1988

Determination of iron, copper and zinc in a single aliquot of serum sample using 4-([5-bromo-2-pyridyl]azo)resorcinol; Fluorometric cyclic assays for pyridine nucleotides which are reduced or oxidized in enzyme coupled assays.

Gerardo Maula. Castillo

University of Windsor

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PART I
DETERMINATION OF IRON, COPPER AND ZINC
IN A SINGLE ALIQUOT OF SERUM SAMPLE
USING 4-[5-BROMO-2-PYRIDYL]AZORESORCINOL

PART II.
FLUOROMETRIC CYCLIC ASSAYS
FOR PYRIDINE NUCLEOTIDES WHICH ARE REDUCED
OR OXIDIZED IN ENZYME COUPLED ASSAYS

by
GERARDO-MAULÁ CASTILLO

A THESIS
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry and Biochemistry in
Partial Fulfillment of the Requirements for the
Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
1987
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FOREWORD

For some chemists, devising a way of analyzing small amounts of material has always been a challenge if a valid excuse could be found to do so. It has always dominated our goal in method development. The fact that cells are very small, limited in availability, and that diagnostically important substances are often present in exceedingly low concentration are enough justification. Moreover, substances in cells, so small in quantity, and never before encountered, may be stumbled upon using sensitive microanalytical techniques and this may shed some light in the endless pursuit of knowledge about the normal and disease processes. Sensitive analytical methods extend our senses to see beyond what is and verify what might be.

G. Castillo

Windsor, Ontario

December, 1987
ABSTRACT

PART I

DETERMINATION OF IRON, COPPER AND ZINC
IN A SINGLE ALIQUOT OF SERUM SAMPLE
USING 4-[(5-BROMO-2-PYRIDYL)AZO]RESORCINOL

by

GERARDO MAULA CASTILLO

The individual and the sequential determinations of iron, copper and zinc using 4-[(5-bromo-2-pyridyl)azo]resorcinol (Br-PAR) were investigated in terms of its applicability in serum samples. The methods obey Beer's law from 25-500 μg dL⁻¹. The methods show agreement between individual and sequential determination. The recovery studies at five different levels of Fe, Cu and Zn averaged (mean ± SD): 99.7 ± 5.1%; 104.4 ± 3.8%; and 100.9 ± 3.2%, respectively. The within-assay CV's taken at six different serum levels for Fe, Cu and Zn averaged (mean ± SD): 3.4 ± 1.2%; 3.7 ± 1.8%; and 3.5 ± 1.9%, respectively. The between-assay CV's for Fe, Cu and Zn in serum taken at four different levels and twenty-one different occasions averaged out to be (mean ± SD): 4.3 ± 1.0%; 5.3 ± 1.7%; and 4.3 ± 1.0%, respectively. The Fe, Cu and Zn determined sequentially using Br-PAR was compared with 5,5'13-2(−pyridyl)-1,2,4-triazine-5,6-diylbis-2-furan sulfonic acid (PTF-600), bathocuproine sulfonate (BCS) and 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP) methods, respectively. The correlation equations for the comparison studies with Fe, Cu and Zn were: y = 0.9918x + 8.052 (r=0.9662); y = 0.8812x + 11.44 (r=0.9603); y = 0.9918x + 1.748 (r=0.9924), respectively.
ABSTRACT

PART II

FLUOROMETRIC CYCLIC ASSAYS
FOR PYRIDINE NUCLEOTIDES WHICH ARE REDUCED
OR OXIDIZED IN ENZYME COUPLED ASSAYS

by

GERARDO MAULA CASTILLO

Almost every substance in a cell is either subject to enzyme attack or can be influenced by enzyme action. Most enzyme action involves reduction or oxidation of pyridine nucleotides or can be caused to do so with the aid of auxiliary enzymes. In fact nearly every substance of biological interest could, in principle, be measured with a pyridine nucleotide system. We have developed sensitive fluorometric cyclic assays for pyridine nucleotides that are reduced or oxidized in enzyme coupled assays. The assays are capable of detecting pyridine nucleotides as low as 100 fmol with very good precision and can further be modified to detect much smaller amounts, if desired.

The cyclic assay is based on the regeneration of substrate being measured in order to achieve a measurable sustained rate of reaction which otherwise is not possible if the concentration of substrate being measured is so low that it usually runs out in less than a second. The approach for optimization of such a method is documented in this thesis.
As analyte models, we determined glucose 6-phosphate dehydrogenase in erythrocytes and ATP in pure solution. The cycling method is capable of detecting 20 nU of glucose 6-phosphate dehydrogenase which is roughly the amount of enzyme in 50 erythrocytes. It was also found that the cycling method is capable of detecting 100 fmol of ATP.
ACKNOWLEDGEMENTS

I would like to express my sincerest thanks and appreciation to Dr. Roger J. Thibert for his patience, financial support and academic guidance throughout my entire program.

I thank Dr. Bennie Zak, my co-advisor, for his patience on going over the first part of this work. Moreover, I would like to thank him and Dr. Narace Seudeal for the reagents that were used in the first part of this work.

I would like to express my appreciation to Dr. Trevor Hyde, Miss Eileen Chape and all the staff of the Chemistry Laboratory, Hotel-Dieu Hospital, Windsor, Ontario, for their assistance during my Clinical Chemistry internship. Warmest thoughts to all of those who became friends to me and who made my internship such an enjoyable experience.

My appreciation also extends to the staff of the Chemistry laboratory, Grace Hospital, Windsor, Ontario for providing serum samples for trace metal analysis.

Thanks also to the rest of my committee, Drs. Keith Taylor and Bruce Virgo for their helpful comments and suggestions.

Warmest thoughts to my colleagues in the Department of Chemistry and Biochemistry, University of Windsor who became close friends to me which I shall always cherish. A special mention must be made to those in my group, Peter Catomeris and Gheorghe Brotea, for their cooperation and sacrifices that enabled successful completion of this work.

I would also like to thank Miss Maeve Doyle for her accurate and expedient typing of this thesis.
DEDICATION

Dedicated to my parents, Romualdo and Remedios, and my brothers and sisters, whose concern and understanding made all this possible.
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PART I

DETERMINATION OF IRON, COPPER AND ZINC
IN A SINGLE ALIQUOT OF SERUM SAMPLE
USING 4-[(5-BROMO-2-PYRIDYL)AZO]RESORCINOL

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<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>AA</td>
<td>ascorbic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALDH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCS</td>
<td>bathocuproine sulfonate</td>
</tr>
<tr>
<td>Br-PADAP</td>
<td>2-((5-bromo-2-pyridyl)azo)-5-diethylaminophenol</td>
</tr>
<tr>
<td>Br-PAR</td>
<td>4-((5-bromo-2-pyridyl)azo)resorcinol</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>( \lambda_{em} )</td>
<td>emission wavelength</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>( \lambda_{ex} )</td>
<td>excitation wavelength</td>
</tr>
<tr>
<td>fmol</td>
<td>femtomoles (= ( 10^{-15} ) moles)</td>
</tr>
<tr>
<td>G-6-P</td>
<td>glucose 6-phosphate</td>
</tr>
<tr>
<td>G-6-PDH</td>
<td>glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HK</td>
<td>hexokinase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>i-MPMS</td>
<td>1-methoxyphenazine methosulfate</td>
</tr>
<tr>
<td>n</td>
<td>number of samples</td>
</tr>
<tr>
<td>NAD(^+)</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide reduced form</td>
</tr>
<tr>
<td>NADP(^+)</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate reduced form</td>
</tr>
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<td>N-EMPES</td>
<td>N-ethylmethoxyphenazine ethosulfate</td>
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PES  phenazine ethosulfate
6-P-G  6-phosphogluconate
pmol  picomole (= $10^{-12}$ moles)
PMS  phenazine methosulfate
PTF-600  5,5'13-2(-pyridyl)-1,2,4-triazine-5,6-diyl]bis-Z-furan sulfonic acid
SD  standard deviation
SDS  sodium dodecyl-sulfate
tCA  trichloroacetic acid
Tris  tris(hydroxymethyl)aminomethane
PART I

DETERMINATION OF IRON, COPPER AND ZINC
IN A SINGLE ALIQUOT OF SERUM SAMPLE
USING 4-((5-BROMO-2-PYRIDYL)AZO)RESORCINOL
CHAPTER I

INTRODUCTION

A. Background

The three most common trace metals present in serum are iron, copper and zinc. Their concentrations are relatively similar in the normal range but values for each metal may vary somewhat differently in pathological circumstances (1). Many reagents have been proposed to determine iron, copper or zinc concentrations in various types of samples (2–8). Several of these have been used together to determine more than one of these metals in a single sample (9–11). The more practical of these procedures involves the determination of copper and iron on a single aliquot of filtrate by sequential reaction using bathocuproine sulfonate for copper, then bathophenanthroline sulfonate for iron, followed by an assay for zinc on a second aliquot by masking all metals with cyanide and selectively demasking the zinc–cyanide complex with chloral hydrate allowing zinconate formation (1). Recently, Seudeal et al. (12) reported a single ligand used for the determination of all three metals sequentially in a single aliquot of sample. The reagent 4-((5-bromo-2-pyridyl)azo)resorcinol (Br–PAR)* (Fig. 1) was originally synthesized by Busev and Ivanov (13) as a sensitive auxochromic derivative of pyridylazoresorcinol for the determination of indium and cobalt (14,15). The procedure for synthesis of this compound was modified and improved by Seudeal et al. (12) with the advantage of obtaining high purity while less time is required for the synthesis. This reagent forms highly colored chelates with iron, copper and zinc and was proposed for use in a sequential procedure

*incorrectly named as 2-amino-5-bromopyridylazo resorcinol (12)
FIGURE 1
STRUCTURE OF Br-PAR SODIUM SALT

Legend

The 4-(5-bromo-2-pyridyl)azoireso®cinol (Br-PAR) is a reagent that forms complexes with iron, copper and zinc giving characteristic absorbance at 510 nm. The addition of CN⁻ to the mixture of these complexes results in the dissociation of the Br-PAR-copper complex but not the iron and the zinc complexes of Br-PAR. The addition of EDTA can cause the dissociation of both the copper and zinc complex of Br-PAR leaving the iron complex of Br-PAR intact.
for the determination of these metals by binding them with Br-PAR under controlled conditions, and selective removal of copper with cyanide and of zinc with EDTA.

The scheme of reactions for the sequential determination of iron, copper and zinc are as follows:

\[
\begin{align*}
\text{Br-PAR} + \text{Fe(II)} + \text{Cu(I)} + \text{Zn(II)} & \quad \rightarrow \quad \text{Fe(Br-PAR)$_2$ + Cu(Br-PAR)$_2$ + Zn(Br-PAR)$_2$} \\
& \quad \text{All these absorb at 510 nm} \\
& \quad \text{CN}^- \\
& \quad \text{Fe(Br-PAR)$_2$ + Zn(Br-PAR)$_2$ + Cu(CN)$_{complex}$} \\
& \quad \text{This does not absorb at 510 nm} \\
& \quad \text{EDTA} \\
& \quad \text{Fe(Br-PAR)$_2$ + ZnEDTA + CuEDTA + Cu(CN)$_{complex}$} \\
& \quad \text{These do not absorb at 510 nm}
\end{align*}
\]

The first absorbance reading will be taken before the addition of cyanide. This absorbance corresponds to the total amount of iron, copper and zinc in the sample. The extent of the decrease in absorbance after the addition of cyanide corresponds to the amount of copper in the reaction mixture. If the treatment of cyanide is followed by the addition of EDTA, the second decrease in absorbance will be observed. The extent of the second decrease in absorbance corresponds to the amount of zinc in the reaction mixture. The remaining absorbance corresponds to the amount of iron in the reaction mixture.
B. Purpose of the Study

The purpose of this study is to explore the application of the Br-PAR reagent in the sequential determination of iron, copper and zinc in human serum as well as its use for the individual determination of any of the three metals. The recovery and precision of the method will be studied. The method will be compared with the 5,5'[3-2(-pyridyl)-1,2,4-triazine-5,6-diyl]bis-z-furan sulfonic acid (PTF-600) for iron, bathocuproine sulfonate (BCS) for copper, and 2-[(5-bromo-2-pyridyl)azol-5-diethylaminophenol (Br-PADAP) for zinc.

