3. Phosphorylcholine, phosphorylethanolamine and glycerylphosphorylcholine have been identified and estimated in the acid-resistant fraction of extracts from pregnant and post-partum rabbit uteri. Glycerylphosphorylethanolamine was found in the pregnant-rabbit uterus.

I should like to thank Dr D. M. Needham, F.R.S., for suggesting this problem and for her guidance and encouragement; Dr R. M. C. Dawson for his helpful advice; and Dr R. van Heyningen and Dr A. Pirie for kindly allowing me to read their paper in manuscript. This work has been supported by grants to Dr D. M. Needham from the Medical Research Council and the Agricultural Research Council.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Appleton, H. D., La Du, B. N., Levy, B. B., Steele, J. M. & Brodie, B. B. (1953). *J. biol. Chem.* **205**, 803.
 Artom, C. (1945). *J. biol. Chem.* **157**, 585.
 Awapara, J., Landua, A. J. & Fuerst, R. (1950). *J. biol. Chem.* **183**, 545.
 Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
 Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
 Dawson, R. M. C. (1955a). *Biochem. J.* **59**, 5.
 Dawson, R. M. C. (1955b). *Biochem. J.* **60**, 325.
 Dawson, R. M. C. (1955c). In *Biochemical Problems of Lipids*, p. 280. Ed. by Popják, G. & le Breton, E. London: Butterworths Scientific Publications.
 Dawson, R. M. C. (1957). *Biol. Rev.* **32**, 188.
 Engel, R. W., Salmon, W. D. & Ackermann, C. J. (1954). In *Methods of Biochemical Analysis*, vol. 1, p. 265. Ed. by Glick, D. New York: Interscience Publishers Inc.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Hollmann, S. (1949). *Hoppe-Seyl. Z.* **284**, 89.
 Kennedy, E. P. & Weiss, S. B. (1955). *J. Amer. chem. Soc.* **77**, 250.
 Kornberg, A. & Pricer, W. E. (1952). *Fed. Proc.* **11**, 242.
 LePage, G. A. (1945). In *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, p. 167. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
 Menkes, J. H. & Csapo, A. (1952). *Endocrinology*, **50**, 37.
 Needham, D. M. & Cawkwell, J. M. (1956). *Biochem. J.* **63**, 337.
 Outhouse, E. L. (1936). *Biochem. J.* **30**, 197.
 Outhouse, E. L. (1937). *Biochem. J.* **31**, 1459.
 Purkayastha, R. & Roy, S. C. (1956). *Ann. Biochem.* **16**, 97.
 Schmidt, G., Hershman, B. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 523.
 Slater, E. C. (1953). *Biochem. J.* **53**, 157.
 van Heyningen, R. & Pirie, A. (1958). *Biochem. J.* **68**, 18.
 Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.

The Reaction of Peptides and Insulin with Methylhydrazine

By J. H. BRADBURY

Wool Textile Research Laboratories, C.S.I.R.O., Geelong, Australia

(Received 10 March 1958)

The hydrazine method for the determination of the C-terminal amino acids of proteins (Akabori, Ohno & Narita, 1952; Ohno, 1953) has been modified by Bradbury (1956) to give improved recoveries of most of the amino acids. Subsequent kinetic studies showed that the increased selectivity of fission of peptide bonds possible in hydrazinolysis, as against acid hydrolysis, is due to the greater importance of steric factors in hydrazinolysis (Bradbury, 1958a). In an attempt to increase still further this selectivity the larger molecule methylhydrazine has been reacted with glycylglycine and DL-leucylglycine. The possibilities of the use of methylhydrazine in the determination of the C-terminal amino acids of proteins and as a protein solvent have been examined.

EXPERIMENTAL AND RESULTS

Materials

Glycylglycine, DL-leucylglycine and trypsin (Light and Co., Colnbrook, Bucks), insulin (Boots Pure Drug Co., Ltd., Nottingham) and lysozyme (Armour and Co., Chicago) were used without further purification. Merino 64's wool was purified as described previously (Bradbury, 1958b). Anhydrous hydrazine was obtained from the hydrate by drying over CaO and distilling (Bradbury, 1958a).

Methylhydrazine sulphate (Fisons Chemicals, Wigmore Street, London, W. 1) and a 100% molar excess of solid A.R. NaOH were placed in a flask fitted with a reflux condenser. Sufficient water was added to dissolve the NaOH and the flask cooled to slow the vigorous reaction. The mixture was then distilled under reduced pressure, two fractions being collected, the first of which was rich in

methylhydrazine. This was dried by the addition of freshly ignited CaO, heating at 100° for 3 hr. and then distilling under reduced pressure (10^{-4} mm. Hg) at room temperature. A second drying over CaO gave a product containing 93.5% of methylhydrazine as measured by titration with iodate (Penneman & Audrieth, 1948). This product was used without further purification because it was found previously that 95% hydrazine gave the same rate constants as the anhydrous materials (Bradbury, 1958a).

