XCVIII. THE MANOMETRIC DETERMINATION OF AMINO-ACIDS

By MORTON F. MASON

From the Physiological Laboratory, Cambridge

(Received 7 February 1938)

IN a recent investigation dealing with the locus of deamination [Mason, 1937] the author estimated amino-acids by a method (not described) involving manometric measurement of the CO_2 split from the carboxyl groups of those acids by heating with ninhydrin, a procedure previously suggested as feasible by Van Slyke & Dillon [1936]. The validity of the gasometric technique developed was established by analyses of many amino-acids and related compounds. It is the purpose of this communication to describe the method employed and to give the results of these tests.

More recently Van Slyke & Dillon [1938] have given a detailed account of the gasometric procedure as they have developed it. The technique which they recommend differs only slightly from that used in obtaining the data submitted in this paper.

The reaction between α -amino-acids and ninhydrin in aqueous solution appears to proceed in a manner analogous to the formation of murexide and is to be formulated as follows [Ruhemann, 1911]:

The cyclic acids proline and hydroxyproline undergo a somewhat different condensation with ninhydrin, in which, however, they are decarboxylated [Grassmann & van Arnim, 1934]. The configurations which are able to participate in the reaction appear to be sharply limited, in so far as the evolution of CO_2 is concerned. They include those substances having a free NH_2 group in the α position adjacent to a carboxyl and certain acids with cyclic imino groups in

positions similar to those of the prolines. The non-reactivity of *N*-methyl-tyrosine (surinamine) is an example contrary to the generalization that all substances with the structure $RCH_2.NH.CH(R).COOH$ react [Van Slyke & Dillon, 1938]. Those acids in which the amino group is in a position other than α do not react, nor do peptides in which the free $COOH$ and NH_2 groups are spatially distant. On the other hand, glutathione, which has a free α - NH_2 group adjacent to $COOH$, yields 1 mol. CO_2 (*vide infra*). It should be noted that certain other cyclic ketones, e.g. isatin, also react in a manner similar to that of ninhydrin with amino-acids [Grassmann & van Arnim, 1934].

Previous applications of the ninhydrin reaction to the quantitative determination of amino-acids have been colorimetric or photometric procedures. These are subject to the criticism that not all amino-acids produce the same colour under the same conditions, that numerous substances other than amino-acids yield colour [Neuberg, 1914], and that various substances, e.g. ammonium salts, interfere greatly with the character and intensity of the colour developed [Polonovski & Moreno-Martin, 1935]. The literature on these methods has been summarized by Mitchell & Hamilton [1929].

Details of procedure

Reagents. $NaOH$ c. 0.5 *N*, prepared as described by Van Slyke *et al.* [1933]. Lactic acid, c. *N*. A sat. solution of KH_2PO_4 , freed from dissolved CO_2 by shaking briefly under reduced pressure or by boiling. Ninhydrin (triketohydrindene hydrate). Glycerol.

Apparatus. The combustion tube and condenser described for procedure "A" in the determination of total C by Van Slyke *et al.* [1933] for use in conjunction with the Van Slyke-Neill manometric gas machine. An Ostwald-Van Slyke pipette calibrated to deliver 1.5 ml. Glass beads.

Procedure. The solution to be analysed is made slightly acid and is freed from dissolved CO_2 by shaking in a flask at reduced pressure or by boiling. A 1–5 ml. aliquot containing 0.05–2.0 mg. available carboxyl CO_2 is introduced into the clean dry combustion tube, followed by 1 or 2 ml. sat. KH_2PO_4 and about 30 mg. ninhydrin. A glass bead is employed to reduce bumping. The combustion tube is immediately connected with the condenser, the walls of the ground glass joint being smeared with glycerol as a sealing fluid. The condenser is then attached to the manometric gas pipette into which have been introduced 2 ml. of the 0.5 *N* alkali as described by Van Slyke *et al.* [1933]. The gas chamber and combustion tube are then connected by turning the stopcock, the mercury levelling bulb resting on the lower support. The contents of the combustion tube are gently heated with a micro-burner. Before the onset of boiling the mercury in the gas chamber is alternately raised and lowered several times, care being exercised to prevent bumping of fluid up into the condenser. After 3 min. gentle boiling the flame is removed and the mercury level is alternately raised and lowered to draw the CO_2 over into the alkali in the gas pipette. This must be done with some care at first in order to avoid bumping, heating of the stopcock and condensation of large amounts of vapour in the gas chamber, with consequent alteration of the volume of fluid over which the CO_2 is ultimately measured.¹ The

