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DETAILED REPORT ON THE USE OF  
ENZYMATIC CYCLING FOR HIGH  
SPECIFICITY AND PRECISION IN THE  
COLORIMETER ANALYSIS OF AMMONIA

By Frederick W. Carson & Helen W. Davies

-October 1980-



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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  $10^{-5}$  M ammonia of the assay described by suitable modifications of the procedure. The ammonia-specific concentrations. The sensitivity could be extended well below the 10 nmol per sample limit of determination 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 the correlation coefficients of 0.997 to 0.9998. The coefficient of variation was 2% for a series of replicate measurements on samples containing  $4.41 \times 10^{-5}$  M ammonium ion. Standard solutions may be carried through the determination of unknown concentrations. Only volumetric equipment and a spectrophotometer or colorimeter are required. The analysis time per sample is 3h. All solutions may be prepared in advance and are stable for at least two weeks.

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## List of Abbreviations

A	Absorbance
ADH	Alcohol Dehydrogenase
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
Bicine	N,N-bis-(2-hydroxyethyl)-glycine
$\epsilon$	Extinction coefficient
$E_o^t$	Standard physiological reduction potential
GDH	Glutamate dehydrogenase
$AG^{ot}$	Standard physiological free energy change
INT	2-(p-Iodophenyl)-3-p-nitrophenyl)-5-phenyl tetrazolium chloride
$K_m$	Michaelis-Menten constant
$\alpha$ -KG	$\alpha$ -Ketoglutarate
	Molar
MITT	3-(4',5'-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
nm	Nanometer
PES	5-Ethylphenazinium ethyl sulfate
PMS	5-Methylphenazinium methyl sulfate
Tris	Tris-(hydroxymethyl)-aminomethane

## INTRODUCTION

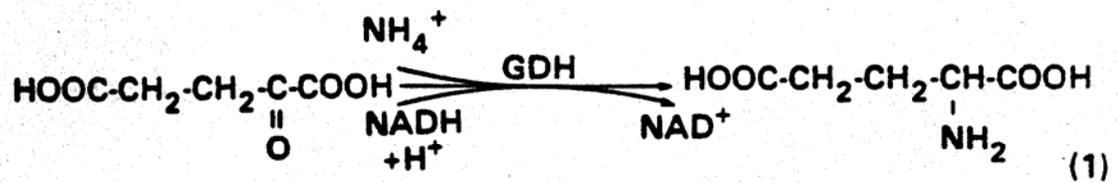
A method which utilizes enzymatic cycling for the determination of ammonia as ammonium ion concentration in water has been developed. It is believed that the method offers a viable alternative to presently available analytical methods for ammonium ion.

Standard Methods for the Examination of Water and Wastewater” for 1971 recommends either the Nessler reaction, the Indophenol Blue method, or the Kjeldahl method for ammonia- nitrogen (1). The Nessler reaction, in which ammonia is reacted with alkaline potassium iodomercurate (Nessler reagent), with the formation of a colored product, is subject to interference from amines, organic chloramines, and a number of other organic chemicals, as well as insoluble magnesium and calcium complexes (2). In the Indophenol Blue method, the ammonia is reacted with phenol and sodium hypochlorite to produce indophenol blue, which is colorimetrically determined. The mechanism of the reaction is not known and the intensity of the color produced varies with the catalyst, pH, and reagent concentrations (3). Furthermore, phenol is poisonous. A recent modification of this method, in which sodium salicylate is used in place of phenol, still gives results which depend on prevailing light and pH conditions (4). The Kjeldahl method measures organic nitrogen in addition to ammonia

nitrogen. In contrast, the method presented here is based on an enzymatic reaction which is absolutely specific for ammonium ion. There is no possibility of interference from amines or other organic chemicals.

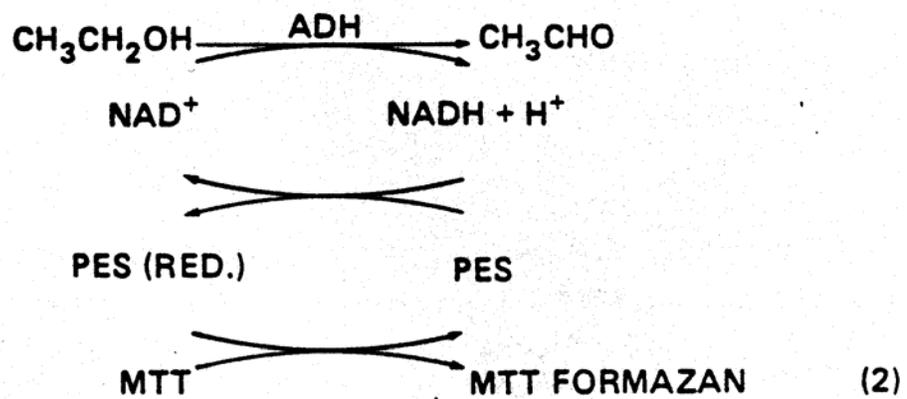
The method which is to be described here involves two steps, shown as Scheme I. Structures are shown in Figure 1. In the first step, Equation 1, the available ammonium ion is quantitatively consumed in the reductive amination of  $\alpha$ -ketoglutarate ( $\alpha$ -KG), in the presence of the enzyme glutamate dehydrogenase (GDH), to produce glutamic acid. This reaction involves the simultaneous, stoichiometric oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD<sup>+</sup>). The amount of NAD<sup>+</sup> produced is equivalent to the amount of ammonium ion originally present. This conclusion follows from the fact that the standard physiological Gibbs free energy change,  $\Delta G^{\text{ot}}$ , for this reaction is -8.30 kcal/mole (calculated using a standard physiological reduction potential, E, of -0.14 volts for the reduction of  $\alpha$ -KG (5)). Thus, the reaction is highly driven in the direction shown, going essentially to completion. The reaction is carried out in the presence of excess  $\alpha$ -KG and NADH, so that the extent of reaction is dependent only upon the available ammonium ion.

At the completion of the first step, the excess NADH remaining in the solution is destroyed by treatment of



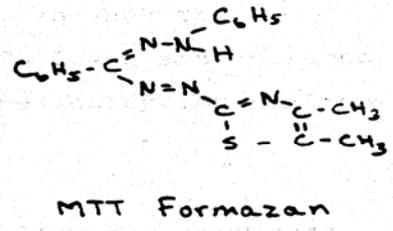
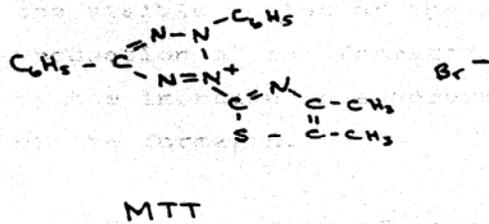
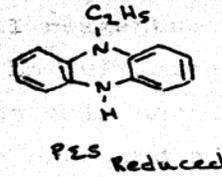
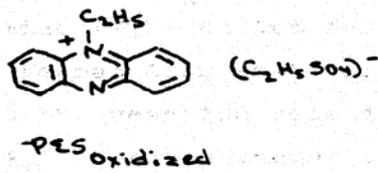
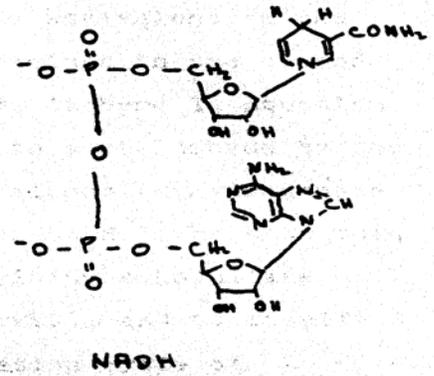
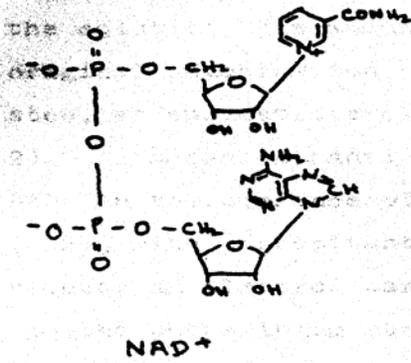
$\alpha$ -KETOGLUTARATE

GLUTAMIC ACID



SCHEME I

Figure 1



the solution with acid, a process which also denatures the enzyme but does not affect the  $\text{NAD}^+$  (6). The  $\text{NAD}^+$  in the solution, the amount of which is equivalent to the original ammonium ion, is then determined in the second step, an enzymatic cycling procedure (Scheme I, Equation 2). As shown, ethanol is oxidized to acetaldehyde by the  $\text{NAD}^+$  in the presence of the enzyme alcohol dehydrogenase (ADH), with concomitant formation of NADH. The NADH then reduces an electron carrier, phenazinium ethosulfate (5-ethylphenazinium ethyl sulfate; PES), and is itself reoxidized. This initiates a repeating cycle of reactions culminating in the final irreversible reduction by PES of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its formazan. All reagents, including the tetrazolium salt, are present in sufficient excess so that the reaction rate is dependent only upon the added  $\text{NAD}^+$ . Then the amount of formazan produced after cycling for a set time is proportional to the initial  $\text{NAD}^+$  concentration.

Tetrazolium formazans absorb light very strongly in the visible region of the spectrum, so that the production of the formazan can be followed and measured by the increase in absorbance at the-absorption maximum of the formazan. The principle of enzymatic cycling has long been

known and has been extensively reviewed (7,8). it is based upon the pairing of two reactions (oxidation of ethanol and reduction of PES here) which utilize a common reactant (NAD<sup>+</sup> here) which is consumed in one reaction and regenerated in the second.

One product (or reactant) is monitored, generally spectrophotometrically or by the formation of a derivative. When other reactants are present in excess, the rate of formation of the measurable product will be proportional to the concentration of a reactant in limited supply (NAD<sup>+</sup> here).

The advantages of enzymatic cycling are numerous. Principle among them is the obvious amplification by the cycling system of the response to an added reactant. This affords greatly increased sensitivity in detection of both low concentrations and small concentration differences. An enzymatic cycling system can, in theory, respond in time to only one or a few molecules of a; reactant. Furthermore, the response of the system is widely adjustable since it is sensitive to such readily- controlled experimental parameters as reaction time, temperature, enzyme concentrations, and pH. Another advantage, important here, is that energetically unfavorable reactions can be driven by the removal of one of the products in the cycling process.  $\Delta G^{\circ}$  for the oxidation of ethanol by NAD<sup>+</sup> is +5.67 kcal/mole (9), but the continuous oxidation of the NADH promotes the reaction.

Other investigators have utilized the  $\alpha$ -ketoglutarate reaction (Equation 1) in the determination of ammonium ion in water (10-13). However, these methods have depended upon measurement of the decrease in NADH concentration, generally measured spectrophotometrically. Thus they are based on the observation of a very small change in a relatively large quantity (as would be expected for the  $10^{-4}$  to  $10^{-5}$  molar concentration of ammonium ion generally present in natural and treated waters (14)). As already noted, the cycling reaction leads to an amplified response to the original NADH-NAD<sup>+</sup> conversion, readily monitored by following the production of formazan.

A search of the literature disclosed that a number of methods have been devised for the measurement of NAD<sup>+</sup> concentration. Among them are references to the use of NAD<sup>+</sup> NADH concentration tetrazolium salt cycling systems, both for measurement of NAD<sup>+</sup> concentration (15, 16) and, more frequently, for assay of enzyme activities (17, 18, 19). Tetrazolium salts are particularly well suited for enzyme cycling procedures because reduction, for which the intermediate electron carrier is required (20), occurs readily under mild conditions and is reversible. The formazan products are stable.

The approach taken in the development of the method presented here (Scheme I) was to break the total proposed procedure down into several steps in order to study separately each reaction involved. Accordingly, the first reaction investigated was the reduction of the tetrazolium salt. This was followed by development of the enzymatic cycling system. The next step was to devise the best possible conditions for conducting the glutamate dehydrogenase reaction. Finally, all of the reactions were combined into the simplest possible total procedure. The resultant scheme was then evaluated with stock ammonium ion solutions. The same sequence is followed in the presentation of the experimental results.

The 1971 Edition of "Standard Methods for the Examination of Water and Wastewater" (14) gives the following values as typical for the ammonium ion content of natural and treated fresh waters:

Low ammonia concentration      1 mg/ 1 N      ( $7 \times 10^{-6}$  M  $\text{NH}_3$ )

Slightly polluted water              N below 1 mg /1 ( $7 \times 10^{-5}$  M  $\text{NH}_3$ )

The procedure presented here was therefore designed for ready applicability in this concentration range. In addition, consideration was given throughout to stability, both during the course of any reaction and during storage, of any reagents used.

## EXPERIMENTAL

### I. Chemicals:

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

Tris (tris-(hydroxymethyl)-aminomethane), ultra pure came from Schwarz/Mann, Orangeburg, New York.

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

Spectro-grade Chloroform was from Mallinckrodt Chemical Works, Saint Louis, Missouri.

Absolute Ethanol, reagent quality, was from U.S. Industrial Chemicals Co., Tuscola, Illinois.

pH Buffer Solutions, 1 N and 10 N Sodium Hydroxide, and 1 N Hydrochloric Acid 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.

### II. Equipment and Techniques

A Beckman Model DB Spectrophotometer equipped with a Hewlett-Packard 7101BM strip chart recorder was used for spectral measurements. Data were recorded as % Transmission and converted mathematically to the corresponding Absorbance values. Quartz cuvettes were used for measurements at wavelengths shorter than 350 nm. All cuvettes, quartz and glass, had 1-cm path lengths.



In determining the solution Absorbances reported here the general procedure was to first measure the Absorbance of the absorption cell and solvent being used at the wavelength of interest, in order to determine the correction to be applied to the final Absorbance after a reaction. Such corrections vary among cells. When  $\Delta A$ 's (Absorbance changes) were to be reported such corrections were of course not necessary, as first and last measurements were made in the same cell.

In general, 5.00 ml and 0.50 ml serological pipets were used to prepare the samples for the experimental measurements to be described. Replicate measurements of weights of typical volumes were made for each type of pipet in order to determine the precision of volumes transferred with that pipet. These determinations led to the standard deviation figures given with the volumes specified in the text.

As indicated in the text, microliter syringes and a fixed-volume-ejector microliter pipet were used in two instances in the final experimental procedure. Standard deviations for the volumes transferred were determined as above.

All pH measurements were made with a Fisher Accumet Model 520 pH/Ion Meter. The standard procedure was to calibrate the pH meter using buffer of pH 7.0 and a

second buffer of either 1 pH unit greater than a higher, or 1 pH unit lower than a lower final pH to be attained. Adjustment for ambient temperature was included. A water bath was used to maintain a constant temperature in any solution in which it was suspected that pH adjustment might result in change of the solution temperature.

Where appropriate statistical analyses of data were carried out using computer program FITOBJ (21). Values reported for slopes, intercepts, and standard deviations were obtained in this way, as were fitted y-values used to construct lines in figures. The reported correlation coefficients (22) were calculated using the equation

$$r = \frac{n \sum x_i y_i - \sum x_i \sum y_i}{\sqrt{(n \sum x_i^2 - (\sum x_i)^2)(n \sum y_i^2 - (\sum y_i)^2)}}$$

### III. Preparation of Solutions:

Solutions for experimental measurements were prepared, using standard volumetric glassware, as follows. They are listed in order of mention in the Results section. Representative actual weights and calculated molecular weights (MW's) and molarities are given.