Ethylhydrazine sulphate (Fisons Chemicals, Wigmore Street, London, W. 1) had an apparent purity of 103.5% on titration with iodate. On conversion into ethylhydrazine by the above method an apparent purity of 104.2% was obtained. The product was used without further purification, as it seemed likely that these high results were due to the presence of a small amount of a substituted hydrazine of lower equivalent weight than that of ethylhydrazine.

Procedure and results of rate measurements

The experimental procedure for the determination of the rate of the uncatalysed and acid-catalysed reaction of methylhydrazine with glycylglycine and DL-leucylglycine was the same as that used previously (Bradbury, 1958a). The rate of reaction of each dipeptide with methylhydrazine was considerably slower than with hydrazine, necessitating much longer times of reaction at 60° (up to 3 days). In spite of this the recoveries of amino acid and unchanged peptide were comparable with those obtained with hydrazine (Bradbury, 1958a). First-order rate constants were obtained graphically for the uncatalysed and the sum of the catalysed and uncatalysed reactions. They were used to calculate the second-order rate constants for the uncatalysed reactions, k_u'' , and catalysed reactions, k_c' , as before (Bradbury, 1958a). The kinetic scheme proposed in that paper may be incorrect because of the possible similarity of the mechanisms of acid hydrolysis (Martin, 1957) and acid-catalysed hydrazinolysis of peptides; but until this point can be clarified it is valid to continue to assume the present mechanism, provided that no conclusions are based thereon. The rate constants for the reaction of the two dipeptides with methylhydrazine are given in Table 1.

Two experiments made with DL-leucylglycine at 80° and 100° indicate that the temperature-dependence of the rate constants is of the same order as that observed with hydrazine. Treatment of the two peptides with ethylhydrazine gave very low yields of amino acid and unchanged peptide. The reason for this is obscure.

Treatment of insulin with methylhydrazine containing hydrazine sulphate

The method used was that already described for the determination of the C-terminal amino acids of proteins by hydrazinolysis (Bradbury, 1958b). Insulin was treated with methylhydrazine containing m-hydrazine sulphate (used instead of methylhydrazine sulphate to increase the rate of the reaction) at 60° and 100° for 16 hr. The yields of the C-terminal amino acid alanine were 1.7 and 5.4% respectively of the theoretical amount, as compared with 36% (uncorrected) for hydrazine. Also present were the non-C-terminal amino acids glycine, serine and glutamic acid in comparable amounts with that obtained previously with hydrazine (see Table 1, Bradbury, 1958b).

Hydrazine and methylhydrazine as solvents for proteins

Samples of insulin, trypsin, lysozyme and wool (2 mg. each) were placed in tubes on a vacuum line and 0.2 ml. of anhydrous hydrazine or methylhydrazine was distilled into each. The tubes were sealed off and shaken at 25°, when it was found that both insulin and trypsin dissolved rapidly in both hydrazine and methylhydrazine, producing 1% solutions, but lysozyme and wool were not dissolved by either solvent even after prolonged shaking.

DISCUSSION

The range of reactivities of different peptide bonds towards hydrazine is much greater than towards aqueous hydrochloric acid, owing largely to the operation of steric factors (Bradbury, 1958a). On the basis of the so-called intuitive approach to steric hindrance of reactions it would be expected that a further increase of the size of the attacking molecule, in this case the replacement of hydrazine by methylhydrazine, would cause further enhancement of this effect. However, Ingold (1957) quotes several cases of the failure of this intuitive method.

In Table 2 the rate constants are summarized for the reaction of glycylglycine and DL-leucylglycine with hydrazine, methylhydrazine and aqueous hydrochloric acid. From previous work with a wider range of peptides (Bradbury, 1958a) it is

Table 1. Rate constants for the uncatalysed and catalysed reactions of methylhydrazine with glycylglycine and DL-leucylglycine at 60°

The catalysed rate constant was determined from measurements in the presence of m-methylhydrazine sulphate. The density of methylhydrazine used in the calculation of $[\text{CH}_2\text{:NH}\cdot\text{NH}_2]$ was taken as 0.90 at 60°; an experiment at 20° gave 0.934. $k_u'' = k_u/[\text{CH}_2\text{:NH}\cdot\text{NH}_2]$; $k_c' = k_c/2$; in evaluating k_c' it is assumed that $k_u' = k_u$. For further details of symbols, see Bradbury (1958a).