¹ Increase of this volume by 0.1 or 0.2 ml. affects the accuracy of the determination only slightly; indeed, bumping of a few drops of the fluid from the combustion tube into the pipette will not invalidate the analysis if the reaction of the absorbing fluid is still alkaline. The error introduced is that entering into the gas factors which are calculated on the basis that the fluid volume over which the CO_2 is measured is 3.5 ml.

mercury level is thus altered 10–15 times allowing it to reach the lower shoulder of the pipette.

The contents of the combustion tube are again gently boiled for 1 min., and the absorption carried out as before. The unabsorbed gas is then ejected as described by Van Slyke *et al.* [1933, p. 641] and the absorption of CO_2 repeated at a greatly reduced pressure by altering the mercury level another 10 times. The unabsorbed gas is again ejected, and a final absorption is made by again altering the mercury level 10 times. Under these circumstances the pressure during the final manipulation is about 50 mm. when the mercury level is at the 50 ml. mark.

The stopcock connecting the condenser and gas chamber is closed, the condenser disconnected, the stopcock capillary sealed with mercury and the last portion of unabsorbed gas ejected. The CO_2 is then liberated with lactic acid and the pressure measured before and after absorption with 5 *N* alkali as described by Van Slyke *et al.* [1933]. Measurements are made at the 0.5 ml. or 2.0 ml. mark depending on the amount of CO_2 present.

Blank analyses are performed similarly except that ninhydrin is omitted. One boiling period is sufficient in the blank determination if there is no reason to suspect the presence of substances decarboxylated by heat alone. Duplicate analyses usually agree within 1–2 %, and with practice somewhat greater precision is obtained. The time required to complete a determination is 15–20 min. Between analyses the condenser is rinsed with distilled water and allowed to drain. The combustion tube is rinsed with water, alcohol and ether, and dried, and the gas chamber is washed with dilute lactic acid in the usual manner.

$$\begin{aligned} \text{Calculation: } P_{\text{CO}_2} &= (p_1 - p_2) - C \\ P_{\text{CO}_2} \times f_{\text{CO}_2} &= \text{mg. CO}_2 \text{ in the sample analysed.} \end{aligned}$$

The $(p_1 - p_2)$ reading in the blank analysis is the *C* correction. The value of this correction has averaged about 10 mm. when measured at the 2.0 ml. mark for the reagents used in this laboratory. The factors f_{CO_2} are those derived by Van Slyke & Sendroy [1927, p. 141].

In relating the CO_2 measured to the amino-acid concentration in mixtures it should be noted that the presence in unknown proportions of aspartic acid and cystine introduces an inaccuracy into the expression of the concentration in millimols, as these acids yield 2 mol. CO_2 (*vide infra*). Analogous difficulties are encountered when a similar calculation is made from the N_2 evolved with HNO_3 . This must be borne in mind in making any comparisons between amino-acid N and amino-acid CO_2 in the same solution.

Results of analyses of solutions of pure amino-acids

The results of analyses of 24 common amino-acids are given in Table I. For reasons irrelevant to this communication these acids were dissolved in saturated solutions of picric acid. Similar results are obtained in aqueous solutions. The recoveries are satisfactory save in three instances, alanine, serine and tryptophan. The reasons for incomplete recoveries of these have not as yet been ascertained. In the analysis of a mixture of amino-acids, e.g. a protein hydrolysate, imperfect behaviour of these three acids would introduce ordinarily only a very small error into the determination of the total amino acid content.