Because of the small sample size required (0.300 mL) which can be further decreased by using smaller reaction volume, this can be an ideal reagent in the clinical laboratory for the determination of any or all of the three metals, i.e., iron, copper, and zinc in biological sample. This method could also be applied to the pharmaceutical industry where zinc determination is used for quality control purposes in insulin preparations (16). It will also ensure that copper and iron are not interfering with zinc determination.
CHAPTER II

MATERIALS AND METHODS

A. Apparatus

A Shimadzu UV-Visible recording spectrophotometer UV-240 with 1-cm cells was used for absorbance measurements. Centrifugation was carried out using an Eppendorf centrifuge 5414 for separation of the filtrate from TCA-precipitated serum protein.

B. Reagents

1. Chemicals. 4-(5-Bromo-2-pyridylazo)resorcinol (Br-PAR) was synthesized in this laboratory as previously described (12). Bathocuproine sulfonate (BCS) was obtained from G.F. Smith Chemical Co., Columbus, OH. 2-(5-Bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP) and the atomic absorption standards Fe in 2% HNO₃ (1000 µg mL⁻¹), Zn in 2% HCl (1000 µg mL⁻¹), and Cu in 2% HNO₃ (1001 µg mL⁻¹) were purchased from Aldrich Chemical Co., Milwaukee, WI. Sodium dodecyl sulfate (SDS) was from Sigma Chemical Co., St. Louis, MO. Other reagents of analytical grade mentioned below were from Fisher Scientific Co., Don Mills, ON, Canada.

2. Metal-free water. The water is distilled and passed through Xenopure Laboratory Water System, Mega-90 from Xenon Environmental, Burlington, ON, Canada.

C. Solutions

1. Borate buffer, pH 9.6-9.8. Dissolve 7.75 g boric acid and 20 mL of 4N NaOH in 250 mL, acid-washed volumetric flask using metal-free water and make up to the mark.
2. Trichloroacetic acid (TCA) for Br-PADAP procedure. Dissolve 10.0 g TCA in a 100-mL, acid-washed, volumetric flask using metal-free water. TCA solutions of other concentrations should be prepared in the same manner.

3. Trichloroacetic acid-ascorbic acid precipitating solution (TCA-AA). For Br-PAR procedure, add 0.38 g ascorbic acid in 50.0 mL of 15% TCA. For BCS procedure, add 0.50 g ascorbic acid in 50.0 mL of 20% TCA. These should be prepared fresh before the assay.

4. 4-[(5-(Bromo-2-pyridyl)azo)resorcinol (Br-PAR), 9.0 x 10^{-4}M. Dissolve 0.030 g in a 100-mL, acid-washed volumetric flask using metal-free water. To make a working solution of Br-PAR-NaOH, mix 25.0 mL Br-PAR and 6.5 mL of 4N NaOH. This should be prepared fresh for each assay.

5. Sodium cyanide. Dissolve 0.150 g sodium cyanide in 100 mL metal-free water. To make a working solution of cyanide, take a 1.0-mL aliquot and dilute to 6.0 mL with metal-free water.

6. Disodium ethylenediaminetetraacetate (EDTA). EDTA (7.0 g) was dissolved in 100 mL of metal-free water.

7. Bathocuproine sulfonate (BCS). Dissolve 0.030 g of BCS in 100 mL of 50% ammonium acetate and warm to dissolve if necessary.

8. 2-(5-Bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP). Add 0.008 g of Br-PADAP to a 25-mL, acid-washed volumetric flask. Add 1.0 mL of N,N'-dimethylformamide and dilute to mark, then mark with SDS solution (see below).

9. Sodium dodecyl sulfate (SDS). Dissolve 15.0 g of SDS in metal-free water and dilute to 100 mL.
10. SDS-Tris buffer. Add 2.4 g of Tris to a beaker containing 40 mL of SDS solution. Titrate to pH 8.0 with HCl, add 0.250 g ascorbic acid and 0.075 g NaCN, then dilute to 50 mL with metal-free water.

11. Chlortal hydrate solution. Dissolve 8.0 g chlortal hydrate and dilute to 100 mL with metal-free water.

12. Metal standards. Use stock metal solutions (50 μg mL⁻¹) to prepare two types of standards. To prepare standards containing only one metal, take an aliquot of the respective metal (either iron, copper or zinc) stock solution and dilute to produce standards with concentrations ranging from 25 to 500 μg dL⁻¹. To prepare "mixed" standards, i.e., each standard contains all three metals in a 1:1:1 ratio, mix equal aliquots of stock metal solutions for iron, copper and zinc in a 100-mL, acid-washed, volumetric flask and dilute to the mark with metal-free water to obtain metal concentrations ranging from 25:25:25 to 500:500:500 μg dL⁻¹.

D. Procedures

1. Filtrate preparation for Br-PAR procedure. Pipet 0.30 mL of sample to be analyzed into a plastic microcentrifuge tube. Add 0.60 mL of TCA-AA precipitating solution. Cap the centrifuge tube, mix well and centrifuge for 10-15 min.

2. Filtrate preparation for BCS procedure. Pipet 0.60 mL of sample to be analyzed into a plastic microcentrifuge tube. Add 0.60 mL of freshly prepared TCA-AA precipitating solution, cap the tube and process as above.

3. Filtrate preparation for Br-PADAP procedure. Pipet 0.30 mL of sample to be analyzed into a plastic microcentrifuge tube. Add 0.30 mL 10% TCA solution, cap the tube and process as above.
4. Determination of iron, copper and zinc in serum using Br-PAR. Add 0.80 mL supernate to 0.63 mL Br-PAR-NaOH solution into a 3-mL cuvet followed by 1.5 mL buffer. Read the absorbance, "A", at 510 nm against a blank. The blank is prepared in the same way as the sample except that the sample is replaced with the metal-free water that was used to make up the standard. The absorbance "A" represents the total absorbance due to Fe(II), Cu(I) and Zn(II) chelates of Br-PAR (12).

The Br-PAR, NaOH and buffer can be premixed if desired, but slightly longer time (~ 2 min) is required for complete color formation rather than instant color formation as above.

a. Determination of iron. To both blank and sample above add 0.40 mL of EDTA and read the absorbance corresponding to Fe(II) chelate of Br-PAR after about 2 min.

b. Determination of copper. To the above reaction mixture that gave absorbance "A", add 80 µL of sodium cyanide solution to both blank and sample and mix gently (to avoid bubble formation) using an acid-washed, plastic, Pasteur pipette. Read the absorbance "b" at 510 nm after about a minute. The absorbance "b" represents Fe(II) and Zn(II) chelates of Br-PAR. Therefore, after volume correction, (1.027 b=B) the difference A-B is the absorbance due to the Cu(I) chelate of Br-PAR.

c. Determination of zinc. Add 0.40 mL EDTA to both the above sample and blank and mix as previously described. Read the absorbance "c" at 510 nm in about 1.5 min. The absorbance "c" represents the Fe(II) chelate of Br-PAR and therefore can be subtracted from "b" after volume correction to give the absorbance due to the Zn(II) chelate of Br-PAR.
5. Determination of iron, copper and zinc sequentially. After determination of the absorbance "A" above, add 80 μL of sodium cyanide solution to both blank and sample and mix gently (to avoid bubble formation) using an acid-washed, plastic pasteur pipette. Read the absorbance "b" at 510 nm 1 min after the addition of cyanide. Then add 0.40 mL EDTA to both blank and sample and mix gently as before. Read the absorbance "c" at 510 nm 1.5 min after the EDTA addition.

To avoid running a blank for every sample, the blank can be run separately against air and the absorbance taken at 3 stages of analysis: before cyanide addition; after cyanide addition; after EDTA addition. The absorbance values thus obtained can be subtracted from the absorbances of the corresponding stages of the sample read against air.

6. Comparison Methods:

a. Determination of iron using PTF.600. The PTF-600 (5,5′[3-2(-pyridyl)-1,2,4-triazine-5,6-diyll bis-2-furan sulfonic acid) method (17) for iron determination was the automated method as described for the Perspective (American Monitor Corp., Indianapolis, Indiana).

b. Determination of copper using BCS. The procedure followed was that of Watkins et al. (1). Pipet 1.0 mL supernate into 0.25 mL BCS and read the absorbance at 484 against a reagent blank made with metal-free water replacing the sample.

c. Determination of zinc using Br-PADAP. The procedure followed was that of Homsher et al. (2). Pipet 1.0 mL of SDS-Tris buffer into 0.40 mL of supernate, mix, then add 1.0 mL of Br-PADAP color reagent. Adjust the spectrophotometer to zero against reagent blank at 555 nm, add 50 μL of chloral hydrate solution
and measure the absorbance of the resulting complex within 3 min. (Note: crystalline chloral hydrate seems to deteriorate if left on the shelf for a long time.)

7. Samples. Blood specimens from individuals, both hospitalized and ambulatory patients, were collected in plain red-top Vacutainers, centrifuged, and were stored at 4°C until use. The red-top Vacutainers were tested and found to release zinc of about 10-20 μg dL⁻¹ to metal-free water and supposedly do the same for blood sample during collection but to what extent has not been determined. Since we are not attempting to determine normal range of zinc in a normal population, samples collected this way can be used for our study.
CHAPTER III

RESULTS AND DISCUSSION

A. Determination of Iron, Copper and Zinc Individually

1. Calibration. Typical equations for lines of regression (Beer's law plot, i.e., absorbance versus concentration in \( \mu g \, dL^{-1} \)) for iron, copper, and zinc standards were: 
\[ y = 1.043 \times 10^{-3}x + 0.0022 \quad (r=0.9999); \quad y = 1.048 \times 10^{-3}x + 0.0200 \quad (r=0.9996); \quad y = 1.258 \times 10^{-3}x + 0.0029 \quad (r=0.9998), \]
respectively. Each of the above equations was taken from six different concentrations ranging from 50–300 \( \mu g \, dL^{-1} \). All three equations obey Beer's law. The wider concentration range, 25–500 \( \mu g \, dL^{-1} \), was also investigated and found to obey Beer's law, using the same volume of sample and reaction mixture. In terms of "reagent sensitivity", the absorbance change of 0.001 unit represents 0.09, 0.08, 0.07 \( \mu g \, dL^{-1} \) change in concentration of iron, copper, and zinc, respectively. The "method sensitivity" depends on pipetting precision since greater variability in pipetting would make it difficult to resolve two values close to each other.

B. Individual Determination Versus Sequential Determination

The determination of serum iron, copper, and zinc individually using Br-PAR was not different from sequential determination, except that for iron the addition of cyanide was not necessary, since EDTA displaces or removes both copper and zinc from their Br-PAR chelates. The twenty-eight serum samples measured both individually and sequentially for iron using Br-PAR gave a regression equation of 
\[ y = 1.004x - 0.4474 \quad (r = 0.9996) \] when compared (see Fig. 2).
FIGURE 2

CORRELATION OF SERUM IRON
DETERMINED INDIVIDUALLY AND SEQUENTIALLY
USING Br-PAR

Legend

The regression equation is: \( y = 1.004x + .4474 \) \( (r = 0.9996) \). The sample size, \( n = 28 \). That is, 28 serum samples were analyzed individually for iron using Br-PAR. See CHAPTER II, p. 9 for procedure. The same 28 serum samples were analyzed sequentially for iron, copper and zinc using Br-PAR (see CHAPTER II, p. 10 for procedure) and the values obtained for iron were plotted against their corresponding value obtained from individual determinations. Each value was a result of duplicate determinations.
C. Determination of Iron, Copper, and Zinc Sequentially

1. Flexibility of the procedure. The determination of iron, copper, and zinc sequentially was modified slightly from the previous study (12) for convenience by premixing the Br-PAR and NaOH, since this does not show any different effect compared to when the reagents were pipetted individually into the cuvette. Another modification was tried by premixing the Br-PAR, NaOH and buffer. This modification, however resulted in a slightly longer time for complete color formation (~2 min) but was feasible, more convenient, and decreased the pipetting variation from aliquot to aliquot. All the determinations in this laboratory were performed using the Br-PAR-NaOH mixture rather than the premixed Br-PAR-NaOH-buffer reagents.

