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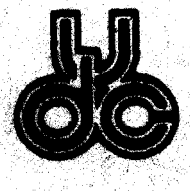

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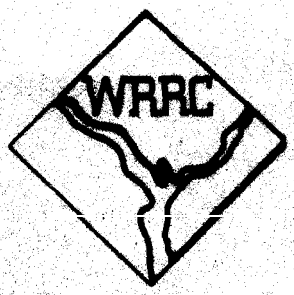

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Determination of Nitrite and Nitrate in Water by Reduction to Ammonia

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September 1982

RMINATION OF NITRITE AND NITRATE IN WATER BY REDUCTION TO AMMONIA FOLLOWED BY ENZYMATIC CYCLING[1] by

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FINAL REPORT

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ABSTRACT

A procedure has been developed to determine the concentration of biologically significant nitrogen present as ammonia, nitrite and nitrate in water samples. In addition, the concentration of each of these constituents may be determined separately if desired. The method is sensitive and not subject to the interferences commonly encountered in nitrate determinations. It involves the reduction of nitrite and/or nitrate to ammonia with Devarda's metal while simultaneously trapping the released gaseous ammonia with dilute hydrochloric acid solution in a modified Conway diffusion cell. Subsequently, the ammonia produced is determined using the enzymatic cycling assay previously developed by Carson and Davies. Standard solutions of ammonium chloride must be carried through.the procedure to prepare a standard curve from which unknown concentrations of nitrate may be determined. Using the established procedure, plots of Absorbance change at 600 nm versus original nitrate concentration were linear, with correlation coefficients ranging from 0.991 to 0.999. A series of replicate measurements had a coefficient of variation of 3% for samples containing 2.70×10^{-5} M nitrate ion when compared to a such a standard curve.

Devarda's metal reduces both nitrite and nitrate. Nonetheless, individual concentrations of nitrogenous components could be determined as follows: the procedure described would give ammonia plus nitrite plus nitrate; treatment of a sample with purified sulfamic acid solution prior to the analysis would destroy the nitrite and the assay would quantify ammonia plus nitrate; omission of the reduction step would lead to detection of ammonia alone in the enzymatic cycling analysis. Hence, each component could be determined by difference.

The equipment required consists of standard volumetric glassware, Eppendorf pipettes, modified Conway diffusion cells and a spectrophotometer or colorimeter. The reagents used are relatively inexpensive and safe to handle. The reduction is carried out at room temperature overnight or for 16 hours using 1.00 ml of sample.

INTRODUCTION

The number of analyses for nitrate-nitrogen is greater than that for any other form of nitrogen in water and waste water because it is the most difficult to determine and subject to the greatest number of interferences. The five main methods for determination of nitrate are reduction to ammonia; manual methods using chromogenic agents; direct spectrophotometry; ion selective electrode methods; and reduction to nitrite.

The measurement of nitrate by UV spectrophotometry is useful only in clean water due to interferences from turbidity and from both inorganic and organic matter (1,2). Colorimetric methods suffer from serious interferences, poor reproducibility, poor sensitivity, or tedious procedures and undesirable reagents (1). Nitrate-specific electrodes show interferences due to $C1^-$ and $HC0_3^-$. Reduction of nitrate

to ammonia involves either a tedious and time-consuming steam distillation to determine ammonia or use of an ammonia electrode. The ammonia electrode is extremely sensitive to temperature changes and there is a problem of loss of ammonia in alkaline solution due to temperature changes (3). The

