interfere with the reaction of catalase, hydrogen peroxide and 3-amino-1:2:4-triazole just as ferri-cyanide was shown to do.

SUMMARY

1. 3-Amino-1:2:4-triazole caused a complete, irreversible and relatively rapid inhibition of crystalline purified preparations of liver and erythrocyte catalase in the presence of low and constant concentrations of hydrogen peroxide. Without hydrogen peroxide the inhibition of catalase by 3-amino-1:2:4-triazole was slight and proceeded much more slowly. Other substances that could replace hydrogen peroxide required the presence of oxygen, and it is considered that they acted by producing peroxide by auto-oxidation.

2. Ferri-cyanide, but not ferrocyanide, prevented this inhibition.

3. The spectrum of inhibited catalase differed from that of catalase. The visible part of the spectrum was shifted towards the longer wavelengths, and the Soret band was slightly lowered.

4. Inhibited catalase was obtained in a crystalline form that did not differ from that of uninhibited catalase.

5. The ability of catalase inhibited by 3-amino-1:2:4-triazole to oxidize ribose 5-phosphate with potassium ferri-cyanide as hydrogen acceptor was unchanged.

6. In the presence of oxygen, but in the absence

of added hydrogen peroxide, 3-amino-1:2:4-triazole caused the inhibition of catalase in liver and kidney suspensions, but had no effect in blood haemolysates. The catalatic activity of blood haemolysates could be inhibited by 3-amino-1:2:4-triazole only in the presence of added hydrogen peroxide.

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REFERENCES

- Brauer, G. (1954). *Handbuch der Präparativen Anorganischen Chemie*, vol. 1, p. 353. Stuttgart: Enke.
Euler, H. von & Josephson, K. (1927). *Liebigs Ann.* **452**, 158.
Feinstein, R. N. (1949). *J. biol. Chem.* **180**, 1197.
Francoeur, M. & Denstedt, O. F. (1954). *Canad. J. Biochem. Physiol.* **32**, 6.
Heim, W. G., Appleman, D. & Pyfrom, H. T. (1955). *Science*, **122**, 693.
Heim, W. G., Appleman, D. & Pyfrom, H. T. (1956). *Amer. J. Physiol.* **186**, 19.
Herbert, D. & Pinsent, J. (1948). *Biochem. J.* **43**, 203.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 148.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Keilin, D. & Hartree, E. F. (1954). *Nature, Lond.*, **173**, 720.
Laser, H. (1955). *Biochem. J.* **61**, 122.
Sugimura, T. (1956). *Gann*, **47**, 159.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Methods and Tissue Metabolism*. p. 136. Minneapolis: Burgess Publishing Co.

The Kinetics of Hydrazinolysis of Simple Peptides in Anhydrous Hydrazine

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The method of Akabori, Ohno & Narita (1952) for the determination of the C-terminal amino acids of peptides and proteins consists in treating with anhydrous hydrazine at 100–120° for about 8 hr. to split the peptide bonds and produce amino acid hydrazides and free amino acids from the C-terminal positions. The latter can be separated from the former by treatment with benzaldehyde, by dinitrophenylation and subsequent extraction with sodium bicarbonate of the dinitrophenyl-amino acid from the dinitrophenyl-amino acid hydrazide (Ohno, 1953). The main disadvantage of this method is that the severe treatment required

for complete hydrazinolysis causes decomposition of cysteine and cystine and poor yields of arginine, aspartic acid and glutamic acid (Locker, 1954; Niu & Fraenkel-Conrat, 1955). The problem of severity of treatment has been largely overcome by the addition of an acid catalyst, hydrazine sulphate, to the reaction mixture, when heating at 60° for 16 hr. brings about complete fission of the peptide bond (in the seven peptides studied) and allows all the common amino acids to be recovered in good yields (Bradbury, 1956).

In order to apply the hydrazine method with confidence it is important to know more about the

hydrazinolysis reaction. One requires to know the rates of the acid-catalysed and uncatalysed reaction for various types of peptide bonds at different temperatures and the influence of water on the system. Such information would be useful in work on the identification of small peptides from partial hydrolysates (Kay, Schroeder, Munger & Burt, 1956; Kroner, Tabroff & McGarr, 1955). It would also be of importance in deciding the appropriate conditions of hydrazinolysis of proteins in order to obtain on the one hand the maximum yield of *C*-terminal amino acid and no appreciable amount of *C*-terminal peptide (Bradbury, 1958), or, on the other, the maximum yield of *C*-terminal peptides [if one is interested in the *C*-terminal sequence of amino acids (Niu & Fraenkel-Conrat, 1955; Ohno, 1955)].

