''THE COLORIMETRIC ESTIMATION OF ALPHA-AMINO NITROGEN IN TISSUE FLUIDS

BY

H. M. RUBINSTEIN* AND J. D. PRYCE

From the Departments of Chemical Pathology and Haematology,
Postgraduate Medical School of London

Various authors have reported methods for the colorimetric estimation of alpha-amino nitrogen with ninhydrin (Moore and Stein, 1948, 1954; Smith and Agiza, 1951; Troll and Cannan, 1953; Yemm and Cocking, 1955; Kalant, 1956; Rosen, 1957). Only Kalant has applied this method to the direct measurement of alpha-amino nitrogen in biological fluids; he reported on the determination of total alpha-amino nitrogen in dog plasma.

This paper describes a colorimetric ninhydrin method for amino-acid determination, based on that of Troll and Cannan. It differs in using disodium ethylenediamine tetra-acetic acid (E.D.T.A.) instead of pyridine to enhance colour development. This improves reproducibility by eliminating trace metal interferences, and yields lower blanks than the pyridine reagent. In addition, we use a separate buffer to maintain the pH optimal at 5.1. For the first time a technique has been evolved for applying the colorimetric ninhydrin procedure to biological fluids generally, and, in particular, it has been successfully applied to urine.

The optimum conditions for pH and concentration of cyanide, ninhydrin, and other reagents have been studied, and the optimal time for heating has been established. With the exception of the E.D.T.A. concentration, all the factors are moderately critical.

A : Method for Simple Solutions of Amino-acids

Buffer.—Add 300 ml. of 2M-sodium acetate (164 g./l. anhydrous salt) to 700 ml. of 2M-acetic acid (120 ml. glacial acetic acid/l.). Add approximately 5 g. of disodium ethylenediamine tetra-acetate and exactly 240 mg. of potassium cyanide. Preserve with chloroform. This solution is stable indefinitely.

Phenol Reagent.—Dissolve 400 g. of phenol in 100 ml. of absolute ethanol by heating. Cool and add approximately 5 g. of "permutit" (Decalso F). Stir well for 10 minutes and filter. Even moderate

*Damon Runyon Fellow in Cancer Research.

colour due to oxidation of the phenol does not interfere with subsequent determinations.

Ninhydrin.—Dissolve 6 g. of ninhydrin in 100 ml. of absolute ethanol by vigorous stirring. Add approximately 2 g. of "permutit" and stir for 10 minutes. Filter and store in a brown bottle. The solution is a pale yellow-green. If the solution is contaminated by protein or amino-acid the colour will change.

Stock Standard.—Any full-colour amino-acid is suitable (Table I). Monosodium glutamate (mol. wt.

TABLE I

COLOUR VALUES OF AMINO-ACIDS AND RELATED COMPOUNDS RELATIVE TO GLUTAMIC ACID ON A MOLAR BASIS

Full (97–103%)	High	Low
Glycine	Lysine 110%	Phenylalanine 94%
Alanine	Ornithine 110%	Tyrosine 88%
Leucine	Cystine 121%	Tryptophan 78%
Isoleucine		Taurine 29%
Valine		Beta alanine 8%
Serine		Ammonia nitrogen 46%
Histidine		Ethanolamine 16%
Citrulline		Ethylenediamine 16%
Threonine		Methylamine 8%
Artinine		Ethylamine 3%
Methionine		
Aspartic acid		Urea 0·09%
Asparagine		Creatinine 0·26%
Glutamine		Uric acid 0·00
Cysteic acid		Hippuric acid 0·07% or less

187.14) has been used in this laboratory. Dissolve 935.7 mg. in distilled water and make up to 100 ml. This solution is 0.05 M. Preserve with chloroform.

Working Standard.—Dilute stock standard 1 in 100 with distilled water. One millilitre of the working standard contains 7.0 μg. alpha-amino nitrogen.