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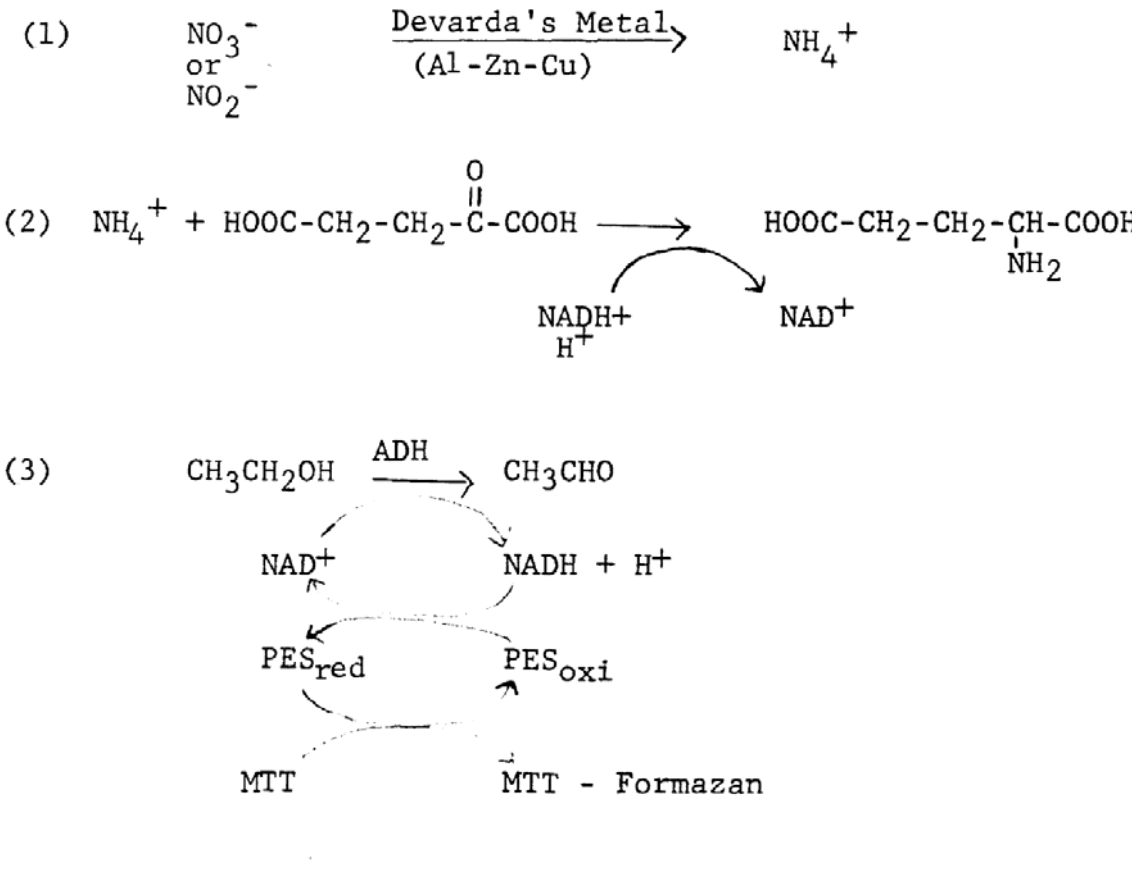
Abbreviations used:
ADH; alcohol dehydrogenase (Alcohol:NAD oxidoreductase, EC
ADP: adenosine 5'-diphosphate;
α-KG, α -ketoglutarate
GDH, L-glutamic acid dehydrogenase (L-Glutamate:NAD(P) oxidoreductase, deaminating, EC 1.4.1.3);
MIT, 3-(4',5'-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
NADH, nicotinamide adenine dinucleotide, reduced;
PES, 5-ethylphenazinium ethyl sulfate.
Tris, tris-(hydroxymethy)-amincmethane

reduction methods to convert nitrate to either ammonia or nitrite typically are applicable only to high concentrations (> 5mg/l $N0_3$-N) or minute levels (< 0.lmg/l NO_3 - N) (4).

The procedure developed here for the determination of N03-N is useful in the intermediate range of nitrate levels (1 -6 mg/l N03-N). This method involves the reduction of nitrate to ammonia by Devarda's metal in the presence of NaOH and trapping of the ammonia produced in dilute hydrochloric acid simultaneously in a modified Conway diffusion cell (5). The ammonia produced is then analyzed by the enzymatic cycling system developed by Carson and Davies (6), as shown in scheme I.

SCHEME I



The ammonia produced is trapped in the center well of the Conway cell. Thus, there are no interferences from Cl^-, $HC0_3$-, or other inorganic or organic matter in the sample unless it can be reduced to ammonia. Nitrite will also be reduced to ammonia, but it can be removed by treating the sample before analysis with purified sulfamic acid (7). The determination of ammonia is based on an enzymatic reaction which is absolutely specific for the ammonium ion.

The reduction of nitrate by Devarda's metal has been used for many years in soil and water analysis (8,9) and in modified Kjeldahl procedures. (10) for concentrations greater than 50 ppm. A microdiffusion method for nitrate in the concentration range of 1-20 ppm has been reported (11). However, the coefficients of variation in the 1-2 ppm range were relatively high. Using enzymatic cycling to determine the ammonia produced gives good results at the 1 ppm concentration level.

