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CONDENSED REPORT ON THE
USE OF ENZYMATIC CYCLING FOR HIGH SPECIFICITY AND
PRECISION IN THE COLORIMETRIC ANALYSIS OF AMMONIA

by Frederick W. Carson

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FINAL- REPORT

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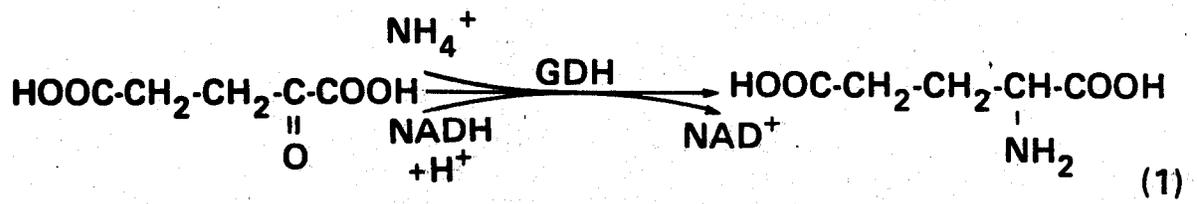
ABSTRACT

A new method for the colorimetric determination of ammonia in water by enzymatic cycling has been developed. It is simple and precise in the range of 10^{-4} to 10^{-5} ammonia concentrations. The sensitivity could be extended well below the 10 nmol per sample limit of determination of the assay described, by suitable modifications of the procedure. The ammonia-specific glutamate dehydrogenase reaction is followed by an enzymatic cycling reaction, to provide controlled; amplification of the response to ammonium ion. The cycling reaction product, a tetrazolium formazan, has a conveniently-measured visible absorption maximum at 600 nm. When the established procedure is followed, plots of absorbance vs. original ammonium ion concentration are linear, with correlation coefficients of 0.997 to 0.9998. The coefficient of variation was: 2% for a series of replicate measurements on samples containing 4.41×10^{-5} M ammonium ion. Standard solutions may be carried through the procedure to construct a calibration curve for the determination of unknown concentrations. Only volumetric equipment and a spectrophotometer or colorimeter are required. The analysis time per sample is 3 h. All solutions may be prepared in advance and are stable for at least two weeks.

INTRODUCTION

The value of the enzymatic method for the determination of ammonia has been well-established for use in the analysis of water, sediments, cell extracts, blood plasma, and urine (Levitzki, 1970; Ishihara *et al.*, 1972; da Fonseca-Wollheim, 1973, Jacobs S Olthuis, 1973; Verdouw, 1973; van Anken & Schiphorst, 1974; Bostick *et al.*, 1976; Bruce *et al.*, 1978; Ijpma *et al.*, 1978; Kalb Jr. *et al.*, 1978; Peshlamm *et al.*, 1978; Wu *et al.*, 1978; Doumas *et al.*, 1979; Humphries *et al.*, 1979). It is based on the production of gluteric acid from the reaction of α -ketoglutarate with ammonium ion catalyzed by the enzyme glutamate dehydrogenase (GDH)⁴, as shown in Equation 1. There is simultaneous, stoichiometric

*Abbreviations used: ADH, alcohol dehydrogenase(Alcohol: NAD oxidoreductase, EC 1.1.1.1): ADP, adenosine diphosphate; GDH, t-glutamic acid dehydrogenase(k-Glutamate:NAD(P) oxidoreductase, deaminating, EC 1. 4. 1.3) ; INT, 2- (p-iodophenyl) -3-p-nitrophenyl) -5-phenyltetrazolium chloride; MTT, 3- (4¹, 5•-dimethylthiazol-2,-yl) -2,5-diphenyltetrazolium bromide; PES, 5-ethylphenazinium ethyl sulfate; NAD⁺, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PHS, 5-methylphenazinium methyl sulfate; Tris, tris-(hydroxymethyl)-aminomethane.



