NOTES

The Solubility of Ethyl Acetate in Water

By A. P. Altshuller[1] and H. E. Everson

Received September 15, 1952

In view of some uncertainties in the literature[2-6] it was found necessary to redetermine the solubility of ethyl acetate at various temperatures for an investigation which was in progress.

The experimental results are given in Table I where t is the turbidity temperature in °C. and s is the solubility in grams of ethyl acetate per 100 g. of water.

Table I

t	s	t	s	t	s
19.2	8.34	25.9	7.93	31.2	7.60
20.4	8.31	26.0	7.93	31.8	7.58
20.5	8.29	26.3	7.94	31.9	7.58
21.3	8.26	27.0	7.89	31.9	7.57
22.0	8.22	28.0	7.80	32.0	7.51
22.6	8.14	28.1	7.81	32.8	7.49
22.7	8.12	28.4	7.82	33.0	7.46
23.0	8.11	28.7	7.81	33.8	7.40
23.4	8.10	29.7	7.71	34.0	7.38
24.7	8.05	29.9	7.71	36.8	7.32
25.1	8.03	30.0	7.72	37.8	7.25
25.4	8.02	30.0	7.69	39.9	7.15
25.4	8.03	30.1	7.69	39.9	7.15
25.4	8.00				

By the application of the method of least squares the following equation is calculated

$$s = (9.552 \pm 0.018) - (0.0618 \pm 0.0006)t$$

The results of this investigation are compared with those previously given in the literature in Table II.

Table II

Comparison of the Solubility Results for Ethyl Acetate in Water

Investigator	20°	25°	30°	35°	40°	45°	50°
This invest.	8.32	8.01	7.70	7.39	7.08	..	..
Schles. and Kub.[3]	8.42	8.03	7.69	7.41	7.18	7.00	6.88
Merriman[4]	8.53	8.08	7.70	7.38	7.10	..	..
Seidel[5]	9.02	8.58	8.24	7.98	7.72	7.53	7.31
Gl. and P.[6]	..	7.39	..	..	..	..	6.04
B. and Gl.[7]	8.40	..	..	..	6.97	..	..

While the earlier results of Glasstone and Pound[5] are far lower than the other results, the more recent values of Beech and Glasstone,[6] determined by a different method, are in better agreement although the value given at 40° is still somewhat low. The high results of Seidel[4] may be caused by appreciable amounts of water and alcohol being present in the ethyl acetate thus increasing the solubility above the correct value.

Consideration of the data above leads to the following average values for the solubility of ethyl acetate (in g. of ethyl acetate per 100 g. of water) as reliable: 20°, 8.42; 25°, 8.04; 30°, 7.70; 35°, 7.39; 40°, 7.12.

Experimental

The solubilities were determined by the synthetic, turbidimetric method.[7] The solubilities of ethyl acetate agree within ±0.1° when determined by heating and then cooling. The appearance of turbidity may be detected within about 0.2° range usually. Temperatures were determined with a $^1/_{20}$° thermometer which gave agreement within $^1/_{100}$° with the sodium sulfate transition point. Each solubility was redetermined two or three times.

The ethyl acetate employed was Mallinckrodt analytical reagent grade ethyl acetate. The percentage of water as determined by Karl Fischer reagent[8] was 0.06%. The ethyl acetate was redistilled from a column of 24 theoretical plates. No change in refractive index ($n^{24.3}$D 1.3697) was found.

(1) National Advisory Commitee for Aeronautics, Lewis Flight Propulsion Laboratory, Cleveland, Ohio.

(2) N. Schlesinger and W. Kubasowa, *Z. physik. Chem.*, **142**, 25 (1929).

(3) R. W. Merriman, *J. Chem. Soc.*, **103**, 1774 (1913).

(4) Landolt-Börnstein, "Physikalisch-chemische Tabellen," 5 Aufl., Bd. I, s. 752 (1923).

(5) S. Glasstone and A. Pound, *J. Chem. Soc.*, **127**, 2660 (1925).

(6) D. Beech and S. Glasstone, *ibid.*, 67 (1938).

(7) A. Weissburger, "Physical Methods of Organic Chemistry," Vol. I, Part I, Interscience Publishers, Inc., New York, N. Y., 1939, p. 319.