A high blank has been reported to be a problem using Devarda's metal with very low nitrate concentrations (12).

This was a potential problem in the work reported here as well. However, the blank was substantially reduced by using sodium hydroxide as the base instead of potassium carbonate (13) and by using more dilute solutions than those reported previously (14).

This method was developed in three steps. First, a quantitative method for reducing nitrate to ammonia was sought; second,-the best analytical conditions were determined; and third, the enzymatic cycling system was employed to determine the ammonia produced. The procedure was then tested using standard $NaNO_3$ solutions and evaluated against standard curves prepared using standard NH_4C1 solutions.

MATERIALS AND METHODS

I. CHEMICALS

Chemicals used except for those listed below were from Sigma Chemical Co., Saint Louis, Missouri, and were used as received.

Tris (tris-(hydroxymethyl)-amino methane), ultra pure, came from Schwartz/Mann, Orangeburg, New York.

Ammonium chloride, granular, reagent, A.C.S. was from Matheson, Coleman and Bell, Norwood, Ohio.

Absolute ethanol, reagent quality, was from U.S. Industrial Chemical Co., Tuscola, Illinois.

pH Buffer Solutions; 1.000 N, 10.00 N, 0.0100 N sodium hydroxide; 1.000 N, 0.0100 N Hydrochloric acid, sodium nitrate, and Devarda's metal were Fisher Certified Reagents, from Fisher Scientific Co., Fair Lawn, New Jersey.

Concentrated hydrochloric acid, analytical reagent, came from Mallinckrodt Chemical Works, Saint Louis, Missouri.

NPX Tergitol (non-ionic wetting agent) came from Union Carbide Chemical Co.

II. EQUIPMENT AND TECHNIQUES

A Beckman Model DB Spectrophotometer was used for spectral measurements. Data were recorded as per cent transmittance and converted mathematically to the corresponding Absorbance

values. Disposable plastic cuvettes (Kartell) were used for measurements at 600 nm and had a 1-cm path length. Quartz cuvettes, were used for measurements at 340 nm.

Some volumetric measurements were made with Eppendorf pipettes (10-100,p1 and 100-1000 Al) and an Oxford Macropipette (1-5 ml). A glass volumetric pipette (3.00 ml) was used for the enzyme solution.

III. PREPARATION OF SOLUTIONS

All solutions were prepared using standard volumetric glassware. Metal-free water (quartz-distilled, with conductivity < 1.0-5-f-L- -1 cm^{-1}) was used in the preparation of all solutions. Solutions were stored at 4^0 C unless otherwise noted. Those expected to be light sensitive were stored in amber glass containers.

Tris buffer (.0.100 M):

(Tris: tris(Hydroxymethyl)amino methane.)

Tris (12.112 g) (M.W. 121.1) was dissolved in conductivity water, adjusted to pH 8.0 with 1.0 N hydrochloric acid and diluted to 1000 ml.

MTT-PES in water (MTT: 3.051×10^{-3} M: PES: 1.254×10^{-4}): (MTT:

3-(4',5'-dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide;

PES: 5-ethyl phenazinium ethyl sulfate.)

MTT was Sigma No. M-2128, PES was Sigma No. P-4883. MTT (63.21 mg) and PES (20.90 mg) were dissolved in conductivity water and diluted to 50.0 m1.

NADH in Tris (6.29×10^{-3} M) (M.W. 745.4):

(NADH: nicotinamide adenine dinucleotide, reduced) NADH was Sigma, disodium salt, Grade II, (.Sigma No. N-8129).

NADH (23.44 mg) was dissolved in Tris buffer (pH 8.0) and diluted to 5.0 ml.

Ethanol in Tris (.1.3706 M):

Absolute ethanol (4.00 ml) was diluted to 50.0 ml in Tris Buffer.

Alcohol Dehydrogenase (ADH) in water (2.963×10^{-6} M): ADH was Sigma:

ADH from Yeast, 89.69% protein, No. A-3263) (M.W. 150,000).

ADH (.12.39 mg) was dissolved in water and diluted to 25 ml. The solution was filtered through a 1 micron polycarbonate membrane (Nuclepore Corp., Pleasanton, CA).