Information of this type was obtained by a study of the kinetics of the acid-catalysed and uncatalysed hydrazinolysis of the peptides glycylglycine, DL-leucylglycine, L-lysylglycine dihydrobromide, L-leucyl-DL-valine and diglycylglycine in anhydrous hydrazine. First-order rate constants were obtained in all cases by use of large excesses of hydrazine and hydrazine sulphate over the amount of peptide present. Energies and entropies of activation were calculated in two cases. As this is probably the first example of kinetic measurements in anhydrous hydrazine as a solvent, some comparison is made between these results and those obtained for the similar case of acid hydrolysis in water. A subsequent paper deals with the application of the hydrazine method to the determination of the *C*-terminal amino acids of proteins.

EXPERIMENTAL

Materials

Peptides. Glycylglycine (British Drug Houses Ltd., Poole, Dorset), DL-leucylglycine and diglycylglycine (L. Light and Co. Ltd., Colnbrook, Bucks) were used without further purification. A sample of ϵ -*N*-benzyloxycarbonyl-lysylglycine ethyl ester (supplied by Dr J. M. Swan, Wool Textile Research Laboratory, Melbourne) was hydrolysed by treatment with *N*-NaOH in acetone at room temperature for 1 hr., acidified and the free acid filtered off and dried (m.p. 204°, uncorr.). This was allowed to react with a small volume of 20% (w/w) HBr in acetic acid at room temperature for 30 min.; the concentration of HBr is not critical. Lysylglycine dihydrobromide was precipitated by the addition of dry ether, filtered off, washed with ether and dried *in vacuo* at 60°. Equivalent weight: theory, 182.4; found, 193. L-Leucyl-DL-valine was kindly prepared by Dr W. E. Savage, Wool Textile Research Laboratory, Melbourne. The purity of these peptides was checked by dinitrophenylation followed by one-dimensional paper chromatography; no spots due to impurities were observed.

Anhydrous hydrazine. Hydrazine hydrate (100%), supplied by Gollin and Co., Melbourne, was dried over CaO and distilled at room temperature and 10⁻⁴ mm. Hg

pressure to give a product containing 95% of N₂H₄ (Penneman & Audrieth, 1948). After a second drying and distillation the analysis was 100 ± 0.3% of N₂H₄.

Preparation, separation and estimation of dinitrophenyl-peptides and dinitrophenyl-amino acids

Amino acids and peptides were dinitrophenylated by at least one of the methods due to Sanger (1945) and Levy (1954) with some slight modifications. In the first method 5 mg. of the amino acid or peptide was dissolved in about 5 ml. of 0.5M-NaHCO₃ soln., 5 ml. of a solution of 1-fluoro-2,4-dinitrobenzene (FDNB) in absolute ethanol (27 mg./ml.) was added and sufficient ethanol to make the ratio of ethanol to water 2:1. The solution was shaken for 3 hr. at room temperature, the alcohol evaporated and 5 ml. of 0.5M-carbonate buffer, pH 9, added. The aqueous solution was extracted with ether (4 × 5 ml.) and the combined ether layers were washed once with carbonate buffer, which was added to the aqueous solution. The latter was acidified and extracted with 5 ml. portions of ethyl acetate until these washings were colourless. In general five washings were sufficient, but in some cases, e.g. dinitrophenyl (DNP)-diglycylglycine, 10–15 washings were necessary. In the second method 5 mg. of the amino acid or peptide was dissolved in about 6 ml. of 0.5M-carbonate buffer, pH 9, and about 50 mg. of FDNB added. The mixture was stirred at 40° for 3 hr., after which it was extracted by the method already described.

The ethyl acetate solution of the DNP-amino acid or DNP-peptide obtained by either method was made up to volume in a standard flask and a portion applied to the paper with a micrometer syringe. The spots were run in one dimension with the toluene, phosphate or 2-methylbutan-2-ol solvent (Fraenkel-Conrat, Harris & Levy, 1955). The paper was dried, the spots together with three blanks were cut out, weighed approximately, eluted with 5 ml. of 2% NaHCO₃ soln. at 50° for 15 min. and their optical density was measured at 360 mμ (385 mμ for DNP-proline) in a 1 cm. cell of the Unicam spectrophotometer. The small blank correction (usually about 0.01/100 mg. of paper) was subtracted from the observed optical density for calculation of the molar-extinction coefficient. Solutions of these materials in ether, ethyl acetate or acetone have been stored in the refrigerator, without noticeable decomposition, for more than a year.

The extinction coefficients obtained by the above methods with one-dimensional chromatography are summarized in Table 1. They have been used in previous work (Bradbury, 1956) and in the present investigation. The average value of 1.47 × 10⁴ for the 17 DNP-amino acids listed is about 20% lower than that obtained for the pure DNP-amino acids in NaHCO₃ soln. (Fraenkel-Conrat *et al.* 1955). The difference is attributed to incomplete dinitrophenylation, losses during extraction and losses on the paper during chromatography. However, as these sources of error are reproduced in later hydrazinolysis experiments it is reasonable to assume that they are cancelled out by the use of the above values of ϵ .