Metal-free water preparation (quartz-distilled, with conductivity  $<1 \times 10^{-5} \text{ n} \cdot \text{cm}^{-1}$ ) was used in the preparation of all solutions. Unless otherwise noted, solutions were stored at 4°C. Those expected to be light-sensitive were stored in amber-colored containers.

Tris buffer (0.100 M):

(Tris: Tris-(hydroxymethyl)-aminomethane) 12.112 g of Tris (MW 121.1) was dissolved in conductivity water, adjusted to pH 8.0 with 1.0 N hydrochloric acid, and diluted to 1000 ml.

MTT Formazan in Chloroform ( $4.293 \times 10^{-5} \text{ M}$ ): MTT Formazan: 3-(4',5'-Dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide formazan) 0.72 mg of MTT formazan (MW 335.4) was dissolved in Spectro-grade  $\text{CHCl}_3$  and diluted to 50 ml.

MTT- PES in Water (MTT:  $3.051 \times 10^{-3} \text{ M}$ ; PES:  $1.254 \times 10^{-4} \text{ M}$ )• (MTT: 3-(4',5'-Dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide; PES: 5-Ethyl phenazinium ethyl sulfate) 63.21 mg of MTT (MW 414.3) and 20.90 mg PES (MW 333.4) were dissolved in conductivity water and diluted to 50 ml.

NADH in Tris ( $3.408 \times 10^{-4} \text{ M}$ ):

(NADH: Nicotinamide adenine dinucleotide, reduced) NADH was from Sigma: Disodium Salt, Grade III, Approx. 980 (No. N-8129) 6.35 mg of NADH (MW 745.4) was dissolved in 0.100 M Tris buffer (pH 8.0) and diluted to 25 ml.

Bicine buffer (0,100 M): (Bicine: N,N-bis-(2-hydroxyethyl)-glycine) 8.165 g of Bicine (MW 163.2) was dissolved in conductivity water, adjusted to pH 8.0 with 0,10 N sodium hydroxide, and diluted to 500 ml.

NAD<sup>+</sup> in Water ( $3.307 \times 10^{-5}$  M):  
(NAD: Nicotinamide adenine dinucleotide)  
NAD<sup>+</sup> was from Sigma: Grade III, Approx. 98% (No. N-7004)  
2.39 mg of NAD<sup>+</sup> (MW 722.7) was dissolved in conductivity water and diluted to 100 ml,

Ethanol in Tris (1.3706 M):  
4,00 ml of absolute Ethanol (MW 46,07), density 0.7893 g/ml, was diluted to 50 ml in Tris buffer (pH 8.0).

Alcohol Dehydrogenase (ADH) in Water ( $2.963 \times 10^{-6}$  M):  
ADH was from Sigma: ADH from Yeast, 89,69; protein, 233 IU/mg, (Product No. A-3263)  
12.39 mg of Yeast ADH (MW 150,000 (23)) was dissolved in 25 ml of conductivity water. The solution was filtered through a 1 micron polycarbonate membrane (Nuclepore Corp., Pleasanton, CA).

ADP in Tris ( $2.033 \times 10^{-3}$  M):  
(ADP: Adenosine 5'-diphosphate)  
ADP was from Sigma: Grade I, Sodium Salt, from Equine Muscle, 95-991 (No. A-0127)  
100.88 mg of ADP (MW 496.1) was dissolved in Tris buffer (pH 8.0) and diluted to 100 ml.

$\alpha$ -Ketoglutarate ( $\alpha$ -KG) in Tris-ADP ( $1.999 \times 10^{-2}$  M)L.; 73.01 mg of  $\alpha$ -KG (MW 146.1) was dissolved in, Tris-ADP ( $2.033 \times 10^{-3}$  M) and diluted to 25 ml:

Glutamate Dehydrogenase (GDH) in Tris-ADP ( $1.406 \times 10^{-6}$  M): GDH was from Sigma: L-Glutamic Dehydrogenase, from Bovine Liver, Type III, Lyophilized powder, 76.001 protein, 44 IU/mg, Free Ammonium Ion content <0,03  $\mu$ g/mg protein (Product No. G-7882)  
6,14 mg of GDH (MW 332,000 (24)) was dissolved in 10 ml of Tris-ADP ( $2.033 \times 10^{-3}$  M). The solution was filtered through a 1 micron polycarbonate membrane (Nuclepore Corp., Pleasanton, CA).

Ammonium Chloride Standard Solution ( $1.1030 \times 10^{-4}$  M) (25): Ammonium Chloride (MW 53.50) was dried for 27 hours at  $110^\circ$  and allowed to cool in an evacuated dessicator over phosphoric anhydride. 0.59011 g of the dried ammonium chloride was dissolved in and then diluted to 1000 ml with conductivity water. 10 ml of this solution was then diluted to 1000 ml with conductivity water.

## RESULTS

### I. Suitability of Tetrazolium Salt for Enzymatic Cycling and Properties of Formazan

Enzymatic cycling systems involving NADH and terminating in the reduction of a tetrazolium salt require the participation of an intermediate electron carrier (20). An intermediate carrier is reduced by cycling NADH and it then reduces the tetrazolium compound, as illustrated in scheme I. Thus, reduction of a tetrazolium salt generally must be studied in the presence of a compatible auxiliary electron carrier.

The electron-accepting system originally thought to be readily adaptable to the contemplated cycling procedure makes use of <sup>2-</sup>(2-iodophenyl)-3-p-nitrophenyl)-5-phenyltetrazolium chloride (INT) with 5-methylphenazinium methyl sulfate (PMS) as the intermediate electron carrier. This pair of compounds has frequently been used in cycling reactions with NADH (18,19). However, in the course of the preliminary experiments to establish the nature of the response of this system to added NADH, a number of drawbacks to its use became apparent. As has been noted by other investigators (17,19), the formazan produced is insoluble in water, which would necessitate the use of a solubilizer such as albumin or Tween 20 to keep the INT

formazan in solution for the necessary spectral absorption measurements. In addition, it was observed that a reaction takes place in a solution of INT and PMS in Tris buffer (pH 8.0) in the absence of any added NADH or other added reducing agent. The nature of the reaction could not be readily determined, but PMS is known to be light-sensitive. For these reasons a different tetrazolium system was sought.

Papers by Nisselbaum and Green (15) and by Bernofsky and Swan (16) describe procedures for the determination of NAD<sup>+</sup> concentration system using 3-(4,5'-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT), sometimes called thiazolyl blue, as the terminal electron acceptor. It was expected that the MTT formazan would be more soluble than the INT formazan due to absence of the polar -NO<sub>2</sub> group found in the INT compound. The procedure of Bernofsky and Swan (16) also involves the use of 5-ethylphenazinium ethyl sulfate (PES) as the intermediate electron carrier. They found PES to be more stable than PMS. Experiments were conducted to determine the suitability of the MTT- PES combination for the proposed procedure.

MTT formazan (obtained from Sigma Chemical Co.) was dissolved in spectro-grade chloroform. The absorbances of solutions of different concentration were measured. Data are listed in Table 1.

TABLE 1

ABSORBANCE, OF MTT-FOMAZAN IN CHLOROFORM  $\lambda_{\text{max}} = 547 \text{ nm}$ 

Molarity MTT-Formazan	Absorbance at 547 nm	Calculated $\epsilon \times 10^{-3}$
$0.72 \times 10^{-5}$	0.130	18.11
$1.43 \times 10^{-5}$	0.252	17.61
$2.86 \times 10^{-5}$	0.490	17.11
$4.29 \times 10^{-5}$	0.750	17.47
	Mean	17.60
	Standard Deviation	0.43



The data show that the absorbance of the formazan, the final product of the cycling reaction, follows Beer's Law in the  $10^{-5}$  molar range. I.e., the absorbance is proportional to the concentration. This result simplifies the procedure. The extinction coefficient was calculated: mean  $\epsilon = 17.60 \times 10^3 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$  (the standard deviation). A value of  $13.0 \times 10^3$  for  $\epsilon$  is reported elsewhere, but it is noted that the color intensity can vary with different commercial preparations (26). The spectra were unchanged after several hours in room light, indicating that the formazan is stable.

In order to evaluate the response of the MTT-PES combination to added reducing agent and to observe the solubility of the resultant formazan, measured quantities of NADH (solution in Tris buffer) were mixed with MTT-PES (solution in water). Reaction occurred immediately, as evidence by the appearance in the absorption spectrum of a broad band centered at 600nm. Absorption spectra of an MTT- PES mixture and of the MTT formazan produced by an addition of NADH are shown in Figure 2. Absorption maxima for MTT and PES alone are 390 nm and 373 nm, respectively. Tris is transparent throughout the visible region of the spectrum. All samples contained 0.50mL. of MTT-PES solution and

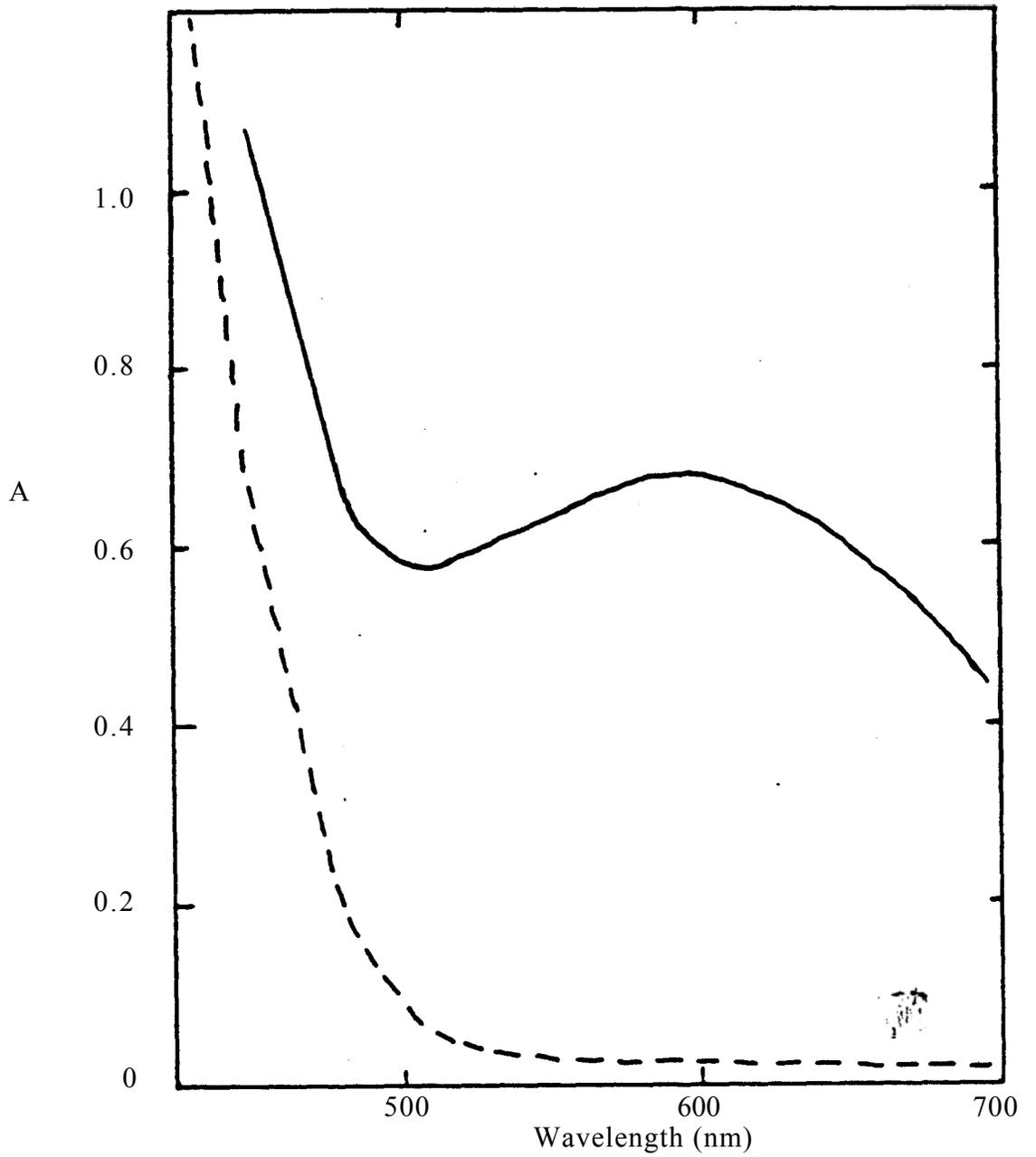


Figure 2: Absorption Spectra

---- MTT ( $4.96 \times 10^{-4}$  M) -- PES ( $2.04 \times 10^{-4}$  14) in Water

\_\_\_ MTT Formazan, produced by  $6.24 \times 10^{-5}$  14 NADH

TABLE 2

## ABSORBANCE OF MTT-PES SOLUTION VS. ADDED NADH

MTT:  $5.03 \times 10^{-4}$  M; PES:  $2.00 \times 10^{-4}$  M  
Reference cell: Water

Molarity NADH	Absorbance at 600 nm
$1.05 \times 10^{-4}$	1.096
$8.36 \times 10^{-5}$	0.851
$6.27 \times 10^{-5}$	0.591
$5.22 \times 10^{-5}$	0.496
$4.18 \times 10^{-5}$	0.383
$3.13 \times 10^{-5}$	0.268
$2.09 \times 10^{-5}$	0.153
$1.57 \times 10^{-5}$	0.098
$1.05 \times 10^{-5}$	0.048



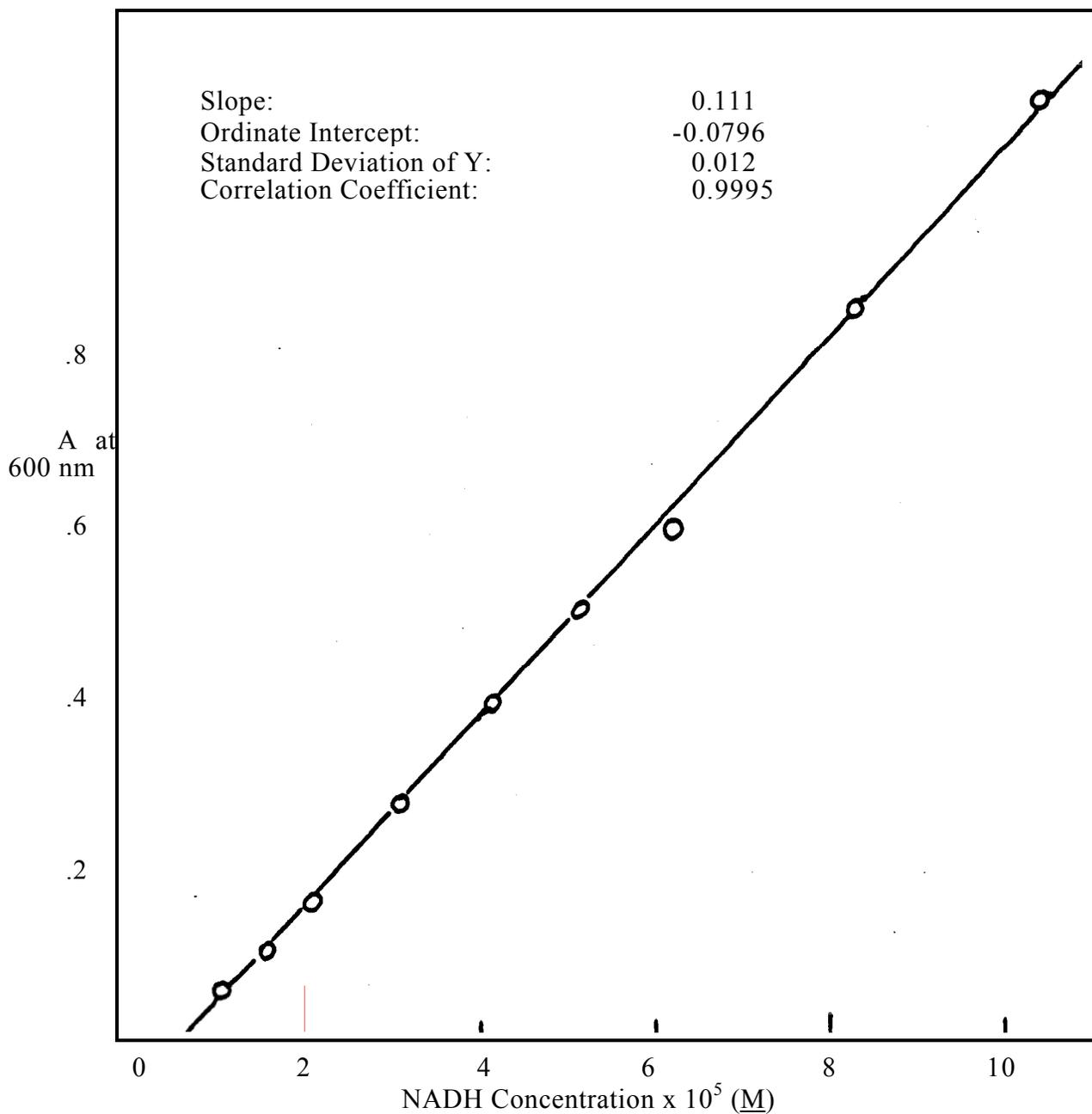


Figure 3: Absorbance (A) at 600 nm vs. NADH Concentration MTT:  $5.03 \times 10^{-4}$  M; PES:  $2.00 \times 10^{-4}$  M  
Reference Cell: Water



sufficient Tris buffer to give a total final sample volume of 3.00 ml. Values for slope, standard deviation, ordinate intercept, and correlation coefficient obtained by linear regression analysis of the data are supplied in the inset.