ADP in Tris (2.033×10^{-3} M) (M.W. 496.1): (ADP:

adenosine 5"-diphosphate)

ADP was Sigma Grade I, sodium salt, from equine muscle, 95-99% protein, No. A-0127.

ADP (100.88 mg). was dissolved in 100 ml of Tris buffer (pH 8.0).

o(-Ketoglutarate (x,-KG) in Tris-ADP ($6.00x10^{-3}$ M) (M.W. 146.1) and Glutamate Dehydrogenase (GDH) ($.4.67x10^{-8}$ M) (M.W. 332,000) in Tris-ADP:

c-/,-KG was Sigma: No. K-1750; GDH was Sigma: L-Glutamic Dehydrogenase, from Bovine Liver, Type III, Lyophilized powder, 76.00% protein, Free Ammonium Ion Content < 0.03)Ug/mg protein, No. G-7882.

of-KG (87.46 mg) and GDH (2.04 mg) were added to 100 ml of Tris-ADP and the solution was filtered through a 1 micron polycarbonate membrane.

Ammonium Chloride Standard Solution ($1.10x10^{-4}$ M) (M.W. 53.50): Ammonium chloride (0.59011 g) was dissolved in and then diluted to 1000 ml with conductivity water.

Several reducing agents were tried because a high blank was obtained in preliminary studies involving reduction of nitrate with Devarda's metal. However, these other reducing agents proved unsuccessful for a variety of reasons. Sn (II) could not be used because it produced a variety of products besides ammonia (.15). Reduced iron gave a colored solution that interfered with the spectrophotometric measurements. Sodium dithionite was investigated as a reducing agent, but the product formed also reduced NAD+ to NADH, which would interfere with the enzymatic cycling (16). Al has been reported as reducing nitrate to ammonia (17). However, it was found that the reduction was not quantitative. It was during the testing of the reduction with aluminum (see below) that it was discovered that part of a blank reaction was due

to an apparent impurity in the potassium carbonate. Hence, sodium hydroxide was employed as base thereafter. The reduction of nitrate to ammonia was done in 43 mm modified Conway diffusion cells in order to prevent the loss of ammonia into the air. The diffusion of the ammonia produced into 0.0100 N HCl alleviates the problems of interferences from compounds in the sample and precipitation of metal hydroxides that form when the pH is adjusted to determine ammonia directly on the sample solution.

The first attempts at nitrate reduction were done following O'Deen and Porter's method in 83 mm modified Conway cells instead of glass tubes (.18). The trapping solution consisted of 2.00 ml of 1.000 N HCl while 1.00 ml of 13.00 N NaOH was in the sample well along with 4.00 ml of sample and 40-50 mg of Devarda's, metal. After 6 or more hours, 0.500 ml aliquots were taken from the center well and neutralized with 0.500 ml of 1.000 N NaOH. After several attempts, it was determined that the calculated quality of NaOH did not neutralize all the acid as it should have on checking the pH with pH paper. It was found that the total volume in the center well had decreased and the acid was more concentrated. Apparently, the strong alkali solution in the sample and closing well was acting as a dehydrating agent. This would not have been a problem when the total excess acid in the center well was titrated (19).

Boric acid was tested as a trapping solution, but it interfered in the enzymatic cycling step. Then 2.00 ml of 0.0100 N HC1 was used in the center well, 4.00 ml of sample plus 70 mg of Devarda's metal and 1.00 ml of 45% w/v K2CO3 in the sample well and 2.00 ml of 45% K2CO3 in the closing chamber. Again, it was found that there was a loss of volume in the center well. In addition, there was a large blank.

Finally, it seemed necessary to take the entire volume from the center well to alleviate this problem. Smaller cells were used with a smaller sample size. After the reduction was tested with elemental aluminum using first NaOH and then K_2CO_3, it was found that there was a substantial decrease in the blank when using the NaOH. The best conditions were then determined

An 0.500 ml aliquot of 0.0100 N HC1 was added to the center well and 1.00 ml of sample containing from 1.34 to 6.70 mg/1 NaN03 was added to the sample well. About 100 mg of Devarda's metal was added to the sample well. To the closing chamber was added 2.00 ml of 2.00 N NaOH and finally 0.50 ml of 2.00 N NaOH was added to the sample chamber and the lid immediately placed on the cell. After 16 or more hours at room temperature, the entire volume was removed from the center well with an Eppendorf pipette. Since the cells and pipette tips are plastic, there is almost no adherence of the solution to the plastic surfaces. The solution was

neutralized with 0.500 ml of .0100 N NaOH and the pH was 7.0 when tested with pH paper. A volume of 70 /cl of H2O was added to bring the total volume to 1.00 ml.