The Br-PAR procedure can be scaled down as follows: To obtain the filtrate, use 100 μL serum and mix with 200 μL TCA-AA and process as in the 3-mL procedure. Premix Br-PAR, 4N NaOH, and borate buffer by mixing 5 mL, 1.3 mL, 15 mL, respectively. For the assay use 0.25 mL of filtrate, 0.67 mL of Br-PAR-NaOH-buffer mix, 25 μL CN⁻ and 125 μL EDTA. This would be convenient in pediatric clinical chemistry.

The concentration of cyanide used in the 3-mL sequential procedure was lower (20 μg per 80 μL delivered to the reaction mixture) than the amount used previously (12) to avoid slight displacement of zinc from Br-PAR-ZnII chelate. The concentration of EDTA masking solution was increased from 6.0 g dL⁻¹ to 7.0 g dL⁻¹ to speed up the rate of displacement of zinc from Br-PAR and eventual reading of the result. The time allowed for second reading to be taken was 1 min (after cyanide addition) and 1.5 min (after EDTA addition) for the third or last reading. Vigorous mixing after addition of the masking agents
should be avoided to prevent bubble formation. After filtrate preparation, this procedure would allow a turn-around-time of less than three minutes. The use of raw serum without protein precipitation has not yet been tried in this laboratory. The elimination of the preparation of a filtrate would make the procedure more amenable to automation.

2. Calculations. The following scheme was used to determine each metal in a single reaction vessel where the matrix of the reaction change is only due to the masking agents' dilution (see Fig. 3). In order to relate subsequent readings of absorbance of different volumes due to the addition of masking agent, correction for dilution was necessary:

**Scheme:**

Steps in sequential analysis

1. \([\text{Fe}^{2+} + \text{Cu}^{+1} + \text{Zn}^{2+}] + \text{Br-} \text{PAR}\)

2. \(\text{A} + \text{CN}^-\)

3. \(\text{b} + \text{EDTA}\)

4. Normalization to 2.93 mL initial volume
   a) Normalized absorbance due to \([\text{Fe}^{2+} + \text{Zn}^{2+}] = b \times 1.027 = B\)
   b) Normalized absorbance due to \([\text{Fe}^{2+}] = c \times 1.164 = C\)

5. \(\text{A-B}\)

6. \(\text{B-C}\)
FIGURE 3

ABSORPTION SPECTRUM CHANGES
UPON ADDITION OF MASKING AGENTS

Legend

Absorption spectrum change upon addition of masking agents to reaction mixture in the cuvette containing 300: 300: 300 µg dL⁻¹ standard of iron, copper, and zinc: — — — spectra found, — — — calculated spectra for correction due to the dilution, (A): initial absorption spectrum, (b): absorption spectra after cyanide addition, (B): spectrum of b if no volume change, (c): absorption spectrum after EDTA addition, (C): spectrum of c if no volume change occurs.
FIGURE 3

**ABSORBANCE**

**WAVELENGTH (nm)**

- Curve A
- Curve B
- Curve C
The 1.027 is the factor that is used to normalize the second absorbance back to original volume of 2.93 mL in order for the subtraction of the first and second readings to be analytically valid. If this correction is not made, there will be a negative error of 2.7% in the combined iron and zinc absorbance found which then goes towards the copper. Similarly, 1.164 is used to normalize the third reading back to original volume of 2.93 mL before subtraction, thus compensating for the 16.4% decrease in iron that would have occurred in the sequential determination.

3. Calibration. Typical equations for the lines of regression (Beer's law plot, i.e., absorbance versus concentration in μg dL⁻¹) for iron, copper and zinc standards determined sequentially (Fig. 4) were: \( y = 1.057 \times 10^{-3} x + 0.0022 \) (\( r = 0.9998 \)); \( y = 1.061 \times 10^{-3} x - 0.0215 \) (\( r = 0.9990 \)); \( y = 1.256 \times 10^{-3} - 0.0050 \) (\( r = 0.9995 \)), respectively. The above equations were taken from six different concentrations determined in duplicate at a concentration range of 50-300 μg dL⁻¹. All three equations obey Beer's law. The wider concentration range 25-500 μg dL⁻¹ was also found to obey Beer's law. It is recommended that all the samples be run together with the standards since a slight variation of standard curves may occur from run to run.

4. Analytical recovery. Table 1 shows the percent recoveries at five different levels of iron, copper, and zinc, and averaged (mean ± SD): 99.7 ± 5.1%; 101.4 ± 3.8%; and 100.9 ± 3.2%, respectively. This indicates that a matrix effect is non-existent because the amount of metals added to the serum were recovered as completely as if they were protein-free standard (12).
FIGURE 4

SEQUENTIAL DETERMINATION OF IRON, COPPER AND ZINC

Legend

Masking agents are added at 5-min intervals and the resulting absorbances at each stage is relatively flat for 5 min. Asterisks indicate the time at which the reading would normally be done. Concentrations of each of the three metals in 1:1 mixtures: (a) 300; (b) 250; (c) 200; (d) 150; (e) 100; (f) 50 µg dL⁻¹. Each line is a result of 8 tracings superimposed on top of each other. The procedure used was the same as in CHAPTER II, p. 10 except that each stage was monitored for 5 min.
<table>
<thead>
<tr>
<th>Level</th>
<th>Original serum value μg/dL</th>
<th>Amount added μg/dL</th>
<th>Found μg/dL</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe</td>
<td>Cu</td>
<td>Zn</td>
<td>Fe</td>
</tr>
<tr>
<td>1</td>
<td>50.7</td>
<td>53.6</td>
<td>78.6</td>
<td>50.0</td>
</tr>
<tr>
<td>2</td>
<td>50.7</td>
<td>53.6</td>
<td>78.6</td>
<td>100.0</td>
</tr>
<tr>
<td>3</td>
<td>50.7</td>
<td>53.6</td>
<td>78.6</td>
<td>150.0</td>
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<td>4</td>
<td>50.7</td>
<td>53.6</td>
<td>78.6</td>
<td>200.0</td>
</tr>
<tr>
<td>5</td>
<td>50.7</td>
<td>53.6</td>
<td>78.6</td>
<td>250.0</td>
</tr>
</tbody>
</table>

\[a\] To 150 μL of serum sample, 150 μL of metal mixed or H₂O was added.\n
\[b\] Each level is a mean of 5 replicates.
5. Within-assay variation. The within-assay coefficient of variation taken at six different serum levels (Table 2) for iron, copper, and zinc averaged (mean ± SD): 3.4 ± 1.2%; 3.7 ± 1.8% and 3.5 ± 1.9%, respectively.

6. Between-assay variation. The between-assay coefficient of variation for iron, copper, and zinc in serum taken at four different levels at twenty-one different runs gave (mean ± SD): 4.3 ± 1.0%; 5.3 ± 1.7%; 4.3 ± 1.0%, respectively (see Table 2). The four levels used are in the range of clinical importance.

D. Comparison with Reference Methods

Thirty-five different serum samples were assayed sequentially using Br-PAR for iron, copper and zinc and then by reference methods for iron (17), copper(1), and zinc(2) (Fig. 5-7). The following regression equations were obtained:
y = 0.9918x + 8.052 (r = 0.9662); y = 0.8812x + 11.44 (r = 0.9603); y = 0.9918x + 1.748 (r = 0.9924)
for iron, copper and zinc, respectively.

The reference method used for copper determination was the BCS method (1). The greater scattering of points that was observed in Fig. 6 could be due to the lower molar absorptivity of BCS rather than the relatively high molar absorptivity of the Br-PAR copper complex. When looking at a regression line, it is obviously important to notice the extent of scatter since you could have a correlation coefficient very close to r = 1.0 and still have a high scatter. This may be worse than r < 1.0 where there is less scattering. The positive y-intercept appears insignificant when compared to the level of trace metals we are dealing with.

The reference method for zinc determination used a highly sensitive Br-PADAP (2) color reagent. The correlation of Br-PAR with Br-PADAP method showed less scatter along the regression line (see Fig. 7).
### TABLE 2

**METHOD PRECISION FOR DETERMINATION OF IRON, COPPER AND ZINC IN SERUM**

<table>
<thead>
<tr>
<th>Level</th>
<th>Fe (µg dL⁻¹)</th>
<th>Cu (µg dL⁻¹)</th>
<th>Zn (µg dL⁻¹)</th>
<th>CV, % Fe</th>
<th>CV, % Cu</th>
<th>CV, % Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-assay (n=5 replicates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50.7(2.7)</td>
<td>53.6(3.6)</td>
<td>78.6(5.4)</td>
<td>5.3</td>
<td>6.7</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>97.5(4.7)</td>
<td>103.4(5.1)</td>
<td>128.0(5.3)</td>
<td>4.8</td>
<td>4.9</td>
<td>4.1</td>
</tr>
<tr>
<td>3</td>
<td>148.9(3.7)</td>
<td>151.8(5.1)</td>
<td>177.1(5.1)</td>
<td>2.5</td>
<td>3.4</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>197.8(6.4)</td>
<td>203.2(5.3)</td>
<td>225.8(7.0)</td>
<td>3.2</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>255.3(5.4)</td>
<td>256.7(5.3)</td>
<td>285.3(5.2)</td>
<td>2.1</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>319.2(8.6)</td>
<td>323.3(7.3)</td>
<td>341.7(6.6)</td>
<td>2.7</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Between-assay (n=21 replicates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>49.8(2.9)</td>
<td>56.8(4.4)</td>
<td>78.9(4.4)</td>
<td>5.8</td>
<td>7.7</td>
<td>5.6</td>
</tr>
<tr>
<td>2</td>
<td>98.1(3.8)</td>
<td>107.6(5.2)</td>
<td>155.0(5.9)</td>
<td>3.9</td>
<td>4.8</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>149.8(5.9)</td>
<td>152.2(7.4)</td>
<td>178.8(8.1)</td>
<td>3.9</td>
<td>4.9</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>198.6(6.9)</td>
<td>202.8(7.6)</td>
<td>225.6(7.3)</td>
<td>3.5</td>
<td>3.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>
FIGURE 5

COMPARISON OF THE PROPOSED METHOD WITH THE REFERENCE METHOD FOR IRON

Legend

Correlation plot between the proposed Br-PAR method for serum iron and the American Monitor Corporation's PTF-600 method for serum iron (17). The equation of the line of best fit is: \( y = 0.9918x + 8.052 \) \( (r = 0.9662) \). The sample size, \( n=35 \). Each point was a result of single determination using both methods. See CHAPTER II, p. 10 for procedure.
FIGURE 6

COMPARISON OF THE PROPOSED METHOD WITH THE REFERENCE METHOD FOR COPPER

Legend

Correlation plot between the proposed Br-PAR method for serum copper and the BCS method for serum copper (1). The equation of the line of best fit is: \( y = 0.8812x + 11.44 \) \((r=0.9603)\). The sample size, \( n = 35 \). Each point was a result of single determination using both methods. See CHAPTER II, p. 10 for procedure.
FIGURE 7

COMPARISON OF THE PROPOSED METHOD WITH THE REFERENCE METHOD FOR ZINC

Legend

Correlation plot between the proposed Br-PAR method for serum zinc and the highly sensitive Br-PADAP procedure for serum zinc (2). The equation of the line of best fit is: \( y = 0.9918 + 1.748 \) \( r = 0.9925 \). The sample size, \( n = 35 \). Each point was a result of single determination using both methods. See CHAPTER II, p. 10 for procedure.
CHAPTER IV