(8) K. Fischer, *Angew. Chem.*, **48**, 394 (1935).

Department of Chemistry
University of Cincinnati
Cincinnati, Ohio

Determination of the Terminal Carboxyl Residues of Peptides and of Proteins[1]

By Victor H. Baptist[2] and Henry B. Bull[2]

Received November 28, 1952

Recently, Fromageot, *et al.*,[3] and Chibnall and Rees[4] have described the reduction of the terminal carboxyl groups of insulin with subsequent identification of the resulting amino alcohols generated by the hydrolysis of the reduced protein. The present note reports a modification similar to that which Waley and Watson[5] have applied to insulin, of the method originally presented by Schlack and Kumpf[6] for the identification of the terminal carboxyl residues of peptides.

In outline, this method involves the creation of the thiohydantoin on the carboxyl end of the peptide chain in an acid medium, the amino end being blocked by acetylation. The thiohydantoin is then hydrolyzed from the peptide in an acid medium and isolated. The purified thiohydantoin is then hydrolyzed in an alkaline medium, thus producing the corresponding amino acid which is then identified and estimated.

(1) From a thesis submitted by V. H. Baptist to the Graduate School of Northwestern University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1952.

(2) Biochemistry Department, State University of Iowa, Iowa City, Iowa.

(3) C. Fromageot, M. Jutisz, D. Meyer and L. Penasse, *Biochim. Biophys. Acta*, **6**, 283 (1950).

(4) A. C. Chibnall and M. W. Rees, *Biochem. J.*, **48**, xlvii (1951).

(5) S. G. Waley and J. Watson, *J. Chem. Soc.*, 2394 (1951).

(6) P. Schlack and W. Kumpf, *Z. physiol. Chem.*, **154**, 125 (1926).

Experimental

The general procedure for the production of the thiohydantoins and the final identification of the amino acids was as follows: Approximately 0.1 millimole of the synthetic peptide was treated with 1 to 1.5 millimoles of anhydrous, powdered ammonium thiocyanate, dissolved in 2 ml. of solvent consisting of 9 parts of acetic anhydride and 1 part of glacial acetic acid by volume and the reaction mixture maintained between 40 and 45° for 4 hours with stirring. Two ml. of 20% hydrochloric acid was then added dropwise with good agitation. After the hydrochloric acid had been added, the mixture was heated on a steam-bath for one hour and then taken to dryness under vacuum. Water was added and the mixture again taken to dryness. The dry mixture was dissolved in 10 ml. of 0.25 molar phosphate buffer at *p*H 6.5 and the solution extracted three times each with 10 ml. of ethyl acetate. The ethyl acetate extract was taken to dryness and the dry mixture hydrolyzed with 2.5 ml. of 1.25 *N* barium hydroxide at 140° in a sealed tube for 5 hours. The cooled hydrolysate was neutralized with carbon dioxide and the resulting solution heated for 10 minutes on a steam-bath to destroy the carbamic acids which may have formed. The resulting solution was made up to an appropriate volume and a quantitative estimate of the amino acids present made with filter paper chromatography as described by Bull, Hahn and Baptist.[7]

It was necessary to check the effectiveness of the various steps in the above procedure. For example, we must know the extent of hydrolysis of the thiohydantoin by the hydrochloric acid used to split the thiohydantoin from the peptide. To this end, 15 mg. of 5-methyl-2-thiohydantoin was hydrolyzed at 100° in a sealed tube with 2 ml. of 10% hydrochloric acid for different lengths of time. Even after 5 hours hydrolysis, the extent of conversion of this thiohydantoin to alanine amounted to less than 2%.

It was also necessary to test the completeness of the extraction of the thiohydantoin from the phosphate buffer with ethyl acetate. Thiohydantoins of alanine, leucine, methionine, phenylalanine, glycine, tyrosine and valine were prepared by the method of Johnson and Nicolet.[8] The partition coefficient between ethyl acetate and phosphate buffer ranged from 0.49 for 2-thiohydantoin to 1.0 for the thiohydantoin of phenylalanine.

It was found that lysine, arginine, alanine, leucine, cysteine, serine, glycine, methionine, phenylalanine, aspartic acid, glutamic acid, threonine, tyrosine and valine were not extracted in any detectable amounts from phosphate buffer by ethyl acetate.