Procedure.—Add 0.5 ml. of the unknown solution (containing not more than 3.5 μg. of alpha-amino nitrogen) to a 6 × ¾ in. test-tube. For the blank use 0.5 ml. ammonia-free distilled water, and for the standard take 0.5 ml. of the working standard (3.5 μg. of alpha-amino nitrogen). Add 0.5 ml. of buffer solution to each tube. Add 5 ml. of phenol reagent and shake until clear. Add 0.5 ml. of ninhydrin solution and shake again. Place the tubes in an actively boiling water bath for four to five minutes. It is unnecessary to stopper the tubes. Remove and cool in

water for at least 10 minutes. Shake the tubes vigorously and equally. After shaking, the blank solution takes on a pale yellow colour, and the test solutions take on the typical purple colour. The colour fades slowly and proportionately for several hours. Allow the tubes to stand for at least 10 minutes after shaking, and read the optical density at 570 mμ or with a yellow filter (Ilford 626). When read against water, the optical density of the blank is always below 0.05.

The standard deviation of 100 consecutive duplicate determinations was ±2.10%. In Table I are listed the relative colour values of the common amino-acids and several other compounds. Proline, hydroxyproline, and cysteine have not been included because they do not give purple colours with ninhydrin (Moore and Stein, 1948).

B : Method for Plasma

Kalant (1956) found that ethanol, trichloro-acetic acid, and sulphosalicylic acid were unsatisfactory as protein-precipitating agents, but that tungstic acid was suitable. Acetone has been tested as a precipitant, but it is unsatisfactory because of the low solubility in acetone of many amino-acids. Picric acid is a satisfactory precipitant, but a considerable amount of extra labour is involved, since the excess picric acid must be removed (Stein and Moore, 1954). Tungstic acid was selected for this study. It is not ideal because it leaves a slight residual turbidity, which necessitates that the blank and standard also be treated with the protein precipitant.

Heparinized plasma was used. Serum is, of course, unsatisfactory owing to the considerable proteolysis which occurs on clotting. Separated plasma is stable indefinitely if frozen.

Protein Precipitant.—Add 1 part of 13.3% (w/v) sodium tungstate to 9 parts of 0.11 N-sulphuric acid (3.1 ml. of conc. sulphuric acid/l.). This mixture should be made up every few days as it rapidly becomes turbid.

Working Standard.—Dilute 4 ml. of stock standard to 100 ml. with distilled water.

Procedure.—Rinse 0.3 ml. of plasma into 0.7 ml. distilled water in a centrifuge tube. Add 3 ml. of the tungstic acid reagent with shaking. Centrifuge. Decant the supernatant through a cotton-wool plug. For the blank, add 3 ml. of the tungstic acid reagent to 1 ml. of ammonia-free distilled water, and for the standard add 3 ml. of tungstic acid reagent to 1 ml. of the working standard. In each case, analyse 0.5 ml. as in section A, reading the optical density against water. The optical density of the blank is always below 0.075.

Calculation.—The sample analysed is equal to 300/8 μl. of plasma ; the standard analysed contains 3.5 μg. alpha-amino nitrogen ; and μg. per 100 μl. equals mg. per 100 ml.

$$\frac{\text{O.D.* test}-\text{O.D. blank}}{\text{O.D. standard}-\text{O.D. blank}}\times\frac{8}{3}\times3\cdot5=\frac{\text{mg. alpha-amino nitrogen}}{\text{per 100 ml. plasma}}$$
* O.D.=optical density.

Results.—The plasma of 29 normal laboratory workers was investigated by this method. Blood was drawn three hours after a light breakfast.

Fifteen Women.—Range 3.68–5.47 mg./100 ml., mean 4.52 mg./100 ml., S.D.±0.51 mg./100 ml.

Fourteen Men.—Range 3.68–5.25 mg./100 ml., mean 4.56 mg./100 ml., S.D.±0.40 mg./100 ml.

Combined Series.—Range 3.68–5.47 mg./100 ml., mean 4.54 mg./100 ml., S.D.±0.45 mg./100 ml.

All specimens were analysed in duplicate and the standard deviation of the difference was ±3.7%.

From a patient with pernicious anaemia, 24 plasma samples were analysed over a period of six weeks. All of the values obtained fell between 3.86 and 5.23 mg./100 ml.

Recoveries of pure amino-acids added to plasma specimens on four occasions were 97%, 102%, 96%, and 103%.