CONCLUSIONS

The individual and the sequential determination of iron, copper and zinc using 4[(5-bromo-2-pyridyl)azo]resorcinol (Br-PAR) were found to have good applicability in serum samples. The new method offers several important advantages. These are: simplicity; very high sensitivity for all three metals; no need for extraction and heating; no solubilizing detergent is required; and a short time requirement for determining all three metals sequentially. The simplicity of the method is reflected by the fact that all the reactions take place in a single aqueous matrix at the same pH and absorbances are read at one wavelength (510 nm) for all three metals. This method is also flexible in terms of sample size due to its high sensitivity. Also the reagent can be used in both individual and sequential determination of any or all three metals. The method shows agreement between individual and sequential determination. The CVs are well below 4% for within run and 6% for between run. The recovery studies show average recovery values within 99-101% indicating that a matrix effect is non-existent. The method also shows good correlation with the established methods; PTF-600 for iron, BCS for copper, and Br-PADAP for zinc. The application of this method in raw serum, although never tried, is a good possibility making the procedure more amenable for automation.
PART II

FLUOROMETRIC CYCLIC ASSAYS
FOR PYRIDINE NUCLEOTIDES WHICH ARE REDUCED
OR OXIDIZED IN ENZYME COUPLED ASSAYS
CHAPTER I

INTRODUCTION

A. Measurement of Pyridine Nucleotides

Many assays for pyridine nucleotides have been previously described (18–24). The absorption of the reduced form at 340 nm has been exploited for many years to study innumerable enzyme reactions (22) but the measurement of the native fluorescence of the reduced form gives much higher sensitivity. One may also take advantage of the fact that the reduced form can be destroyed by acid (25,26) and hence, measurement of the oxidized form is possible by reducing them enzymatically or by hydrosulfite (23) followed by measurement in the reduced form as mentioned above. The reduced form can be used to reduce equivalent or proportional amounts of dye causing a color change that can be measured spectrophotometrically. Another way is to react the oxidized form with cyanide at pH 11 to give a compound very much like the reduced form in its absorption and fluorescence properties (21). Other researchers (23,24) measured pyridine nucleotides by enzyme systems causing evolution of CO₂ which was then measured manometrically. The oxidized form of pyridine nucleotides can also be condensed with hydroxide or acetone to form a highly fluorescent compound (18–20,27). The most sensitive and a less cumbersome way is by cyclic assay (28–33). All the sensitive cyclic assays developed so far for pyridine nucleotides are discontinuous and some need a separate indicator reaction to quantitate the cycling product.

B. Purpose of the Study

The purpose of this thesis is to develop fast, sensitive, fluorometric, cyclic assays for pyridine nucleotides that are reduced or oxidized in enzyme coupled assays. This is based on the premise that almost every substance in a cell is
either subject to enzyme attack or can influence enzyme action. Most enzyme action involves oxidation or reduction of pyridine nucleotides or can be caused to do so with the aid of auxiliary enzymes. In fact, nearly every substance of biological interest could in principle, be measured using a pyridine nucleotide coupled assay system.

C. Principle of the Proposed Methods

The catalytic or cyclic assay can be defined as, an assay based on the regeneration of substrate being measured in order to achieve a detectable sustained rate of reaction which otherwise is not possible if the substrate concentration being measured is so low that it usually runs out in less than a second. One can look at it as if substrate acts as a catalyst which may be the reason why Bergmeyer (34) called the assay based on this principle "Catalytic Assay". The first cycling procedure was described in 1935 by Warburg et al. as quoted by Lowry et al. (29); subsequently others (28–33) made improvements in the application of the cycling principle. Passonneau and Lowry (34) made further contributions in the kinetics of "enzyme cycling". The kinetic calculation of these authors implied the fact that for pyridine nucleotide determination using cycling, the rate can be expressed as:

1. Triphosphopyridine nucleotide determination (see Fig. 1)

\[
\text{Rate} = k([\text{NADP}^+] + [\text{NADPH}]) \quad \text{(eqn. 1)}
\]

2. Diphosphopyridine nucleotide determination (see Fig. 1)

\[
\text{Rate} = k([\text{NAD}^+] + [\text{NADH}]) \quad \text{(eqn. 2)}
\]
FIGURE 1

SCHEME OF REACTIONS

FOR THE DETERMINATION OF PYRIDINE NUCLEOTIDES

Legend

If one wishes to determine NADP\(^+\) or NAD\(^+\) the sample has to be treated with HCl to destroy the NADPH and NADH. In order to determine NADPH or NADH, the sample must be treated with NaOH. The cycling schemes are under the condition that G-6-P, G-6PDH, resazurin and 1-MPMS are in sufficiently large amounts to give the desired cycling rate. Similarly, the ethanol, ALDH, resazurin and 1-MPMS in the second scheme below are also under the same condition. The pyridine nucleotides are the only limiting substance in these reactions. The rate of cycling will be proportional to pyridine nucleotide concentration.
\[
\text{FIGURE 1}
\]

\[
\text{NADP}^+: \text{HCl treated}
\]
\[
\text{NADPH}: \text{NaOH treated}
\]

\[
\begin{align*}
\text{G-6-P} & \xrightarrow{\text{G-6-PDH}} \text{6-PG} \\
\text{NADP}^+ & \xleftrightarrow{\text{1-MPMS}} \text{NADPH} \\
\text{Resorufin} & \xrightarrow{} \text{Resazurin} \\
\end{align*}
\]

\[
\text{Rate} = k \ (\text{[NADPH]} + \text{[NADP]})
\]

\[
\begin{align*}
\text{NAD}^+: \text{HCl treated} \\
\text{NADH}: \text{NaOH treated}
\end{align*}
\]

\[
\begin{align*}
\text{Ethanol} & \xrightarrow{\text{ALDH}} \text{Acetaldehyde} \\
\text{NAD}^+ & \xleftrightarrow{\text{1-MPMS}} \text{NADH} \\
\text{Resorufin} & \xrightarrow{} \text{Resazurin} \\
\end{align*}
\]

\[
\text{Rate} = k \ (\text{[NADH]} + \text{[NAD]}])
\]
This is true if the concentration of pyridine nucleotide to be measured is distinctly lower than its Michaelis constant, \( K_m \), and the concentration of catalysts and other substrates are in sufficiently high concentrations to give the desired cycling rate.

The \( k \) can be defined as:

\[
    k = \frac{k_1 \times k_2}{k_1 + k_2}
\]

(eqn. 3)

Where \( k_1 \) and \( k_2 \) are the first order rate constant of each reaction with respect to its substrate pyridine nucleotide. One can see that the decisive kinetic factor for each reaction is its apparent first order rate constant, \( k_x = \frac{V_{\text{max}}}{K_m} \). It follows that by increasing the \( V_{\text{max}} \) (i.e., the enzyme concentration) you can increase the constant \( k \) (eqn. 3) and hence the sensitivity of the method (see eqn. 1 and 2).

If one uses organic catalysts like phenazine methosulfate (PMS), 1-methoxyphenazine methosulfate (1-MPMS) or \( N \)-ethylmethoxyphenazine ethosulfate (\( N \)-EMPES), one does not usually deal with \( K_m \). But the fact that the concentration of the catalyst determines the rate constant of the reaction if the substrate is limiting, still holds.

Figure 1 shows the scheme of reactions that are going to be used in this study. Notice the fact that the system does not distinguish between the reduced and oxidized forms of pyridine nucleotides. If one wishes to measure the reduced forms of pyridine nucleotide, the sample can be treated with \( \text{NaOH} \) which destroys the oxidized forms of the pyridine nucleotide. To measure the oxidized form, the sample should be treated with \( \text{HCl} \) before cyling. In general, any coupled assay which produces oxidized or reduced pyridine nucleotide can
be treated appropriately (with NaOH or HCl) after a desired time and the cycling mixture can then be added. In these cycling assays (Fig. 1) it is not necessary to have separate indicator steps, since the increase in fluorescence due to rescrufin production can be followed directly.
CHAPTER II

MATERIALS AND METHODS

A. Apparatus

**Fluorometer:** The fluorescence measurements and spectral scans of fluorescence were made using a Perkin-Elmer model 204 fluorescence spectrophotometer equipped with a Perkin-Elmer 150 Xenon power supply and a model 56 recorder, obtained from Perkin-Elmer Corp. Downsview, Ontario.

**Spectrophotometer:** A Shimadzu UV-visible recording spectrophotometer UV-240 connected to a Shimadzu Graphic Printer PR-4, distributed by Teckscience Ltd., Toronto, Ontario, was used to obtain absorption spectra and absorbances.

**Cuvets:** Quartz fluorometer cuvetts (dimension: 1.00 x 0.20 x 4 cm) obtained from Heima (Canada) Ltd., Concord, Ontario were used to measure fluorescence rather than the larger cuvetts (1.00 x 1.00 x 4.0 cm) to reduce significantly the inner filter effect observed with the larger cuvetts. A plastic spacer can be placed at the bottom of the cuvet holder to elevate the cuvet for small volume measurements.

Quartz cuvetts of 1.00-cm path length purchased from Beckman Instruments, Inc., Toronto, Ontario were used to obtain absorption spectra and absorbances.

**Balances:** For weights above 1.0 g, a Mettler PC 4400 Delta Range electronic balance was used and for weights below one gram, a Mettler H16 semiautomatic balance was used. Both balances were from Fisher Scientific Co., (Canada), Don Mills, Toronto, Ontario.

**pH Meter:** A Fisher Accumet pH meter supplied by Fisher Scientific Co., (Canada) was used to measure pH. It was equipped with a glass electrode from
Graphic Controls, Buffalo, NY.

**Micropipettors:** The Oxford fixed volume pipettors (10 μL, 50 μL, 100 μL, 200 μL) from Canadian Laboratory Supplies, Ltd., Toronto, Ontario, and Gilson Pipetman Models P-200D and P-100D with disposable tips C-20 and C-200 from Mandel Scientific Co., Ltd. Ville St. Pierre, PQ, were used in this study.

**Glassware and Plasticware:** The disposable borosilicate tubes (10 x 75 mm) and all other glassware were obtained from Fisher Scientific Co., (Canada). The plastic containers for buffer storage were obtained from Sarstedt Inc., Montreal, Quebec. The disposable plastic scintillation vials from Packard Instrument Canada Ltd., Mississauga, Ontario, and the disposable 1.9 mL polypropylene microcentrifuge tubes with caps from Elkay Products Inc., Boston, MA were used as containers for solutions of smaller volumes.

**B. Reagents**

**Enzymes:** The following enzymes were purchased from Sigma Chemical Co., St. Louis, MO; hexokinase (HK) from bakers yeast [ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1]; glucose-6-phosphate dehydrogenase (G-6-PDH) from bakers yeast [D-glucose 6-phosphate; NADP + oxidoreductase EC 1.1.1.49]; alcohol dehydrogenase (ALDH) from bakers yeast [alcohol: NAD + oxidoreductase, EC 1.1.1.1]

The enzyme activities quoted are those of the supplier. Unit definitions are as follows: HK, one unit is the amount of enzyme which will phosphorylate 1.0 μmol of glucose per min at pH 8.5 (± 0.5) at 25°C; G-6-PDH, one unit is the amount of enzyme which will oxidize 1.0 μmol of glucose 6-phosphate to 6-phosphogluconate per min at 25°C at pH 7.4 in the presence of NADP +; ALDH, one unit will convert 1.0 μmol of ethanol to acetaldehyde per min at pH 8.8
at 25°C.