~~PES (RED.)~~

~~PES~~

~~MITT~~

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(2)

oxidation of reduced nicotinamide adenine dinucleotide (NADH), the enzyme cofactor, to oxidized nicotinamide adenine dinucleotide (NAD⁺). This enzymatic reaction is specific for ammonium ion, with no known interference from amines, glutamine, or other organic compounds. Furthermore, it has been found to be free of interference from cations, a problem frequently encountered in water analysis (Bostick et al., 1976). The ammonia concentration is determined by monitoring the decrease in the 340 nm NADH absorption in most cases. Consequently, the sensitivity and precision of this method is limited by the precision with which small decrease in the large initial absorbance can be measured.

The method presented here utilizes enzymatic cycling (Lowry, 1973) for controlled amplification of the response of the glutamate dehydrogenase reaction to ammonium ion. Enzymatic cycling systems afford very high specificity. Furthermore, they offer the potential for quantitative detection of truly minute quantities of compounds through the adjustment of such experimental parameters as cycling time, temperature, and reactant and enzyme concentrations. The work described here utilizes the enzymatic reaction shown in Equation 1 to achieve selectivity and the cycling scheme shown in Equation 2 for amplification to produce a product absorbing strongly in the visible region of the spectrum. This combination was found to meet the following goals. It provides a specific, simple, inexpensive, reliable procedure for the routine analysis of ammonia at

concentrations typically found in biological fluids and polluted water. Furthermore, it has enough flexibility to offer the possibility of substantial improvement of its limit of determination it is more sensitive and specific than the chemical derivatization method previously developed in this laboratory, which responds to both ammonia and primary amines (Gross S Carson, 1977).

In the first. step, excess α - ketoglutarate and NADH are used to force the production of glutamic acid from, ammonia to completion.. This is feasible because of the large driving force for the reaction., $\Delta G^{\circ} = -8h3 \text{ kcal/mol}$. (Lehninger, 1975). As a result, NAD^+ is produced in an amount stoichiometric with the initial quantity of ammonium ion. After completion of the first step, the remaining excess of NADH is destroyed by treatment of the solution with acid, a process which also denatures the enzyme but does not affect the NAD^+ (Lowry et al., 1961). Thereafter, the NAD^+ is used as a catalyst in the cycling phase, Equation 2. The oxidation of ethanol by NAD^+ is catalyzed by alcohol dehydrogenase (ADH). Oxidation of the resultant NADH is coupled to the irreversible reduction of 3-(4',5'-dinethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (NTT; thiazolyl blue) to its formazan through the electron carrier 5-ethylphenazinium ethyl sulfate (PES).

Use of these components in a cycling assay for NAD^+ has been described previously (Bernofsky E Swan, 1973). NTT formazan absorbs strongly in the visible region of the spectrum, is

stable, and does not precipitate under the conditions used here. All reagents in the cycling mixture, including the tetrazolium salt, are present in sufficient excess so that the reaction rate is dependent only upon the added NAD⁺. Then the rate of formation of the formazan, followed by the increase in the absorbance at 600 nm, is proportional to the NAD⁺ concentration.

MATERIALS AND METHODS

Chemicals and enzymes. Chemicals were purchased from Sigma Chemical Co., Sigma Chemical Co., Saint Louis, MO, unless otherwise indicated. ADH was Sigma Product No. A 3263, Alcohol Dehydrogenase (233 IU/mg), from Yeast, 90% protein. ADP was Sigma Product No. A 0127, Adenosine 5'-Diphosphate, Grade I: Sodium Salt, from Equine Muscle, 95-99%. Ammonium Chloride, granular reagent, ACS grade, was purchased from Matheson, Coleman and Bell; Norwood, OH. Ethanol, Absolute, Reagent quality, was purchased from U.S. Industrial Chemicals Co., Tuscola, IL. GDH was Sigma Product No. G 7882, L-Glutamic Dehydrogenase (44 IU/mg). Type III: from Bovine Liver, lyophilized powder, 76% protein, free Ammonium Ion content <0.03 µg/mg protein. Hydrochloric Acid, concentrated analytical reagent, came from Mallinckrodt Chemical Works, Saint Louis, MO. Hydrochloric Acid, Standardized 1.000 N was a Fisher Certified Reagent, from Fisher Scientific Co., Fair Lawn, NJ. α -Ketoglutaric Acid was Sigma Product No. K 1750, Free Acid. MTT was Sigma Product No. M 2128, 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium Bromide; Thiazolyl Blue. NADH was Sigma Product No. M 8129, β -Nicotinamide Adenine Dinucleotide Reduced Form, Disodium Salt, Grade III: approx. 984. PES was Sigma Product No. P 4883, Phenazine Ethosulfate. Sodium Hydroxide, Standardized 1.000 N and 10.00 N Solutions were Fisher Certified Reagents, from Fisher Scientific Co., Fair Lawn,

NJ. Tris was Ultra-pure Tris (hydroxymethyl) aminomethane from Schwarz/Mann, Orangeburg, NY.

