a peptide molecule at different stages of hydrolysis, as follows:

Expressed as percentage of total N of gramicidin, if x = α-amino-N of free amino-acids, $(73{\cdot}3-x)$ the α-amino-N of amino-acid residues in peptide linkage (i.e. difference of free amino-acids before and after complete hydrolysis), y the amino-N of the partial hydrolysate, and z the 'hydroxyamino-N' (i.e. amino-N of free ethanolamine), then $y-x-z$ = free amino-N of peptides, and the average number of amino-acid residues per peptide molecule is $(73{\cdot}3-x)/(y-x-z)$. These values are shown in the

Table 2. *Course of hydrolysis of gramicidin in a mixture of equal volumes of* 10N-HCl *and glacial acetic acid at* 37°

((*a*) Values are N as percentage of total N of gramicidin.
(*b*) Values are as percentage of 'theoretical' values for a complete hydrolysate. These are taken to be: amino-N, 80% of total N of gramicidin; α-amino-N of amino-acids, 73·3% of total N of gramicidin; 'hydroxyamino'-N, 6·7% of total N of gramicidin.)

Time of hydrolysis (days)	Amino-N		α-Amino-N of free amino-acids		'Hydroxyamino'-N determined at			Average no. of amino-acid residues per molecule of peptide
					(*a*)		(*b*)	
	(*a*)	(*b*)	(*a*)	(*b*)	2 hr.	24 hr.	24 hr.	
0	0*	0*	(0)	(0)	0	0	0	—
1	16	20	0·8	1	2·4	2·8	42	5·7
2	23	29	1·6	2	2·0	2·2	33	3·7
6	33	41	7·2	10	3·6	3·8	57	2·9
11	41	51	12	16	4·1	4·2	63	2·4
19¾	47	59	20	27	3·1	3·3	49	2·2
47	55	68	29	39	3·2	3·4	51	2·0

* Hotchkiss (1941).

complete hydrolysate, assuming the stoichiometric minimum molecule postulated by Gordon, Martin & Synge (1943*a*; cf. Synge, 1944, 1945). The actual

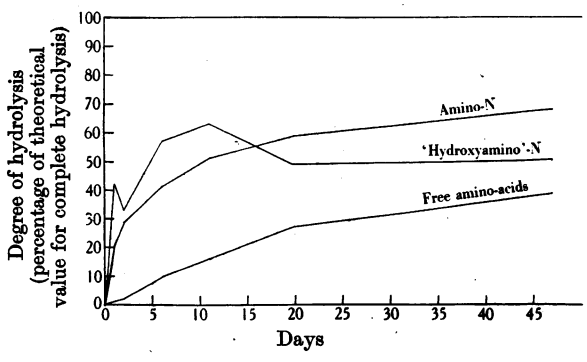


Fig. 3. Course of evolution of amino groups, 'hydroxyamino' groups and free amino-acids during low temperature acid hydrolysis of gramicidin.

values of amino-N and ninhydrin-CO_2 for a 'complete' hydrolysate approximate closely to these figures (Hotchkiss, 1941).

It has been possible to obtain an estimate of the number of residues other than ethanolamine per last column of Table 2. For similar calculations of peptide size during protein hydrolysis compare Gordon, Martin & Synge (1941), Van Slyke, Dillon *et al.* (1941) and Stein, Moore & Bergmann (1944). In the present instance, glycyl peptides are probably absent and the amino-N values are therefore unlikely to be too high.

The course of evolution of amino-N, α-amino-N of free amino-acids and 'hydroxyamino-N' as percentages of the theoretical maximum for each quantity is plotted in Fig. 3.

DISCUSSION

When the synthetic dipeptides here studied are arranged in order of ease of hydrolysis by acid, it is seen (Table 1) that this sequence is the same (for those peptides common to both investigations) as that found for ease of hydrolysis by alkali (Levene *et al.* 1932). It should be noted, however, that when the velocity constants are expressed on a common scale (those for glycylglycine being taken as 1 both in acid and alkali) the differences are relatively greater for alkali than for acid. The influence of various alkyl side-chains on the hydrolyzability of an adjacent peptide bond is seen to be in the same

sense for acid as for alkaline hydrolysis.. In view of the specially large inhibitory effects of the 'spreading' side-chains of valine and α-amino*iso*-butyric acid residues it seems reasonable to attribute the effects, at least in part, to steric limitation by the side-chains of the access of ions to the peptide bond.