The second part of the procedure was to determine the ammonia produced by enzymatic cycling.

The procedure is as follows:

Tris (12.112 g) was dissolved in about 400 ml of conductivity water and the pH was adjusted to 8.0 with 1.00 N HC1. The volume was brought to 1000 ml. ADP (0.10088 g) was dissolved in the Tris buffer and diluted to 100 ml. After thorough mixing, the Tris-ADP was transferred to a 125 ml Erlenmeyer flask. (α-KG (0.08746) and GDH (0.00204 g) were dissolved together in a 100 ml aliquot of Tris buffer. NADH (0.02345 g) was dissolved in 5 ml of Tris buffer. A 2.00 ml aliquot of this solution was added to the GDH solution and allowed to stand at room temperature for 2.0 hours. The pH was adjusted to 12.0 with 10.00 N NaOH and the solution was heated for 15 minutes in a 60^0 C water bath. The solution was cooled to room temperature and the pH adjusted to 8.0 with concentrated HC1. Then 0.00230 g of GDH was added and the solution was filtered through a 1 micron polycarbonate membrane. To the 1.00 ml of solution from the nitrate reduction was added 3.00 ml of GDH solution. The solution was mixed and allowed to stand at room temperature for 2.5 hours.

Reagents for the second part of the enzymatic cycling are as follows:

(1) Absolute ethanol (4.00 ml) dissolved in 50 ml of Tris buffer.

(2) ADH (0.01239 g) in 25 ml of conductivity water.

(3) MTT (0.06321 g) plus PES (0.02090 g) in 50 ml of conductivity water.

After 2 hours, 0.24 ml of 1.000 N HC1 was added to each sample and mixed. After 5.0 minutes, 0.24 ml of 1.000 N NaOH was added to each sample and mixed. Then 0.100 ml of sample was added to a plastic cuvette containing 2.60 ml of ethanol in Tris buffer, 0.100 ml of ADH in water, and 0.200 ml of MTT-PES in water. The samples were mixed and the Absorbance was measured at time zero and at 1.00 hour. Samples were kept in the dark at room temperature during this waiting period.4 A was calculated by subtracting the Absorbance at time zero from the Absorbance at 1.00 hour. Absorbance was plotted versus original nitrate concentration and versus calculated ammonia concentration in the GDH solution. The nitrate reductions were done in triplicate and the final AA's at 600 nm were averaged.

Standard ammonium chloride solution was made by dissolving 0.59011 g of NH_4C1 in conductivity water and diluting to 1000 ml to give $1.10x10^{-4}$ M ammonium ion. A standard curve was prepared using 0.100 ml to 1.000 ml of this standard solution diluted to 1.000 ml with conductivity water in the assay. Samples for the standard curve were carried through the two parts of the enzyme cycling procedure exactly as explained above.

RESULTS

Table I lists, for each of four concentrations, the ammonium ion concentration in the GDH reaction, the !Q A at 600 nm for the nitrate reduction, and the A A at 600 nm for the NH4C1 standards, both after 60 minutes of cycling. The data from Table I are plotted in Figure 1.

As can be seen from Figure 1, there is a linear response of A A to the nitrate concentration following reduction in the enzymatic cycling procedure. The higher value of the intercept of the nitrate reduction compared to the NH_4C1 standards is due to the blank in the diffusion step. Similar results were obtained in three different runs. The best correlation is obtained when comparing the nitrate reduction and the standard curves made on the same day or adjacent days. As reported previously, there is a steady increase in the blank reaction with the length of time of storage of the reagents due to decomposition of the NADH (20,21) Therefore, samples and standard curves should be run on the same day.

Table II lists similar results, but the ammonium chloride standards are carried through the entire procedure including the diffusion in Conway cells without Devarda's metal. Listed are the ammonium ion concentrations in the GDH reaction, and A A at 600 nm for both the standards and the reduced nitrate samples, both after 60 minutes of cycling time. The data are plotted in Figure 2. As can be seen from the graph, the

response is also linear for standards carried through the entire procedure. The lines are very close to coincident when the- standards are measured in this way.