It was not possible to dinitrophenylate glycylglycine or diglycylglycine quantitatively by Levy's (1954) method and in this respect these two peptides seem to be anomalous, since a number of other dipeptides (some not included in Table 1) reacted quite satisfactorily under these conditions. However, on prolonged treatment at pH 9 at

Table 1. *Molar-extinction coefficients of dinitrophenyl-amino acids and dinitrophenyl-peptides*For explanation of the abbreviations used for amino acids in this Table see *Biochem. J.* (1953), 55, 5.

Method of dinitrophenylation ...	Extinction coefficient $\times 10^{-4}$		
	Levy (1954) (pH 9 40°)	Sanger (1945) (pH about 8)	Sanger (1945) (NaHCO ₃ replaced by 0.4M- phosphate buffer, pH 6.4)
DNP-amino acid or DNP-peptide			
Ala; Arg; Asp; CySO ₃ H; Glu; Gly; His (bis); His (α -mono-); Leu; Met; Pro; } Phe; Ser; Thr; Try; Tyr (bis); Val }	1.47 $\pm$ 0.03	—	—
L-Cystine (bis)	2.28	—	—
L-Lysine (bis)	2.20	—	—
Glycine	1.49	1.40	0.52 (variable)
Glycylglycine	0.45 (variable)	1.14	1.07
Diglycylglycine	0.48* (variable)	1.08	1.08
DL-Leucylglycine	1.45	1.51	—
L-Leucyl-DL-valine	—	1.30	—
Lysylglycine (bis)	—	2.0	—

* Obtained $\epsilon = 1.07$ on shaking at room temperature in ethanol-water (2:1) for 66 hr. with buffer, pH 9, and FDNB.

room temperature in the mixed solvent, a quantitative yield of DNP-diglycylglycine was obtained. It is interesting to note that glycylglycine and diglycylglycine with pK_a 8.0 can be dinitrophenylated quantitatively at pH 6.4, whereas glycine (pK_a 9.6) reacted only partially. This behaviour suggests that FDNB reacts only with the uncharged form of the amino group.

Procedure for rate measurements

To a small amount of peptide (usually 2 mg.) was added 30 times the weight of hydrazine sulphate (AnalaR) and the materials were dried for about 3 hr. at 10^{-4} mm. Hg pressure. An accurately known volume of anhydrous hydrazine was distilled in from a calibrated reservoir at room temperature, and the tube sealed and heated under vacuum for the appropriate time and temperature ($\pm 0.2^\circ$). It was then opened, excess of hydrazine removed in a vacuum desiccator over conc. H₂SO₄ and the residue dissolved in hot water and shaken with about 0.2 ml. of benzaldehyde (AnalaR) for 2 hr. to remove hydrazine sulphate and amino acid hydrazides as the benzal compounds. The mixture was centrifuged, the benzaldehyde layer washed twice with water, the aqueous layers were dinitrophenylated by Sanger's (1945) method and the alcohol was removed at the pump and 5 ml. of carbonate buffer, pH 9, added. This solution was extracted with 4 $\times$ 5 ml. portions of ether-ethyl acetate (1:1, v/v); the organic layers were diluted with 20 ml. of ether and extracted with 3 $\times$ 10 ml. of buffer, pH 9. The combined aqueous layers were acidified with HCl and extracted with 5 ml. portions of ethyl acetate until the washings (usually five) were colourless. The solution was made up to volume in a standard flask and at least two portions were run on a paper chromatogram with a suitable solvent to separate the unchanged peptide from the C-terminal amino acid and the other products of reaction. The spots were cut out and measured as previously described and the percentage yield was calculated with the extinction coefficients of Table 1.

The extraction procedure described above is simpler than the three-stage process used by Ohno (1953) and Niu & Fraenkel-Conrat (1955) or the rather involved procedures recently described by Akabori *et al.* (1956). It has the additional advantage over the former that the bis-DNP-amino acids of tyrosine and cysteine, which are not completely recovered by their method [it involves extraction from ether-ethyl acetate (1:1, v/v) by NaHCO₃ soln.], are extracted quantitatively from ether-ethyl acetate (3:1, v/v) by buffer solution, pH 9.