C : Method for Cerebrospinal Fluid

The reagents are those for plasma. Rinse 0.6 ml. of cerebrospinal fluid into 0.4 ml. of distilled water in a centrifuge tube. Continue exactly as for plasma, preparing the blank and standard in the same manner.

Calculation.—The sample analysed contains 600/8 μl. cerebrospinal fluid. Hence,

$$\frac{\text{O.D.* test}-\text{O.D. blank}}{\text{O.D. standard}-\text{O.D. blank}}\times\frac{8}{6}\times3\cdot5=\frac{\text{mg. alpha-amino nitrogen}}{\text{per 100 ml. C.S.F.}}$$
* O.D.=optical density.

Results.—Five fresh specimens of cerebrospinal fluid normal with respect to protein and cell content were investigated. The values were 1.08, 1.39, 1.44, 1.45, and 1.25 mg./100 ml.

D : Method for Urine

The principal problem in measuring urinary amino-acids by this method is completely to rid the sample of ammonia. This can be achieved by adding alkali to the urine sample, and evaporating to dryness over concentrated sulphuric acid.

For free alpha-amino nitrogen, the reagents are as follows:

Alkali Solution.—0.05 M-potassium carbonate (6.9 g./l. of the anhydrous salt).

Working Standard.—Dilute stock standard 1 : 1 with distilled water.

Procedure.—Rinse 0.2 ml. urine into 2 ml. 0.05 M-potassium carbonate solution in a small beaker. For the blank, substitute 0.2 ml. distilled water for the urine, and for the standard, substitute 0.2 ml. of the working standard. Place all the beakers in a large vacuum desiccator, the bottom of which contains 300–400 ml. of concentrated sulphuric acid. Evacuate the desiccator to a pressure of 20–30 mm. Hg. Allow the solutions in the beakers to go to dryness. This takes several hours, but it is most convenient to do this step overnight. Dissolve the residue in 10 ml. distilled water. Take 0.5 ml. of each for analysis as in section A. The optical density of the blank is always below 0.05.

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Calculation.—The sample analysed contains 10 μl. of urine, and the standard 3.5 μg. of alpha-amino nitrogen. Thus,

$$\frac{\text{O.D.* test}-\text{O.D. blank}}{\text{O.D. standard}-\text{O.D. blank}} \times 10 \times 3 \cdot 5 = \frac{\text{mg. free alpha-amino}}{\text{nitrogen per 100 ml. urine}}$$

* O.D. = optical density.

Results and Discussion.—This method was applied to 24-hour urine collections from 20 normal laboratory workers. The specimens were preserved with 6 ml. each of toluene and chloroform, and stored at 4° until analysed. Results are given in Table II.

TABLE II

URINARY FREE, TOTAL, AND BOUND ALPHA-AMINO NITROGEN IN 20 NORMAL PEOPLE

Urinary Alpha-amino Nitrogen	Range	Mean (±S.D.)	Significance of Difference between Male and Female Values
Free			
Concentration 8*	8·06– 15·54	10·73	
(mg./100 ml.) 12†	6·95– 17·52	10·14	
combined	—	10·37	
Total 8	75·8 –197·1	130·9 ±39·9	
(mg./24 hours) 12	78·0 –203·1	132·2 ±35·1	
combined	—	131·7 ±37·1	
Mg./kg./24 hours 8	1·39– 3·44	2·22± 0·63 ⎱	
12	0·98– 3·27	1·88± 0·63 ⎰	P=0·26
combined	—	2·01± 0·67	
Total			
Concentration 8	14·51– 29·34	22·66	
(mg./100 ml.) 12	14·24– 31·60	23·33	
combined	—	23·06	
Total 8	192·0 –426·2	274·2 ±70·2	
(mg./24 hours) 12	186·4 –423·0	299·4 ±66·2	
combined	—	289·3 ±69·0	
Mg./kg./24 hours 8	3·52– 7·44	4·67± 1·16 ⎱	
12	2·75– 6·20	4·15± 0·97 ⎰	P=0·32
combined	—	4·36± 1·07	
Bound			
Total 8	81·8 –229·1	143·3 ±46·2	
(mg./24 hours) 12	100·9 –259·6	167·2 ±44·2	
combined	—	157·6 ±45·0	
Mg./kg./24 hours 8	1·24– 4·00	2·45± 0·79 ⎱	
12	1·62– 3·06	2·28± 0·44 ⎰	P=0·56
combined	· —	2·35± 0·60	
Ratio			
Total/free 8	1·50– 2·67	2·17± 0·39 ⎱	
(mg./24 hours) 12	1·65– 2·82	2·32± 0·36 ⎰	P=0·41
combined	—	2·26± 0·38	

* Female.   † Male.