Reagent solutions: Metal-free water with conductivity $<1 \times 10^{-4}$ (1^{-1} -cm $^{-1}$) was prepared by deionizing tap water using a Corning 400377 Ultra High Purity mixed bed resin and distilling the product twice through a quartz apparatus. All aqueous solutions were prepared from the purified water described above and stored at 4° C. Tris buffer was adjusted to pH, 8.0 and a final concentration of 0.100 M with, 1.000 N hydrochloric acid.. Ammonium chloride dried at 110° was used to prepare a 1×10^{-3} M (5.901" mg/liter) stock solution (Taras et al., 1971). This was diluted as necessary with metal-free water.

Reagent A. The assay reagent required for the reaction shown on in Equation 1 is prepared as follows . A 2.00 ml aliquot of a 6.29×10^{-3} M solution of NADH in 0.100 M Tris, pH 8.0 (prepared by dissolving 4.689 g of NADH in stock Tris solution and making it up to a volume of 1.000 liter) is added to 100 ml of a solution containing 2.02×10^{-3} M ADP (1.001 g/liter), 4.67×10^{-8} M GDH (Me 332 000; 0.0204 g/liter), and 5.99×10^{-3} M α -ketoglutarate (0.8746 g of α -ketoglutaric acid/liter) in stock Tris. The ADP is included to stabilize the enzyme (Frieden, 1963, 1965). This mixture is stored in a dark container for at least 2 h at room temperature. Protection from light assures maximum NADH stability (Bergmeyer, 1974). The result of this treatment is quantitative conversion of ammonia, which

contaminates most ADP preparations, to glutamic acid. At this point, sufficient concentrated sodium hydroxide (approximately 1.3 ml of 10.00 N base) is added to raise the pH to 12.0 and the solution is heated to 65°C for 15 min in order to destroy NAD⁺ produced in the preceding reaction (Lowry & J-L, 1961). This treatment denatures the GDR. After cooling the solution to room temperature, enough concentrated hydrochloric acid (approximately 1.0 ml) is added to lower the pH to 8.0. Fresh, solid GDH (2.26 mg) is added to give a final active enzyme concentration: of 4.96×10^{-8} M. Approximate final concentrations of other components are: ADP; 1.94×10^{-3} M; α -ketoglutarate, 5.74×10^{-3} M, and NADH, 1.20×10^{-4} M in 0.098 M Tris, pH 8.0. The Solution is filtered through a polycarbonate membrane of 1 μ m pore size and stored under argon at 4° C in an amber glass container. The reagent prepared in this way now gives a stoichiometric reaction with added ammonium ion, with little or no blank reaction, and is stable at 4°C for at least two weeks.

Preparation of reagents required for the enzymatic cycling reaction shown in Equation 2 is described below.

Reagent B. Dissolve 12.45 mg (7.47×10^{-6} mol) of yeast ADH, MW 150 000, in 25.00 ml of water. The resultant 2.99×10^{-6} M solution of ADH is filtered through a polycarbonate membrane of 1 μ m pore size and stored under argon at 4°C in an amber glass container.

Reagent C. Dilute 0.80 ml (1.371×10^{-2} mol) of absolute

ethanol to 50.00 ml with 0.1000 M Tris buffer, pH 8.0, to give 0.274 I ethanol in 0.098 a Tris, pH 8.0. Store the solution at 4⁰C.

Reagent D: Dissolve 63.49 mg (1.532×10^{-4} mol) of MTT and 21.2 mg (6.355×10^{-5} mol) of PES in water and make up the volume to 50.00 ml. The resultant solution of 3.06×10^{-3} M MTT and 1.27×10^{-3} M PES must be stored at 4° C under argon in an amber glass container to avoid decomposition.