Inspection of the data and Figure shows that the quantity of formazan produced, as measured by the absorbance at 600nm, varies linearly with the amount of NADH added. All solutions appeared to be clear when the reactants were mixed and the resultant spectra were unchanged for at least fifteen minutes. There was no evidence of precipitation in that time. In other words, the formazan to be produced by the enzymatic cycling is soluble in a concentration range where absorbances can be readily determined. In addition, the absorption maximum of the formazan is sufficiently removed from the absorption peaks of the MTT and PES that there is no interference from absorption of other components of the reaction mixture at the wavelength of measurement. Conductivity water can be used in the reference cell.

To evaluate the stability of MTT-PES solutions under a variety of conditions, aqueous MTT- PES was diluted with a fixed volume of a solvent in question, and the spectra of the resulting solutions were recorded after various time intervals. The absence of any change in the spectrum was accepted as an indication of stability. It

was observed that the MTT-PES mixture was stable for at least 24 hours in water, and for at least 3 hours (more than sufficient for the enzymatic cycling reaction), but not for 24 hours, in Tris buffer, all samples having been kept at room temperature in the dark. A small spectral shift was observed for solutions in Bicine, the buffer used by Bernofsky and Swan (16). (When allowed to stand in room light, gradual changes were observed in all MTT-PES solutions, as was also observed in a solution of PES alone, presumably due to light-sensitivity of PES.) Solutions in conductivity water can be kept at 4°C in dark containers for at least three weeks.

## II. Enzymatic Cycling

The primary requirement of the enzymatic cycling reaction (Equation 2) is that the NAD<sup>+</sup> be present in concentrations less than the enzyme's (alcohol dehydrogenase's) K<sub>m</sub> value for NAD<sup>+</sup>. When this condition is met, the reaction rate has a linear dependence on the NAD<sup>+</sup> concentration. This is evident from consideration of the Michaelis-Menten equation, the rate equation for the reaction of a substrate in an enzyme-catalyzed reaction. This equation is

$$V = V_{\max} [S] / K_m + [S]$$

where v is the initial reaction velocity (or rate), V<sub>max</sub> is the maximum velocity at saturating substrate concentration, (S) is the initial substrate concentration, and K<sub>m</sub> is the Michaelis-Menten constant.

K<sub>m</sub> and V<sub>max</sub> are characteristic of a particular enzyme-substrate pair. It can be readily seen that when the substrate concentration is much lower than K<sub>m</sub> the above equation reduces to

$$V = V_{\max} / K_m \times [S]$$

and the rate is directly proportional to the substrate concentration. K<sub>m</sub> of Yeast ADH for NAD<sup>+</sup> is 7.4x10<sup>-5</sup> M (27). Other reactants must be present in excess and the quantity of tetrazolium salt must be sufficient so that

the supply is not exhausted by conversion to the formazan during cycling. When all of these conditions are satisfied, the amount of formazan produced after cycling for a given length of time should be proportional to the NAD<sup>+</sup> added to the reaction mixture. Experiments were conducted to verify this.

NAD<sup>+</sup>, up to 0.16 ml of solution in water, was added to Tris solutions containing 0.50 ml of ethanol, 0.20 ml of MTT-PES solution, and 0.10 ml of ADH solution. The final sample volume was 3.00 ml. Concentrations in the samples were: ethanol, 0.23 M; ADH,  $9.88 \times 10^{-8}$  M; MTT,  $2.02 \times 10^{-4}$  M; PES,  $8.42 \times 10^{-5}$  M; and NAD<sup>+</sup>,  $2.20 \times 10^{-7}$  to  $1.76 \times 10^{-6}$  M. The cycling reaction was allowed to proceed in the dark at room temperature and the absorbances of the solutions at 600 nm were recorded. Figure 4 shows the absorbance change at 600 nm with time, up to 60 minutes, for a solution containing  $1.10 \times 10^{-6}$  M NAD<sup>+</sup>. It can be seen that, as predicted, the increase in absorbance with time is linear, with a correlation coefficient of 0.9999.

In Table 3 are listed the absorbances at 600 nm at specified cycling times of several solutions containing verifying concentrations of NAD<sup>+</sup>. These data are plotted on Figure 5, with the cycling time for each set of points indicated on the graph. Linear regression analysis results are also in Table 3. Clearly, the absorbance produced by cycling for a given length of time is a

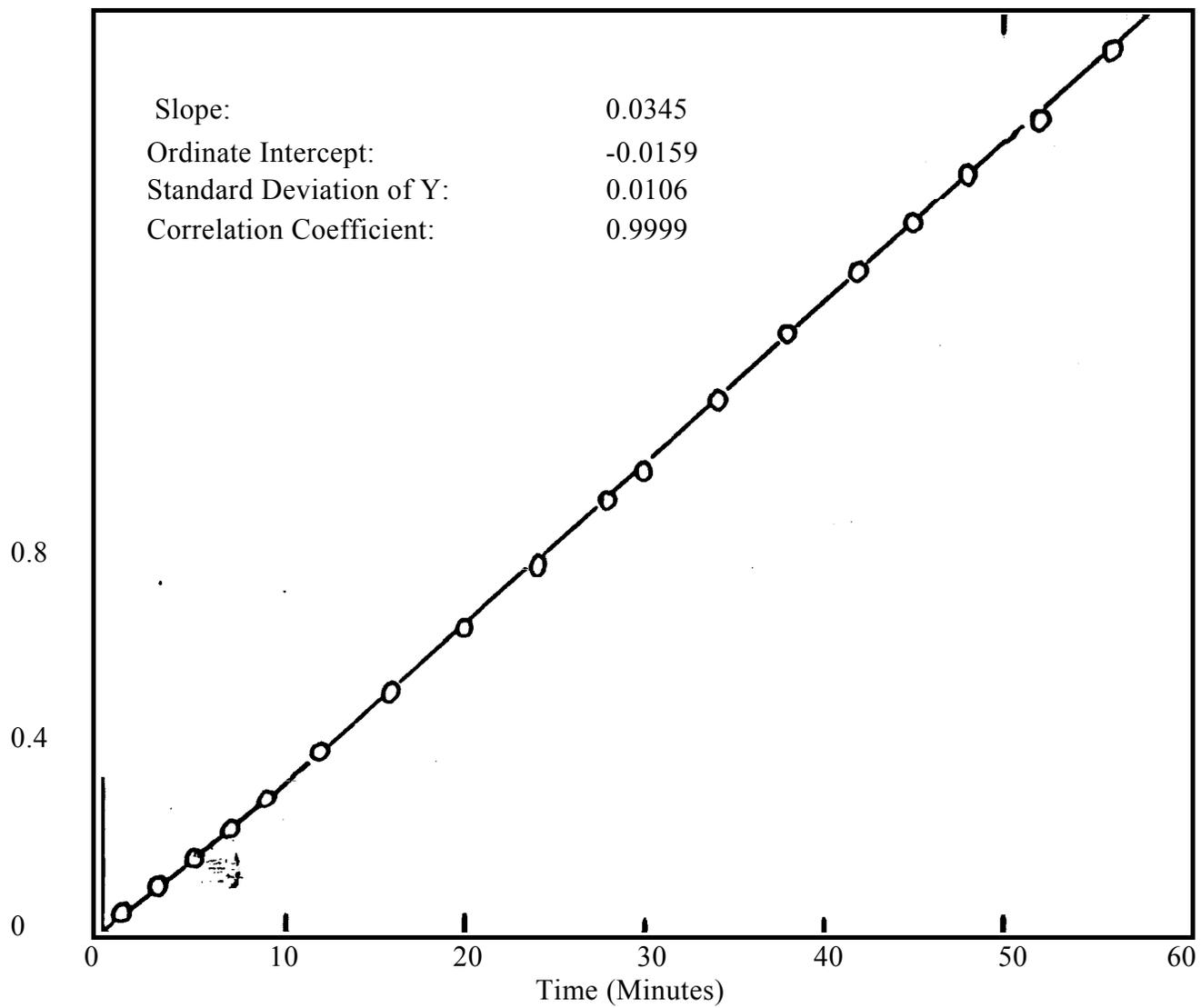


Figure 4: Absorbance at 600 nm vs. Time for a  $1.10 \times 10^{-6}$  (M) NAD<sup>+</sup> Solution in the Enzymatic Cycling Reaction

TABLE 3

ABSORBANCE OF ADH-MTT-PES SOLUTIONS -VS. ADDED NAD<sup>+</sup>

Ethanol: 0.2 M; ADH:  $9.88 \times 10^{-8}$  M MTT:  $2.02 \times 10^{-5}$  M; PES:  $8.42 \times 10^{-5}$  N

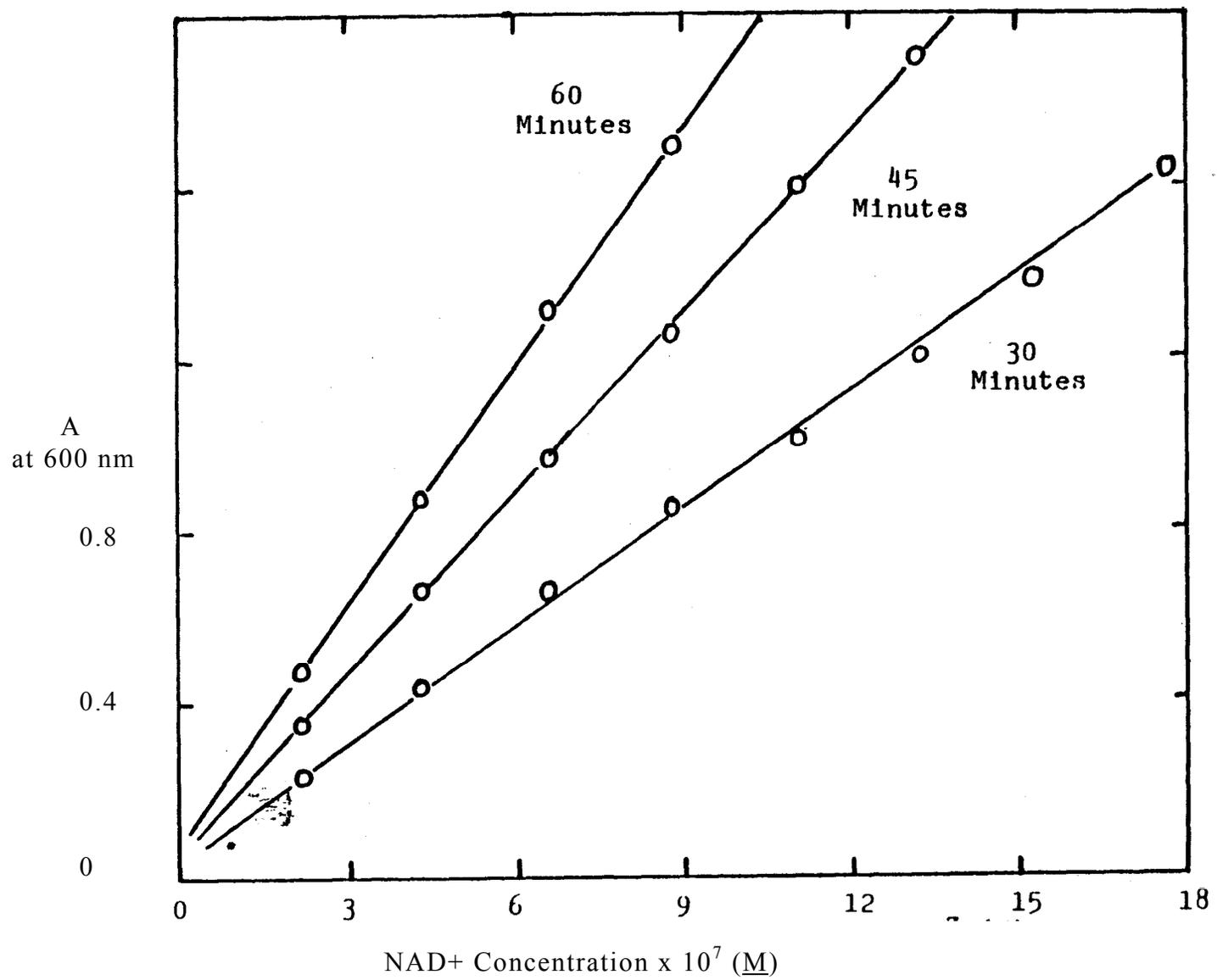
Molarity NAD <sup>+</sup>	<u>Absorbance at 600 nm</u>			
	30 Minutes	45 Minutes	60 Minutes	90 Minutes
$2.20 \times 10^{-7}$	0.23	0.35	0.47	0.70
$4.41 \times 10^{-7}$	0.43	0.66	0.85	1.34
$6.61 \times 10^{-7}$	7.66	0.97	1.31	2.00
$8.82 \times 10^{-7}$	0.85	1.26	1.70	2.30
$1.10 \times 10^{-6}$	1.01	1.59	2.07	
$1.32 \times 10^{-6}$	1.21	1.89		
$1.54 \times 10^{-6}$	1.38	2.10		
$1.76 \times 10^{-6}$	1.64			

## Linear Regression Analysis:

Slope:	0.0890	0.1400	0.1840	0.2948
Ordinate Intercept:	0.0451	0.0408	0.0640	0.0477
Standard Deviation of Y	0.0247	0.0093	0.0260	0.0094
Correlation Coefficient	0.9989	0.9999	0.9994	0.9999



Figure 5: Absorbance at 600 nm vs. NAD<sup>+</sup> Concentration for the Enzymatic Cycling Reaction



linear function of the amount of added NAD<sup>+</sup>, indicating; that this system is suitable for the proposed procedure.