Chemicals: β-NADP⁺ (monosodium salt, synthetic from yeast β-NAD⁺),
β-NADPH (tetrasodium salt, by chemical reduction of β-NADP⁺), β-NAD⁺ (from
yeast), β-NADH (disodium salt, prepared enzymatically from yeasts β-NAD⁺ using
ALDH), ATP (disodium salt, from equine muscle), G-6-P (monosodium salt), maleimide
and Sephadex G-25 were purchased from Sigma Chemical Co., St. Louis, MO.

Resazurin and resorufin were purchased from Eastman Organic Chemicals,
Rochester, NY.

1-MPMS and N-EMPES were purchased from Research Organics, Cleveland,
OH.

All other chemicals were of analytical grade and obtained from BDH
Chemicals, Toronto, Ontario.

Water: The water is distilled and passed through a Zenopure Laboratory
water system Mega-90 from Zenon Environmental, Burlington, Ontario, Canada.
This was the water used over the entire study.

C. Solutions

Phosphate Buffer (250 mM) pH 6.8: Equal volumes of 250 mM Na₂HPO₄ and
250 mM Na₂HPO₄ were mixed. This is stable for several months at room
temperature. Refrigeration is not recommended since crystallization may occur.

Tris-HCl Buffer (250 mM) pH 7.5: Tris(hydroxymethyl)aminomethane (7.6 g)
was dissolved in water, titrated to pH 7.5 with HCl and made up to 250 mL
with water.

Citrate Buffer (5 mM) pH 7.5: Citric acid (2.1 g) was dissolved 1.8 L in
of water and allowed to stand for a day to destroy all the reduced pyridine
nucleotides if any. It was then made basic to pH 13 to destroy the oxidized
form of pyridine nucleotide by the addition of NaOH and left standing for a
day followed by addition of HCl to pH 7.5 and made up to 2.0 L. This was used
for chromatography and reconstitution of G-6-PDH and hexokinase.

**Resazurin (0.39 mM):** Resazurin (10 mg) of 98% purity was dissolved in 100
mL water. This reagent was stable for months in the dark, although it was
made every week since slight conversion of resazurin to resorufin was observed
when exposed to light.

**1-MPMS (3.9 mM):** 1-MPMS (150 mg) of 88% purity was dissolved in 100 mL
of water. This solution was stable for 6 months at room temperature. **NOTE:**
The purity of 1-MPMS was obtained from its absorbance at 386 nm. The literature's
millimolar absorption coefficient is 15.9 (35). It was found that 0.0422 mg/mL
gives absorbances of 1.763, therefore, the concentration is 0.111 mM. The
concentration gives us an apparent molecular weight of 380 whereas the actual
is 336; therefore, the purity is 336/380 x 100 = 88%.

**G-6-P (35 mM):** G-6-P (10 mg) of 98% purity was dissolved in 1.0 mL of
water. This was made fresh before the assay although it can be stored for
weeks if desired.

**Glucose (1.0 M):** Glucose (180 mg) was dissolved in 1.0 mL of water. This
was made fresh before the assay, although it can be stored for a longer time
if desired.

**Ethanol (70% v/v):** Absolute ethanol (7 mL) was made up to 10 mL with
water. This was made fresh before the assay.

**G-6-PDH (100 U/mL):** The vial containing 250 U G-6-PDH was reconstituted
with 2.5 mL of 5 mM citrate buffer, pH 7.5. This was stable for several weeks.
at 4°C.

**ALDH (1 mg/mL or 260 U/mL):** ALDH (1.0 mg) was reconstituted with 1.0 mL water. This was made fresh before the assay.

**HK (100 U/mL):** The vial containing 200 U was reconstituted with 2.0 mL of 5 mM citrate buffer, pH 7.5. This was stable for weeks at 4°C.

**Standards:** β-NADP+, β-NADPH, β-NAD+, β-NADH and ATP standards were prepared fresh before the assay using plain water as solvent. The serial dilutions were performed to give calculated final concentrations in the cuvet as required.

**G-6-PDH Control Samples:** The normal control samples from Sigma Chemical Co., were reconstituted with 1.0 mL water as directed. To obtain samples which would have lower G-6-PDH activity, one of the reconstituted vials was left at 60°C until no more enzyme activity could be detected as assayed using cycling procedure described below. This was then used as diluent for the normal control to obtain samples with a similar matrix to the normal control but with lower G-6-PDH activity, specifically 0.25 to 0.75 times the original G-6-PDH activity of the control normal.

**G-6-PDH Assay Solution:** The assay reagent obtained from Sigma Chemical Co., was reconstituted as directed. That was, G-6-PDH reagent vial (Catalog no. 345-8) was reconstituted with 1.0 mL water and mixed to dissolve the contents. After reconstitution of the reagent according to Sigma, it was said to contain NADP⁺ (1.5 mM), maleimide (12 mM), buffer, stabilizer and lysing agents. This was stable for 8 h at room temperature or 5 days at 4°C.

**G-6-PDH Substrate Solution:** The substrate solution obtained from Sigma Chemical Co., (catalog no. 345-g) contains G-6-P (1.05 mM), buffer, magnesium salt, and sodium azide (0.1%) as preservative.
Magnesium Chloride Solution (0.5 M): MgCl₂·H₂O (10.2 g) was dissolved in 100 mL water. This was prepared once over the entire study.

ATP assay solution. Tris-HCl buffer (240 μL), 150 μL of 3.5 mg/mL or 4.0 mM NADP⁺, 15 μL of MgCl₂ solution, 30 μL of G-6-PDH solution, 75 μL of glucose solution, 120 μL of HK solution above and 370 μL of water were added together in 1.9 mL polypropylene microcentrifuge tubes with cap, capped and kept on ice until use. This was made fresh just before use. All solutions used for making ATP assay solution were described above.

D. Procedures

1. Triphosphopyridine nucleotides, NADPH, cycling:

   Pipet into 10 x 75 mm test tubes:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Final concentration in the assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Sample or H₂O (blank) 100 μL</td>
<td>0.2-20 nM</td>
</tr>
<tr>
<td>b. Phosphate buffer (250 mM) 200 μL</td>
<td>100 mM</td>
</tr>
<tr>
<td>c. Resazurin 0.39 mM 50 μL</td>
<td>39 μM</td>
</tr>
<tr>
<td>d. 1-MPMS (3.9 mM) 50 μL</td>
<td>0.39 mM</td>
</tr>
<tr>
<td>e. G-6-P (35 mM) 50 μL</td>
<td>3.50 mM</td>
</tr>
<tr>
<td>f. G-6-PDH (100 U/mL) 50 μL</td>
<td>5.0 U</td>
</tr>
</tbody>
</table>

   Mix and transfer to a fluorometer cuvet using a disposable glass Pasteur pipette and monitor the increase in fluorescence (λₑₓ: 560 nm, λₑₘ: 580 nm) for 2 to 3 min at 25°C or 37°C.

   Unless otherwise indicated, the fluorometer setting is as follows: sensitivity control = X4, selector switch = X10, 0% adjustment knob = variable, 100%
adjustment knob = fully clockwise, analyzer wavelength control = 580 nm and exciter wavelength control = 560 nm.

One can single out NADPH (or NADP+) by treating the sample with enough base (or acid) to bring the pH to 12 (or 2) for 5-10 min (or 3-6 min) at 60°C (or 25°C). The amount of acid or base to be used will depend on whether the sample is highly buffered.

The phosphate buffer for cycling (Step b of Procedure 1 above) at pH 6.8 can usually return the acidic or basic sample to pH 6.8 and therefore, neutralization of the sample is in most cases unnecessary.

If one wishes to increase the sensitivity, to detect concentrations lower than 1 fmol, the cycling may be run for longer time (i.e., 0.5 h) followed by heating at 100°C to destroy the enzyme and stop the cycling. The fluorescence can then be read against blank in which the sample is replaced by water and treated the same way.

The sensitivity may also be increased further by increasing the amount of enzyme used. Before this can be done, the enzyme has to be purified further from contaminating triphosphopyridine nucleotide. The following purification was performed to analyze pyridine nucleotide below 100 fmol.

The 1.5 mg of crystallized G-6-PDH (250 U) was reconstituted with 0.200 mL of 5 mM citrate buffer at pH 7.5. This solution was chromatographed on Sephadex G-25 (bed size 0.8 x 25 cm) which had been washed thoroughly and equilibrated with 5 mM citrate buffer (pH 7.5) free from any triphosphopyridine nucleotides. The sample was eluted with the same 5 mM citrate buffer, whereupon the two 500-μL fractions with highest activities were pooled. This enzyme can be used without further treatment. From the 1.0-mL collected fraction, we
obtained 85% of the original enzyme activity. This treatment gives the same results as the charcoal treatment but with much higher recovery of the enzyme in agreement with that reported by Cox et al. (31).

The assay for activity within each fraction was performed by diluting 10 µL from each fraction with 1.0 mL citrate buffer. From the diluted fraction 10 µL is added to 1.0 mL of the G-6-PDH assay solution described above, and 2.0 mL of substrate solution, also described above, were added. The rate of increase in absorbance at 340 nm was measured.

If one doubles the G-6-PDH in the above cycling procedure, one does not have to increase the 1-MPMS because the level of 1-MPMS when 5 U of G-6-PDH was used was on the higher end of the optimum. If 10 U of G-6-PDH were used, the 1-MPMS was only slightly below the optimum. (See CHAPTER III, p. 70.)

2. Diphosphopyridine nucleotide, NAD(H), cycling:

   Pipet into 10 x 75 mm test tubes:

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
<th>Final concentration in the assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Sample or H₂O (blank)</td>
<td>100 µL</td>
<td>1-40 nM</td>
</tr>
<tr>
<td>b. Tris-HCl buffer (250 mM) pH 7.5</td>
<td>200 µL</td>
<td>100 mM</td>
</tr>
<tr>
<td>c. Resazurin (0.39 mM)</td>
<td>50 µL</td>
<td>39 µM</td>
</tr>
<tr>
<td>d. 1-MPMS (3.9 mM)</td>
<td>50 µL</td>
<td>0.39 mM</td>
</tr>
<tr>
<td>e. Ethanol (70% v/v)</td>
<td>50 µL</td>
<td>7.0% or 1.2 M</td>
</tr>
<tr>
<td>f. ALDH (1 mg/mL = 260 U/mL)</td>
<td>50 µL</td>
<td>13 U</td>
</tr>
</tbody>
</table>

   Mix and transfer to a fluorometer cuvet using a disposable glass Pasteur pipette and monitor the increase in fluorescence (λ<sub>ex</sub>: 560 nm, λ<sub>em</sub>: 580 nm) for 2-3 min at 25°C or 30°C.
One can single out NADH (or NAD+) by treating the sample with enough base (or acid) to bring the pH to 12 (or 2) for 5-10 min (or 3-6 min) at 60°C (or 25°C). The amount of acid or base to be used will depend on whether or not the sample is highly buffered. The Tris-HCl buffer for cycling (in Step b of Procedure 2 above) at pH 7.5 is, in most cases, capable of returning the pH of acidic or basic samples to pH 7.5 and therefore neutralization of the sample was unnecessary.

Again as in NADP(H) cycling, the sensitivity can be increased by increasing the ALDH and 1-MPMS.

3. Application of cycling in the determination of G-6-PDH activity in erythrocytes

a. NADPH calibration curve was obtained as in Procedure 1. See CHAPTER II, p. 43.

b. The G-6-PDH control samples were diluted 150-fold with water and 10-μL aliquots of these samples were further diluted 100-fold with reconstituted Sigma G-6-PDH assay solution. Two 10-μL aliquots (one for time = 0 min and one for time = 5 min) of each of the above 15,150-fold diluted samples were pipetted into 10 x 75 mm test tubes and 20 μL of Sigma substrate solution were added and mixed. The zero-time tubes were stopped immediately with 30 μL 0.2 N NaOH. The 5-min time tubes were incubated at 37°C for 5 min after which they were stopped with 30 μL 0.20 N NaOH. After stopping the reactions, the tubes were incubated at 60°C for 10 min to speed up the destruction of NADP+ substrate, leaving the NADPH product intact. All the tubes were made up to 100-μL samples for cycling by adding 40 μL water. The assay was continued from step b of Procedure 1.
Δ Rate = (Rate at t = 5 min) - (Rate at t = 0 min)

The Δ Rate obtained was used in the regression equation of the NADPH cycling calibration curve to obtain the number of picomoles of NADPH produced in 5 min, \( \text{NADPH}_5 \text{ min} \), by the enzyme in the sample.

\[
\text{G-6-PDH activity in U/g Hb} = \frac{\text{NADPH}_5 \text{ min} \times 303}{126 \text{ g Hb/L}}
\]

The 303 is a factor which accounts for the dilution and conversion to units of enzyme activity. The 126 g Hb per liter was the amount of hemoglobin (Hb), in the blood samples as given by the Sigma Chemical Co.