Peptide	Uncatalysed rate constant	Catalysed rate constant	$10^5 k_u''$ (l.mole ⁻¹ min. ⁻¹)	$10^5 k_c'$ (min. ⁻¹)	$10^5 k_c''$ (l.mole ⁻¹ min. ⁻¹)
	$10^5 k_u$ (min. ⁻¹)	$10^5 (k_u' + k_c')$ (min. ⁻¹)			
Glycylglycine	41.6	99	2.12	57.4	28.7
DL-Leucylglycine	2.20	6.6	0.112	4.40	2.20

Table 2. Comparison of second-order rate constants for the reaction of glycylglycine and DL-leucylglycine with hydrazine, methylhydrazine and aqueous hydrochloric acid

The rate constants for the reactions with hydrazine (see Table 7, Bradbury, 1958*a*) and methylhydrazine were obtained at 60°. Harris, Cole & Pon (1956) determined the hydrolytic rate constants in HCl at 99°.

Peptide	Uncatalysed rate constants 10 ⁵ <i>k</i> " (l.mole ⁻¹ min. ⁻¹) in		Catalysed rate constants 10 ⁵ <i>k</i> " (l.mole ⁻¹ min. ⁻¹) in		Hydrolytic rate constants 10 ⁵ <i>k</i> " (l.mole ⁻¹ min. ⁻¹)
	Hydrazine	Methylhydrazine	Hydrazine	Methylhydrazine	
Glycylglycine	41	2.12	3040	28.7	3.5
DL-Leucylglycine	1.81	0.112	145	2.20	0.7

reasonable to suppose that the ratio *k*"(Gly.Gly)/*k*"(Leu.Gly) gives an approximate estimate of the relative selectivities of the reagents towards the splitting of peptide bonds. A simple calculation from the results in Table 2 gives a value of the ratio of 5 for aqueous hydrochloric acid and average values of 16 for methylhydrazine and 22 for hydrazine. Therefore the selectivity of the reagents towards peptide bonds is in the order aqueous acid < methylhydrazine < hydrazine.

The lower reactivity of methylhydrazine as compared with hydrazine towards peptide bonds precludes its use as a reagent for the determination of the C-terminal amino acids of proteins, as shown by the failure of the two experiments with insulin. However, it may be useful as a solvent for proteins, since it appears to combine the good solvent properties of hydrazine (as shown by the solubility tests with four proteins) with decreased reactivity towards peptide bonds. For physical measurements on proteins it may be possible to use methylhydrazine in place of hydrazine in order to avoid the degradation which accompanies the use of the latter (Rees & Singer, 1956; Bradbury, 1958*a*).

SUMMARY

1. The kinetics of the uncatalysed and acid-catalysed reaction of glycylglycine and DL-leucyl-

glycine with methylhydrazine were examined by techniques recorded in a previous paper (Bradbury, 1958*a*).

2. Contrary to expectations based on an intuitive approach to steric effects it has been found that methylhydrazine is no more selective in its fission of peptide bonds than is hydrazine.

3. The slow fission of peptide bonds by methylhydrazine, as compared with hydrazine, precludes its use for the determination of the C-terminal amino acids of proteins, but is advantageous in its application as a protein solvent.

It is a pleasure to thank Mr H. Groll for assistance with the experimental work.

REFERENCES

- Akabori, S., Ohno, K. & Narita, K. (1952). *Bull. chem. Soc. Japan*, **25**, 214.
 Bradbury, J. H. (1956). *Nature, Lond.*, **178**, 912.
 Bradbury, J. H. (1958*a*). *Biochem. J.* **68**, 475.
 Bradbury, J. H. (1958*b*). *Biochem. J.* **68**, 482.
 Harris, J. I., Cole, R. D. & Pon, N. G. (1956). *Biochem. J.* **62**, 154.
 Ingold, C. K. (1957). *Quart. Rev. chem. Soc., Lond.*, **11**, 1.
 Martin, R. J. L. (1957). *Aust. J. Chem.* **10**, 256.
 Ohno, K. (1953). *J. Biochem., Tokyo*, **40**, 621.
 Penneman, R. A. & Audrieth, L. F. (1948). *Analyt. Chem.* **20**, 1058.
 Rees, E. D. & Singer, S. J. (1956). *Arch. Biochem. Biophys.* **63**, 144.

Fractionation of Proteins of the Microsomes of Rat Liver by Means of a Non-Ionic Detergent

By P. COHN' AND J. A. V. BUTLER

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3

(Received 27 February 1958)

Labelled amino acids have been shown to be rapidly incorporated into the proteins of the microsome fraction and particularly into those which are closely associated with ribonucleic acid (Allfrey, Daly & Mirsky, 1953; Hultin, 1955; Littlefield, Keller, Gross & Zamecnik, 1955; Simkin & Work, 1957). Little is known of the nature of the micro-

somal proteins into which this incorporation occurs. Littlefield *et al.* (1955) found that during short periods after the administration of a radioactive amino acid to a rat, proteins of high specific radioactivity remained insoluble after treatment of the microsomes with sodium deoxycholate. Hultin (1955) and Simkin & Work (1957) obtained micro-