Equipment. A Beckman DB Spectrophotometer equipp4d Pith, a Hewlett-Packard 7101BM strip chart recorder and a cell compartment thermostated at $25.0 \pm 0.1^{\circ}$ C was.. used: for spectral measurements. Quartz cuvettes had 1.000 cm path lengths. Kartrell disposable polystyrene 1 cm cuvettes, Cat. No. 219, from Dynalab Corp., Rochester, NY, with a stated precision of $\pm 1\%$, were used for cycling assay measurements.

A number, of assays were performed- by running reactions,: in a constant temperature bath thermostated at $25.0 \pm 0.1^{\circ}$ C. However, it was found that reproducible results could be obtained without temperature control of either the reaction mixtures or the cell compartment so long aS standards and analytical samples were assayed concurrently at ambient temperature.

Polycarbonate membranes with a 1.4 μ m pore size for filtering enzyme solutions were products of Nuclepore Corp., Pleasanton, CA.

Assay Procedure. Application of the enzymatic cycling method for the assay of ammonia is summarized in the four steps below. The volumes used in Steps 1 and 2 may be proportionately decreased by as much as a factor of twenty if the sample size is a limiting factor.

Step 1: The reaction shown in Equation 1 is run by mixing 0.50 ml of the ammonia sample to be analyzed with 1.50 ml of Reagent A. The reaction is allowed to proceed to completion for 2.5 h, at room temperature in the dark.

Step 2: Excess NADH is destroyed by adding 0.12 ml of 1,000 hydrochloric acid to the sample from Step 1 and allowing it to stand at room temperature for 5 min. Thereafter, the reaction mixture is neutralized with 0.12 ml of 1.000 N sodium hydroxide.

Step 3: The enzymatic cycling reaction shown in Equation may be run in a spectrophotometer cuvette. A 0.10 ml aliquot of the reaction mixture from Step 2 is added to a mixture of 0.10 ml of Reagent B, 2.60 ml of Reagent C, and 0.20 ml of Reagent D. Final reactant concentrations in the resultant 3.00 ml sample are: ADH, 9.96×10^{-8} M; ethanol, 0.238 M; MTT, 2.04×10^{-4} M; and PES, 8.47×10^{-5} M in 0.087 M Tris, pH 8.0.

Step 4. The absorbance at 600 nm is measured at the beginning of the cycling reaction. Subsequently, the sample is maintained at 25°C in the dark for 30 or 60 min, at which time the 600 nm absorbance is measured again.

RESULTS

Results obtained by following the above procedure with standard solutions of ammonium chloride are presented in Figure 1. They demonstrate a linear response of the system at cycling times of 30 and 60 min. Slopes are 0.058 ± 0.002 and 0.121 ± 0.004 liter-mol⁻¹, ordinate intercepts are 0.04 ± 0.01 and 0.11 ± 0.03 , and correlation coefficients are 0.997 and 0.998, respectively. The precision of each estimate above is given as + 1 SD. Initial values of the 600 nm absorbance ranged from 0.009 to 0.019 in these experiments. Thus, samples of unknown ammonium ion concentration may be assayed with good precision by reference to standard curves, similar to those plotted in Figure 1, constructed from results with standards run concurrently.

Results of experiments to assess the stability of the reagents during storage are presented in Figure 2, which covers a period of two weeks. The absorbance change at 600 nm after 30 minutes resulting from the enzymatic cycling reaction is plotted as a function of the original ammonium ion concentration using a set of solutions prepared on the same day and stored under identical conditions. It is seen that the lines are displaced from each other slightly but the response is still linear. Slopes range from 0.053 to 0.059 liter-mol⁻¹ with SD's from 0.001 to 0.002 liter-mol⁻¹; ordinate intercepts lie between 0.10 and 0.15 with SD's

