

## Release of Nitrogen from Amino Acids with Ninhydrin for $^{15}\text{N}$ Analysis<sup>1</sup>

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Received September 4, 1964

The reaction with indane-1,2,3-trione-2-hydrate (ninhydrin) is firmly established for the colorimetric determination of  $\alpha$ -amino acids (1-3). The intensity of the blue-violet color produced is measured at about 570 m $\mu$ . This color is most intense when the reaction with ninhydrin is carried out at about pH 5 (1). At this pH, the products of the reaction are usually carbon dioxide, an aldehyde containing one less carbon atom than the parent amino acid, and the blue pigment, diketohydrindylidene-diketohydrindamine.

When the reaction is performed at pH less than 3, however, the pigment is not formed, and the  $\alpha$ -amino group is released as ammonia. MacFadyen (4) describes a method for the quantitative assay of several amino acids with ninhydrin based on estimation of ammonia produced at pH 2.5. It is necessary to remove the ninhydrin completely while the solution is acid, since the complex is re-formed under neutral or alkaline conditions, and variable recoveries of ammonia result. MacFadyen achieved this by reduction of ninhydrin with hydrogen sulfide to insoluble hydrindantin, which was filtered. Adsorption on charcoal has also been employed (5), but the most convenient procedure is by oxidation with strong hydrogen peroxide (6).

In this paper a method for the release of amino groups as ammonia after formation of the colored complex at pH 5.4 is described. Performing the reaction at pH 5.4 extends the range of amino acids from which complete recovery of amino nitrogen as ammonia can be achieved. For most amino acids, the accuracy and precision is comparable to that achieved by Kjeldahl analysis; however the method is much less sensitive than the colorimetric procedures. It is easily adapted to the recovery of  $\alpha$ -amino nitrogen as ammonia for mass spectrometry after colorimetric analysis or detection on paper chromatograms.

<sup>1</sup>Supported by research grants from C.S.I.R.O. and the University of Western Australia.

## EXPERIMENTAL

Laboratory reagent-grade amino acids were obtained from several commercial sources, dissolved in 0.1 *N* hydrochloric acid, neutralized with sodium hydroxide, and adjusted to contain about 50  $\mu$ moles/ml of solution. Analytical grade ninhydrin was obtained from British Drug Houses.

*Reagents*

(a) pH 5.4 acetate buffer—a mixture of 270 gm sodium acetate-trihydrate, 200 ml water and 50 ml glacial acetic acid is diluted with ammonia-free water to 750 ml. This buffer is identical with that described by Rosen (3).

(b) 3% ninhydrin solution—3 gm of ninhydrin is dissolved in deionized water to give 100 ml of solution.

(c) 2 *N* hydrochloric acid solution—32% acid is added to four times its volume of water.

(d) 30% (100 vol) hydrogen peroxide.

*Procedure*

For mass spectrometry, a sample containing between 2 and 50  $\mu$ moles of amino acid is suitable. A 1-ml sample of amino acid in a 20  $\times$  150 mm test tube is mixed with 1.0 ml acetate buffer and 2.0 ml ninhydrin solution. The tube is heated in a boiling water bath for 10 min and destruction of the ninhydrin-ammonia complex is achieved by addition of 4.0 ml 2 *N* hydrochloric acid, with a further 10-min heating. Ninhydrin is destroyed by adding 0.25 ml hydrogen peroxide with an additional period of 5 min at 100°C. Ammonia is then recovered by steam distillation at pH 10, with the phosphate-borate buffer system of Pucher *et al.* (7) except that 1.0 *N* sodium hydroxide containing 5 gm borax per 100 ml is used to adjust pH. Twelve ml of this solution with 10 ml of phosphate-borate buffer produce a pH of 10 when mixed with the reacted amino acid sample. In the case of amino acids which contain only one nitrogen atom the ammonia can be distilled by using 6 ml 3 *N* sodium hydroxide instead of the buffer solution.

Thirty ml of distillate is collected in 5 ml of 0.2 *N* sulfuric acid and ammonia is determined by Nesslerization. The presence of ethanol in the original amino acid sample is undesirable, since this distils over with the ammonia, and interferes with Nesslerization. In addition, ethanol gives rise to a peak at mass 29 in the mass spectrometer, which is unsatisfactory for  $^{15}\text{N}$  determination. These interferences can be eliminated by vacuum rotary evaporation of the original sample or of the sulfuric acid solution to dryness.

The techniques for production of  $^{15}\text{N}_2$  and for  $^{15}\text{N}$  analysis were substantially as described by Burris and Wilson (8).  $^{15}\text{N}$  determinations were made on an Associated Electrical Industries MS-3 mass spectrometer.