Valylglycine is seen to be much the most resistant to acid hydrolysis of the dipeptides studied, and it is therefore easily understood why it was found in high yield, and substantially unaccompanied by free valine or glycine in a 10-day acid hydrolysate of gramicidin (Synge, 1944). Valylvaline is probably still more resistant; its occurrence after vigorous acid hydrolysis of gramicidin (Christensen, 1943, 1944) is thus readily explained.

It may be noted that the average number of amino-acid residues per peptide molecule in the 10-day gramicidin hydrolysate previously studied (Synge, 1944) is about 2½ (Table 2).

The resistance to acid hydrolysis exhibited by peptides of the higher monoamino-acids with fatty side-chains, such as leucylleucine and valylvaline, suggests that if such amino-acids occurred directly linked to one another, corresponding dipeptides would have been found by Gordon, Martin & Synge (1943*b*) in the acid hydrolysates of gelatin studied by them and strengthens their suggestion that such linkages do not occur in gelatin.

It is reasonable, as a first approximation, to assume that the stability to hydrolysis of each bond in a long peptide chain will depend mainly on the nature of the amino-acid residues directly involved in it, in such a way that the relative stability of the various bonds will be correlated with that of the corresponding dipeptides. On this view, long peptide chains would, at least in respect of their monoamino monocarboxylic residues, be subject to initial attack at much the same points both by acid and alkali; a disappointing prospect, as already noted (Synge, 1944), for studies of protein structure by partial hydrolysis methods. However, although the same bonds in a long peptide chain are likely to be attacked initially, the subsequent effects of acid and alkali are likely to be different, since in acid ionic repulsion will protect the bond next to that broken and involving the residue whose amino group has been set free, while in alkali the corresponding bond involving the residue with a free carboxyl group will be protected.

This enhanced stability of dipeptides to acid and alkaline hydrolysis in comparison with larger peptides embodying the same amino-acid residues was postulated by Gordon *et al.* (1941; cf. Synge, 1943) on the basis of physico-chemical theory and of impressions gained from a wide survey of the literature. It is now possible to adduce some more direct evidence in respect of the reality of the effect in acid hydrolysis:

(1) The observations of Stein *et al.* (1944) on the low temperature acid hydrolysis of silk show that consistently fewer free amino-acids and more dipeptides are present in the hydrolysate than would be the case if the splitting of peptide bonds were random, on which assumption, when $1/n$ of the bonds in an original long-chain peptide had been split, $1/n^2$ of the amino-acid residues present would be free. (However, these effects were not so marked with the proteins studied by Gordon *et al.* (1941).)

(2) The present study with gramicidin shows that the absolute rate of hydrolysis of gramicidin in its initial stages greatly exceeds the rate of hydrolysis of dipeptides corresponding to bonds possibly present, and further that the number of free amino-acids at any single stage is less than the 'random' expectation. Christensen (private communication)* has also noticed the latter phenomenon with low temperature acid hydrolysis of gramicidin, and states that it is less marked with hydrolysis at higher temperatures. This diminution of the effect on raising the temperature is to be expected with the ionic repulsion mechanism already suggested.

(3) Christensen (1943, 1944) has found the peptide bond in valylvaline much more resistant to acid hydrolysis than the same bond in benzoylvalylvaline.

In comparing the course of hydrolysis of silk (Stein *et al.* 1944) with that of gramicidin, it is interesting to note that peptides higher than dipeptides persist to a later stage of the hydrolysis of gramicidin than of silk. This may perhaps be correlated with the different amino-acid composition of the two substances. Kinetic interpretation of the data of Stein *et al.* is rendered difficult by the fact that they employed only a slight excess of acid for hydrolysis.

For studies of the structure of peptides and proteins by the recognition of partial hydrolysis products, it would in general be desirable to employ a hydrolytic agent that did not discriminate in its attack on different peptide bonds, were it not for technical difficulties of separating the multitude of representative compounds, thus produced. A good alternative would be to employ in separate studies a number of more selective hydrolytic agents, each preferentially attacking different bonds in the compound under investigation. Until the synthetic powers of proteolytic enzymes are better understood, acid and alkali are the main hydrolytic agents of practical use, and it is hoped that the considerations presented here may be of help towards using them with good effect. The disadvantage that both may tend to attack initially the same bonds may turn out to be offset by their subsequent preferential attack on different bonds; further, the ten-

* (*Note added in proof.*) See Christensen, H. N. & Heysted, D. M., *J. biol. Chem.* (1945), **158**, 593.

dency of any bond in the original molecule to acquire increased stability to acid or alkali once it is embodied in a dipeptide molecule should be definitely advantageous.