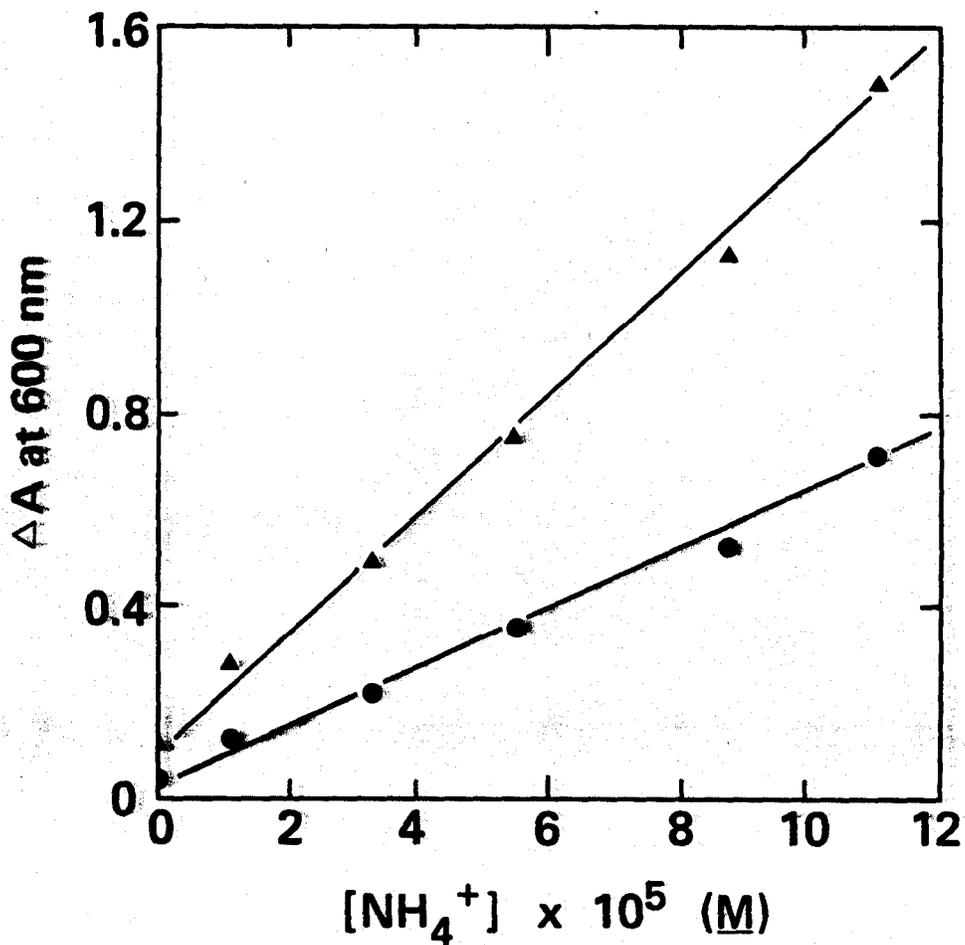


Fig. 1. Absorbance change at 600 nm at cycling times of 30 (•) and 60 (&) min observed in the analysis of standard ammonium chloride solutions. The concentrations refer to ammonium ion in the samples prior to any of the steps involved in the assay. Refer to the Materials and Methods section for reagent concentrations and the procedure.