Using this approach, it is possible to determine nitrate concentrations in samples by carrying samples and standards through the entire procedure. A standard curve would be constructed from which the unknown concentrations could be read.

TABLE I

ΔA AT 600 run AFTER 60 MINUTES OF THE ENZYMATIC CYCLING REACTION

Procedure and Reactant Concentrations in Text

Calculated Molarity of NH4+ in GDH reaction	NO_3^- Reduction	NH_4C1 Standards
0	0.205	0.074
1.08×10^{-5}	0.406	0.385
1.62×10^{-5}	0.614	0.522
2.16×10^{-5}	0.699	0.649
2.70×10^{-5}	0.867	0.803
Linear Regression Results:		
Slope:	0.246	0.250
Ordinate Intercept:	0.185	0.111
Standard Deviation of Y:	0.036	0.022
Correlation Coefficient:	0.992	0.996

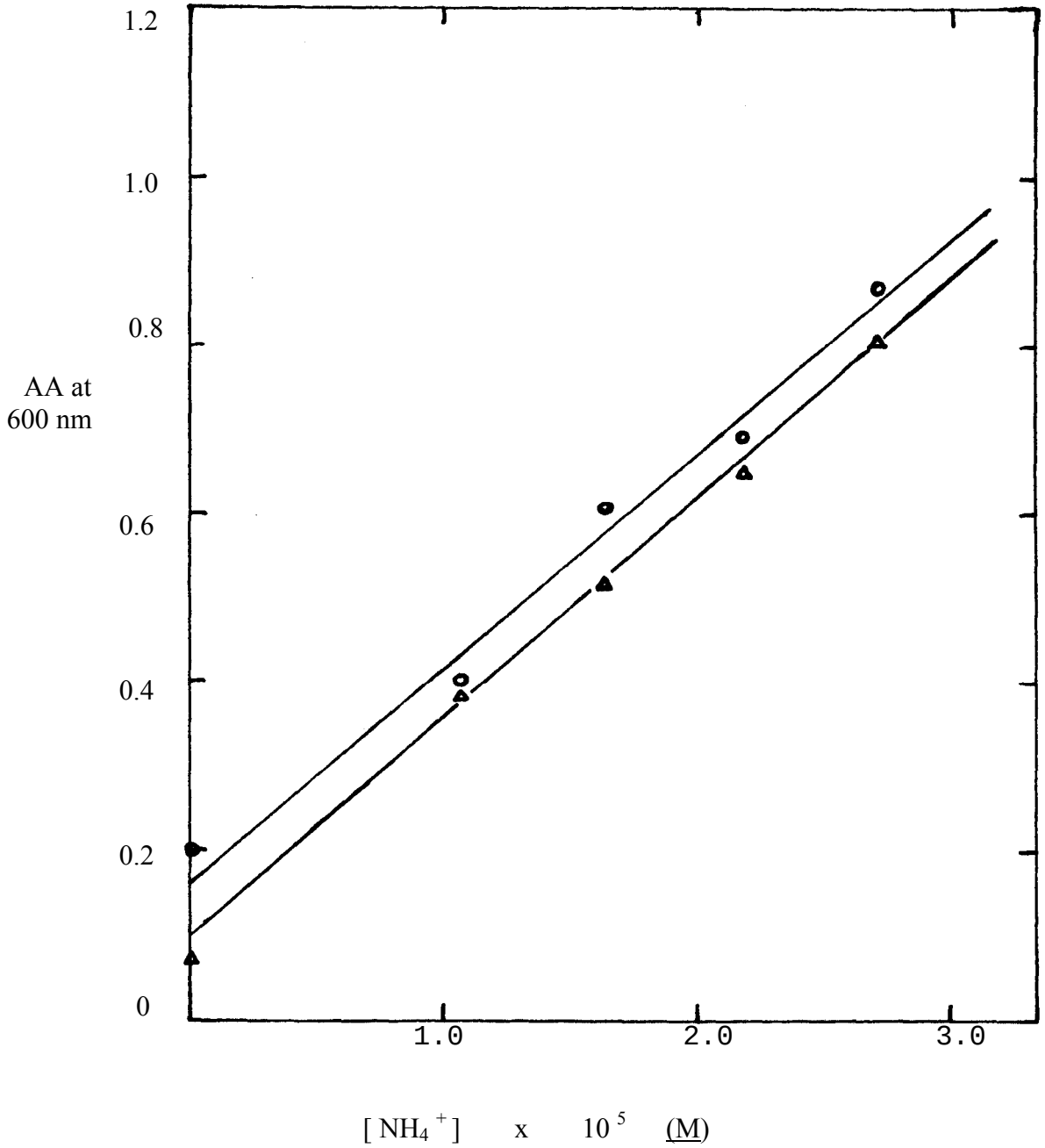


Figure 1: ΔA at 600 nm vs. calculated NH4+ concentration

o $NaN0_3$ Reduction

Δ NH_4 Cl Standards in GDH Reaction

TABLE II

Δ AT 600 nm AFTER 60 MINUTES OF THE ENZYMATIC CYCLING REACTION

Procedure and Reactant Concentrations in Text

Calculated Molarity NH4+ in GDH Reaction	Diffused NH4C1 Standards	Duplicate N03 Reductions	
0	0.239	0.225	0.245
1.08×10^{-5}	0.546	0.598	0.551
1.62×10^{-5}	0.713	0.641	0.717
2.16×10^{-5}	0.858	0.861	0.884
2.70×10^{-5}	0.956	0.986	1.039
Linear Regression Analysis:			
Slope:	0.265	0.278	0.296
Ordinate Intercept:	0.253	0.242	0.240
Standard Deviation of Y:	0.021	0.046	0.006
Correlation Coefficient:	0.998	0.991	0.999