## RESULTS AND DISCUSSION

Recoveries of ammonia from a range of amino acids when subjected to degradation with ninhydrin are shown in Table 1. The amino acids examined include all the common protein constituents, and several known

TABLE 1  
MOLES AMMONIA EVOLVED PER MOLE AMINO ACID<sup>a</sup> REACTED WITH NINHYDRIN

Amino acid	No. N atoms/molecule	pH 5.4 acetate buffer	pH 2.5 citrate buffer <sup>b</sup>
DL- $\alpha$ -Alanine	1	0.99	—
L-Arginine-HCl	4	1.02	0.96
DL-Aspartic acid	1	0.99	0.98
L-Asparagine	2	1.02	0.98
L-Cysteine-HCl	1	0.34	0.61
L-Citrulline	3	1.14	1.09
$\gamma$ -Amino- <i>n</i> -butyric acid	1	0.92	0.09
DL- $\alpha$ -Amino- <i>n</i> -butyric acid	1	1.00	1.00
L-Glutamic acid	1	1.00	0.98
L-Glutamine	2	1.20	1.04
Glycine	1	0.99	1.00
L-Histidine-HCl-H <sub>2</sub> O	3	1.02	1.09
4-Hydroxy-L-proline	1	0.21	0.08
DL-Isoleucine	1	0.99	—
DL-Leucine	1	1.02	—
L-Lysine-HCl	2	1.01	1.02
DL-Methionine	1	0.97	—
L-Ornithine	2	0.94	1.04
DL-Phenylalanine	1	0.94	—
L-Proline	1	0.08	0.05
DL-Serine	1	1.09	—
DL-Threonine	1	0.91	—
DL-Tryptophan	2	1.03	0.53
L-Tyrosine	1	0.97	—
Urea	2	0.04	0.12
L-Valine	1	1.00	—

<sup>a</sup> Moles of amino acid were computed on the nitrogen content of the solutions, determined by Kjeldahl digestion (8). Ammonia was steam-distilled from 52% sodium hydroxide solution (w/v), containing 8% sodium thiosulfate (w/v), into 1% boric acid, and titrated with 2.5 mM hydrochloric acid using Tashiro's indicator.

<sup>b</sup> pH 2.5 sodium-citrate buffer (2 M) was prepared from citric acid and sodium hydroxide solutions and 1.0 ml substituted for the pH 5.4 acetate buffer. Treatment with hydrochloric acid was omitted, and 0.25 ml hydrogen peroxide added after 15 min heating at 100°C.

to occur in the free state in plant tissues. Excepting the imino acids and cysteine, only minor deviations from the theoretical equimolar yield of ammonia occur.

In most cases, acidification with 3 ml of 2 *N* hydrochloric acid was adequate to ensure complete destruction of the colored complex. However 4 ml was added routinely since recovery from  $\gamma$ -amino-*n*-butyric acid was often incomplete with less — indicated by return of the blue color on addition of alkali. Whenever the blue colored complex is re-formed, poor recoveries can be anticipated. Percentage recoveries of ammonia from glutamic acid when hydrochloric acid was added to achieve final pH of 4.0, 0.8, and 0.2 (1, 2, and 3 ml) were 59, 89, and 100, respectively. The quantity of ninhydrin recommended (60 mg/analysis) proved adequate for maximal recoveries of ammonia when no more than 50  $\mu$ moles of amino acid was involved but reduced recoveries were observed when less than 40 mg was supplied.

Under these conditions, the action of ninhydrin is not completely specific for  $\alpha$ -amino nitrogen: thus  $\gamma$ -amino-*n*-butyric acid yields almost all its nitrogen as ammonia. Citrulline and glutamine produce more ammonia than can be accounted for on their  $\alpha$ -amino nitrogen content alone, though this effect is reduced by performing the reaction with ninhydrin at pH 2.5 (molar recoveries from several of the acids at this pH are also indicated in Table 1). If absolute specificity is required for the amides the amide nitrogen can be removed (7) before the  $\alpha$ -amino nitrogen is released.

Unequivocal evidence for specific attack would require the use of pure amino acids labeled with isotopic nitrogen. Rittenberg (9) reports that histidine labeled with  $^{15}\text{N}$  in the imidazole ring (32 atom per cent excess) produced ammonia with less than 0.02 atom per cent excess  $^{15}\text{N}$  on treatment with ninhydrin at pH 2.5, indicating that only the  $\alpha$ -amino nitrogen was released.