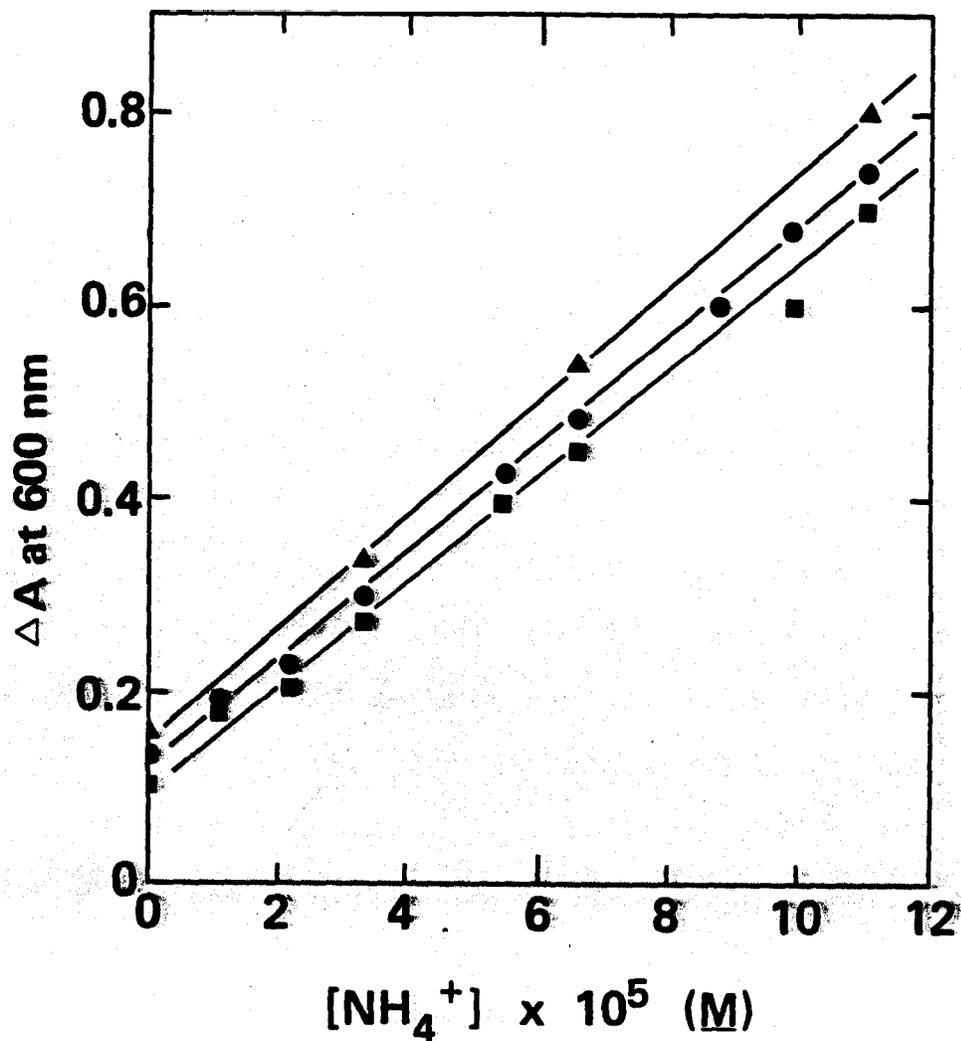


Fig. 2. The effect of aging on the absorbance change at 600 nm at a cycling time of 30 min observed in the analysis of standard ammonium chloride solutions. The concentrations refer to ammonium ion in the samples prior to any of the steps involved in the assay. Refer to the Materials and Methods section for reagent concentrations and the procedure. Reagents were prepared on the same day, stored under identical conditions at 4°C in the dark, and aliquots were used as follows: a , 1 day;* , 7 days; and,14 days after preparation.

Between 0.006 and 0.01; and correlation coefficients are 0.997 to 0.9998. Initial values of A at 600 nm ranged from -0.014 to 0.009 in these studies. Enzyme preparations became cloudy after about 3 weeks of storage at 4° C, suggesting significant protein denaturation.

To evaluate the precision of the method, 8 samples containing 4.4×10^{-5} M ammonium ion were carried through the entire procedure, giving a coefficient of variation (CV) of 2% after a cycling time for 30 min. Initial values of the 600 nm absorbance ranged from 0.003 to 0.020 in these experiments. An error analysis indicated that volumetric errors could account for 0.2% of this variation, a 15-second cycling time error would contribute an additional 0.8%, and spectrophotometer precision errors were 1.6% at A=0.75 and increased with decreasing absorbance.

DISCUSSION

The conditions chosen for the GDH reaction shown in Equation 1 were dictated in part by; the desire to use reagents which are stable for at least one week at 4⁰C. GDH in 0.100 g Tris buffer was found to denature significantly overnight at 4⁰C (R. D. Huhn, unpublished observations), in accord with the findings of Bruce et al.; (1978). Furthermore, this difficulty is exacerbated by low concentrations of NADH (Di Prisco E Strecker, 1966; Smith et al., 1975; Cross & Fisher, 1970)

However, ADP is known to be one of the best stabilizers of this hexameric enzyme. It activates GDH and prevents NADH-induced dissociation by binding to an allosteric regulatory site (Frieden, 1963, 1965; Di Prisco & Strecker, 1966; Cross & Fisher, 1970; Fisher et al., 1972; Fisher, 1973; Schmidt, 1974; Smith et al., 1975). Hence, ADP was included in the GDH preparation.