4. Application of cyclic assay in the determination of ATP

ATP assay solution (20 µL) was pipetted into 10 x 75 mm test tube at 37°C. 10 µL of ATP standard (giving 0.50, 1.00, 1.50, 2.00 and 2.50 pmol ATP/assay), sample or water as blank was added. The tube was allowed to incubate for 10 min followed by the addition of 30 µL of 0.2N NaOH and incubation at 60°C for 10 min. The tube was made up to 100-µL samples for cycling by addition of 40 µL water and were kept on ice until measurement by the cycling procedure outlined in Procedure 1 above. The amount of ATP in samples was obtained from the calibration curve run in the same way.
CHAPTER III

RESULTS AND DISCUSSION

A. Advantages of the Catalysts Used

PMS is a versatile electron mediator in the assay and activity staining for various enzymes of diagnostic importance but its photochemical instability significantly discourage the use of this dye in routine methods. Even under scattered light in normal laboratories at a concentration of 0.025 mM in a clear bottle, 98% of it is destroyed in a matter of days (36) let alone using it under the very intense excitation light of a fluorometer. So far PMS has been proposed to be substituted for by diaphorase or Meldora blue (36). Meldora blue however has been reported to have a very intense blue color (36), insoluble in its reduced form and it is not certain whether it would work with the resazurin system. Diaphorase is an enzyme and is by no means stable once dissolved in aqueous solution (37,38). Furthermore, commercial preparations of diaphorase, not only are expensive but can cause significant problems in terms of the blank rate because of contaminating pyridine nucleotides.

In 1977, Hisada et al. (35) reported that the compound they synthesized, 1-MPMS, is a very stable, non-enzymatic electron mediator between NADH and electrodes. It was found in this laboratory that 1-MPMS and N-EMPES would also catalyze the non-enzymatic reduction of resazurin to resorufin by NADH or NADPH. The 1-MPMS and N-EMPES, unlike PMS, are very stable in water solution for as long as six months and probably beyond at room temperature. These compounds are ideal for cycling since they, unlike the enzymes, are least likely to be contaminated by pyridine nucleotides. For both types of cycling (Fig. 1), the only components which are direct cell extracts and more likely be
contaminated with pyridine nucleotides are the enzymes. But in this case the enzymes, G-6-PDH and ALDH, had already been purified to such an extent that for every mole there are less than 5 mmol of NADPH and NADH, respectively, as indicated by the supplier's data sheets. This was verified in this laboratory and was found that for every 5U of G-6-PDH there is a corresponding blank rate of 0.8 pmol, hence the enzyme is contaminated by 0.8 pmol or less of NADPH. For ALDH, 13 U (or 0.05 mg) have shown a blank rate corresponding to 2 pmole hence one can say that contamination arising from the enzyme is 2 pmole or less.

B. The Use of Resazurin

The NADPH (or NADH) produced in the cycling irreversibly reduces the non-fluorescent compound, resazurin, to a highly fluorescent compound, resorufin, in the presence of the electron mediating compound, 1-MPMS or N-EMPES (Figs. 2 and 3). The resorufin is about 1000-fold more fluorescent than NADPH or NADH (39) and, therefore, is a better fluorophore to use for quantitative analysis.

As indicated in the procedure, one does not need an extra indicator step to determine the product of cycling, since fluorescence increase can be followed directly without the worry of photosensitivity of the reagent and, therefore, no time consuming incubation step of the cycling mixture was necessary. The lack of incubation of the cycling reaction and the lack of separate indicator step are the major advantages of this cycling method as compared to all other cyclic assays previously done (28-33).
FIGURE 2

INDICATOR REACTION FOR THE PROPOSED CYCLING ASSAYS

Legend

The reaction between NADH (or NADPH) and resazurin is catalyzed by either 1-MPMS or N-EMPES to give NAD$^+$ or NADP$^+$ and the highly fluorescent compound resorufin.
FIGURE 2

\[
\text{NAD(P)H} + H^+ + \text{RESAZURIN} \rightleftharpoons \text{RESORUFIN}
\]

or

\[
\text{NAD(P)^+} + H_2O + \text{RESORUFIN}
\]

1-MPMS
FIGURE 3

EMISSION AND EXCITATION SPECTRA OF RESORUFIN

Legend

Left panel: The fluorescence emission of resorufin in Tris-HCl buffer pH 7.5 at different wavelengths as shown on the x-axis while exciting at all wavelengths.

Right panel: The fluorescence emission of resorufin at 580 nm while exciting at different wavelengths as shown in the x-axis. (- - - -) shows the predicted decrease in fluorescence but not observed because of increased detection of scattering as the excitation wavelength gets closer to the analyzer wavelength. The fluorometer setting is as indicated in procedure 1, p. 40 except that the 0% and 100% adjustment knob are both varied in such a way as to fit the spectra onto the paper. The predicted decrease in fluorescence can be observed at lower sensitivity setting (i.e., X1) of the fluorometer (see Chapter III, p. 38 and 43).
FIGURE 3

EMISSION

EXCITATION

FLUORESCENCE

WAVELENGTH (nm)

550 580 600 530 560
It should also be indicated that the irreversibility of the resazurin reduction insures unidirectional cycling which is capable of a long sustained reaction if deemed necessary, for example, when extremely high sensitivity is required.

It was found that the optimum resazurin concentration which would give maximum rate is 39 μM (Fig. 4). Concentrations higher than this caused a decrease in the observed rate probably due to quenching (see Fig. 5) of resorufin fluorescence by resazurin.

C. Specificity of the Cycling

The use of G-6-PDH and ALDH induces specificity for NADP(H) and NAD(H), respectively. The cycling system does not distinguish between oxidized and reduced forms. It is, therefore, an exceedingly fortunate circumstance that the reduced forms of these nucleotides are unstable in acid, whereas the oxidized forms are unstable in alkali (29,25). In fact, it is now well known that the reduced pyridine nucleotides may be destroyed by acid without damaging the oxidized form and, conversely, the oxidized nucleotides may be destroyed by alkali without the slightest loss of the reduced forms (25). These reports were confirmed in this laboratory (Table 1). The differences in stability reported by others (40) are enormous; at pH 2.0, the reduced form is said to be 100,000 times more stable than the oxidized forms and at pH 3 there is a difference of the same order of magnitude in the opposite direction. These extreme differences in behaviour are the key to their enormous analytical potential.
FIGURE 4

OPTIMIZATION OF RESAZURIN CONCENTRATION FOR NADP(H) CYCLING

Legend

The reaction volume is 500 µL and contains the following: 100 mM Tris-HCl, pH 7.5; 3.5 mM G-6-P; 0.39 mM or 0.15 mg/mL 1-MPMS; 5 U G-6PDH; 50 pmol NADP+. Resazurin concentration is as indicated on the x-axis. Each point is an average of duplicate determination.
FIGURE 5

ABSORPTION SPECTRA OF COLORED COMPONENTS IN THE ASSAY MIXTURE

Legend

The absorption spectra of colored substances in the assay mixture:

1) Resorufin (1.1 μM); 2) 1-MPMS (0.13 mM); 3) Resazurin (20 μM); 4) Sum of 1-MPMS and resazurin; 5) Sum of resorufin, 1-MPMS, and resazurin. The 560 nm and the 580 nm are the excitation and emission wavelengths of the fluorescent resorufin, respectively. Therefore, the inner filter effect is expected to occur at both excitation and emission wavelengths of resorufin which would result in apparent quenching.
FIGURE 5

[Graph showing absorbance vs. wavelength (nm) with curves labeled 1, 2, 3, 4, 5 and peaks at 560 nm and 580 nm]
### TABLE 1

**EFFECT OF pH ON PYRIDINE NUCLEOTIDES**

<table>
<thead>
<tr>
<th>Concentration(^a) of pyridine nucleotide</th>
<th>% loss(^b) after HCl treatment to pH 2.0 at 25°C for</th>
<th>% loss(^b) after NaOH treatment to pH 12.5 at 60°C for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 min</td>
<td>6 min</td>
</tr>
<tr>
<td>5.28 µM NADPH</td>
<td>99.98%</td>
<td>100.14%</td>
</tr>
<tr>
<td>5.86 µM NADP(^+)</td>
<td>0.34%</td>
<td>0.51%</td>
</tr>
<tr>
<td>6.46 µM NADH</td>
<td>99.99%</td>
<td>100.01%</td>
</tr>
<tr>
<td>7.06 µM NAD(^+)</td>
<td>0.43%</td>
<td>0.54%</td>
</tr>
</tbody>
</table>

\(^a\) The concentration indicated is the final concentration during the treatment process.

\(^b\) The concentrations at the end of each treatment were determined in duplicate by cycling assay immediately after the due time.
As a direct consequence of the above-mentioned behaviour, one can measure, using cycling, any form of product pyridine nucleotide coenzyme (reduced or oxidized) by destroying the excess substrate nucleotide coenzyme.

D. Effects of Cycling Volume

The use of large volumes can be a disadvantage not only because it can cause a significant inner filter effect and hence decrease the observed signal but can also be costly in terms of reagents being used. So we switched to the use of a smaller volume (i.e., 0.500 mL). The observed signal was found to be five times higher and we used six times less reagent than in a 3.0-mL cuvet. The small volume technique had been exploited extensively by Lowry et al. (22,29) to analyze small sample volumes. They went as far as making constriction pipettes capable of measuring 50 pL with 1% CV (40). The fluorometry can also be adapted to volume as low as 10 μL (22). This indicates the possibility that the proposed method (with sensitivity already comparable to that of Lowry et al. (29) which uses a 1-μL cycling volume, longer cycling time, and separate indicator reaction) has a potential for tremendous improvements. As it stands now though, the proposed methods presented in this thesis are better, since they are adaptable to a large sample volume by decreasing the reagent volume, that is, a 500-μL cycling volume gives more freedom in terms of sample to reagent ratio than that of 1-μL cycling volume. Hence, very dilute samples can be analyzed in which Lowry's (29) method, which was a 1-μL or 100-μL cycling volume, would not be able to accommodate.

E. Effects of Magnesium Chloride

G-6-PDH is known to be activated by magnesium chloride (41). Therefore we had planned to include magnesium chloride in the NADP(H) cycling. Figure
6 shows that 50 mM magnesium chloride can increase the G-6-PDH activity to 60%. But it was found that magnesium chloride inhibits cycling (see Fig. 7) presumably through the 1-MPMS catalyzed reaction, and hence the MgCl₂ was not used.

The NAD(H) cycling uses ALDH and it was reported (28) that ALDH is sensitive to divalent cations hence we did not advocated the use of divalent cations in NAD(H) cycling. But the use of EDTA in the cycling mixture was not implemented here because there is enough cycling rate capable of detecting pmol levels of NAD(H). The inhibition by divalent cation, if any, would be minimal in this case since we did not put divalent cation in the cycling reagent. Although, if this assay is used in conjunction with a coupled assay that has very high magnesium chloride concentration, one may advocate the use of EDTA or other chelating agents. It was also observed that a freshly prepared solution of EDTA appeared to have, in it a compound that can reduce resazurin in the presence of light to resorufin.