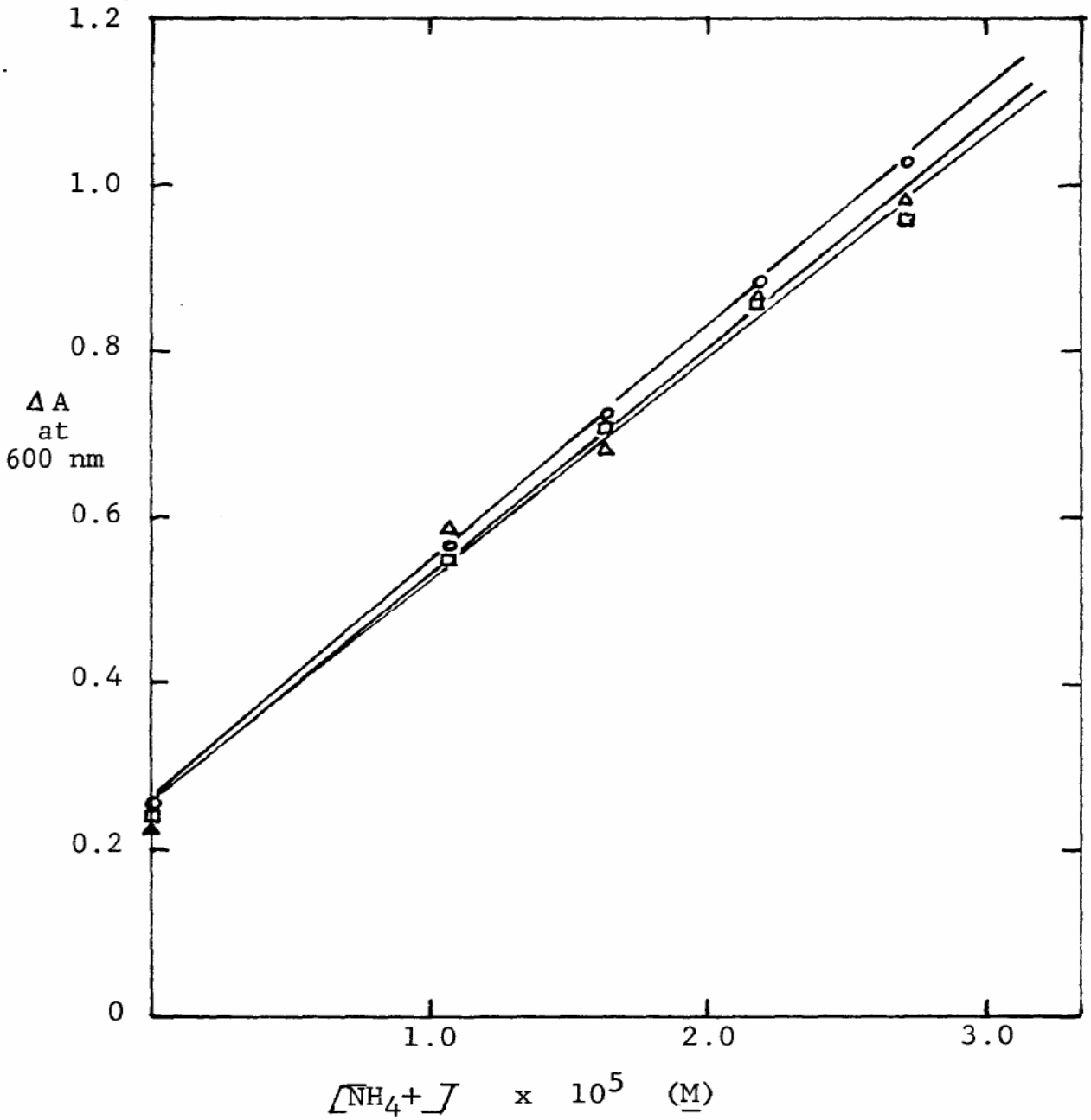


Figure 2: ΔA at 600 nm vs. calculated NH_4^+ concentration
▫ NH_4Cl Standards carried through entire procedure
△ $NaNO_3$ Reduction
○ $NaNO_3$ Reduction

DISCUSSION

The results reported above demonstrate that the procedure involving reduction with Devarda's metal followed by enzymatic cycling analysis of the ammonia produced provides a useful method for the determination of nitrate-N. Devarda's metal reduces nitrate to ammonia in the presence of base and the ammonia is trapped by hydrochloric acid simultaneously in a Conway diffusion cell. Then the ammonia is determined by the enzymatic cycling procedure which has already been shown to be successful in a previous report (22). The response is linear and proportional to the original nitrate concentration. Since Devarda's metal reduces both nitrite and nitrate to ammonia, presumably this procedure would be applicable to nitrite or nitrite plus nitrate determinations as well.

A summary of the method follows:

(1) Using Conway diffusion cells with 0.0100 N HCl in the center well and 2.00 N NaOH in the sample well and closing chamber, nitrate is reduced to ammonia with Devarda’s metal and the ammonia produced is trapped in the hydrochloric acid.

(2) The pH of the acid is adjusted to 7 with o.50 ml of 0.0100 N NaOH and the total volume is brought to 1.00 ml with conductivity water. This sample is added to 3.00 ml of the prepared reagent containing α-ketoglutarate, NADH, and glutamate dehydrogenase. The reaction proceeds to completion in the dark.

(3) Next, 0.240 ml of 1.000 N HC1 is added to destroy the excess NADH followed by 0.240 ml of 1.000 N NaOH to bring the pH back to 8.