TABLE I

OVER-ALL RECOVERIES OF AMINO ACIDS FROM PURE AMINO ACIDS AND FROM SYNTHETIC PEPTIDES

Starting material	Amino acid recovered	Recovery, %
Alanine	Alanine	63
Serine	Serine	12[a]
Threonine	Threonine	16[b]
Methionine	Methionine	48
Lysine	Lysine	0
Arginine	Arginine	0
Glutamine	Glutamic	0
Glutathione	Glutamic	0
Glutathione	Glycine	7
Leucylglycylglycine	Glycine	28
Glycylglycylalanine	Alanine	50
Glycyltyrosine	Tyrosine	33
Glycylleucine	Leucine	57
Dicarbobenzoxycystinylvaline	Valine	41
Glycylvaline	Valine	61
Glycylphenylalanine	Phenylalanine	39
Diphenylacetyllysine	Lysine	54
Benzoxyarginine	Arginine	>1

[a] Recovered as alanine. [b] Recovered as glycine (8%) and as α-amino-*n*-butyric acid (8%).

(7) H. B. Bull, J. W. Hahn and V. H. Baptist, THIS JOURNAL, **71**, 550 (1949).

(8) T. B. Johnson and B. H. Nicolet, *ibid.*, **33**, 1973 (1911).

Finally, the hydrolysis of 5-methyl-2-thiohydantoin with 1.25 *N* barium hydroxide at 140° for 5 hours produced a 75% conversion of this thiohydantoin to alanine indicating the effectiveness of the hydrolysis of the thiohydantoins by barium hydroxide.

The over-all recoveries of the terminal carboxyl residues as amino acids from synthetic peptides and from pure amino acids as starting materials are shown in Table I. In no case were other than carboxyl terminal amino acids from peptides detected.

The above method for the terminal carboxyl residues was applied to three purified proteins. 0.126 g. of crystalline pork insulin (Armour) was dissolved in 10 ml. of acetic anhydride–acetic acid–ammonium thiocyanate mixture and the reaction allowed to proceed overnight at room temperature and the reaction mixture treated as described above for synthetic peptides. 0.134 g. of beef insulin (Armour) was treated in the same way as for pork insulin. One gram of lyophilized crystalline egg albumin reacted with 0.3 g. of ammonium thiocyanate in 77 ml. of the acetic acid–acetic anhydride solvent. The amounts of the recovered amino acids were multiplied by recovery factors. The recovery factors were arrived at by adding a known amount of a given amino acid to a second sample of the protein previous to treatment and determining the percentage of the added amino acid recovered. The recovery factors together with the corrected amounts of the amino acids as terminal α-carboxyl residues are shown in Table II.

TABLE II

CORRECTED MICROMOLES OF AMINO ACIDS AS TERMINAL CARBOXYL GROUPS PER GRAM OF PROTEIN

Amino acid	Correction factor	Pork insulin	Beef insulin	Egg albumin
Alanine	2.7	77	59	..
Tyrosine	7.3	129	118	..
Phenylalanine	2.2	149	258	..
Alanine	5.0	...	...	17.7
Valine	3.6	...	...	21.4
Leucine	3.8	...	...	65.4

Discussion

As can be seen from Table I, the thiohydantoin method for the detection and estimation of the free α-carboxyl residues of peptides and of proteins is not a complete method. The thiohydantoin method fails to detect free α-carboxyl residues of glutamic acid, of aspartic acid, of lysine and of arginine. Although a 54% recovery of lysine was obtained using diphenylacetyllysine, it is believed that this high recovery resulted from the incomplete hydrolysis of the diphenylacetyllysine to lysine and thus the diphenylacetyl or monophenylacetyl lysine was extracted and subsequently hydrolyzed to lysine in the last step. The method as employed also leads to another ambiguity, because as pointed out by Bremner[9] and confirmed by us, serine is converted to alanine and threonine into glycine and into α-amino-*n*-butyric acid during alkaline hydrolysis. The destruction of threonine is not so serious, because α-amino-*n*-butyric acid does not ordinarily occur in proteins and, accordingly, this amino acid can be identified and assumed to arise from threonine.