However, all commercial preparations of ADP tested proved to cause large blank reactions, presumably because of contamination with significant amounts of ammonium ion. Therefore, the stratagem described for the preparation of reagent A was devised. Incubation of the ADP with α -ketoglutarate and NADH in the presence of GDH eliminates the contaminant within 2 h. This was established by monitoring the disappearance of NADH spectrophotometrically at 340 nm. Subsequent treatment with base destroys not only the GDH, but also both NAD⁺ generated: in this reaction and any pre-existing NAD⁺ contaminating the NADH as supplied commercially (Margolis et al, 1976). Finally, addition of fresh enzyme provides a reagent which gives a tolerably small blank reaction, as shown in Figure 1.

The reaction pH of 8.0 was chosen because it is the pH optimum for GDH in the presence of ADP (Smith et al., 1975). At the same time it is high enough to assure adequate stability of the NADH without danger of denaturing the enzyme. The reaction time of 2.5 h was established by monitoring the disappearance of NADH at 340 nm in samples

Containing high concentrations of ammonia.

PES was chosen as the intermediate electron carrier of Equation 2 on the basis of the following considerations. A combination of 5-methylphenazinium methyl sulfate (PMS) and 2-(p-iodophenyl)-3-p-nitrophenyl) -5-phenyltetrazolium chloride (INT) is commonly used for electron transport from NADH in enzymatic cycling systems (Nachlas et al., 1960; Babson & Babson, 1973). However , preliminary work showed that PMS gave variable results, presumably because it is both unstable and photolabile (McIlwain, 1937; Ghosh& Quayle, 1979). PES appeared to be more stable and gave more consistent results. The superiority of PES over PMS found in this study confirms the conclusions of Bernofsky and Swan (1973) and reinforces the recommendation of Ghosh and Quayle (1979) that PES be substituted for PMS wherever possible.

Preliminary work using INT as the final electron acceptor was complicated by the necessity of employing albumin, gelatin or detergents to prevent precipitation of the formazan product (Nachlas er al., 1960; Babson & Babson, 1973; Mollering et al., 1974). In contrast, it was found that the formazan resulting from reduction of MTT did not produce a noticeable precipitate under the conditions used here. Consequently, the PES-MTT electron transfer system was adopted.

The most troublesome feature of the assay encountered during its development was a persistent blank reaction. Although the process used to prepare Reagent A eliminated

Most of this problem, presumably by destroying adventitious ammonia and NAD^+ , a significant blank reaction persists, as shown by the nonzero ordinate intercepts in Figures 1 and 2. Probable causes of this difficulty are under investigation. Reasonable possibilities include contamination of reagents and glassware with ammonia (Folbergrova et al., 1969; Kalb et al., 1978; Nazar & Schoolwerth, 1979), slow oxidation of the NADH in Reagent A to NAD^+ (Lowry et al., 1961; miksic & Brown, 1977), contamination of the ADH in Reagent B with NAD^+ (Bernofsky & Swan, 1973), and decomposition of the PES-MTT system, both in Reagent D and in the assay mixture (Bernofsky & Swan, 1973; Gohsh & Quayle, 1979).

The reproducibility of the blank reaction seen in Figures 1 and 2 is a factor limiting the sensitivity of the assay described. According to the definition given by Currie (1968), the limit of determination in an assay is dependent on the value for any blank reaction plus 10 times the SD of the net signal. The variance of the net signal, SD^2 , is the sum of the variance of the blank and that of the observed signal. These were found to be relatively constant, the mean blank SD being 0.0060 and the mean observed signal SD being 0.0092 for the 30 min assays. Thus, $\text{SD}^2 = (0.0060)^2 + (0.0092)^2$ and $10\text{SD} = 0.110$. In other words, the limit of determination for this procedure is the ammonia concentration giving a response 0.110 absorbance units that of the blank. This is just 10SD divided by the slope of each line in Figure 1 or 1.85×10^{-5}

to 2.06×10^{-5} M. Thus, the limit of determination for this assay is 2×10^{-5} A or 10 nmol of ammonia per sample. A volume reduction of a factor of ten in Steps 1 and 2 could be expected to lower this limit to 1 nmol per sample. The limit of detection (Currie, 1968) may be calculated similarly by dividing 3SD by the slope and is expected to be 150 pmol if samples of minimum volume, 0.025 ml, are used. Further possibilities for improving the sensitivity of the assay described are under investigation.