RESULTS

Kinetic analysis

The equation for the reaction between a dipeptide and anhydrous hydrazine is given by reaction 1, Table 2. Since the concentration of the hydrazine does not alter during the reaction the rate of reaction is proportional to the concentration of dipeptide present. Integration of the simple first-order rate equation gives

$$k_u t = 2.303 \log c_0/c, \quad (1)$$

where c_0 is initial concentration of dipeptide and c is concentration of dipeptide at time t . k_u is calculated from the slope of the straight line obtained by plotting $\log c$ against time and the second-order rate constant k_u'' by the equation $k_u'' = k_u/[N_2H_4]$.

In the presence of hydrazine sulphate, which reacts with hydrazine according to the equation $N_2H_4 \cdot H_2SO_4 + N_2H_4 = 2N_2H_5^+ + SO_4^{2-}$ (Audrieth & Ogg, 1951), reactions 2 and 3 (Table 2) occur simultaneously. By using a large excess of acid catalyst over the amount of peptide present reaction 3 becomes first-order.

Table 2. Summary of hydrazinolysis reactions and their rate constants

Reaction no.	Equation	Rate constants	
		Pseudo-first-order	Second-order
Dipeptide			
1	$\text{H}_2\text{N}\cdot\text{CHR}_1\cdot\text{CO}\cdot\text{NH}\cdot\text{CHR}_2\cdot\text{CO}_2\text{H}^* + \text{N}_2\text{H}_4 = \text{H}_2\text{N}\cdot\text{CHR}_1\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + \text{H}_2\text{N}\cdot\text{CHR}_2\cdot\text{CO}_2\text{H}$	k_u	k_u
2	$\text{H}_3\text{N}^+\cdot\text{CHR}_1\cdot\text{CO}\cdot\text{NH}\cdot\text{CHR}_2\cdot\text{CO}_2\text{H} + \text{N}_2\text{H}_4 = \text{H}_3\text{N}^+\cdot\text{CHR}_1\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + \text{H}_3\text{N}^+\cdot\text{CHR}_2\cdot\text{CO}_2\text{H}$	k'_u	—
3	$\text{H}_3\text{N}^+\cdot\text{CHR}_1\cdot\text{CO}\cdot\text{NH}\cdot\text{CHR}_2\cdot\text{CO}_2\text{H} + \text{N}_2\text{H}_5^+ = \text{H}_3\text{N}^+\cdot\text{CHR}_1\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + \text{H}_3\text{N}^+\cdot\text{CHR}_2\cdot\text{CO}_2\text{H}$	k_c	k'_c
Diglycylglycine (g_3)			
4	$g_3 + \text{N}_2\text{H}_4 = \text{H}_2\text{N}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + g_2$	k_{1u}	—
5	$g_3 + \text{N}_2\text{H}_4 = \text{H}_2\text{N}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + g$	k_{2u}	—
6	$g_3 + \text{N}_2\text{H}_4 = \text{H}_2\text{N}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + g^\dagger$	k_{3u}	—

* This may exist as $\text{H}_3\text{N}^+\cdot\text{CHR}_1\cdot\text{CO}\cdot\text{NH}\cdot\text{CHR}_2\cdot\text{CO}_2^-$ in anhydrous hydrazine.

† The further reaction $\text{H}_2\text{N}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2 + \text{N}_2\text{H}_4 = 2\text{gNH}\cdot\text{NH}_2$ is of no interest here since it cannot be measured.

The subscripts u and c denote the uncatalysed and catalysed reactions respectively; g , glycine; g_2 , glycyglycine.

Table 3. Uncatalysed hydrazinolysis of glycyglycine and DL-leucylglycine at 60°

Time (hr.)	Glycine (%)	Dipeptide remaining (%)	Total	(Dipeptide %) corr., assuming total = 100 %	log (Dipeptide %) corr.
Glycylglycine					
0	0	100	100	100	2.000
0.167	22.9	91.0	113.9	$\frac{91.0 \times 100}{113.9} = 80.0$	1.903
0.50	40.2	67.0	107.2	62.7	1.797
1.50	56.0	39.7	95.7	41.5	1.618
4.00	99.0	4.8	103.8	4.6	0.670
DL-Leucylglycine					
0	0	100	100	100	2.000
16.25	33.3	60.4	93.7	64.5	1.810
24	33.6	39.3	72.9	54.0	1.732
30	48.1	22.2	70.3	31.6	1.501

These two reactions then satisfy the equation

$$-dc/dt = (k'_u + k_c) c, \quad (2)$$

where c is concentration of dipeptide at any time t . Integration of this first-order rate equation gives an equation similar to equation (1) in which $(k'_u + k_c)$ is calculated from the slope of a graph of $\log (\% \text{ dipeptide})$ against time. Since $k'_u \simeq k_u$ (see below), k_c is calculated by subtraction and k'_c by the equation $k'_c = k_c/[\text{N}_2\text{H}_5^+]$.