F. Effects of pH on Cycling

The optimum pH of G-6-PDH is 8.0-9.0 because of the fact that at this pH the lactone produced breaks down rapidly (41) rather than from the direct effect of pH on the enzyme. In Figure 8, one can see that the optimum pH for the cycling involving G-6-PDH is 6.8. This may be due to the fact that 1-MPMS catalyzed reaction favours the acidic pH since it involves the use of NADP⁺ + H⁺ as substrate (see Fig. 2) and hence high H⁺ concentration will favour a reaction which utilizes H⁺. This also has the effect of using up the G-6-PDH
FIGURE 6

EFFECT OF MgCl₂ ON G-6-PDH ACTIVITY

Legend

Each reaction mixture contains: 50 mM Tris-HCl buffer, pH 7.5; 0.40 mM NADP⁺; 3.30 mM G-6-P; 5.0 mM maleimide; 0.02 U G-6-PDH; and MgCl₂ concentration as indicated on the x axis. The increase in absorbance at 340 nm was monitored. Each point is an average of duplicate determinations.
FIGURE 6

% OF ORIGINAL ACTIVITY

MgCl₂ (mM)

230

115
FIGURE 7

THE EFFECT OF MgCl₂ IN NADP(H) CYCLING

Legend

The reaction volume of 500 μL contains the following: 100 mM Tris·HCl, pH 7.5; 39 μM resazurin, 3.5 mM G-6-P; 0.39 mM or 0.15 mg/mL 1-MPMS; 5U of G-6-PDH; 300 pmol NADP⁺; and MgCl₂ as indicated on x-axis. Each point is an average of duplicate determination.
FIGURE 8

EFFECT OF pH ON THE RATE OF NADP(H) CYCLING

Legend

The final reaction mixture of 500 μL contains the following: 100 mM Tris-HCl of variable pH as indicated in the x-axis; 39 μM resazurin; 0.39 mM or 0.15 mg/mL 1-MPMS; 3.5 mM G-6-P, 5U G-6-PDH; and 25 pmol NADP⁺. Each point is an average of duplicate determinations.
catalytic product (i.e., NADPH) analogous to the lactone breaking down rapidly at pH 8.0-9.0 causing an increase in enzyme catalysis. It was also observed, separately, that the 1-MFMS-catalyzed reaction reacts at much slower rate under basic conditions than under acidic conditions. At a pH lower than 6.8 the observed cycling rate starts to decrease probably due to the decrease in fluorescence of resorufin and/or enzyme inactivation. The decrease in resorufin fluorescence was observed at pH lower than 6.8 but whether or not this is the only reason for decrease remains uncertain.

For NAD(H) cycling, which uses ALDH, it was found that the rate also increased with decreasing pH down to pH 6.8 but unlike NADP(H) cycling the blank rate of NAD(H) cycling becomes significantly higher. So it was decided to keep the pH at 7.5 where the blank rate is reasonably low.

G. Effects of Buffer on Cycling

For NADP(H) cycling where the optimum pH is 6.8, different types of buffers were tried and it was found that phosphate buffer seems to be the buffer of choice (see Fig. 9). Other buffers available in this laboratory such as triethanolamine and citrate were tried but freshly made solutions of these buffers reduced significant amount of resazurin. Although long standing of these buffers in the refrigerator at 4°C seems to decrease this observed effect, we decided not to use them because of the resazurin reduction problem. It was speculated that this could be due to reducing compounds contaminating the buffer rather than the buffer itself. The choice of phosphate as buffer seems to be ideal since the pK_a (7.2 at 25°C) of phosphate is close to pH 6.8 and the acidic and basic sample of oxidized and reduced pyridine nucleotide can be buffered to pH 6.8.
FIGURE 9

EFFECT OF BUFFERS AND pH ON THE NADP(H) CALIBRATION CURVES

Legend

Note the different slopes of calibration curves for NADP⁺ using different buffers and pH's of cycling. The reaction mixture of 500 μL has 0.39 mM 1-MPMS, 39 μM resazurin, 3.5 mM G-6-P, 5U G-6-PDH, and NADP⁺ as indicated on the x-axis. The buffers concentration for all is 100 mM and of the following types: A, B, C and F are phosphate buffers with pH's 6.85, 7.00, 6.45 and 7.4, respectively. D, E, G and H are Tris-HCl buffers with pHs 6.85, 7.00, 7.2 and 7.4, respectively. Each point is an average of duplicate determinations.
For NAD(H) cycling we decided to use the Tris-HCl buffer, pH 7.5. Phosphate buffer was tried and found to decrease the NAD(H) cycling rate with time. Furthermore, it was reported by Colowick et al. (21) that phosphate accelerates the rate of NAD⁺ cleavage by heat. The decrease in cycling rate with time was never observed in phosphate buffer for NADP(H) cycling under the cycling conditions described.

H. Inner Filter Effect Caused by 1-MPMS and Resazurin on Resorufin Fluorescence

It was observed in the early part of the research that resazurin, and to a lesser extent 1-MPMS, decrease the observed resorufin fluorescence because both the 1-MPMS and resazurin absorb at the emission and excitation wavelengths of resorufin (see Fig. 5). This observed quenching is also known as the inner filter effect (42). It was found that the negative logarithm of fluorescence is a linear function of the concentration of the inner filtering substance. Despite this relationship the fixed amount of filtering substance still leaves the observed fluorescence linear with resorufin concentration (see Fig. 10). Therefore, for the cycling assay the observed quenching is not a problem. In Figure 10, line D has the amount or extent of quenching observed under our assay condition. Thus, if one allows 0.1 nmol of NADPH or NADH to convert resazurin to resorufin, assuming 100% conversion, one would see three units increase in fluorescence. This indicates that under the assay conditions which exhibit the same amount of quenching it is virtually impossible to measure less than 0.1 nmol of metabolite without cycling, let alone measuring it kinetically in a coupled assay.
FIGURE 10

EFFECT OF QUENCHING ON THE RELATIONSHIP BETWEEN RESORUFIN FLUORESCENCE AND ITS CONCENTRATION

Legend

Note the change in slope of the resorufin fluorescence curves with different amounts of 1-MPMS and resazurin. The amount of resorufin in the mixture of 500 µL is indicated on the x-axis. In addition, the mixture contains the following (each point is an average of duplicate determinations):

<table>
<thead>
<tr>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Tris-HCl buffer, pH 7.5</td>
</tr>
<tr>
<td>B: A + 0.39 mM 1-MPMS</td>
</tr>
<tr>
<td>C: A + 7.8 µM resazurin</td>
</tr>
<tr>
<td>D: A + 39 µM resazurin</td>
</tr>
<tr>
<td>E: B + 39 µM resazurin</td>
</tr>
</tbody>
</table>

Note: This is not a cycling reaction but rather solutions of resorufin with different amounts of inner filtering substances to see how much inner filter effect there is under different conditions.
I. Concentration Dependence of G-6-PDH Versus 1-MPMS and ALDH Versus 1-MPMS

There are two reactions involved in each type of cycling where each reaction is dependent on the other because the product of each reaction is the substrate for the other reaction. The overall rate, therefore, will be determined by the slowest rate among the two reactions (see Fig. 1). The goal is to make both reactions have more or less equal rate constants so as to take advantage of all the catalyst that is in the system, that is, not to put more of one catalyst than what the other catalyst is capable of handling.

At any fixed level of enzyme it is possible to approach a saturation point with respect to 1-MPMS (Figs. 11 and 12) and vice versa. As a consequence, it is virtually impossible to simultaneously saturate the system with both components. Thus, increasing the level of enzymes increases the amount of 1-MPMS required to saturate the assay and vice versa. As can be seen in Fig. 11, the 5U of enzyme is fully saturated with 0.075 mg 1-MPMS. This level of catalyst is capable of determining 0.100-15 pmol of NADP(H). If one wishes to determine much lower concentrations, one has to increase both catalytic components. If the 1-MPMS is increased beyond the optimum (highest point on the curve, see Fig. 11) one sees a decrease in rate which is explainable by the quenching of resorufin fluorescence by 1-MPMS (Figs. 5 and 10). A similar pattern was observed in Fig. 12 for NAD(H) cycling. If one looks at the spectrum of 1-MPMS (Fig. 5), one can see that it absorbs at both the excitation and emission wavelengths of resorufin. Therefore, it was expected to quench the fluorescence.
FIGURE 11

SATURATION WITH 1-MPMS AT DIFFERENT LEVELS OF G-6-PDH

Legend

Each assay was carried out in a volume of 500 μL and contains the following: 100 mM phosphate buffer, pH 6.85; 39 μM resazurin; 3.5 mM G-6-P; 5.8 pmol NADP⁺; 1-MPMS as indicated on the x-axis (conversion: 0.39 μmol = 0.15 mg). The G-6-PDH contents were as follows: A to K have 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 and 0.5 units of G-6-PDH, respectively. The asterisk is the condition under which we normally perform our assay. Each point is an average of duplicate determinations.
FIGURE 12

SATURATION WITH 1-MPMS AT DIFFERENT LEVELS OF ALDH

Legend

Each assay was carried out in a volume of 500 µL and contains the following: 100 mM Tris-HCl, pH 7.5; 39 µM resazurin; 7% (v/v) ethanol; 15 pmol NAD⁺; and 1-MPMS as indicated in the x-axis. The ALDH contents of tubes are as follows: A = 0.10 mg or 26 U, B = 0.05 mg or 13 U. Each point is an average of duplicate determinations.
FIGURE 12

![Graph showing rate of 1-MPMS (mg/cuvet) versus rate (fluorescence/min). Lines A and B are plotted.]
J. Calibration Curves

Figures 13 and 14 shows the calibration curves for NADP+ and NAD+ at the pmol level. These calibration curves were obtained with 4.0 Δ fluorescence/min and 3.0 Δ fluorescence/min blank rates for NAD(H) and NADP(H) cycling, respectively. If one wishes to increase the sensitivity further, one can increase the sensitivity of the fluorometer four times (see Figure 15) but as one can see the blank rate increase to 14/min. To increase the sensitivity even further, one can increase the sensitivity of the fluorometer much more, but the blank rate would also increase and it would be very difficult to read from the recorder tracing. Another way of increasing the sensitivity is by increasing the amount of enzyme and 1-MPMS, but this would also increase the blank rate, not only due to an increase in sensitivity of the cycling system but also due to NADP(H)+ that the enzyme is contaminated with. It was therefore decided to purify the enzyme using Sephadex G-25 and the blank rate was reduced by 65%. The doubling of the amount of enzyme can now be achieved without dramatic increase in blank rate, and as shown in Fig. 16, the sensitivity was increased and a reasonable blank rate was maintained. As we can see, there is still lots of room for improvement towards the determination of much lower concentrations of pyridine nucleotides.