The alkali solution employed decreases the anticipated colour of the standard by about 5%, an effect not present when a weaker alkali solution is substituted. Using the 0.05 M-carbonate solution, up to 15 times the amount of ammonia normally present in urine may be completely removed. Since the maximum urinary ammonia may exceed the average by 10 to 12-fold, it seems advisable to use the 0.05 M-solution. Studies at various alkali concentrations have shown that standard and test solutions are proportionally affected, and the analytical results are unchanged. Recovery of pure amino-acids added to urine on five successive occasions gave results of 104%, 101%, 95%, 99%, and 102%.

The 20 specimens listed were analysed in duplicate, with a standard deviation of the difference of ±2.8%.

From the same patient mentioned earlier, seven 24-hour collections were made over a two-week period. The free alpha-amino nitrogen ranged from 74.6 to 99.7 mg. per 24 hours.

Total Alpha-amino Nitrogen

After acid hydrolysis, the urine ammonia level may rise over 50-fold due to decomposition of urea. Consequently, a higher alkali concentration is necessary for complete removal of ammonia by diffusion-absorption.

Alkali Solutions.—These are 0.40 M-potassium carbonate (55.2 g./l. of the anhydrous salt), and 0.25 M-potassium carbonate (34.6 g./l. of the anhydrous salt).

Working Standard.—This is the same as for free alpha-amino nitrogen.

Procedure.—To 2 ml. of urine in a 6 × ½ in. " pyrex " test-tube add 1 ml. of concentrated hydrochloric acid. Seal the tube in a gas-oxygen flame and hydrolyse in an oven at 150° overnight. Allow to cool. Open the tube cautiously and remove the humin by decanting the hydrolysate through a cotton-wool plug.

Rinse 0.15 ml. of the hydrolysate into 2 ml. of 0.40 M-carbonate solution in a small beaker. At this point prepare the blank and standard by adding 0.2 ml. distilled water and 0.2 ml. working standard respectively to 2 ml. of 0.25 M-carbonate solution in individual small beakers. Proceed now exactly as listed under free alpha-amino nitrogen.

Calculation.—As 150 μl. of hydrolysate equals 100 μl. of the original sample, and 5 μl. of urine is in the final sample, therefore,

$$\frac{\text{O.D.* test}-\text{O.D. blank}}{\text{O.D. standard}-\text{O.D. blank}} \times 20 \times 3 \cdot 5 = \frac{\text{mg. total alpha-amino}}{\text{nitrogen per 100 ml. of urine}}$$

* O.D. = optical density.

Results and Discussion.—The 20 normal urine specimens were also analysed for total alpha-amino nitrogen: the results are given in Table II. The standard deviation of the variation of the duplicate estimations was ±3.2%. The seven specimens from the patient mentioned earlier showed values between 266.0 and 303.2 mg./24 hours.

The higher alkali concentration used for the test solution is calculated to equal the concentration employed for the blank and standard solutions after neutralization of the hydrochloric acid.

As noted for free alpha-amino nitrogen, treatment with alkali produces fading of the standard, generally from 8 to 10%. For the reasons given earlier, the analytical results are unaffected, and two recoveries with pure amino-acids added to urine before hydrolysis were 98% and 99%.

Hydrolysis at 150° gave results about 5% higher than hydrolysis at 125°.

When glutamic acid and glycine were hydrolysed under the conditions above, there was a 4 to 5% loss. It was thought improbable that the various

amino-acids in the hydrolysed test solution would behave quantitatively similarly, and there would still exist uncertainty about the actual extent of amino-acid destruction. For this reason the standard was not routinely brought through the hydrolysis procedure. Consequently it is quite possible that the values for total alpha-amino nitrogen are underestimated to the extent of a small percentage.