The kinetics of the uncatalysed hydrazinolysis of diglycylglycine are complicated by the fact that there are two peptide bonds and hence three possible reactions (reactions 4–6, Table 2) as compared with only one (reaction 1) for the dipeptide. The rate of removal of diglycylglycine ($-dg_3/dt$) is given by the equation

$$-dg_3/dt = (k_{1u} + k_{3u}) g_3,$$

where g_3 is concentration of diglycylglycine at any time t . The sum of the uncatalysed rate constants $(k_{1u} + k_{3u})$ can be obtained as described above. Similarly, one can write differential equations

describing the rate of formation of glycyglycine (g_2) and glycine (g) and these can be integrated to give more complicated equations relating k_{1u} , k_{2u} and k_{3u} with measurable quantities. However, it has not been possible to refine the experimental technique sufficiently to obtain reasonable values for the separate rate constants k_{1u} or k_{3u} . The rate constant k_{2u} is obtained from the uncatalysed hydrazinolysis of glycyglycine. The hydrazinolysis of diglycylglycine in the presence of hydrazine sulphate involves the three rate constants for the uncatalysed reactions k_{1u} , k_{2u} , k_{3u} and three more for the analogous catalysed reactions k_{1c} , k_{2c} , k_{3c} . By use of the same treatment as above one obtains the sum $(k_{1u} + k_{3u} + k_{1c} + k_{3c})$ from the rate of disappearance of diglycylglycine and, since $(k_{1u} + k_{3u})$ is known, $(k_{1c} + k_{3c})$ is obtained by subtraction.

Uncatalysed reaction

The detailed results for the hydrazinolysis of glycyglycine and DL-leucylglycine at 60° are given in Table 3. As the experimental error involved in

Table 4. Rate data for the uncatalysed hydrazinolysis of peptides

Peptide	% water in N_2H_4 (w/w)	$10^3 k''_u$ l.mole $^{-1}$ min. $^{-1}$ at the temperatures stated				E (kcal. mole $^{-1}$)	$-\Delta S^*$ cal. deg. $^{-1}$ mole $^{-1}$
		40°	60°	80°	100°		
Diglycylglycine	0.0	—	0.58 ^a	—	—	—	—
Glycylglycine	0.0	—	0.41	—	—	—	—
L-Lysylglycine dihydro- bromide	0.0	—	0.032	—	—	—	—
DL-Leucylglycine	0.0	0.0025	0.018	0.064	—	17.8	36
DL-Leucylglycine	5.0	—	0.018 ^b	—	—	—	—
DL-Leucylglycine	35.0	—	Approx. 0.0033 ^c	—	—	—	—
L-Leucyl-DL-valine	0.0	—	<0.0003	0.0014	0.0057	17.8	44

^a Value of $(k''_{1u} + k''_{3u})$.^b The amount of leucine was found to be 0.6 % of the total leucylglycine after separation on a buffered Celite column at pH 6.5 with ether as the mobile phase (Perrone, 1951).^c 1.4 % of leucine present. Fig. 1. Kinetics of uncatalysed hydrazinolysis at 60°. O, Leu.Gly; Δ , Lys.Gly; $\square$, Gly.Gly; $\blacktriangle$, Gly.Gly.-Gly. All were in 100 % N_2H_4 . $\bullet$, Leu.Gly in 95 % N_2H_4 . For explanation of the abbreviations used for amino acids in this and subsequent figure legends see *Biochem. J.* (1953), 55, 5. Fig. 2. Hydrazinolysis of diglycylglycine in anhydrous hydrazine at 60°. O, Gly; Δ , Gly.Gly; $\square$, Gly.Gly.-Gly.

the determination of the percentage of glycine or of unchanged dipeptide is about $\pm 10\%$ (Bradbury, 1956), it is clear that variations of about $\pm 20\%$ in the total are to be expected. In some cases the total yield falls below 80 %, owing to experimental losses which occur to some extent in the benzaldehyde treatment. These relatively large experimental errors make for inaccurate rate constants but the error can be reduced by the determination, in all cases, of the unchanged peptide and the amino acid produced by the reaction. In view of the known stability of these amino acids and peptides to the hydrazinolysis conditions it is reasonable to assume a 100 % total recovery and to correct the figure for the percentage peptide on this basis (see Table 3). The first-order rate constant is then determined from the gradient of a graph of $\log (\text{dipeptide } \%)_{\text{corr.}}$ against time (see Fig. 1). In Table 4 are recorded the second-order rate constants k''_u , calculated by the equation $k''_u = k_u / [N_2H_4]$, together with the energies (E) and entropies (S) of activation calculated from the values of k''_u at different temperatures (Laidler, 1950). The behaviour of diglycylglycine during hydrazinolysis is shown in Fig. 2.