(4) An aliquot (0.100) of this solution is added to a mixture of ethanol, ADH, MTT and PES for the enzymatic cycling reaction. The absorbance at 600 nm is measured at time zero and at 60 minutes. Cycling is carried out in the dark at room temperature.

A typical plot is shown in Figure 2. The ΔA at 600 nm is plotted versus the concentration of NH4+ in the GDH reaction. The plots are linear and the correlation coefficients ranged from 0.991 to 0.999. This procedure is suitable for nitrate concentrations ranging from 1 mg-6 mg N03-N/l or 10^{-5} to 10^{-4} M. It has several advantages over other commonly used procedures in that it is not subject to the common interferences found in waste water such as organic material, chloride ion, and bicarbonate. It is useful over a concentration range commonly encountered and is simple and inexpensive to use. All of the chemicals are commercially available and a colorimeter may be used for the measurements. The cells are easy to clean and the only additional apparatus-required is standard volumetric equipment and a colorimeter or spectrophotometer. This procedure does not require the constant attention of a laboratory technician, as would be the case if a steam distillation were required. The solutions may be made up in advance and are stable for at least 2 weeks at 4^0 C.

The biggest limitation is the blank due to the Devarda's metal and the breakdown of the NADH on storage. However, the blank does not affect the linear response. It is a problem only in trying to extend the procedure to less concentrated samples.

ACKNOWLEDGEMENTS

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