Cystine and aspartic acid each yield 2 mol. CO_2 whereas only one COOH group of glutamic and hydroxyglutamic acids reacts. Cystine, of course, presents two $\alpha\text{-NH}_2$ groups adjacent to COOH . In the case of aspartic acid, the malonyl

Table I. *Analyses of picric acid solutions containing known amounts of pure amino-acids*

Compound	Quantity analysed	Recovery calculated from CO ₂ evolved
	mg.	%
Glycine	0.61	98.2
<i>dl</i> -Valine	0.89	98.8
<i>l</i> -Leucine	1.34	99.9
<i>dl-iso</i> -Leucine	0.93	101.0
<i>dl</i> -Cysteine	0.88	100.2
<i>dl</i> -Methionine	0.80	102.7
<i>dl</i> - α -Amino-isobutyric	0.62	99.7
<i>dl</i> -Phenylalanine	0.56	100.3
<i>dl</i> - α -Amino-phenylacetic	0.72	97.2
<i>l</i> -Tyrosine	0.48	98.0
<i>l</i> -Dihydroxy-phenylalanine	1.34	99.5
<i>dl</i> -Lysine	1.84	101.2
<i>dl</i> -Arginine	7.15	98.0
Asparagine	1.33	99.6
<i>l</i> -Histidine	1.07	98.3
<i>l</i> -Proline	0.59	102.6
<i>l</i> -Hydroxy-proline	0.66	101.7
<i>dl</i> -Glutamic acid	0.87	102.5
<i>i</i> - β -Hydroxyglutamic acid	2.84	103.7
<i>l</i> -Aspartic acid (2 mol. CO ₂)	0.45	100.6
<i>l</i> -Cystine (2 mol. CO ₂)	0.36	99.9
<i>dl</i> -Alanine	—	75–90
<i>dl</i> -Serine	—	87–97
<i>l</i> -Tryptophan	—	88–96

aldehyde resulting from oxidation by ninhydrin presumably immediately decarboxylates as a result of boiling to yield the second mol. CO₂. The second COOH of glutamic and hydroxyglutamic acids is not entirely insensitive, and gradually gives up CO₂ on prolonged boiling. With the boiling periods specified, recoveries are constantly obtained within 4% of theoretical for one COOH. The second COOH of hydroxyglutamic acid appears to be somewhat more reactive than that of glutamic acid.

This behaviour of aspartic and glutamic acids affords a rapid means of determining the quantities of each when they are separated as the mixed silver salts in the partition of protein hydrolysates. If both the NH₂-N (HNO₂ reaction) and the carboxyl CO₂ are estimated, the mol. CO₂ in excess of the mol. N₂ represent the aspartic acid.

Behaviour of ninhydrin with peptides and amino-acids other than α -amino-acids

Table II gives the results of analyses of a series of peptides. Preliminary analyses with 6 min. boiling indicated that a small amount of CO₂ equivalent to 1–4% of one COOH group was evolved. With the shorter boiling periods, totalling 4 min., much less is obtained. It seems probable that there is some hydrolysis of the peptide linkage with prolonged heating, although a very slow reaction with the ninhydrin is not excluded. Glutathione, which has an adjacent α -NH₂ and COOH group, yields 1 mol. CO₂ as anticipated. Also included in this table are results on two *N*-methylamino-acids, a β -NH₂ and ϵ -NH₂ acid. *N*-methyltyrosine was entirely unreactive; however, sarcosine evolved variable amounts (10–30%) of CO₂. The conditions employed by Van Slyke & Dillon [1938] apparently permit complete decarboxylation of the latter acid. The β - and ϵ -acids did not react.