Catalysed reaction

All experiments were made in the presence of a large excess of hydrazine sulphate, hence first-order rate constants ($k'_u + k_e$) were obtained in all cases. These are recorded in Table 5 for the hydrazinolysis of glycylglycine at three different concentrations of hydrazine sulphate. Assuming that $k'_u = k_u$, the value of k_e is calculated and also the second-order rate constant k''_e . The constancy of k''_e , within experimental error, confirms the validity of the assumption which is now used in the calculation of k''_u for the other peptides. These are summarized in Table 6, together with values of E and ΔS^* calculated from experiments at three

Table 5. *Effect of concentration of hydrazine sulphate on rate constants for glycylglycine at 60°*

$[\text{N}_2\text{H}_5^+]$	$10^3 k_u$ (min. ⁻¹)	$10^3 (k'_u + k_e)$ (min. ⁻¹)	$10^3 k_e$ (min. ⁻¹) assuming $k_u = k'_u$	$10^3 k'_e = 10^3 k_e/[\text{N}_2\text{H}_5^+]$ (l.mole ⁻¹ min. ⁻¹)
0	12.4	—	—	—
0.50	—	25.4	13.0	26.0
1.00	—	47.5	35.1	35.1
2.00	—	72.4	60.0	30.0

Mean 30.4 ± 2.6 Table 6. *Rate data for the catalysed hydrazinolysis of peptides*

Peptide	Water in N_2H_4 (%, w/w)	$10^3 k'_e$ (l.mole ⁻¹ min. ⁻¹)				E (kcal. mole ⁻¹)	$-\Delta S^*$ cal. deg. ⁻¹ mole ⁻¹
		40°	60°	80°	100°		
Diglycylglycine	0.0	—	37.3 ^a	—	—	—	—
Glycylglycine	0.0	—	30.4	—	—	—	—
L-Lysylglycine dihydrobromide	0.0	—	1.87	—	—	—	—
DL-Leucylglycine	0.0	0.38	1.45	4.9	—	13.5	40
DL-Leucylglycine	5.0	—	1.45 ^b	—	—	—	—
DL-Leucylglycine	35.0	—	Approx. 0.9 ^c	—	—	—	—
L-Leucyl-DL-valine	0.0	—	0.045	0.085	0.200	9.2	60

^a Value of $k'_{1e} + k'_{3e}$.^b Leucine content 0.8% of total leucylglycine.^c Leucine content 1.3%.Table 7. *Comparison of rate data for hydrazinolysis and hydrolysis*

Peptide	Hydrazinolysis at 60°			Hydrolysis in HCl at 99°*
	$10^3 k'_u$ (l.mole ⁻¹ min. ⁻¹)	$10^3 k'_e$ (l.mole ⁻¹ min. ⁻¹)	k'_e/k'_u	$10^3 k_2$ l.mole ⁻¹ min. ⁻¹)
Diglycylglycine	0.58†	37.3†	64	—
Glycylglycine	0.41	30.4	74	3.5
L-Lysylglycine dihydrobromide	0.0316	1.87	59	—
DL-Leucylglycine	0.0181	1.45	80	0.7
L-Leucyl-DL-valine	<0.0003	0.045	>150	—
L-Leucyl-D-leucine	—	—	—	0.2

* Harris, Cole & Pon (1956).

† $(k'_{1u} + k'_{3u})$.‡ $(k'_{1e} + k'_{3e})$. Fig. 3. Hydrazinolysis of diglycylglycine in anhydrous hydrazine containing $\text{m-N}_2\text{H}_4\cdot\text{H}_2\text{SO}_4$ at 60°. O, Gly; Δ , Gly.Gly; $\square$, Gly.Gly.Gly.

different temperatures. Fig. 3 shows the course of the catalysed hydrazinolysis of diglycylglycine at 60° in *m*-hydrazine sulphate.

DISCUSSION

From Table 7 it is seen that, for the peptides studied, $k'_e/k'_u < 59$, hence the overall rate of reaction at 60° in *m*-hydrazine sulphate ($[\text{N}_2\text{H}_5^+] = 2$) is up to five times as fast:

$$1 + (k'_e \cdot [\text{N}_2\text{H}_5^+]/k'_u \cdot [\text{N}_2\text{H}_4]) = 1 + (59 \times 2/30.4) \approx 5$$

as in the absence of catalyst. It is therefore advantageous to use an acid catalyst in determinations of the *C*-terminal amino acids of a peptide or protein by the hydrazine method in order to obtain the maximum amount of peptide bond breakage with the minimum decomposition of the *C*-terminal amino acid (Bradbury, 1956). Thus the treatment in anhydrous hydrazine containing