The assay described was developed for ready applicability to the 10^{-4} to 10^{-5} M ammonium ion concentrations generally found in natural and polluted fresh water as well as in blood (Routh, 1976; Bruce et al., 1978; Imler et al., 1979). Consequently, the sensitivity is comparable to that of other spectrophotometric methods based on the GDH reaction (Kalb et al., 1978). However it employs colorimetric monitoring of the NBT formazan production in the visible portion of the spectrum at 600 nm ($\epsilon = 11,100 \text{ liter-mol}^{-1} \text{ cm}^{-1}$) and is inherently more precise than methods which monitor the disappearance of NADH or NADPH in the ultraviolet region of the spectrum at 340 nm. This comes about because NADH absorbs strongly at 340 nm, $E = 6.22 \times 10^3 \text{ liter-mol}^{-1} \text{ cm}^{-1}$ (Beaucamp et al., 1974), and is always supplied in large excess. Consequently, small differences between large absorbances must be measured. Furthermore, the output of tungsten-filament lamps used in the most economical colorimeters is much higher at 600 nm

than at 340 nm (Willard et al, 1974), permitting operation at such lower lamp temperatures at 600 nm, with consequent improvement in output stability and lamp life.

A number of other methods for the determination of ammonia have been developed. For example, fluorometric analyses which monitor the GDH reaction have been developed (Bubin & Knott, 1967; Folbergrova et al, 1969). Nazar and Schoolwerth (1979) have reported nanomole sensitivity for the latest version of this assay. Application of the rigorous conservative statistical analysis of Currie (1968) to their data, shows that their limit of determination is somewhat above 25 nmol, considerably higher than the 10 nmol, value calculated for the method; reported in this paper. In this respect, the assay described here requires far simpler apparatus and is more economical than any methods of higher sensitivity, such as those based on radioassay (Kalb et al., 1978), high pressure liquid chromatography (Chen & Farquharson, 1979), or the modified protein sequencer of Ankapiller and Hood (1980). Furthermore, it does not suffer from the interferences and lack of specificity encountered in strictly chemical assays, which have been reviewed (Bostick et al., 1976; Kalb et al., 1978). Lastly, by variation of such parameters as reactant volumes, enzyme concentrations, and cycling time, the system may be made responsive to a wide variety of concentrations. Therefore, this method offers the potential for extremely high sensitivity.

In conclusion, the colorimetric, enzymatic cycling assay for ammonia described here offers a number of advantages: simple, inexpensive apparatus is involved; precision is good (CV 296) to at least 2×10^{-5} M or 10 nmol per 0.5 ml sample and suitable modifications could be expected to reduce the sample size requirement by at least a factor of 10; specificity is high and interferences are unknown; all chemicals and enzymes are commercially available; and all reagents may be prepared in advance and are usable for at least two weeks when stored at 4° C.

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FOOTNOTES

¹Contents of this publication do not necessarily reflect the views and policies of the office of Water Research and Technology, U. S. Department of the Interior, nor does mention of trade names or commercial products constitute their endorsement or recommendation for use by the U. S. government.

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⁴Abbreviations used: ADH, alcohol dehydrogenase (Alcohol: NAD oxidoreductase, EC 1. 1. 1. 1); GDE L-glutamic acid dehydrogenase (L-Glutamate; NAD(P) oxidoreductase, deaminating, EC 1.4. 1.3) ; INT, 2-(p-iodophenyl)-3-p-nitrophenyl)-5-phenyltetrazolium chloride; MTT, 3-(4',5'-dinethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, 5-ethylphenazinium ethyl sulfate; PMS, 5-methylphenazinium methyl sulfate.