Increasing the enzyme concentration leads, as would be expected, to a higher cycling rate. The reactant concentrations used in the measurements ,yielding the data of Table 3 and Figures 4 and 5 are those which earlier experiments had shown provided conveniently measured absorbances for 10<sup>-6</sup> to 10<sup>-7</sup> M NAD<sup>+</sup>. These NAD<sup>+</sup> concentrations are, of course, below the enzyme K<sub>m</sub> concentration, and could at the same time be readily attained by dilution of the 10<sup>-4</sup> to 10<sup>-5</sup> M NAD<sup>+</sup> expected in the initial ammonium ion reaction.

The series of measurements summarized in Table 3 was repeated at weekly intervals (using a freshly- prepared NAD<sup>+</sup> solution each time) to test the stability of alcohol dehydrogenase solutions on storage at 4<sup>0</sup> C. The pattern of change is shown in Figure 6, which covers a period of three weeks. It is typical of that observed with at least four different ADH solutions. Solutions became somewhat cloudy, indicating protein denaturation, and had somewhat decreased activity between the second and third weeks after preparation. It was therefore concluded that a solution of ADH in water can be safely stored for up to two weeks. ADH solutions were prepared in unbuffered water because early experience had shown that an ADH

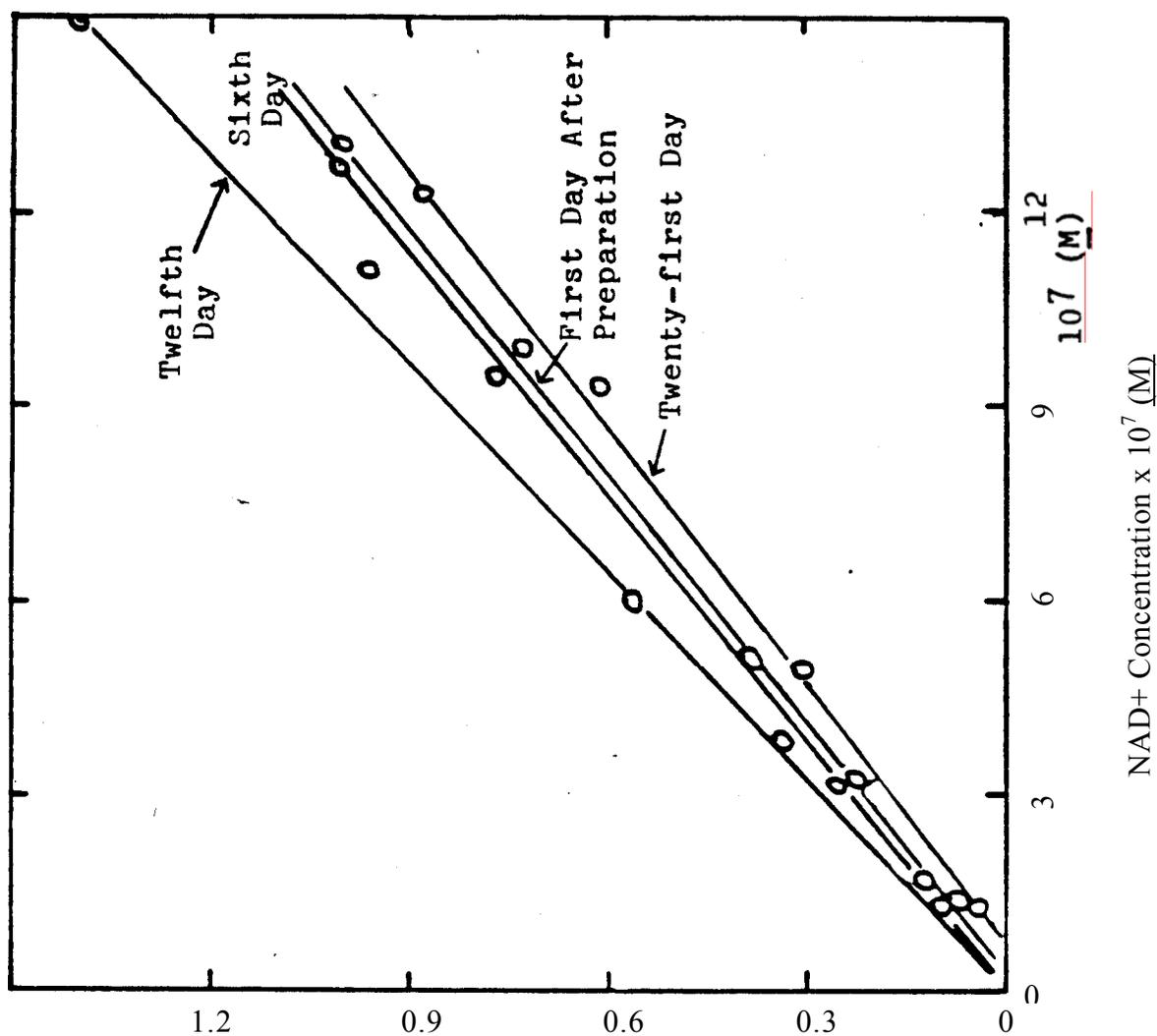


Figure 6;  $\Delta A$  at 600 nm vs. NAD<sup>+</sup> Concentration for ADH Solutions Stored at 4°C

	Slope	Ordinate Intercept	Standard Dev. of Y	Correlation Coefficient
First Day	0.0794	-0.0340	0.0114	0.9997
Sixth Day	0.0837	-0.0206	0.0183	0.9992
Twelfth Day	0.0926	-0.0170	0.0188	0.9995
Twenty-first Day	0.0736	-0.0512	0.0158	0.9901



solution in Tris buffer (pH 8.0) loses activity after several hours.

Attempts to combine the reagents used in the cycling reaction were not successful. As already noted, an MTT-PES solution in Tris would not be stable on storage. Combining the aqueous MTT-PES and ADH solutions led to loss of ADH activity on storage. Therefore, three separate solutions are used for the cycling reaction: ethanol in Tris, ADH in water, and MTT-PES in water.

### III. Glutamate Dehydrogenase Reaction

In devising the conditions for the glutamate dehydrogenase reaction (Equation 1), the intent was, as noted earlier, to have other reagents (NADH and a-ketoglutarate) present in excess, so that the extent of reaction would depend only upon the available ammonium ion. At the completion of the reaction the quantity of NAD<sup>+</sup> formed would then be equivalent to the quantity of ammonium ion originally present. The enzyme concentration would be adjusted so as to produce complete reaction at room temperature within a convenient length of time.

Glutamate dehydrogenase is regulated by complex interactions among allosteric effectors, some of which also promote or retard dissociation of the enzyme (28). ADP (adenosine diphosphate) is an important effector in the presence of NADH. NADH by itself can cause dissociation of the enzyme (29, 30). ADP both stabilizes the enzyme by preventing NADH from binding at the site at which it promotes dissociation (30) and also activates the enzyme at the same time (31, 32). Therefore ADP was included in the reaction mixture. For the purpose of comparing different reaction conditions, the consumption of NADH upon addition of ammonium was monitored by the decrease in the 340nm NADH absorption. Lack of further change in the 340 nm absorption was taken as an indication that the reaction was complete.

Figure 7 shows results obtained using the conditions which were found, after considerable experimentation, to be optimum for the reaction. The change in the absorbance at 340 nm is plotted as a function of the ammonium ion concentration. The data are in Table 4. The procedure was based on four-fold dilution (0.50 ml to a final sample volume of 2.00 ml) of  $10^{-4}$  to  $10^{-5}$  M ammonium ion, the target concentration range.  $1.08 \times 10^{-4}$  M stock solution thus provided  $2.69 \times 10^{-5}$  M ammonium ion in the reaction mixture. The 2.00 ml samples contained, in addition to the 0.50 ml of ammonium ion, 0.90 ml of Tris-ADP, 0.45 ml of  $2.00 \times 10^{-2}$  M  $\alpha$ -KG in Tris-ADP, 0.05 ml of  $4.00 \times 10^{-3}$  M NADH in Tris, and 0.10 ml of  $1.41 \times 10^{-6}$  M GDH in Tris-ADP.

Final concentrations of NADH and  $\alpha$ -ketoglutanate were respectively,  $1.00 \times 10^{-4}$  M and  $4.50 \times 10^{-3}$  M, both in considerable excess over the ammonium ion concentration to be determined. The reaction mixture also contained  $1.47 \times 10^{-3}$  M ADP and  $7.03 \times 10^{-8}$  M GDH in Tris buffer. The ADP concentration is near that recommended in "Methods of Enzymatic Analysis" (33) for a similar system used for assay of GDH. The enzyme concentration was set after it was observed that higher concentrations of enzyme led to decreased overall reaction (as measured by the NADH

Figure 7:  $\Delta A$  at 340 nm vs.  $\text{NH}_4^+$  Concentration in the Glutamate Dehydrogenase Reaction

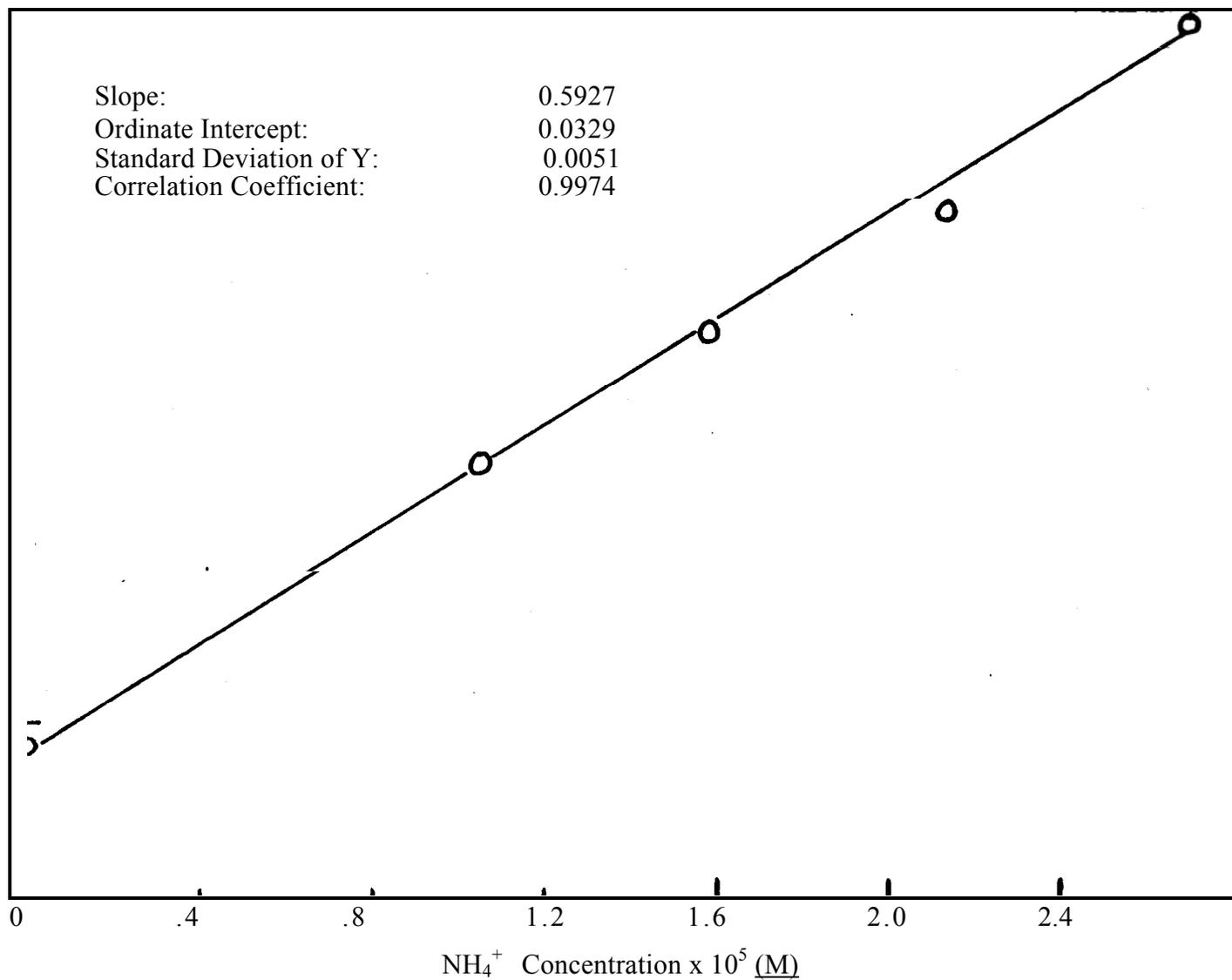


TABLE 4

ABSORBANCE CHANGE AT 340 nm VS. ADDED  $\text{NH}_4^+$  $\alpha$ -KG:  $4,50 \times 10^{-3}$  M; NADH:  $1,00 \times 10^{-4}$  M  
GDH:  $7,03 \times 10^{-8}$  M; ADP:  $1,47 \times 10^{-3}$  IT

Molarity $\text{NH}_4^+$	$\Delta A$ at 340 nm	$\Delta A$ - blank	Calculated $\Delta A$
$2,69 \times 10^{-5}$	0.1975	0.1635	0.1676
$2,15 \times 10^{-5}$	0.1544	0.1204	0.1339
$1,61 \times 10^{-5}$	0.1266	0.0926	0.1003
$1,0,8 \times 10^{-5}$	0.0978	0.0638	0.0670
0	0.0340		



consumption). Below this concentration, total reaction, although slower, was independent of enzyme concentration. Using this  $7 \times 10^{-8}$  M enzyme the reaction was complete in about 80 minutes.

Upon inspection of the data and of Figure 7, two things are obvious. First, the absorbance change was indeed proportional to the ammonium ion concentration, as anticipated. This indicates that these reaction conditions are appropriate to produce the desired dependence of NADH to NAD<sup>+</sup> conversion on ammonium ion concentration. But second, there was a considerable blank reaction. I.e., there was NADH to NAD<sup>+</sup> conversion in the reaction system in the absence of added ammonium ion. Using the literature value of  $\epsilon_{340 \text{ nm}} = 6.22 \times 10^3$  for NADH (34), the AA's expected for complete reaction of ammonium ion in each solution were calculated (AA =  $\epsilon \times \text{concentration}$ ). These calculated AA's are listed in Table 4. Subtraction of the blank value from the observed AA's yielded net AA's (also in Table 4) close to these calculated values. It thus appeared that if the blank reaction could be eliminated the reaction system would operate to produce NAD<sup>+</sup> equivalent to the original ammonium ion

Considerable effort was expended in an attempt to discover the source of the blank reaction. It was noted

that the blank reaction, as measured by the change in the 340 nm absorbance of the solution, took place when the NADH,  $\alpha$ -KG and glutamate dehydrogenase solutions were mixed. There was no appreciable further reaction on dilution of the mixture with water, indicating that the blank was attributable to one of these reactants and was not due to contamination of the water by ammonium ion. (Addition of ammonium ion, on the other hand, to a NADH  $\alpha$ -KG GDH mixture in which the blank reaction had already been allowed to occur, produced a further absorbance change close to that predicted by calculation based on the added ammonium ion.) The blank reaction was not reduced by ten-fold reduction in the enzyme concentration, or by halving the concentrations of either NADH or  $\alpha$ -KG, or when recrystallized Tris was used in the preparation of the buffer solution.