Table 8. *Summary of energy and entropy of activation data*

Peptide	E (kcal. mole ⁻¹)		$-\Delta S^*$ (cal. deg. ⁻¹ mole ⁻¹)	
	Catalysed	Uncatalysed	Catalysed	Uncatalysed
DL-Leucylglycine	13.5	17.8	40	36
L-Leucyl-DL-valine	9.2	17.8	60	44

m-hydrazine sulphate at 60° for 16 hr. leaves only 4% of DL-leucylglycine but over 90% of L-leucyl-DL-valine unreacted. Since DL-leucylglycine is a fairly stable peptide this treatment should be suitable for most proteins and is adequate for insulin and lysozyme (Bradbury, 1958). For a protein with a C-terminal sequence such as valyl-leucine mild treatment at 60° would liberate a large amount of a C-terminal peptide which could be identified. This would be preferable to treatment for 100 hr. at 100°, which would be necessary in order to liberate valine completely but would cause some decomposition of the C-terminal amino acid and also increase the amount of non-C-terminal amino acids produced by side reactions (Bradbury, 1958).

It is found that there is no increase of the amount of the non-C-terminal amino acid leucine from DL-leucylglycine when hydrazinolysis is carried out in the presence of 5% of water. Even when 35% of water is present the leucine content is only doubled, although the rates of both the catalysed and uncatalysed reactions are markedly decreased. These results are in good agreement with those obtained with insulin (Bradbury, 1958), and show that there is no need to take elaborate precautions to exclude water during hydrazinolysis. At least 5% of water can be tolerated [Niu & Fraenkel-Conrat (1955) state that up to 10% does not cause appearance of non-C-terminal amino acids] but hydrazine hydrate as used by Schlogl, Wessely & Wawersich (1954) should be avoided.

Lawrence & Moore (1951) found that in the acid hydrolysis of diglycylglycine the first peptide bond reacted about eight times as fast as in the corresponding hydrolysis of glycylglycine. This has been interpreted as due to stabilization of the peptide bond of glycylglycine owing to repulsion between the adjacent NH_3^+ group and the attacking H_3O^+ ion (Harris, Cole & Pon, 1956). Such an electrostatic effect cannot be operative in the acid-catalysed hydrazinolysis of glycylglycine since the sum of the rate constant ($k''_{1c} + k''_{3c}$) for diglycylglycine is less than twice k''_c for glycylglycine (the value expected if both peptide bonds in diglycylglycine had the same reactivity as the one in glycylglycine).

The prime importance of the steric effect in the hydrazinolysis reaction is shown by consideration of the results for energy and entropy of activation summarized in Table 8. The approximately 40-fold

decrease of the rate constant in going from DL-leucylglycine to L-leucyl-DL-valine (for either the catalysed or uncatalysed reaction) is reflected mainly in the decrease of the entropy of activation. This means that there is increased steric hindrance of reaction due to the introduction of the bulky isopropyl side chain of valine in place of the hydrogen atom of glycine. The same effect, on a greatly reduced scale, is noted in the hydrolysis of glycylglycine and glycyl-L-leucine, where the introduction of the bulky isobutyl side chain of leucine sterically hinders the reaction (Lawrence & Moore, 1951).

It is interesting to compare the relative rates of the acid-catalysed hydrazinolysis and hydrolysis reactions for various peptides. From Table 7 it is seen that in going from glycylglycine to DL-leucylglycine there is a 21-fold decrease of the rate constant of hydrazinolysis but only a fivefold decrease for hydrolysis. An even greater contrast is obtained between DL-leucylglycine and L-leucyl-DL-valine (L-leucyl-D-leucine in hydrolysis can be taken as similar to the latter) where there is a 32-fold decrease in rate constant of hydrazinolysis and only 3.5 for hydrolysis. This difference of behaviour is mainly due to steric factors, which are much more important in hydrazinolysis than in hydrolysis. This is only to be expected since a simple calculation shows that at 50° a water molecule occupies $30.3\text{\AA}^3$ compared with $54.2\text{\AA}^3$ for a molecule of hydrazine. The ionic volumes will be somewhat larger but it is clear that it is more difficult for the bulkier N_2H_5^+ group to approach the reactive centre than for the much smaller H_3O^+ ion.

The wide range of reactivity of various peptide bonds towards hydrazine, if reproduced in the protein molecule, may allow the selective fission of peptide bonds by careful control of the reaction conditions. A possible application would be an attempt to elucidate amino acid sequences of proteins by partial hydrazinolysis followed by separation and identification of the peptide hydrazides. One would expect a simpler mixture of peptide fragments than would be obtained after partial hydrolysis.