SUMMARY

1. The hydrolysis velocity constants for a number of dipeptides in acid at 37° have been determined, and are compared with the data of Levene *et al.* (1932) for similar dipeptides in alkali.

2. The course of hydrolysis of gramicidin by acid under the same conditions has been followed by determination of the evolution of amino groups, free amino-acids and 'hydroxyamino' groups.

3. The nature of the process of hydrolysis by acid of long peptide chains is discussed in relation to the available data.

I wish to thank Mr W. Wolff for his technical assistance.

REFERENCES

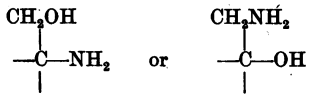
Abderhalden, E. & Bahn, A. (1932). *Hoppe-Seyl. Z.* **210**, 246.
Abderhalden, E. & Bahn, A. (1935). *Hoppe-Seyl. Z.* **234**, 181.
Abderhalden, E. & Brockmann, H. (1927). *Hoppe-Seyl. Z.* **170**, 146.
Abderhalden, E. & Kempe, M. (1907). *Ber. dtsch. chem. Ges.* **40**, 2737.
Abderhalden, E. & Köppel, W. (1927). *Hoppe-Seyl. Z.* **170**, 226.
Abderhalden, E. & Mahn, H. (1927). *Hoppe-Seyl. Z.* **169**, 196.
Abderhalden, E. & Mahn, H. (1928). *Hoppe-Seyl. Z.* **174**, 47.
Abderhalden, E. & Sickel, H. (1927). *Hoppe-Seyl. Z.* **170**, 134.
Axhausen, W. (1905). *Liebigs Ann.* **340**, 128.
Christensen, H. N. (1943). *J. biol. Chem.* **151**, 319.
Christensen, H. N. (1944). *J. biol. Chem.* **154**, 427.
Fischer, E. (1904). *Ber. dtsch. chem. Ges.* **37**, 2486.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943*a*). *Biochem. J.* **37**, 86.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943*b*). *Biochem. J.* **37**, 92.
Hotchkiss, R. D. (1941). *J. biol. Chem.* **141**, 171.
Levene, P. A., Steiger, R. E. & Rothen, A. (1932). *J. biol. Chem.* **97**, 717.
Steiger, R. E. (1934). *Helv. chim. Acta*, **17**, 573.
Stein, W. H., Moore, S. & Bergmann, M. (1944). *J. biol. Chem.* **154**, 191.
Synge, R. L. M. (1943). *Chem. Rev.* **32**, 135.
Synge, R. L. M. (1944). *Biochem. J.* **38**, 285.
Synge, R. L. M. (1945). *Biochem. J.* **39**, 355.
Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
Van Slyke, D. D., Hiller, A. & MacFadyen, D. A. (1941). *J. biol. Chem.* **141**, 681.

The Hydroxyamino Component of Gramicidin Hydrolysates

By R. L. M. SYNGE, *The Lister Institute of Preventive Medicine, London*

(*Received* 12 *June* 1945)

The presence of a compound in acid hydrolysates of gramicidin, containing the groupings



was inferred by Hotchkiss (1941) from the fact that periodate liberates formaldehyde and NH_3 from such hydrolysates (cf. Christensen, Edwards & Piersma, 1941; Synge, 1944*a*). Hotchkiss further showed that the material responsible for these reactions with periodate was not an α-amino-acid, since the α-amino-acid content of hydrolysates (determined by the ninhydrin-CO_2 procedure) was unaffected by treating them with periodate. Serine was not therefore responsible for the reaction (cf. Gordon, Martin & Synge, 1943*b*). Hotchkiss rejected the idea that the material might be *iso*serine or hydroxylysine, and noted its tendency to decompose, with liberation of NH_3, during isolative manipulations. Failure to isolate even small amounts of aldehydo-acid as acidic hydrazone after oxidation with periodate led Hotchkiss later (1944) to doubt that the hydroxyamino component was a carboxylic acid. As a result of chromatographic experiments with acetylated hydrolysates of gramicidin, the same doubt had occurred to the present author, and experiments were undertaken to test for the presence in gramicidin hydrolysates of volatile amino-alcohols.