The only thing possible remaining source of the problem was the ADP. Addition of ADP to mixtures in which the blank reaction had already occurred did produce further small absorbance changes. Therefore, on the assumption that the ADP (the sodium salt from equine muscle had been used) contained ammonium ion, a number of different ADP preparations were investigated. Solutions were prepared using, successively, ADP- barium salt. ADP- Tris salt both from equine muscle and ADP sodium salt from yeast. The blank reaction varied slightly, but in no case was it eliminated. The effects of addition of ADP to the

2)  $\alpha$ -Ketoglutarate (87.46 mg) and glutamate dehydrogenase (2.04 mg) were dissolved in the Tris-ADP solution ( $\alpha$ -KG:  $6.00 \times 10^{-3}$  M; GDH:  $4.67 \times 10^{-8}$  M).

At this point a new approach was taken. As mentioned earlier, NADH in a solution can be destroyed by treatment of the solution with acid. NADH is stable in alkaline media but is more than 99.99% destroyed when the pH is lowered to 2 for two minutes at 25°C (6). NAD<sup>+</sup>, on the other hand, is stable in acid solutions but is more than 99.99% destroyed when the pH is increased to 12 and the solution is heated for four minutes at 60°C. In addition, it had been noted that after the blank reaction had occurred in the glutamate dehydrogenase reaction mixture, addition of ammonium ion led to the desired equivalent consumption of NADH.

The plan devised was to allow the blank reaction to occur in a solution containing all reactants except ammonium ion, to destroy the NAD<sup>+</sup> produced by the blank reaction by treating the solution with base, and to then readjust the pH to 8.0, adding more enzyme if necessary (It was expected that the enzyme would also be destroyed by the pH increase.). The resulting solution should now be ready for stoichiometric reaction with added ammonium ion, with no further blank reaction. Accordingly, the following procedure was employed:

1) A solution of ADP in pH 8.0 Tris buffer ( $2.02 \times 10^{-3}$  M) was prepared (100.10 mg of ADP in 100 ml of Tris).

glutamate dehydrogenase reaction were examined again later with the cycling procedure, and are described in a subsequent section.

3) A solution of NADH in Tris ( $6.29 \times 10^{-3}$  M) was prepared (46.89 mg of NADH in 10 ml of Tris).

4) NADH solution (2.00 ml) was added to the ADP-a-KG-GDH solution. The final concentrations were:

NADH	$1.23 \times 10^{-4}$ M
a-KG	$5.87 \times 10^{-3}$ M
GDH	$4.58 \times 10^{-8}$ M
ADP	$1.98 \times 10^{-3}$ M

5) The blank reaction was allowed to proceed at room temperature for 2 hours - until there was no further change in the 340 nm absorbance.

(As a check on the adequacy of the time chosen, ammonium ion (0.50 ml) was added to one of the solutions (1.50 ml) at this point. This led to a further absorbance change of 0.166 (calculated AA: 0.168), indicating that 2 hours is adequate to eliminate ammonium ion contamination).

6) Sodium hydroxide (10 N) was added dropwise (at the pH meter) to increase the pH of the solution to 12.0.

7) The solution was heated in a 60° water-bath for 15 minutes, and then cooled to room temperature.

8) Concentrated hydrochloric acid was added dropwise (at the pH meter) to lower the solution pH to 8.0.

(There was no reaction (no 340 nm absorbance change) on addition of ammonium ion (0.50 ml) to the

solution (1.50 ml) at this point, indicating that, as expected, the enzyme had been destroyed by the base treatment.)

9) Additional glutamate dehydrogenase was added and the solution (now containing  $5.17 \times 10^{-8}$  M GDH) was filtered and stored overnight at 4°C.

On the following day, the reactions with ammonium ion were carried out.

1.50 ml samples of the treated enzyme solution were mixed with 0.50 ml of ammonium ion solution. Absorbances at 340 nm were measured. After 2.5 hours at room temperature, the reaction appeared to be complete (no further change at 340 nm), and the absorbances were measured again. The data are presented in Table 5 and plotted in Figure 8 -- as AA at 340 nm vs. concentration of ammonium ion.

It can be seen that after this treatment of the enzyme solution the NADH consumption was still proportional to the added ammonium ion, the absorbance changes were still near the calculated values, and the blank reaction had been nearly eliminated. This procedure was repeated a number of times, with similar results. It was observed furthermore that the enzyme solutions prepared in this way could be stored in the refrigerator for two weeks. The 340 nm absorbance change on addition of ammonium ion did not change appreciably in that time.

TABLE 5

ABSORBANCE CHANGE AT 340 nm VS. ADDED NH<sub>4</sub><sup>+</sup> FOR ENZYME  
SOLUTION TREATED TO ELIMINATE THE BLANK REACTION

$\alpha$ -KG:  $4.40 \times 10^{-3}$  M; NADH:  $0.92 \times 10^{-4}$  M  
GDH:  $3.43 \times 10^{-8}$  M; ADP:  $1.48 \times 10^{-3}$  M

Molarity NH <sub>4</sub> <sup>+</sup>	$\Delta A$ at 340 nm	Calculated $\Delta A$
$2.757 \times 10^{-5}$	0.1697	0.1718
$2.206 \times 10^{-5}$	0.1324	0.1374
$1.654 \times 10^{-5}$	0.1051	0.1031
$1.103 \times 10^{-5}$	0.0752	0.0687
$5.514 \times 10^{-6}$	0.0471	0.0344
0	0.0060	

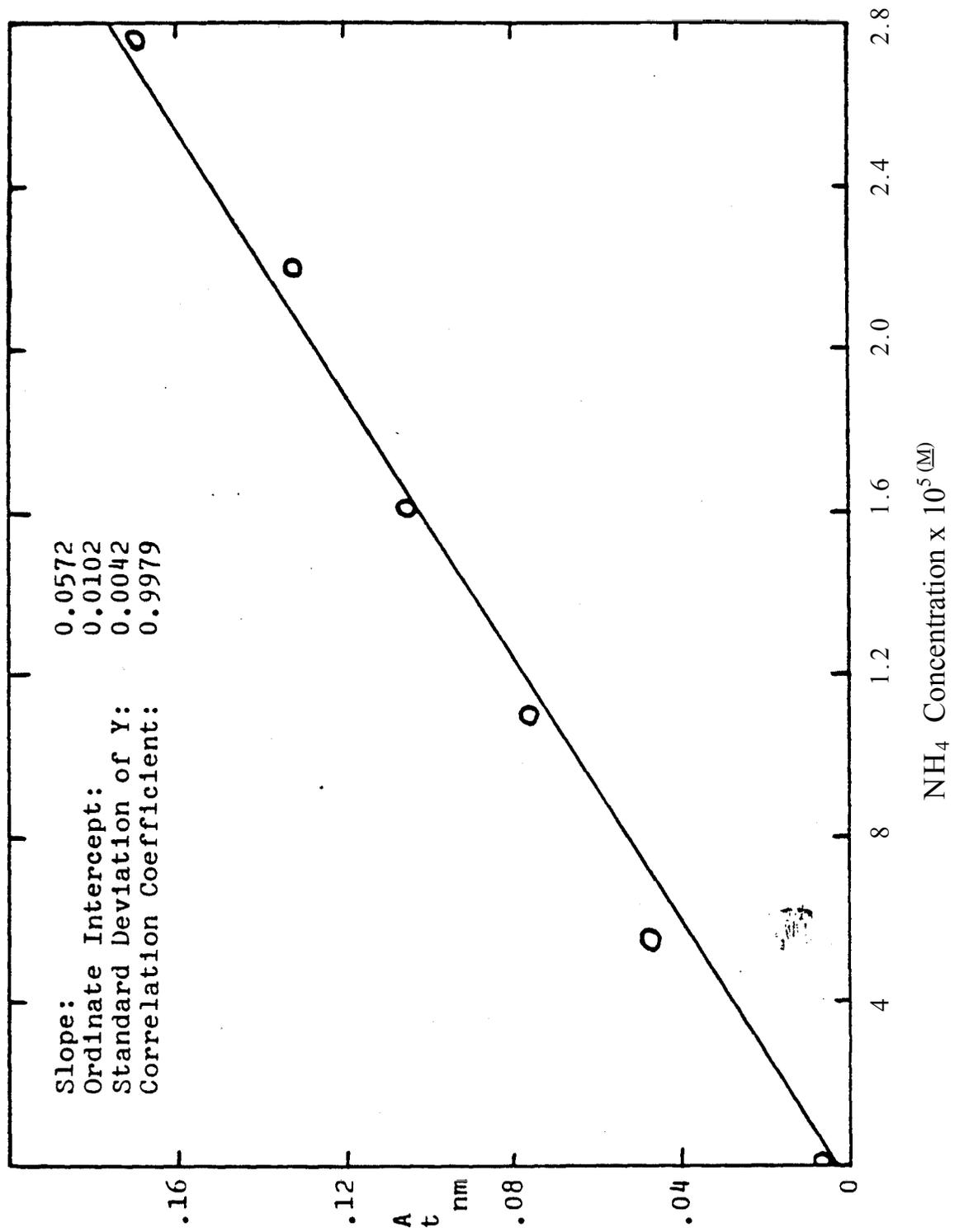


Figure 8:  $\Delta A$  at 310 nm vs.  $\text{NH}_4^+$  Concentration for Glutamate Dehydrogenase Solution Treated after the Blank Reaction

At this point, it appeared that suitable conditions for both the first and second reactions had been established.



#### IV. Coupling of the First and Second Reactions

To combine the first and second steps of the procedure, i.e., to get the NAD<sup>+</sup> produced in the original ammonium ion reaction into the enzymatic cycling reaction, two things had to be considered. First, the solution must be acidified after the first reaction to destroy the remaining excess NADH. And second, the amount of NAD<sup>+</sup> added to the cycling mixture must always be below the enzyme's (ADH's) K<sub>m</sub> value for NAD<sup>+</sup> ( $7,4 \times 10^5$  M).

The stability of NADH decreases drastically with decreasing pH. In fact, as noted earlier, NADH is more than 99.99% destroyed when the pH of the solution is lowered to 2 for two minutes at 25°C (6). This destruction can be followed by the decrease to zero in the 340 nm NADH absorption. Using the pH meter, 1 N hydrochloric acid was added to the 2.00 ml samples (1.50 ml of treated enzyme solution plus 0.50 ml of water or ammonium ion solution) from Step 1. It was found that addition of 0.12 ml of acid caused the pH of the sample to decrease from 8.1 to 1.8. Monitoring the NADH destruction with the spectrophotometer showed that the reaction was complete within four minutes. Slightly less acid could be used to lower the pH to 2.0, but the reaction then required considerably more time. Therefore, 0.12 ml of 1 N hydrochloric acid was used



subsequently, and after allowing; 4-5 minutes for total destruction of the NADH, 0.12 ml of 1 N sodium hydroxide was added to raise the solution pH back to 8. This was done to ensure that the pH of the enzymatic cycling mixture (also at pH 8) would not be perturbed by the addition of the NAD<sup>+</sup> solution. (That the addition of 0.12 ml of 1 N sodium hydroxide did indeed raise the pH back to 8.1 was verified using a pH meter.)

The remaining consideration was the dilution of the NAD<sup>+</sup> solution from Step 1 for the enzymatic cycling reaction. It seemed logical to conduct the cycling reactions in spectrophotometer. The cycling reaction was accordingly planned for a total volume of 3.00mL – a volume conveniently contained in a 1cm. spectrophotometer cell. In addition, earlier experiments had shown that the cycling system works well with  $10^{-6}$  to  $10^{-7}$  M NAD<sup>+</sup>. (See Table 3 and Figure 5.)

Calculations showed that if a 0.50 ml sample of  $10^{-4}$  to  $10^{-5}$  ammonium ion were diluted to 2.24 ml by the end of the first step and 0.10 ml of this solution were then diluted to 3.00 ml for the cycling reaction, the NAD<sup>+</sup> concentration in the cycling mixture would then be  $7.440 \times 10^{-7}$  to  $7.440 \times 10^{-8}$  M.



(original concentration)(0.5)/(2.0+0.12+0.12)(30) concentration in cycling reaction)

This dilution scheme was adopted.



## V. Testing of the Complete Procedure

A standard  $1.1030 \times 10^{-4}$  M ammonium chloride stock solution was prepared according to the procedure given in "Standard Methods for the Examination of Water and Wastewater" (25). The preparation of the solution is described above in the Experimental section.

The complete procedure was tested using the standard ammonium ion solution as follows.

1) The glutamate dehydrogenase solution was prepared as previously described.

In brief, the blank reaction was allowed to occur, the resultant  $\text{NAD}^+$  was destroyed, and fresh enzyme was added. After filtering, the solution was stored in the refrigerator all overnight.

2) For the first reaction, 2.000 ml ( $\pm 0.0053$  ml, the standard deviation) samples were prepared using 1.500 ml ( $\pm 0.0052$  ml) of the glutamate dehydrogenase solution and 0.500 ml ( $\pm 0.0012$  ml, transferred with microliter syringes) of ammonium ion solution, or conductivity water, or a combination of the two to provide the desired ammonium ion concentration. The samples were kept covered, in the dark, at room temperature for the 2.5 hours required to assure complete reaction of the ammonium ion. Absorbances at 340 nm were measured at the start and at the finish of the reaction. Concentrations in these samples were (in 0.10 M Tris buffer):  $\text{NADH}$ :  $9.432 \times 10^{-5}$  M; a -KG:M;  $\text{GDH}$  :  $3.754 \times 10^{-8}$  M;  $\text{ADP}$ :

$1.478 \times 10^{-3}$  M;  $\text{NH}_4^+$  :  $2.758 \times 10^{-5}$  to  $2.758 \times 10^{-6}$  M.

3) Hydrochloric acid (0.120 ml ( $\pm 0.0014$  ml) of 1 N HCl) was added to and mixed with each sample.

4) After 5 minutes, 0.120 ml ( $\pm 0.0014$  ml) of 1 N sodium hydroxide was added to and mixed with each sample.

5) For the enzymatic cycling reaction, 0.1000 ml ( $\pm 0.00075$  ml) of each sample was transferred using fixed- volume- ejector microliter pipet to a mixture spectrophotometer cell) of 1) ethanol in Tris buffer ( $2.600 \text{ ml} \pm 0.0052 \text{ ml}$ ), 2) ADH in water ( $0.100 \text{ ml} \pm 0.0014 \text{ ml}$ ), and MTT-PES in water ( $0.200 \text{ ml} \pm 0.0014 \text{ ml}$ ).

Reactant concentrations in each 3.00 ml ( $\pm 0.0056$  ml) sample were (in 0.10 M Tris buffer):

Ethanol:	0.238	
ADH:	$0.993 \times 10^{-7}$	<u>M</u>
PES:	$8.498 \times 10^{-5}$	<u>M</u>
MTT:	$2.043 \times 10^{-4}$	<u>M</u>
NAD <sup>+</sup> :	$8.207 \times 10^{-7}$	to $8.207 \times 10^{-8}$ <u>M</u>

Immediately following the addition of the NAD<sup>+</sup>, each sample was quickly mixed (by inverting the covered cuvette several times) and the absorbance at 600 nm was recorded. The cuvette was then placed in a 25°C water-bath, which was then covered to exclude light. Absorbances were measured again after 30 and 60 minutes.