Another application of anhydrous hydrazine in protein chemistry concerns its possible use as a solvent. Rees & Singer (1956) found that it dissolved all the proteins tested at room temperature, but there was a continuous decrease with time of the specific viscosity of a sample of γ -globulin in

anhydrous hydrazine at 25°. This is probably due to reaction between the hydrazine and reactive peptide bonds in the protein as shown by the following calculation with glycylglycine. In this case $k_a = 12.4 \times 10^{-3} \text{ min.}^{-1}$ at 60° and, assuming $E = 17 \text{ kcal. mole}^{-1}$, it is found that, at 20°, 68 % of the glycylglycine would have reacted after 50 hr. whereas after the same time at 2° (the freezing point of hydrazine) only 15 % would have reacted. The reactivity towards hydrazine of this peptide bond in the protein is probably not very different from that in the dipeptide, hence it may be concluded that, in general, some degradation of proteins will occur even at 2° after prolonged contact with anhydrous hydrazine.

SUMMARY

1. The kinetics of the uncatalysed and acid-catalysed hydrazinolysis of five simple peptides was studied by an experimental technique involving hydrazinolysis, treatment with benzaldehyde, dinitrophenylation and paper chromatography.

2. First-order rate constants were obtained for the uncatalysed reaction and also for the catalysed hydrazinolysis in the presence of a large excess of catalyst. In *m*-hydrazine sulphate at 60° the reaction is about five times as fast as in anhydrous hydrazine.

3. On the basis of the rate constants it is possible to make recommendations about the use of the hydrazine method for the determination of the *C*-terminal amino acids of proteins.

4. The energies and entropies of activation calculated for two peptides show that the approximately 1000-fold range of rate constants encountered in the catalysed hydrazinolysis at 60° is due largely to steric effects. This may allow selective hydrazinolysis of peptide bonds in proteins.

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REFERENCES

- Akabori, S., Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Haruna, I., Tsugita, A., Sugae, K. & Matsushima, T. (1956). *Bull. chem. Soc. Japan*, **29**, 507.
- Akabori, S., Ohno, K. & Narita, K. (1952). *Bull. chem. Soc. Japan*, **25**, 214.
- Audrieth, L. F. & Ogg, B. K. (1951). *The Chemistry of Hydrazine*, p. 201. New York: John Wiley and Sons, Inc.
- Bradbury, J. H. (1956). *Nature, Lond.*, **178**, 912.
- Bradbury, J. H. (1958). *Biochem. J.* **68**, 482.
- Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). In *Methods of Biochemical Analysis*, vol. 2, p. 360. Ed. by Glick, D. New York: Interscience Publishers Inc.
- Harris, J. I., Cole, R. D. & Pon, N. G. (1956). *Biochem. J.* **62**, 154.
- Kay, L. M., Schroeder, W. A., Munger, N. & Burt, N. (1956). *J. Amer. chem. Soc.* **78**, 2430.
- Kroner, T. D., Tabroff, W. & McGarr, J. J. (1955). *J. Amer. chem. Soc.* **77**, 3356.
- Laidler, K. J. (1950). *Chemical Kinetics*, p. 76. New York: McGraw Hill.
- Lawrence, L. & Moore, W. J. (1951). *J. Amer. chem. Soc.* **73**, 3973.
- Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
- Locker, R. H. (1954). *Biochim. biophys. Acta*, **14**, 533.
- Niu, C. & Fraenkel-Conrat, H. (1955). *J. Amer. chem. Soc.* **77**, 5882.
- Ohno, K. (1953). *J. Biochem., Tokyo*, **40**, 621.
- Ohno, K. (1955). *J. Biochem., Tokyo*, **42**, 615.
- Penneman, R. A. & Audrieth, L. F. (1948). *Analyt. Chem.* **20**, 1058.
- Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
- Rees, E. D. & Singer, S. J. (1956). *Arch. Biochem. Biophys.* **63**, 144.
- Sanger, F. (1945). *Biochem. J.* **39**, 507.
- Schlögl, K., Wessely, F. & Wawersich, E. (1954). *Mh. Chem.* **85**, 957.

The Hydrazinolysis of Insulin, Lysozyme, Wool Proteins and Wool

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Quantitative studies of the hydrazinolysis of proteins have been made by Ohno (1953, 1954, 1955), Locker (1954) and Niu & Fraenkel-Conrat (1955). Ohno (1953) reported yields of up to 68 % of leucine from lysozyme which corrected to about 100 % after allowance for decomposition of leucine during hydrazinolysis. Locker (1954) obtained only about 25 % yields of isoleucine and phenylalanine from tropomyosin and actin respectively,

and found that some amino acids were completely decomposed by the hydrazine treatment whereas others could be only partially recovered. Niu & Fraenkel-Conrat (1955) confirmed this observation and, in their work on a number of proteins, corrected for the amount of *C*-terminal amino acid decomposed by the hydrazine (usually more than half). They found that the recovery of amino acids from amino acid plus protein was not appreciably