The precision study was performed using G-6-PDH which has not been passed through Sephadex G-25 and the CV's were found to be quite low for both within-assay and between-assay (see Table 2).
FIGURE 13

TYPICAL CALIBRATION CURVE FOR NADP(H) CYCLING ASSAY

Legend

The final reaction volume is 500 µL and contains the following: 100 mM phosphate buffer, pH 6.85; 0.39 mM resazurin; 3.9 mM 1-MPMS; 3.5 mM G-6-P; 5U G-6-PDH; and NADP+ as indicated on the x-axis. Each point is an average of duplicate determinations.
FIGURE 13

$y = 3.54x - 1.1$

$R^2 = 0.999$

RATE (Fluorescence/Min)

NADP+ (pmol/cuvet)
FIGURE 14

CALIBRATION CURVE FOR NAD(H) CYCLING ASSAY

Legend

The final reaction volume is 500 µL and contains the following: 100 mM Tris-HCl, pH 7.5; 0.39 mM resazurin; 3.9 mM 1-MPMS; 7% ethanol; 13 U ALDH; and NAD⁺ as indicated on the x-axis. n = 21 samples.
\[ y = 1.97x + 0.22 \]
\[ r = 0.999 \]
FIGURE 15

COMPARISON OF BLANK RATE WITH SAMPLE RATE USING UNPROCESSED G-6-PDH

Legend

The figure shows the extent of the blank rate as compared with the sample rate using the Sigma preparation of G-6-PDH. The level of 0.100 pmol can be precisely determined. Each line was drawn based on the following average rates from duplicate determinations:

<table>
<thead>
<tr>
<th>[NADP⁺], pmol</th>
<th>Rate (ΔF/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 0.300</td>
<td>4.5</td>
</tr>
<tr>
<td>B: 0.200</td>
<td>3.0</td>
</tr>
<tr>
<td>C: 0.100</td>
<td>1.0</td>
</tr>
<tr>
<td>D: 0 (Blank)</td>
<td>0</td>
</tr>
</tbody>
</table>

The regression equation of [NADP⁺] vs rate is: \( y = 17.50x - 0.67 \) (\( r = 0.997 \))

The fluorometer setting is the same as in Procedure 1 (see CHAPTER II, p. 43) except that the sensitivity control = X 6.
FIGURE 16

COMPARISON OF BLANK RATE WITH THE SAMPLE RATE
USING PROCESSED G-6-PDH

Legend

The figure shows the increase in sensitivity at double the amount of enzyme, G-6-PDH. Note also the blank rate relative to the sample rates. The blank rate is significantly lowered (as compared to Fig. 15) after passing the enzyme through Sephadex G-25 column. Each line was drawn based on the following average rates from duplicate determinations.

<table>
<thead>
<tr>
<th>[NADP⁺], pmole</th>
<th>Rate (ΔF/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 0.400</td>
<td>10.0</td>
</tr>
<tr>
<td>B: 0.300</td>
<td>8.0</td>
</tr>
<tr>
<td>C: 0.200</td>
<td>5.0</td>
</tr>
<tr>
<td>D: 0.100</td>
<td>3.0</td>
</tr>
<tr>
<td>E: 0 (Blank, H₂O)</td>
<td></td>
</tr>
</tbody>
</table>

The regression equation of [NADP⁺]’s rate is: \( y = 24.00 \times -0.50 \ r = 0.997 \).

The fluorometer setting is the same as in Procedure 1 (see CHAPTER II, p. 43) except that the sensitivity control = X 6.
### TABLE 2
**METHOD PRECISION FOR THE DETERMINATION OF NADP**

#### I. Within-assay (n=5 replicates)

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean (SD), pmol/cuvet</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.24 (0.08)</td>
<td>0.34</td>
</tr>
<tr>
<td>2</td>
<td>17.42 (0.08)</td>
<td>0.45</td>
</tr>
<tr>
<td>3</td>
<td>11.68 (0.07)</td>
<td>0.60</td>
</tr>
<tr>
<td>4</td>
<td>5.87 (0.05)</td>
<td>0.085</td>
</tr>
<tr>
<td>5</td>
<td>2.93 (0.05)</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>2.44 (0.06)</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>1.90 (0.04)</td>
<td>2.1</td>
</tr>
<tr>
<td>8</td>
<td>1.48 (0.03)</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>0.99 (0.05)</td>
<td>5.1</td>
</tr>
<tr>
<td>10</td>
<td>0.48 (0.03)</td>
<td>6.2</td>
</tr>
</tbody>
</table>

#### II. Between-assay (n=5 replicates)

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean (SD), pmol/cuvet</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.97 (0.06)</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>2.46 (0.06)</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>1.87 (0.05)</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>1.46 (0.04)</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>0.96 (0.05)</td>
<td>5.2</td>
</tr>
<tr>
<td>6</td>
<td>0.47 (0.04)</td>
<td>8.5</td>
</tr>
</tbody>
</table>
K. Application of the Cycling Method to Enzyme Activity Determination

As a model it was decided to measure G-6-PDH in erythrocytes after the 1:1 hemolysates have been diluted 15,150 fold. The scheme of measurement is outlined in Fig. 17. Based on the amount of hemoglobin present in the Sigma G-6-PDH control sample, the amount of G-6-PDH in units per milligram of hemoglobin were calculated from the rate of increase in fluorescence caused by the NADPH produced by G-6-PDH in the sample in 5 minutes. The precision of the method was studied and found that for both within-run and between-run the CV's were much less than 2% (see Table 3).

L. Application of the Cycling Method to the Determination of Metabolites

For the metabolite application of the cycling methods, it was decided to use ATP as a model. Using the scheme outlined in Fig. 18, the calibration curve for the determination of ATP was constructed (Fig. 19). As can be seen in Table 4, the present most sensitive methods for ATP determinations are bioluminescent, isotachophoretic and radioenzymatic methods. The ATP method described here surpasses all of these methods in terms of sensitivity. The sensitivity of the method for ATP proposed here is sensitive enough to detect 100 fmol of ATP with very good precision (CV < 10%). This sensitivity can be extended to much lower concentration if much purer G-6-PDH is used for cycling and purer water in terms of bacterial contamination.
FIGURE 17

SCHEME FOR THE APPLICATION OF CYCLING IN THE DETERMINATION OF G-6-PDH IN ERYTHROCYTES

Legend

For the application of cycling to the determination of enzyme activity, G-6-PDH is used as a model in this enzymatic scheme. The G-6-PDH is allowed to catalyze the reduction of NADP\(^{+}\) by G-6-P for 5 min. The product NADPH is singled out by NaOH treatment. The resulting solution is used as a sample for cycling. The production of resorufin from cycling can be followed fluorometrically, the rate of which is proportional to the amount of NADPH produced by G-6-PDH in 5 min.
Application in Enzyme's Activity Determination

Determination of G-6-PDH in Erythrocytes

\[ G-6-P + NADP^+ \xrightarrow{G-6-PDH} 6-PG + NADPH \]

Single out NADPH by NaOH treatment

\[ G-6-P \xrightarrow{G-6-PDH} 6-PG \]

\[ NADP^+ \xrightarrow{1-MPMS} NADPH \]

Resorufin \[ \xrightarrow{1-MPMS} \] Resázurin
TABLE 3

METHOD PRECISION FOR THE DETERMINATION OF G-6-PDH IN ERYTHROCYTES

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean (SD), U/gHb</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.86 (0.06)</td>
<td>1.55</td>
</tr>
<tr>
<td>2</td>
<td>7.82 (0.07)</td>
<td>0.90</td>
</tr>
<tr>
<td>3</td>
<td>11.82 (0.09)</td>
<td>0.76</td>
</tr>
<tr>
<td>4</td>
<td>15.67 (0.11)</td>
<td>0.70</td>
</tr>
</tbody>
</table>

II. Between-assay (n = 5 replicates)

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean (SD), U/gHb</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.90 (0.07)</td>
<td>1.79</td>
</tr>
<tr>
<td>2</td>
<td>7.84 (0.08)</td>
<td>1.02</td>
</tr>
<tr>
<td>3</td>
<td>11.82 (0.10)</td>
<td>0.85</td>
</tr>
<tr>
<td>4</td>
<td>15.67 (0.11)</td>
<td>0.70</td>
</tr>
</tbody>
</table>
FIGURE 18

SCHEME FOR THE APPLICATION OF CYCLING TO THE DETERMINATION OF ATP

Legend

Application of cycling in determination of a metabolite. ATP is used as a model in this enzymatic scheme. An equivalent amount of ATP is converted to NADPH which is then treated with NaOH to single out NADPH followed by cycling. The increase in the amount of resorufin is monitored as an increase in fluorescence at 580 nm (excitation 560 nm).
Application in Metabolite Determination

Determination of ATP

\[ \text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G-6-P} \]

\[ \text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH} \]

Single out NADPH by NaOH treatment

\[ \text{G-6-P} \xrightarrow{\text{G-6-PDH}} \text{6-PG} \]

\[ \text{NADP}^+ \xrightarrow{\text{Resorufin}} \text{NADPH} \xrightarrow{1\text{-MPMS}} \text{Resazurin} \]
FIGURE 19

CALIBRATION CURVE FOR ATP DETERMINATION USING A CYCLIC ASSAY

Legend

Calibration curve for the determination of ATP. The assay is performed as follows: 10 μL sample or H₂O (blank) are added to 20 μL ATP assay mixture (see CHAPTER III, p. 38) and allowed to react to completion for 10 min at 37°C followed by addition of 30 μL 1.5 N NaOH and incubation at 60°C for 10 min. Then, 40 μL of H₂O is added to make up a sample of 100 μL for cycling and the cycling reagents are added subsequently (see CHAPTER III, p. 39). The increase in fluorescence is monitored at 580 nm (excitation 560 nm). The fluorometer setting is the same as in procedure 1 except that the sensitivity control = X 5. The regression equation for the calibration curve is:

\[ y = 6.36x - 0.06 \quad (r = 0.998) \]

Each point is an average of duplicate determinations.
FIGURE 19

ATP (pmol/cuvet) vs. RATE (Fluorescence/min)

$y = 6.36x - 0.06$

$r = 0.998$
<table>
<thead>
<tr>
<th>Method</th>
<th>Lower limit (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic</td>
<td>$10^9$</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Chémical</td>
<td>$10^4$</td>
</tr>
<tr>
<td>HPLC</td>
<td>250</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>130</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>3.1</td>
</tr>
<tr>
<td>Isotachophoresis</td>
<td>1</td>
</tr>
<tr>
<td>Radioenzymatic</td>
<td>1</td>
</tr>
<tr>
<td>Bioluminescent</td>
<td>1.0</td>
</tr>
<tr>
<td>Proposed</td>
<td>0.10</td>
</tr>
</tbody>
</table>

a Refers to the limit of detection of ATP.

b Reference 43 except for the proposed method.
CHAPTER IV

CONCLUSIONS

Sensitive fluorometric cyclic assays for pyridine nucleotides have been developed for the determination of pyridine nucleotides which are reduced or oxidized in enzyme coupled assays. The use of 1-MPMS is introduced because of its utility as an electron mediator between resazurin and reduced pyridine nucleotides. The use of 1-MPMS eliminated the use of diaphorase and lowered the blank rate. Because 1-MPMS is from non-biological source and is less likely to be contaminated by pyridine nucleotides as compared to diaphorase. The photostability of 1-MPMS allows direct monitoring of the increase in fluorescence as cycling goes on, which has not previously been accomplished either because no indicator reaction is built into the design of cycling system or those that have one PMS or PES which are photolabile are utilized.

The use of resazurin helped increase the sensitivity and hence the amount of catalyst needed to obtain high sensitivity is less than with other cycling methods. The use of resazurin reduction as an indicator reaction introduced irreversibility in the cycling assay and the use of G-6-PDH and ALDH introduced specificity.

The methods have shown good precision down to 100 fmol under the condition used and great potential for much higher sensitivity exists.

Since the methods for the applications can be performed in one test tube, the ATP method as well as the G-6-PDH method are amenable to automation. The bioluminescent method requires expensive instrumentation (a luminometer), the isptachophoresis takes a long time, and the radioenzymatic procedure requires the use of radioactive materials. Therefore, the proposed method seems to be the most suitable for ATP determination.
REFERENCES


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CITIZENSHIP: Canadian

EDUCATION:
1981-1985 B.Sc. Honours Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan.

AWARDS:
1984 Summer Studentship Award, Canadian Cancer Society.
1984 Honour's Scholarship, University of Saskatchewan
1983 Undergraduate Scholarship, University of Saskatchewan
1982 Academic Proficiency Award, Saskatchewan Government
1981 Medal for Academic Excellence, Miller Comprehensive High School

PUBLICATIONS:


PRESENTATION:
Sensitive Fluorometric Catalytic Kinetic Assays for Pyridine Nucleotides that are Reduced or Oxidized in Enzyme Coupled Assays. American Association for Clinical Chemistry, Michigan Section, Windsor, Ontario (October 22, 1987).

PROFESSIONAL MEMBERSHIPS:
American Association for Clinical Chemistry
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