Table II. *Analyses of solutions of peptides and amino-acids other than α -amino-acids*

Compound	Quantity analysed mg.	Recovery calculated from CO ₂ evolved %
Glycyl-glycine	3.69	<3.0
<i>dl</i> -Leucyl-glycine	4.98	0
Glycyl- <i>l</i> -tyrosine	2.92	0
<i>dl</i> -Leucyl-glycyl-glycine	4.14	<1.4
Glycyl- <i>l</i> -tryptophan	2.94	0
<i>dl</i> -Alanyl-glycine	6.26	<2.0
Leucyl- <i>l</i> -tyrosine	3.32	<2.0
Glycyl- <i>dl</i> -leucine	3.96	<2.0
Glycyl-glycyl-glycine	4.18	<2.0
Glutathione (1 mol. CO ₂)	6.53	98.4
<i>dl</i> - β -Amino- β -phenylpropionic	2.20	0
ϵ -Amino- <i>n</i> -hexanoic acid	3.23	0
<i>N</i> -Methyltyrosine	1.63	0
Methylglycine (sarcosine)	4.15	10-30

Development of intense colour is not a criterion of decarboxylation, and may occur without any CO₂ evolution. The colour reactions of ninhydrin with amino-acids and related substances under various conditions are discussed elsewhere [Neuberg, 1914; Grassmann & van Arnim, 1934; Mason, to be published].

Possible interfering substances

A number of common carboxylic acids, keto-derivatives of amino-acids and other compounds, were tested in order to ascertain whether or not they evolved CO₂ or interfered with the determination in any way. Most of these were entirely unreactive (α -ketoglutaric, mesoxalic, lactic, citric, fumaric, succinic, oxalic, acetic, butyric and benzoic acids) although several (pyruvic, oxaloacetic and acetoacetic acids) were decarboxylated by heating only, as evidenced by the value of the blank analysis. No additional CO₂ was evolved with ninhydrin with any of these acids; a trace (<1.0%) of additional CO₂ obtained from pyruvic acid is probably due to some variation in amount of decomposition during the blank determination. However, in the case of glucosamine some of the aldehyde may be oxidized by ninhydrin and the resulting α -amino-acid behave in the usual way.

These data indicate that the measurement of carboxyl CO₂ liberated by ninhydrin should afford a highly specific means of estimating amino-acids in biological materials. The application of the method to analysis of blood filtrates, etc., is being studied further, and will be discussed in a subsequent report.

SUMMARY

1. Amino-acids heated with ninhydrin in aqueous solution are decarboxylated.
2. A technique for measuring manometrically the CO₂ evolved in the Van Slyke-Neill gas machine has been described.
3. Of 24 common amino-acids tested, all yielded 1 mol. CO₂ save aspartic and cystine which yielded 2, and alanine, serine and tryptophan, which evolved slightly less than 1.

4. Peptides do not react unless they have free adjacent α -NH₂ and COOH groups.

5. Various common organic acids other than amino-acids were found not to yield CO₂ upon treatment with ninhydrin.

REFERENCES

- Grassmann & van Arnim (1934). *Liebigs Ann.* **509**, 288.
Mason (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 111.
Mitchell & Hamilton (1929). The Biochemistry of the Amino Acids, A.C.S. Monograph, No. 48.
Neuberg (1914). *Biochem. Z.* **59**, 249.
Polonovski & Moreno-Martin (1935). *Anales soc. espan. fis. quim.* **33**, 574.
Ruhemann (1911). *J. chem. Soc. (Trans.)*, **99**, 792, 1486.
Van Slyke & Sendroy (1927). *J. biol. Chem.* **73**, 127.
—— Page & Kirk (1933). *J. biol. Chem.* **102**, 635.
—— & Dillon (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 362.
—— ——— (1938). *C.R. Trav. Lab. Carlsberg*, **22**, 480.