It will be noted that the results shown in Table II are not in complete agreement with those of Fromageot, *et al.*,[3] who reported two alanine and two glycine residues for the free α-carboxyl residues per 12,000 g. of beef insulin, Chibnall and Rees[4] who reported two alanine and one glycine and one unknown; Harris[10] with the use of carboxypepti-

(9) J. Bremner, *Nature*, **168**, 518 (1951).

(10) J. I. Harris, THIS JOURNAL, **74**, 2944 (1952).

dase reported alanine for the B-fraction and asparagine for the A-fraction.

Our results agree with the results of Sanger,[11] Waley and Watson,[5] and all of the above-named authors that alanine does occur as a terminal α-carboxyl residue. It is true that insulin also yielded small amounts of terminal glycine residues for us, but the amounts were too little to be estimated by our technique. It has been shown above that if aspartic acid occurred as a free carboxyl terminal residue it could not be detected by the present modification of the 2-thiohydantoin method.

The method as modified shows that the terminal α-carboxyl residues of both pork and beef insulin apparently arise principally from alanine, tyrosine and phenylalanine. It is conceivable, however, that the tyrosine and phenylalanine residue could have arisen from impurities common to the three samples of crystalline insulin available to us.

Acknowledgments.—It is a pleasure to acknowledge, with thanks, the gifts of synthetic peptides by Drs. J. P. Greenstein, L. C. King, H. Neurath, E. L. Smith and C. Wachtl. One of us (V. H. Baptist) was supported on a fellowship from a grant-in-aid from Swift and Company of Chicago, Illinois.

(11) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

BIOCHEMISTRY DEPARTMENT
NORTHWESTERN UNIVERSITY MEDICAL SCHOOL
CHICAGO, ILLINOIS

The Base-Catalyzed Decomposition of N-Nitroso-N-cyclopentylurethan[1]

BY FREDERICK W. BOLLINGER,[2] F. N. HAYES AND SAMUEL SIEGEL

RECEIVED NOVEMBER 13, 1952

A previous paper[3] has described the base-catalyzed decomposition of N-nitroso-N-cyclohexylurethan. In continuation of this work this paper reports the base-catalyzed decomposition of N-nitroso-N-cyclopentylurethan. N-Cyclopentylurethan, prepared from cyclopentylamine and ethyl chloroformate, was treated with excess nitrous acid to yield N-nitroso-N-cyclopentylurethan. This product was allowed to decompose in methanol which was in contact with a catalytic amount of potassium carbonate.

Nitrogen, carbon dioxide and methyl nitrite were evolved in the course of the reaction. Cyclopentene, ethanol, methyl ethyl carbonate, cyclopentanol, ethyl cyclopentyl carbonate and N-cyclopentylurethan were obtained by careful fractional distillation. These decomposition products are analogous to those obtained from the base-catalyzed decomposition of N-nitroso-N-cyclohexylurethan.

The formation of these compounds from N-nitroso-N-cyclopentylurethan can be rationalized in terms of a scheme already proposed for the decomposition of N-nitroso-N-cyclohexylurethan: (1) the solvolysis of N-nitroso-N-cyclopentylurethan by methanol or water (formed in the reaction) and (2) a displacement of cyclopentyldiazotate ion by methoxide or hydroxide ion. The five-membered carbon ring skeleton does not undergo contraction in this decomposition. This is in agreement with the work of Hückel, *et al.*,[4] who on diazotization of cyclopentylamine in aqueous solution observed that cyclopentanol and cyclopentene were obtained. The dearth of derivatives of cyclopentylamine in the chemical literature has prompted us to place several on record.

Experimental[5]

N-Cyclopentylurethan.—Following the method of Hartman and Brethen[6] for alkylurethans this compound was obtained in 83% yield, m.p. 7.0–8.5°, b.p. 125° (20 mm.), $n^{25}D$ 1.4605, d^{25}_4 1.0232.

Anal. Calcd. for $C_8H_{15}O_2N$: N, 8.91. Found: N, 9.12.

N-Nitroso-N-cyclopentylurethan.—Following the method of Bollinger, Hayes and Siegel[3] this compound was obtained in 98% yield, an orange oil, $n^{25}D$ 1.4656, d^{25}_4 1.1016. Unlike N-nitroso-N-methylurethan N-nitroso-N-cyclopentylurethan is not a skin irritant.