Advantages gained by reaction at pH 5.4 are: (1) The amino nitrogen of  $\gamma$ -amino-*n*-butyric acid can be recovered at this pH. Less than 10% is released at pH 2.5. Recovery of ammonia from tryptophan is also improved by reaction at pH 5.4. However, recoveries from cysteine, proline, and hydroxyproline remain low—hence the method is unsuitable for degradation of these acids. (2) The procedure can be conveniently applied to amino acid samples after reaction with ninhydrin for colorimetry or for spot detection on chromatograms.

In this laboratory the method is being used in a study of the incorporation of  $^{15}\text{N}_2$  into amino acids by detached serradella (*Ornithopus sativus*) root nodules. Amino acids from nodules are first separated by ion-exchange chromatography (10), followed by analysis of the fractions

with ninhydrin in a pH 5.4 acetate-cyanide buffer (3). Samples containing amino acids are then bulked and taken to dryness in a rotary evaporator. This is necessary since 2-methoxyethanol, present as a solvent for ninhydrin during analysis, occasionally carries over with the ammonia sample and a gradual increase in the mass 29/28 ion current ratio is observed, giving rise to spurious enrichments of  $^{15}\text{N}$ . For the same reason, it is important to exclude the possibility of ethanol contaminating the ammonia sample. These sources of error are particularly likely when small amounts of nitrogen are analyzed, and it is necessary to concentrate the ammonia sample before liberation of nitrogen gas with alkaline hypobromite for mass spectrometry.

Separations of amino acids are also achieved by one-way paper chromatography on acid-washed Whatman No. 3 grade paper. Ninhydrin-

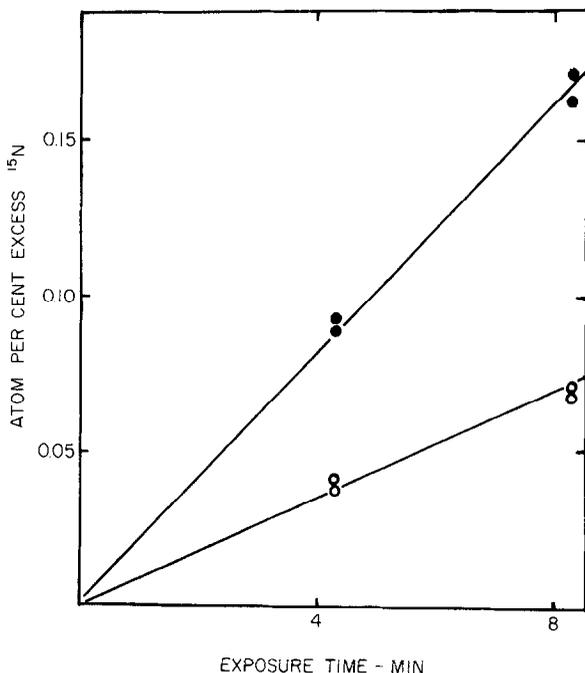


FIG. 1. Comparison of ninhydrin and Kjeldahl methods for release of  $^{15}\text{N}$ -enriched ammonia from nodule supernatants. Duplicate samples of 5 gm of serradella nodules were exposed to an  $^{15}\text{N}$ -enriched gas mixture (argon, 65%; oxygen, 25%; nitrogen, 10% containing 70 atom %  $^{15}\text{N}$ ). Reaction was terminated at times indicated by plunging nodules into liquid nitrogen; subsequently the frozen nodules were dropped into five times their volume of boiling 80% ethanol, and crushed in a mortar. The supernatant fluid from 15 min centrifugation at  $20,000 \times g$  was used for ammonia production with ninhydrin at pH 5.4 (●), or by Kjeldahl digestion (○).

positive bands are eluted with water and the ammonia is released as described in the "Experimental" section.

The reaction with ninhydrin has been found convenient for assay of the rate of  $^{15}\text{N}_2$  fixation with detached serradella nodules. At early times after exposure to isotopic nitrogen most of the label is located within amino compounds in the 80% ethanol-soluble fraction of crushed nodules. The rate of  $^{15}\text{N}_2$  fixation by legume root nodules is usually measured by  $^{15}\text{N}$  analysis of ammonia produced by Kjeldahl digestion of the acid-soluble supernatant from crushed nodules (8). Increased sensitivity is achieved by ammonia production with ninhydrin. This is shown in Fig. 1. In addition, a more specific release of ammonia for mass spectrometry assists in interpretation of the labeling pattern observed in individual amino acids.

#### SUMMARY

A quantitative method for the specific release of  $\alpha$ -amino nitrogen of amino acids as ammonia is described. The amino nitrogen of  $\gamma$ -amino-n-butyric acid is also released. The method has been applied to the determination of  $^{15}\text{N}$  enrichment in amino acids of legume root nodules exposed to  $^{15}\text{N}_2$ .

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