The results obtained by following the procedure outlined above are presented in Table 6. The Table lists, for each of six samples, the original ammonium ion concentration in the sample, the  $\Delta A$ 's at 340 nm observed

TABLE 6

OBSERVED  $\Delta A$ 'S VS. (NH<sub>4</sub><sup>+</sup>) FOR THE GLUTAMATE DEHYDROGENASE  
AND THE ENZYMATIC CYCLING REACTIONS

Procedure and Reactant Concentrations in Text						
Molarity NH <sub>4</sub> <sup>+</sup> in GDH Reaction	Observed $\Delta A$ at 340 nm	Calculated $\Delta A$ at 340 nm	$\Delta A$ at 600 nm- 30 Min.	$\Delta A$ at 600 nm- 60 Min.	Calculated Molarity NAD <sup>+</sup>	
$2.758 \times 10^{-5}$	0.1766	0.1718	0.712	1,493	$8.207 \times 10^{-7}$	
$2.206 \times 10^{-5}$	0.1404	$0.1374^1$	0.534	1.135	$6.566 \times 10^{-7}$	
$1.379 \times 10^{-5}$	0.0935	0.0859	0.354	0.756	$4.103 \times 10^{-7}$	
$8.273 \times 10^{-6}$	0.0566	0.0515	0.224	0.494	$2.462 \times 10^{-7}$	
$2.758 \times 10^{-6}$			0.129	0.287	$8.207 \times 10^{-8}$	
0	0.0053	0	0.045	0.108	0	
Linear Regression Analysis for $\Delta A$ at 600 nm vs. Calculated Molarity NAD <sup>+</sup> (Figure 9):						
Slope:			30 Minutes	60 Minutes		
			0.0782	0.1624		
Ordinate Intercept:			0.04,43	0.1123		
Standard Deviation of Y:			0.0221	0.0411		
Correlation Coefficient:			0.9970	0.9976		



for the glutamate dehydrogenase reaction, the  $\Delta A$  at 340 nm calculated for complete reaction of the ammonium ion in the glutamate dehydrogenase reaction, and the  $\Delta A$  at 600 nm after 30 minutes and after 60 minutes of the enzyme cycling reaction. The last column lists the calculated  $NAD^+$  concentration for the cycling reaction (obtained by assuming production of  $NAD^+$  equivalent to the original ammonium ion and allowing for dilution, i.e.,  $(NH_4^+)/33.60$ ); Comparison with Table 5 shows that the 340 nm  $\Delta A$ 's are very close to those obtained previously. Data from Table 5 were plotted in Figure 8.

In Figure 9 are plotted the  $\Delta A$ 's at 600nm after 30 and 60 minutes of cycling as a function of the calculated sample  $NAD^+$  concentrations. The linear regression results are Table 6. Also plotted in Figure 9 are data obtained from calibration on the same day of the enzyme cycling reaction with a known  $NAD^+$  solution. To do this, a fresh solution of  $NAD^+$  in conductivity water ( $3.918 \times 10^{-5} \text{ M}$ ) was prepared. 0.10mL quantities of this  $NAD^+$  solution (or a combination of it and conductivity water) were used in the enzymatic cycling reaction, exactly as in Step 5 of the procedure just given. The data and linear regression results are in Table 7.

Examination of the data and figures shows that good results were obtained by the procedure described. Most importantly, there was good correlation between the response in the final step -- the enzymatic cycling



Figure 9: AA at 600 nm vs. Calculated NAD+ Concentration in the Enzymatic Cycling Reaction, with NAD+ Calibration

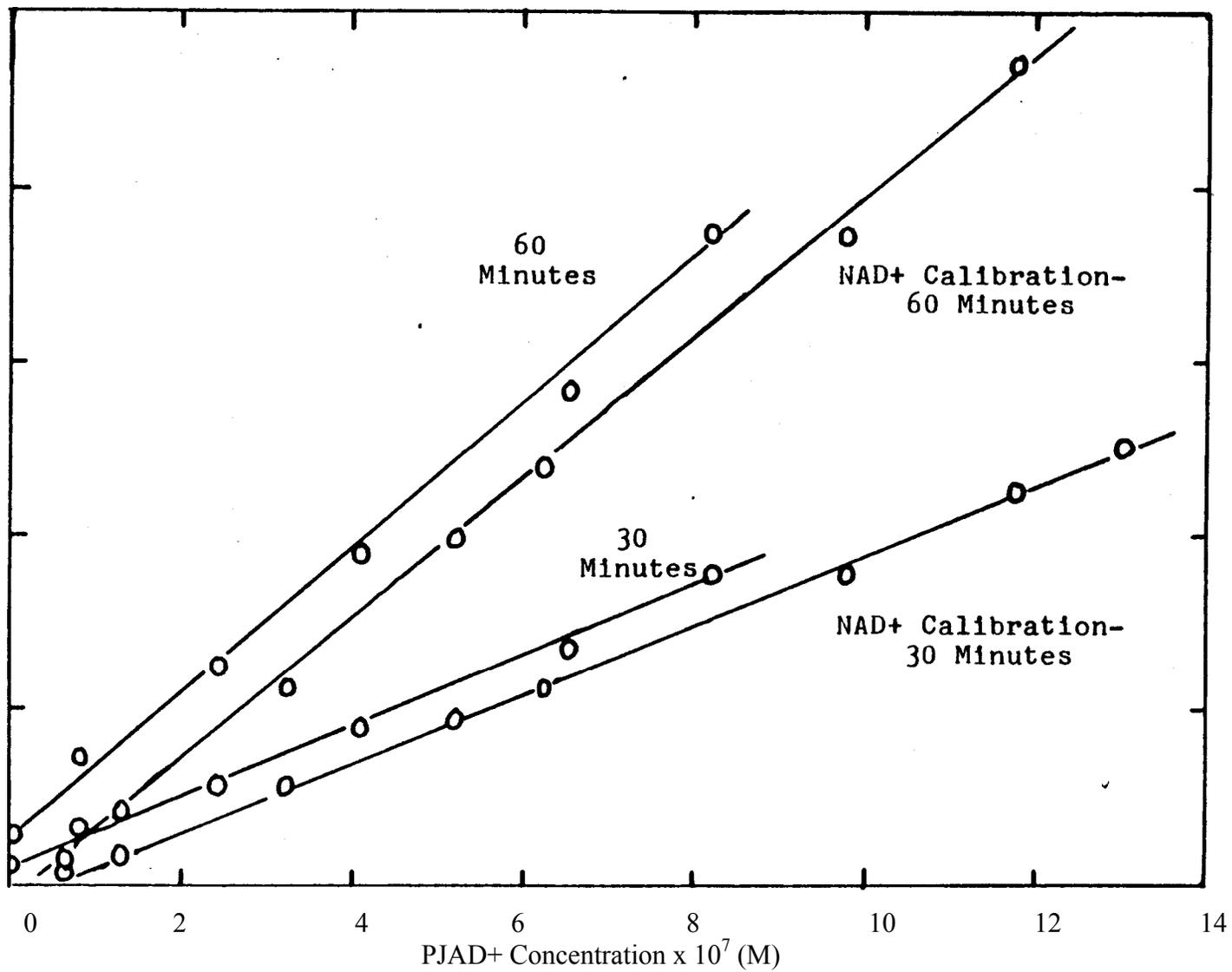


TABLE 7

OBSERVED  $\Delta A$  VS. (NAD<sup>+</sup>) FOR THE ENZYMATIC CYCLING REACTION

Procedure and Reactant Concentrations in Text

Molarity NAD <sup>+</sup>	$\Delta A$ at 600 nm- 30 Minutes	$\Delta A$ at 600 nm 60 Minutes
$1.306 \times 10^{-6}$	1.0163	2.1174
$1.175 \times 10^{-6}$	0.9077	1.8863
$9.795 \times 10^{-7}$	0.7136	1.4871
$6.530 \times 10^{-7}$	0.4488	0.9529
$5.224 \times 10^{-7}$	0.3770	0.7877
$3.265 \times 10^{-7}$	0.2109	0.4496
$1.306 \times 10^{-7}$	0.0680	0.1549
$6.530 \times 10^{-8}$	0.0283	0.0620

Linear Regression Analysis:

Slope:	0.0796	0.1649
Ordinate Intercept:	-0.0419	-0.0760
Standard Deviation of Y:	0.0200	0.0378
Correlation Coefficient:	0.9988	0.9990



reaction -- and the ammonium ion originally added. The measurements of the 340 nm DA's for the first step and the cycling system calibration with known NAD<sup>+</sup> solution had been carried out as 'checks' to ensure that the system was responding, as intended. The 340 nm  $\Delta A$  data, when compared with the calculated  $\Delta A$ 's, verified that the NADH to NAD<sup>+</sup> conversion was complete, and that the blank reaction was small. The parallel relationship (see Figure 9) between the final cycling results and the NAD<sup>+</sup> calibration lines indicates that NAD<sup>+</sup> produced in Step 1 is accurately reflected in the response of the cycling system. That these lines do not coincide would have to be attributed to the small remaining blank reaction still present in the first reaction.

It thus appears that it would be possible to determine unknown ammonium ion concentrations by carrying standard solutions through the procedure along with the unknown solutions. Data from the standard solutions would be used to construct a calibration line from which the unknown concentrations would then be derived.

The complete procedure Just described was repeated a number of times. Reactant concentrations varied slightly, of course, but the results consistently matched those given above. Consideration then had to be given to the reproducibility of response for a set of solutions prepared at the same time and stored under the same

conditions. As noted earlier, studies using; standard NAD<sup>+</sup> solutions had shown that the solutions used in the enzymatic cycling reaction could be kept for two weeks with little change in the response to added NAD<sup>+</sup>. Also, the response of the glutamate dehydrogenase solution, as measured by the NADH consumption with added ammonium ion, was known to be stable for two weeks.

Three separate sets of data were collected for the purposes of assessing the reproducibility of the complete procedure. In each case, all solutions (except the standard ammonium ion) were freshly prepared just prior to the first measurements, and were subsequently stored at 4°C. One collection of data, typical of the three, and covering a period of two weeks, is presented in Table 8. Component concentrations in the solutions prepared for the GDH reaction were: NADH:  $0.944 \times 10^{-4}$  M;  $\alpha$ -KG:  $4.393 \times 10^{-3}$  M; GDH:  $3.508 \times 10^{-8}$  M; ADP:  $1.481 \times 10^{-3}$  M. The cycling solutions contained 0.238 M ethanol;  $1.007 \times 10^{-7}$  M ADH;  $2.044 \times 10^{-4}$  M MTT and  $8.482 \times 10^{-5}$  M PES. The data are plotted in Figure 10. The enzymatic cycling system calibrated with standard NAD<sup>+</sup> on each day. The results for the total procedure were consistently parallel to the calibration lines.

In each case it was observed that the blank reaction gradually increased on storage. The final response to added ammonium ion remained linear and the lines for successive weeks were parallel to the cycling reaction

TABLE 8

 $\Delta A$  AT 600nm AFTER 30 MINUTES OF THE ENZYME CYCLING REACTION

ALL SOLUTIONS PREPARED ON SAME DAY

Procedure and Reactant Concentrations in Text

Molarity Calculated NH <sub>4</sub> <sup>+</sup> in Molarity NAD <sup>+</sup> - GDH Rcn. Cycling Hcn.	$\Delta A$ at 600 One ay After Prep. of Solns.	nm after Days After Prep. of Solns.	30 minutes Days After Prep. of Solns.
$2.758 \times 10^{-5}$ $8.207 \times 10^{-7}$	0.7167	0.740b	0.0055
$2.482 \times 10^{-5}$ $7.386 \times 10^{-7}$	0.605y	0.6831	
$2.206 \times 10^{-5}$ $6.566 \times 10^{-7}$		0.6047	
$1.655 \times 10^{-5}$ $4.924 \times 10^{-7}$	0.4505	0.4857	0.5414
$1.379 \times 10^{-5}$ $4.103 \times 10^{-7}$	0.3940	0.4274	
$8.273 \times 10^{-6}$ $2.462 \times 10^{-7}$	0.2723	0.2994	0.3372
$5.515 \times 10^{-6}$ $1.641 \times 10^{-7}$	0.2039	0.2287	
$2.758 \times 10^{-6}$ $0.821 \times 10^{-7}$	0.1740	0.1940	
0 0	0.1116	0.1331	0.1549
Linear Regression Results:			
Slope:	0.0714	0.0747	0.0797
Ordinate Intercept:	0.1023	0.1221	0.1492
Standard Deviation of Y:	0.0179	0.0102	0.0072
Correlation Coefficient:	0.9970	0.9991	0.9990



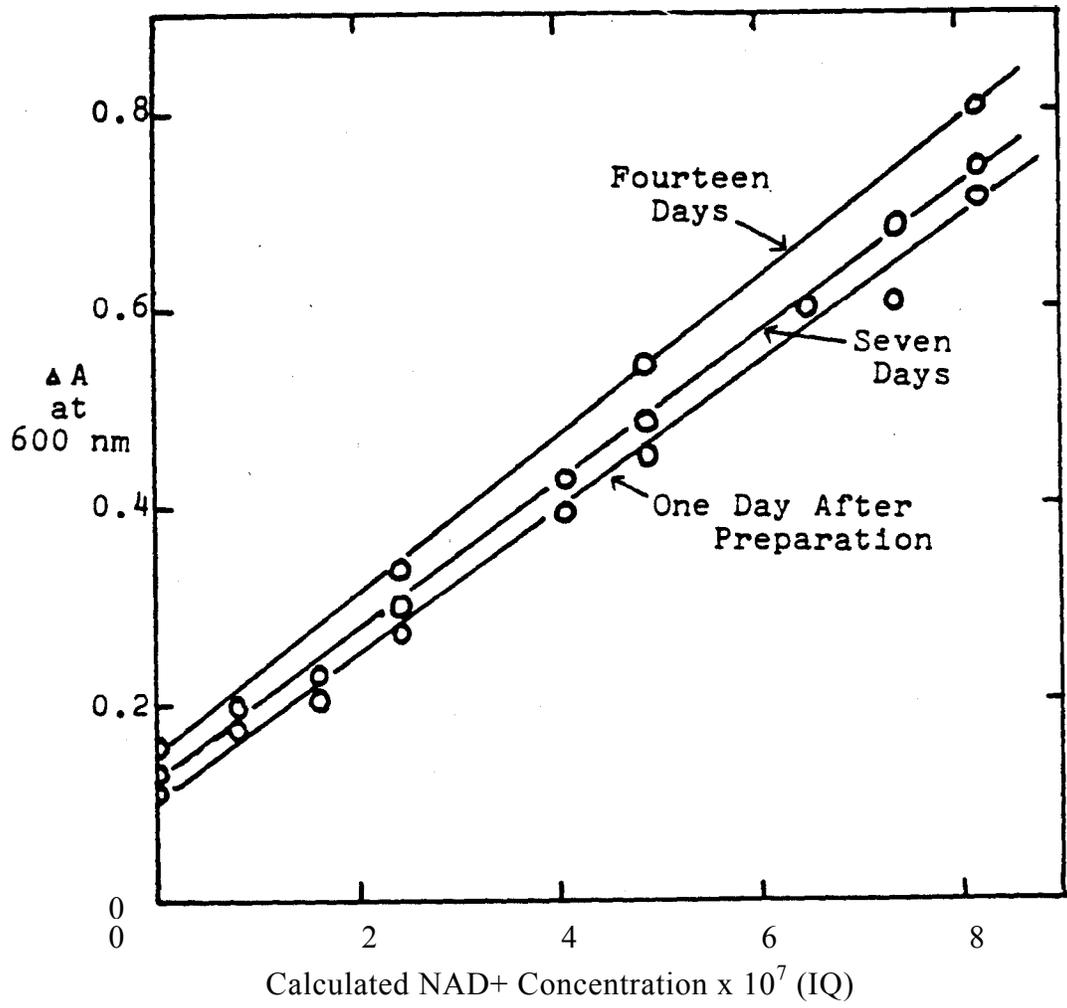


Figure 10:  $\Delta A$  at 600 nm vs. Calculated  $\text{NAD}^+$  Concentration. All Solutions Stored at  $4^\circ\text{C}$



calibration lines but the separation between the calibration lines and the experimental lines increased. A reasonable explanation seemed to be that there was a gradual oxidation of the NADH in the solution prepared for the first reaction. NAD<sup>+</sup> was accumulating in the solution and increasingly contributing to the blank reaction.