Anal. Calcd. for $C_8H_{14}O_3N_2$: N, 15.36. Found: N, 15.67.

Decomposition of N-Nitroso-N-cyclopentylurethan.—The decomposition of N-nitroso-N-cyclopentylurethan and the identification of gaseous and solid products were carried out by methods previously described.[3]

By a careful fractional distillation, cyclopentene, ethanol, methyl ethyl carbonate, cyclopentanol, ethyl cyclopentyl carbonate and N-cyclopentylurethan were obtained. Cyclopentene (crude yield 25%, purified yield 22%), b.p. 45–47°, $n^{20}D$ 1.4165 (lit.[7] b.p. 44.3–44.4° (761 mm.), $n^{20}D$ 1.42246) gave the 2-chlorocyclopentyl 2′,4′-dinitrophenyl sulfide derivative, m.p. and mixed m.p. with an authentic derivative 76.5–77°. In addition cyclopentene was brominated to yield *trans*-1,2-dibromocyclopentane, b.p. 98–102° (43 mm.), $n^{25}D$ 1.5417 (lit.[8] b.p. 105–105.5° (45 mm.), $n^{24}D$ 1.5444[9]). Ethanol (crude yield 78%, purified yield 23%), b.p. 73–76°, $n^{25}D$ 1.3653 (lit.[10] b.p. 78.5°, $n^{20}D$ 1.3610) gave an iodoform derivative, m.p. 119° (lit.[11] m.p. 119°). Methyl ethyl carbonate (crude yield 4%), b.p. 105–110°, $n^{20}D$ 1.3782 (lit.[12] b.p. 107.2–107.8°, $n^{20}D$ 1.3779) was not purified further. Cyclopentanol (crude yield 4%, purified yield 3%), b.p. 61–63° (19 mm.), $n^{25}D$ 1.4480 (lit.[13] b.p. 139°, $n^{20}D$ 1.4530) gave an α-naphthylurethan derivative, m.p. and mixed m.p. with an authentic derivative 118–119° (lit.[14] m.p. 118°). Ethyl cyclopentyl carbonate (crude yield 6%, purified yield 3%), b.p. 90–91° (19 mm.), $n^{25}D$ 1.4300, was identical with the compound made by independent synthesis. This was confirmed by infrared spectra of the two materials. N-Cyclopentylurethan (crude yield 7%, purified yield 4%), b.p. 122–125° (19 mm.), $n^{25}D$ 1.4605 was identical with the product previously prepared. This was confirmed by infrared spectra of the two materials.

2-Chlorocyclopentyl 2′,4′-Dinitrophenyl Sulfide.—This compound, m.p. 76.5–77°, was obtained from cyclopentene and 2,4-dinitrobenzenesulfenyl chloride by the method of Kharasch and Buess.[15]

(1) Abstracted in part from a dissertation submitted by Frederick W. Bollinger to the Graduate School of Illinois Institute of Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Merck & Co., Inc., Rahway, New Jersey.

(3) F. W. Bollinger, F. N. Hayes and S. Siegel, THIS JOURNAL, **72**, 5592 (1950).

(4) W. Hückel, E. Kamenz, A. Gross and W. Tappe, *Ann.*, **533**, 1 (1937).

(5) Melting points and boiling points are uncorrected. Microanalyses were performed by Micro-Tech Laboratories, Skokie, Ill.

(6) W. W. Hartman and M. R. Brethen, "Organic Syntheses," John Wiley and Sons, Inc., New York, N. Y., Coll. Vol. II, 1943, p. 278.

(7) A. I. Vogel, *J. Chem. Soc.*, 1323 (1938).

(8) J. Wislicenus and C. Gärtner, *Ann.*, **275**, 332 (1893).

(9) M. W. Lister, THIS JOURNAL, **63**, 145 (1941).

(10) F. H. Getman and V. L. Gibbons, *ibid.*, **37**, 1995 (1915).

(11) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 3rd ed., 1948, p. 258.

(12) M. H. Palomaa, E. J. Salmi and K. Suoja, *Ber.*, **72**, 313 (1939).

(13) C. R. Noller and R. Adams, THIS JOURNAL, **48**, 1084 (1926).

(14) Reference 11, p. 226.

(15) N. Kharasch and C. M. Buess, THIS JOURNAL, **71**, 2724 (1949).