Discussion

The standard procedure for determining plasma alpha-amino nitrogen is the ninhydrin-CO_2 method of Hamilton and Van Slyke (1943). Normal values by this technique have been published by the originators and by a number of other investigators (Woodruff and Man, 1945 ; Bonsnes, 1947 ; Brewer, Brown, Harvey, and Horwitt, 1947 ; Burt, 1953). The values given for plasma range from 3.3 to 5 mg./100 ml., with a mean of 4.1 to 4.2. The present method gives a mean and range about 10% higher than the gasometric technique. While the manometric method is probably more specific, the colorimetric procedure is far simpler and seems adequate for clinical applications.

The few figures presented here on alpha-amino nitrogen in cerebrospinal fluid agree with those of Christensen, Cooper, Johnson, and Lynch (1947), who report ninhydrin-CO_2 determinations on six specimens, with results ranging from 1.04 to 1.43 mg./100 ml.

Concerning urine levels, accurate data are limited. Thompson and Abdulnabi (1950), using the ninhydrin-CO_2 technique, give the average 24-hour output of free alpha-amino nitrogen as 126 ± 67 mg. for females (11) and 101 ± 39 mg. for males (13), with a combined mean of 113 mg./24 hours.

Eckhardt and Davidson (1949), by the same method, studied one case on varying protein intake, and found levels ranging from 63 mg. to 140 mg./24 hours. On a moderate protein intake the excretion was 118 mg./24 hours.

The gasometric method used in the two studies cited above does not estimate taurine (Van Slyke, Dillon, MacFadyen, and Hamilton, 1941). If one wishes to include this compound, which is excreted in large amounts, about 17 mg./24 hours must be added to the previous figures (Stein, 1953).

Stein (1953), using column separation and colorimetric ninhydrin estimation, has given figures for free urinary amino-acids on six normals. The values range from 97 to 145 mg. alpha-amino nitrogen/24 hours, with a mean of 120.

The present series gives a combined mean of 131.7 ± 31.1 mg. free alpha-amino nitrogen/24

hours, with a range of 75.8 to 203.2. It must be recalled (Table I) that only about one-third of the taurine is estimated by the present method. This error is roughly cancelled out by the error due to urea (see below). In view of the difference in technique and material, and the relatively small series reported in every case, the agreement between the previous and the present data is good.

Concerning the total alpha-amino nitrogen of urine, Thompson and Abdulnabi (1950) report the 24-hour output as 321 ± 138 mg. for females and 251 ± 66 mg. for males, with a combined mean of 283 mg./24 hours. Eckhardt and Davidson (1949) report a value of 575 mg./24 hours on their single case while on a moderate protein intake. Stein (1953) gives results on two cases as 356 and 455 mg./24 hours respectively.

The present series gives a mean of 289.3 ± 69 mg./24 hours, with a range of 186.4 to 426.2. In the present series, unlike the results reported by Thompson and Abdulnabi (1950), no significant sex difference was found, either for free or total alpha-amino nitrogen, when the results were calculated on a weight basis.

The data in Table I show that urea, creatinine, and hippuric acid all react to a slight extent. It can readily be calculated that the interference of the last two, even when present in abnormally large amounts, is negligible. With average urinary outputs of amino-acid and urea, the positive error due to urea is about 5%. If the amino-acid excretion were very low and the urea excretion very high, the error could reach 10%. In the reverse case of a low urea and high amino-acid excretion, the error would fall to about 2%. The error due to urea does not affect the results for total alpha-amino nitrogen, for urea is destroyed by the hydrolysis.

No attempt was made to remove urea from the sample before testing, or to correct for the actual urea content, because the principal use of a method for measuring free urinary amino-acids is in the screening for, and investigation of, cases of pathological amino-acid urea. In cases where the free amino-acid excretion is above the normal range, the error due to urea will drop to 1% or less, and may be ignored.

Summary

A simple variation of the colorimetric ninhydrin technique for estimating alpha-amino nitrogen is presented. Its application to plasma, cerebrospinal fluid, and urine is described. A series of normal values for these fluids obtained by the

present method is given. The present technique gives approximately the same values as other methods already described.

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