Slow oxidation of NADH in solutions of pH 8 could, unfortunately, be expected. Bergmeyer discusses non-enzymatic NADH oxidation at pH 7.8 (35). Other investigators have noted it as well. In a study of the NADH breakdown in acid solutions, Miksic and Brown (36) remark that "In basic solution, oxidation of NADH to NAD can occur as well as cleavage at the sugar or phosphate groups." Margolis et. al. (37), in describing a procedure using high pressure liquid chromatography to assess the purity of NADH, note a slight increase in the peak attributed to NAD<sup>+</sup> on storage of their alkaline 14ADH solutions. Lowry (38) states simply that DPNH (an obsolete abbreviation for NADH), while "...stable toward direct destruction in alkali", is "...susceptible to oxidation even at low temperature." And McComb (39) reports a AA/hour at 340 nm of 0.0005 for 0.1 mmol/l NADH at 25° and pH 7.8 in 0.1 mol/l Tris-Mg<sup>+2</sup> buffer, but does not attribute it to a specific reaction.

Studies on the stability of 14ADH in solution (6,37)

list among the optimum storage conditions the use of buffers of pH 10. That pH cannot be used here, however, as it would in all likelihood cause denaturation of the enzyme. Glutamate dehydrogenase is reported to lose activity rapidly at pH's greater than 8 (40, 41).

As verified by observations here, oxidation of NADH in the glutamate dehydrogenase solution would not affect the first reaction. Because NADH is present in excess, there would still be consumption of NADH equivalent to added ammonium ion. It is only when the two steps are combined that the NAD<sup>+</sup> resulting from that oxidation becomes important. What is transferred to the enzymatic cycling reaction then is not only the NAD<sup>+</sup> produced in the ammonium ion reaction but also NAD<sup>+</sup> resulting from non-enzymatic NADH oxidation. This of course would lead to a non-zero blank as well as increased cycling in each solution. It should be noted, however, that the amount of increase in every sample is constant, so that the linearity of the response is maintained. In an early paper describing an enzyme cycling procedure involving NADH, Lowry (42) acknowledges this condition, noting that "...if the DPNH has become oxidized, it may contribute a much greater blank".

Measurements with the enzymatic cycling system appeared to confirm the suspected NAD<sup>+</sup> accumulation in

the glutamate dehydrogenase solution. On successive weeks, 1.50 ml samples of the GDH solution were analyzed directly on removal from the refrigerator, according; to the established procedure. One-half milliliter of conductivity water was added to maintain the dilution scheme, followed by the acid rain and base treatment and addition of 0.10 ml to the cycling reaction mixture. This procedure was analogous to the blank (zero-ammonium ion) reactions carried out in the total analytical scheme, except that there was no time allowed for the GDH reaction. Thus NAD<sup>+</sup> detected would presumably have originated only in the stored GDH solution. Two preparations were studied in this way, with similar results. On the day following preparation of the GDH solution the  $\Delta A$  at 600 nm measured after 30 minutes of the enzyme cycling reaction was 0.039. After six days the AA was 0.071 and on the thirteenth day it was 0.154. Using the appropriate NAD<sup>+</sup> calibrations of the cycling; system (the calibrations shown in Figure 6) and correcting for dilution, these figures could be interpreted as representing; the accumulation of, respectively,  $3.11 \times 10^{-6}$ ,  $3.71 \times 10^{-6}$ , and  $6.22 \times 10^{-6}$  M NAD<sup>+</sup> on the second, seventh and thirteenth days -- out of an original  $0.947 \times 10^{-4}$  TAI NADH concentration. The 340 nm NADH absorbance of the stored GDH solution was also measured periodically. In the thirteen day period just mentioned the, 340 nm absorbance of that solution

decreased from 0.807 to 0.770. This  $\Delta A$  corresponds to a  $6 \times 10^{-6}$  M NADH decrease ( $\Delta c = \Delta A/c$ ), a value in the same range as the  $6.22 \times 10^{-6}$  M increase in NAD<sup>+</sup> determined from the cycling results.

(Interestingly, this same approach was taken to assess the importance of the base treatment used in originally preparing the GDH solution – to remove the blank attributed mainly to the ADP. Results with the cycling reaction ( $\Delta A = 0.045$ ) indicated that the original blank reaction had led to the buildup of  $4.0 \times 10^{-6}$  M NAD<sup>+</sup> in the solution (originally  $0.944 \times 10^{-4}$  M in NADH). Had this NAD<sup>+</sup> not been destroyed by the base treatment, all subsequent measurements would have been increased by this amount.)

Attempts were made to inhibit non-enzymatic NADH oxidation. Experiments in which glutathione (GSH) was added to the NADH solution -- on the assumption that the GSH would be preferentially oxidized -- did not produce the desired results. Reports in the literature (6, 35, 37) indicate that the optimum conditions for storage of NADH solutions, aside from the use of pH 10 buffers, are a temperature of 4°C and exclusion of light and oxygen. Therefore the practice was to use sealed dark glass containers for the NADH solutions and to bubble argon through the solutions for several minutes (to drive off

oxygen) before storing the solutions in the 4°C refrigerator.

In order to evaluate the precision of the total procedure, eight replicate measurements were made using the same ammonium ion concentration. The samples were carried through the entire procedure as described. The data are in Table 9. The original ammonium ion concentration was  $1.1030 \times 10^{-5}$  M. The mean for the eight measurements was 0.438. The standard deviation was 0.009, or 2.11% of the mean.

Consideration of the sources of error in the procedure led to the conclusion that spectrophotometer precision and, to a lesser extent, cycling time variations were the principle contributors to error. Errors in sample volumes were very small. Standard deviations in the volumes of samples prepared for both the first and second reactions (2.00 ml and 3.00 ml, respectively) were only 0.0053 and 0.0056 ml (0.265% and 0.187%, respectively).

Spectrophotometer error increases with decreasing absorbance. For a series of replicate measurements at 600 nm, the standard deviation was 0.0025 (0.33%) when the absorbance was 0.750 (a final value typically obtained for solutions originally  $1 \times 10^{-4}$  M in ammonium ion). But when the absorbance was 0.022 the standard deviation was 0.0004, or 1.55%. This latter absorbance value is typical of those obtained for the initial measurement (zero-time) for the enzymatic cycling reaction.

TABLE 9

REPLICATE MEASUREMENTS OF  $\Delta A$  AT 600 nm AFTER  
30 MINUTES OF THE ENZYMATIC CYCLING REACTION

Sample	$\Delta A$ at 600 nm	Sample	$\Delta A$ at 600 run
1	0.4355	5	0.4466
2	0.4260	6	0.4465
3	0.4300	7	0.4492
4	0.4291	8	0.4431

Mean: 0.438

Standard Deviation: 0.009



Using a value of 0.750 as typical of the change in absorbance at 600 nm after 30 minutes of the enzymatic cycling reaction for the most concentrated ammonium ion solution ( $2.758 \times 10^{-5}$  M in the GDH reaction), it can be seen that the absorbance change per minute is 0.025. A fifteen-second error in the cycling time would thus produce an error of 0.006 ( $0.025/4$ ), or 0.83%, in the  $\Delta A$ 's obtained. Since the reaction rate is proportional to the concentration, the error would be the same at lower concentrations.

In summary, sample volume errors contribute about 0.2% error, and cycling time errors can contribute 0.8% error to experimental measurements. Spectrophotometer errors contribute about 1.6% error at the highest ammonium ion concentrations used here. At lower concentrations spectrophotometer errors increase when two measurements of low absorbance are made, each has an error approaching 1.55%.

As mentioned earlier, the enzymatic cycling reaction was used to estimate the amount of NAD<sup>+</sup> produced in the original blank reaction encountered in preparing the glutamate dehydrogenase reaction mixture. It is felt that contamination of the ADP added to stabilize the enzyme is primarily responsible for that reaction. The ADP concentration used in the GDH reaction mixture is



$2.0 \times 10^{-3}$  M, or about 1 mg/ml. The estimate of the NAD<sup>+</sup> produced in the solution was  $4.0 \times 10^{-6}$  M.

A series of measurements was made later, using the entire procedure, to see if addition of ADP to the treated GDH solution would lead to an increase in the NAD<sup>+</sup> detected by the cycling reaction. ADP was added to a GDH solution (treated, after the blank reaction) to double the ADP concentration -- to 2 mg/ml. Samples of this GDH solution were carried through the procedure along with samples of GDH solution under the standard conditions. The results, presented in Table 10, showed that for each ammonium ion concentration the enzymatic cycling results were higher for the samples which had contained additional ADP. This can be readily seen the plot of the data in Figure 11, which also contains the NAD<sup>+</sup> calibration line for the cycling reaction. The two experimental lines are parallel (slopes shown in the linear regression results, in Table 10—agree within 3%) and are separated by about 0.06 Absorbance units, a distance produced by an NAD<sup>+</sup> concentration change of about  $0.75 \times 10^{-7}$  M—or about  $2.5 \times 10^{-6}$  M in the solution of the first reaction. In other words, adding ADP led to increase NAD<sup>+</sup> at the completion of the GDH reaction. This supported the supposition that contamination of the ADP was responsible for at least a major portion of the original blank reaction.

TABLE 10

A AT 600 run AFTER 31 MINUTES OF THE ENZYMATIC CYCLING  
REACTION -- WITH AND WITHOUT ADDED ADP

Calculated Molarity NAD+	Absorbance at 600 nm	
	1 mg/ml ADP	2 mg/ml ADP
$8.207 \times 10^{-7}$	0.8055	0.8665
$4.924 \times 10^{-7}$	0.5414	0.6021
$2.462 \times 10^{-7}$	0.3372	0.3958
0	0.1549	0.1904

Linear Regression Analysis:

Slope:	0.0797	0.0824
Ordinate Intercept:	0.1492	0.1923
Standard Deviation of Y:	0.0072	0.0035
Correlation Coefficient:	0.9998	0.9999



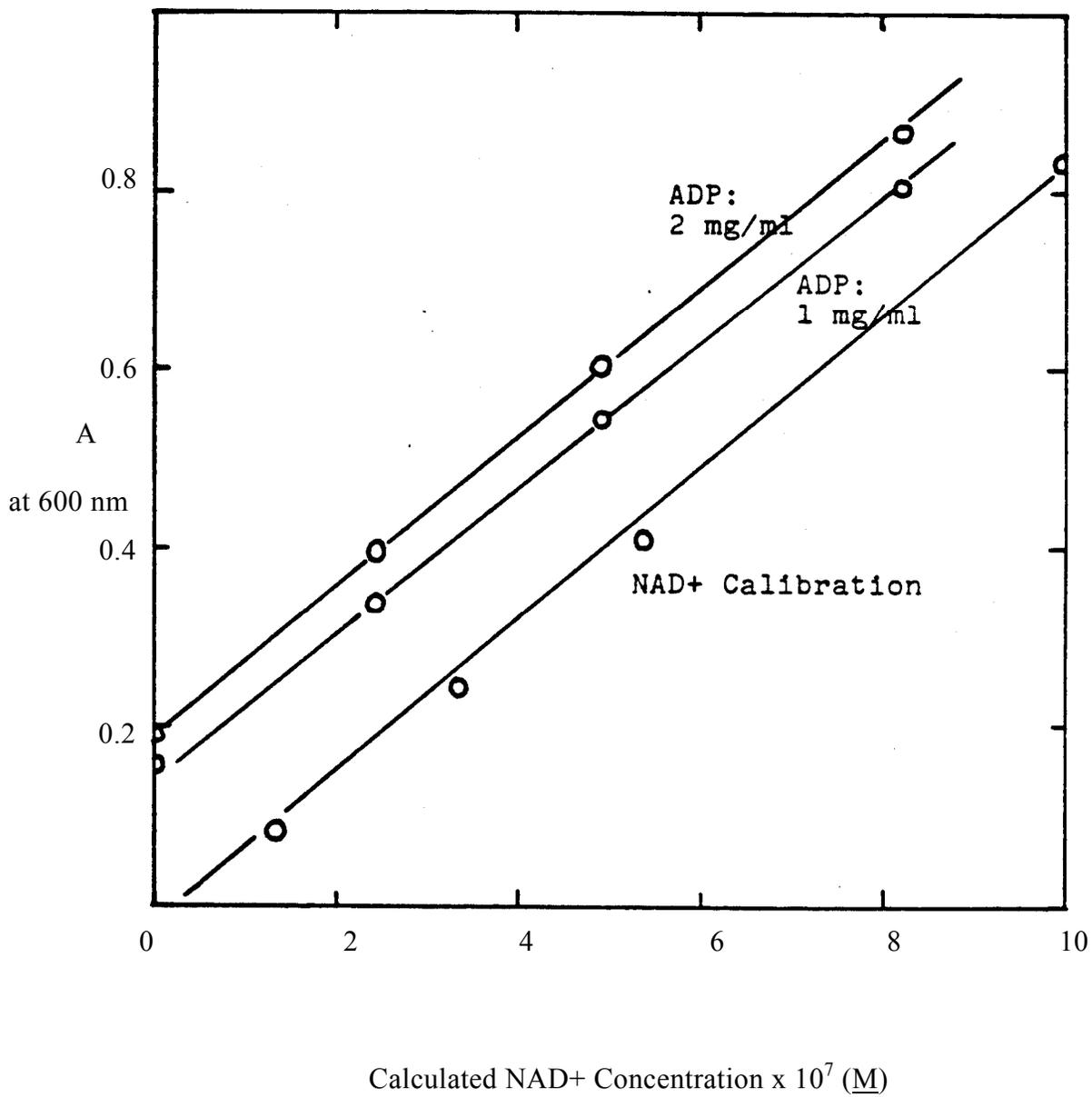


Figure 11: A at 600 nm vs. Calculated NAD+ Concentration, Before and After Doubling of ADP Concentration



One possibility is that the ADP preparation contained ammonium ion. Procedures given in "Biochemical Preparations" (43) and in "Methods in Enzymology" (44) for preparation of ADP from muscle tissues involve initial isolation of ATP (adenosine triphosphate). The procedure in "Methods in Enzymology" includes the subsequent use of ammonium ion-containing solvents in chromatographic purification of the ADP to remove ATP and AMP (adenosine monophosphate). Similar use of an ammonia-containing solvent for separations of AMP, ADP, and ATP is described elsewhere (45). If these procedures were used in the purification of the commercial ADP used in this study, they could conceivably have led to contamination of the ADP by ammonium ion.

Alternatively, Bergmeyer notes that ADP preparations are sometimes contaminated with NADP<sup>+</sup> (46). NADP<sup>+</sup> would not, however, be expected to be detected in the cycling reaction. It has been reported that Yeast alcohol dehydrogenase does not use NADP<sup>+</sup> as a cofactor (47). This possibility was not tested experimentally in the present study.

Further work would be necessary to determine the specific ADP contaminant responsible for the blank reaction encountered in the original glutamate dehydrogenase solution. In any event, the procedure developed here involves treatment of the GDH solution to remove the undesired product of this blank reaction, so that the resultant GDH solution provides the desired linear response to added ammonium ion with only a very small blank.

A final group of experiments were carried out to see what modifications of the procedure would be required to extend the concentration range down to  $10^{-6}$  M ammonium ion. The first approach taken was to dilute the glutamate dehydrogenase solution prepared for the first reaction with buffer, adding GDH to maintain the same enzyme concentration. The cycling reaction conditions were unchanged, except that a larger sample from the GDH reaction was added to the cycling reaction mixture (replacing Tris). But when  $10^{-5}$  to  $10^{-6}$  M ammonium ion solutions were analyzed in this way, the results were not satisfactory. The blank reaction was very large.

In a second attempt, a fresh glutamate dehydrogenase solution was prepared in which the NADH and  $\alpha$ -ketoglutarate concentrations were cut approximately tenfold while the enzyme and ADP concentrations were unchanged.

The blank reaction was allowed to occur and the solution was then treated in the usual way. On the following day 1.50 ml samples were allowed to react with  $10^{-5}$  to  $10^{-6}$  M ammonium ion (prepared by diluting 10 ml of the standard ammonium ion solution to 100 ml with conductivity water) as before, and then treated with acid and base. Concentrations in the GDH reaction were: NADH:

$1.046 \times 10^{-5}$  M; a-KG:  $5.939 \times 10^{-4}$  M; GDH:  $3.484 \times 10^{-8}$  M; ADP:  $1.473 \times 10^{-3}$  M. For the enzymatic cycling reaction, 0.20 ml of each sample was added to a mixture of 0.20 ml of ADH (twice the usual amount) 0.20 ml of MTT-PES, and 2.50 ml of ethanol in Tris. Absorbances at 600 nm were measured at the beginning of the enzymatic cycling reaction and after 120 minutes of cycling. (A previous experiment had established that for this enzyme concentration in the reaction the 600 nm absorbance change with time was indeed linear for at least 150 minutes for  $10^{-7}$  M NAD<sup>+</sup>.) The results are presented in Table 11 and are plotted in Figure 12. It can be seen that the response of the cycling system to added ammonium ion is still linear in this concentration range, but the blank reaction comprises a substantial proportion of that response.



TABLE 11

Δ A AT 600 nm AFTER 120 MINUTES OF THE ENZYMATICAL  
CYCLING REACTION: 10<sup>-5</sup> TO 10<sup>-6</sup> M NH<sub>4</sub><sup>+</sup> ION

Enzyme Cycling Reaction		
Molarity NH <sub>4</sub> <sup>+</sup> In GDH Reaction	Calculated ΔA at	
	Molarity NAD <sup>+</sup>	600 nm 120 Minutes
2.758x10 <sup>-6</sup>	1.588x10 <sup>-7</sup>	0.892
2.482x10 <sup>-6</sup>	1.430x10 <sup>-7</sup>	0.817
2.206x10 <sup>-6</sup>	1.271x10 <sup>-7</sup>	0.794
1.655x10 <sup>-6</sup>	9.535x10 <sup>-8</sup>	0.737
1.379x10 <sup>-6</sup>	7.942x10 <sup>-8</sup>	0.658
8.273x10 <sup>-7</sup>	4.765x10 <sup>-8</sup>	0.604
5.515x10 <sup>-7</sup>	3.177x10 <sup>-8</sup>	0.581
2.758x10 <sup>-7</sup>	1.588x10 <sup>-8</sup>	0.553
0	0	0.497



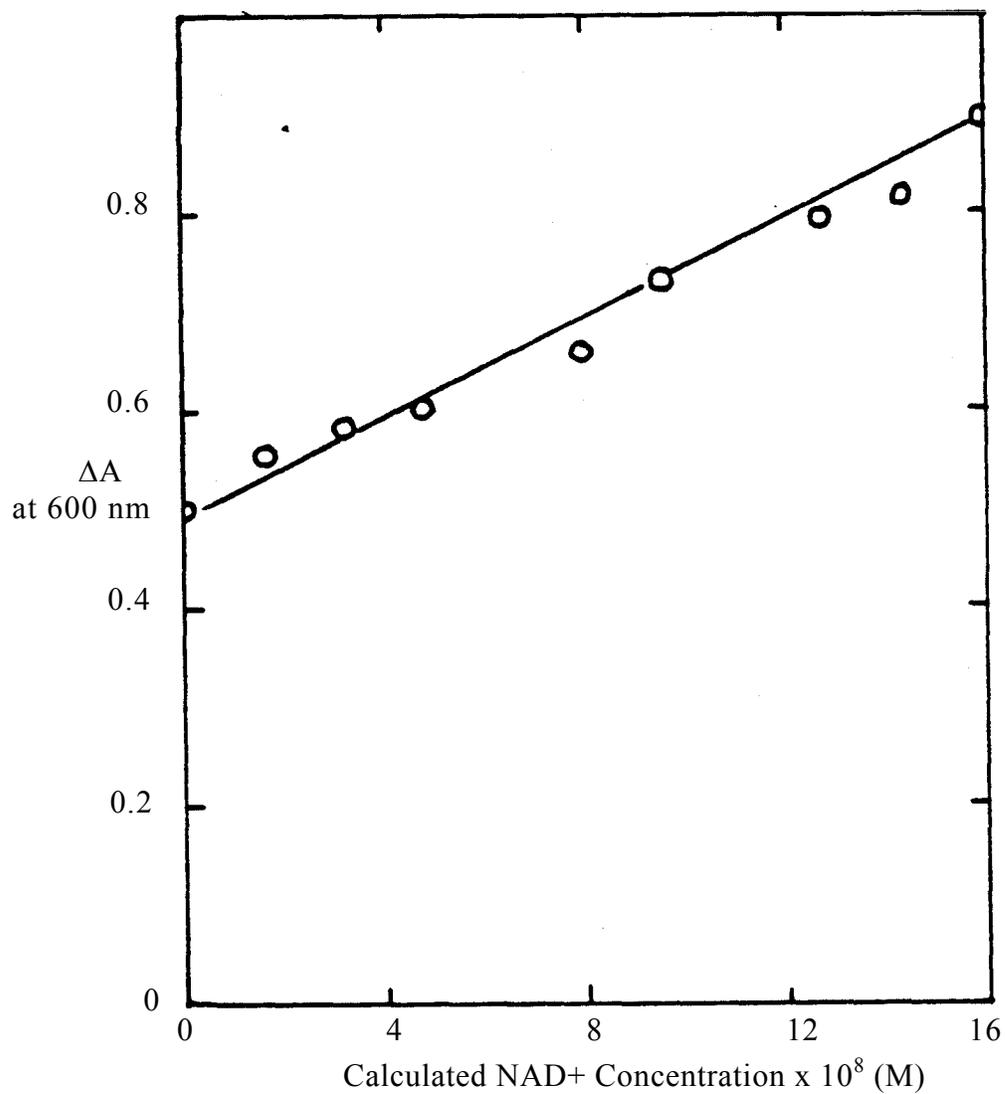


Figure 12:  $\Delta A$  at 600 nm vs. Calculated NAD<sup>+</sup> Concentration After 120 Minutes of Enzymatic Cycling

Slope:	0.0233
Ordinate Intercept:	0.501
Standard Deviation of Y:	0.0172
Correlation Coefficient:	0.993

## CONCLUSIONS

Experimental evidence has been presented to show that the two-step method presented here provides a simple and reliable means of determination of ammonium concentrations in water. It has a number of advantages over other methods. The first step, in which ammonium ion is reacted with  $\alpha$ -ketoglutarate, with simultaneous conversion of NADH to NAD<sup>+</sup>, is an enzymatic reaction.

The reaction is absolutely specific for ammonium ion originally present and is measured in the second step—an enzymatic cycling reaction. Enzymatic cycling systems can, by adjustment of various experimental parameters, be made responsive to a very wide range of reactant concentrations, down to minute quantities. The enzymatic cycling reaction used here culminates in the formation of a soluble, colored product. The overall result is a specific, sensitive procedure by which ammonium ion concentrations are readily determined with a spectrophotometer or colorimeter.

Experimental measurements have been described which verify that the following desirable prerequisites for successful application of the procedure are met.

A) The absorbance of the MTT Formazan, the final product of the cycling reaction, is proportional to its concentration.

B) The amount of formazan produced is proportional to added reducing agent.

The formazan is soluble and the color is stable:

C) The experimental conditions established for the enzymatic cycling reaction provide the desired linear increase with time in absorbance of the formazan. Furthermore, the absorbance after cycling for a set time is proportional to the added NAD<sup>+</sup>.

D) Using the procedure devised for the glutamate dehydrogenase reaction, the consumption of NADH varies linearly with added ammonium ion with only a small blank reaction in the system. The reaction goes to completion, as evidenced by the fact that NADH consumption equals calculated amounts within experimental error.

E) Coupling of the first and second reactions does indeed give a time dependence of the final absorbance measurement which is proportional to the starting ammonium ion concentration.

Application of the method can be summarized in the following four steps.

1) For the first reaction, a reagent containing  $\alpha$ -ketoglutarate, NADH, and glutamate dehydrogenase is prepared. A blank reaction of the system is allowed to occur and the solution is treated with base to destroy

the resultant NAD<sup>+</sup>. The solution is neutralized and fresh enzyme is added.

2) One-half milliliter of ammonium ion solution is added to 1.50 ml of the reagent.

The reaction is allowed to proceed to completion at room temperature, in the dark.

3) Hydrochloric acid (0.12 ml of 1 N HCl) is added to destroy the excess NADH, followed by 0.12 ml of 1 N sodium hydroxide.

4) One-tenth milliliter of the solution obtained from Step 3 is added to a mixture of ethanol, alcohol dehydrogenase, MTT, and PES (total final volume 3.00 ml) for the enzyme cycling reaction. The absorbance at 600 nm is measured at the beginning of the cycling reaction and again after 30 or 60 minutes. Cycling is carried out at 25°C, in the dark.

Standard solutions would be carried through the entire procedure along with samples of unknown concentrations in order to construct a calibration line. The unknown concentrations would be determined from this calibration.

Typical results are shown in Figure 13. The  $\Delta A$  at 600 nm after 30 minutes of the enzymatic cycling reaction is plotted as a function of the concentration of ammonium ion in the standard solution added to the reagent for the first reaction. These plots are linear when the established procedure is followed. Correlation

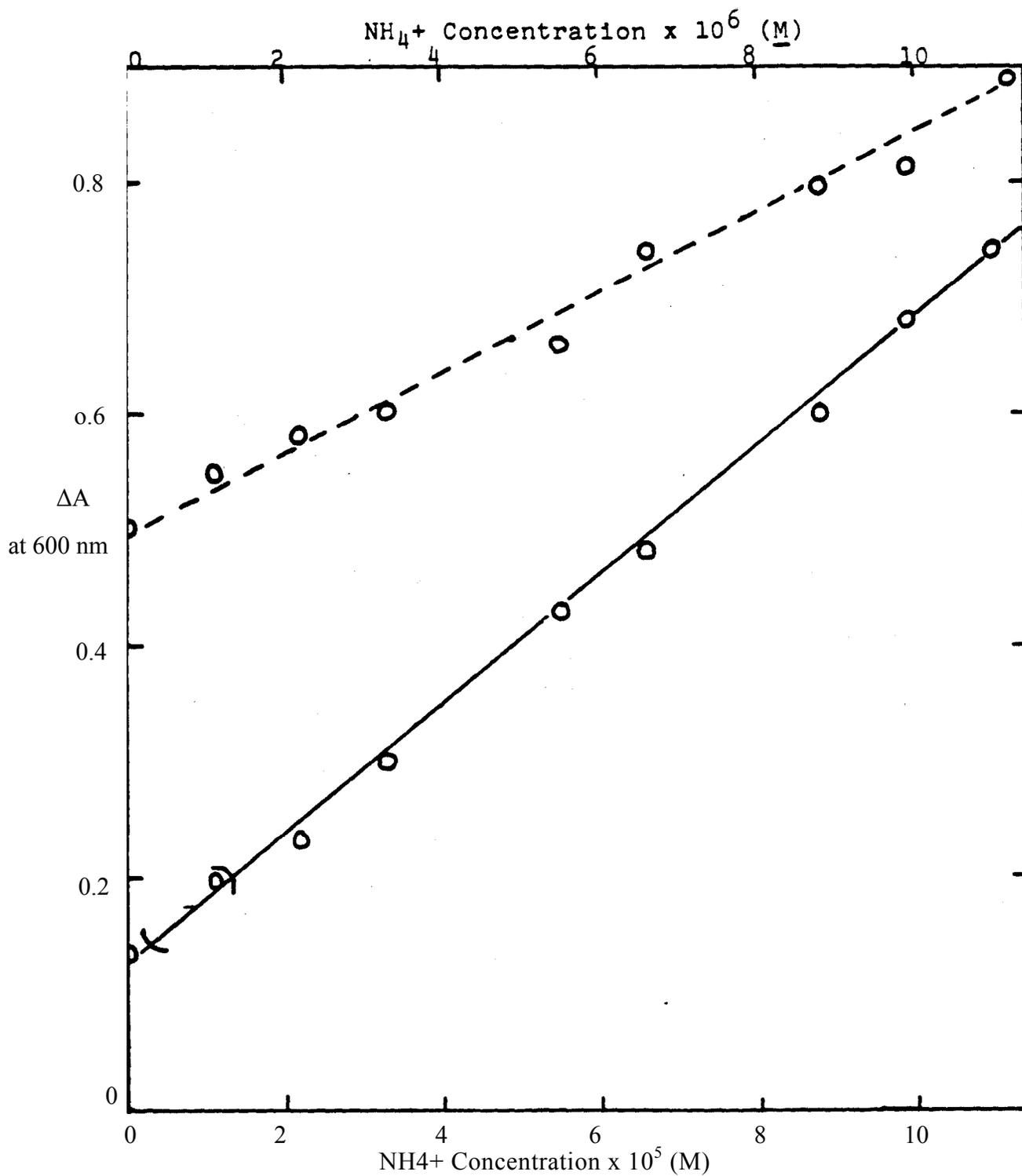


Figure 13:  $\Delta A$  at 600 nm vs. Concentration  $\text{NH}_4^+$  in the Solution Used for the First Reaction

\_\_\_\_\_  $10^{-4}$  to  $10^{-5}$  M  $\text{NH}_4^+$

-----  $10^{-5}$  to  $10^{-6}$  M  $\text{NH}_4^+$



coefficients are generally 0.998 to 0.999. The standard deviation was 2% for a series of replicate measurements. The procedure was designed for ready applicability to the  $10^{-4}$  to  $10^{-5}$  M ammonium ion concentrations generally found in fresh water

The dashed line in Figure 13 shows results obtained for  $10^{-5}$  to  $10^{-6}$  M ammonium ion (concentrations given at the top of the Figure). The dashed line thus represents expansion of the bracketed portion of the solid line. Extension of the concentration range is accomplished by variation of reactant concentration and of the enzyme concentration and time for the cycling reaction.

As stated at the outset, it is believed that this method for determination of ammonium ion concentrations offers a viable alternative to those currently available. Important advantages are its specificity for ammonium ion, with no possibility of interference from amines or other chemicals, and the sensitivity afforded by the enzymatic cycling reaction. Analyses are simple to perform, requiring only standard volumetric equipment and a spectrophotometer or colorimeter. The time allotted to the enzymatic cycling reaction can be adjusted to suit the convenience of the analyst. All solutions can be prepared in advance and can be stored under refrigeration for at least two weeks. In addition, all chemicals are commercially available.

The major limitation is the apparent breakdown of the NADH in the solution prepared for the first reaction. The result is a gradually increasing blank reaction.

This becomes more important at very low ammonium ion concentrations. It should be noted, however, that even though the blank reaction is increasing, the response of the system to added ammonium ion is still linear.



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