NEW FLUOROMETRIC METHODS FOR THE DETERMINATION OF ALUMINUM, PYROPHOSPHATE, AND ADENOSINE 5'-TRIPHOSPHATE IN BIOLOGICAL SYSTEMS.

NARACE DYAL. SEUDEAL
University of Windsor

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NEW FLUOROMETRIC METHODS FOR THE DETERMINATION OF ALUMINIUM, PYROPHOSPHATE, AND ADENOSINE 5'-TRIPHOSPHATE IN BIOLOGICAL SYSTEMS

by

Narace Dyal Seudeal

A Dissertation
Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada
1987
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PART I
THE DEVELOPMENT OF A FLUOROMETRIC TECHNIQUE TO MEASURE ALUMINIUM IN BIOLOGICAL SPECIMENS

PART II
AN INVERSE FLUOROMETRIC TECHNIQUE TO DETERMINE PYROPHOSPHATE IN PLATELETS USING THE ALUMINIUM-MORIN CHELATE

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PART IV
THE SEQUENTIAL DETERMINATION OF ADENOSINE 5'-TRIPHOSPHATE AND PYROPHOSPHATE FROM ONE ALIQUOT OF PLATELETS
ABSTRACT

PART I

THE DEVELOPMENT OF A FLUOROMETRIC TECHNIQUE
TO MEASURE ALUMINIUM IN BIOLOGICAL SPECIMENS

by

Narace Dyal Seudeal

A fluorometric assay was developed to measure aluminium in pure aqueous solutions and protein-free serum filtrates. A modified procedure utilizing nitric acid was used to precipitate proteins.

The principle of the assay is based on the fact that aluminium forms a highly fluorescent chelate with morin (a pentahydroxyflavone). The chelate has an excitation maximum wavelength of 435 nm and an emission maximum wavelength of 510 nm. It fluoresced very strongly at a pH of 5.0 ± 0.75. A buffer of monochloroacetic acid and sodium acetate (pH, 4.1) was used to produce maximum fluorescence. The optimum Al:morin ratio ranged from 1:8-1:20 and maximum fluorescence in this range was produced in 15-20 min. A plot of fluorescence versus aluminium concentration yielded a straight line for concentrations of 200-1500 nmol/L, with a correlation coefficient of 0.998.
Cationic and anionic substances which interfered significantly with the analysis were removed by using Chelex-100 (Na⁺) and Dowex-1 (Cl⁻) resins, respectively.

Ten serum specimens were analysed by this method and showed aluminium concentrations closely correlating \( r = 0.936 \) with those obtained by atomic absorption spectrophotometry.

PART II
AN INVERSE FLUOROMETRIC TECHNIQUE TO DETERMINE PYROPHOSPHATE IN PLATELETS USING THE ALUMINIUM-MORIN CHELATE

by

Narace Dyal Seudeal

A non-enzymatic fluorometric procedure was developed to measure pyrophosphate in platelets. The method is based on the principle of forming a highly fluorescent chelate of aluminium and morin, and then reacting this chelate with phosphate generated from pyrophosphate hydrolysis. This reaction results in a decrease in fluorescence due to the formation of aluminium phosphate, thus correlating with the amount of pyrophosphate originally present.

The procedure was carried out in 50% (v/v) ethanol and a pH of 5.5, which was provided with sodium acetate-acetic acid buffer. The excitation and emission wavelengths were 435 and 510 nm, respectively. Platelets were isolated by a
centrifugation technique and lysed with water. Interferences were removed by passage of the sample through a Dowex-1(Cl⁻) resin. Recovery studies showed an average of 98% pyrophosphate recovered, and precision studies gave average coefficients of variation of 4.0 and 6.1% for within-run and between-run studies, respectively. The mean pyrophosphate found was 2.9 nmol/10^8 platelets and the method gave a good correlation (r = 0.98) with an enzymatic technique.

PART III

THE DEVELOPMENT OF A SIMPLE AND SENSITIVE ASSAY TO MEASURE ADENOSINE 5'-TRIPHOSPHATE IN PLATELETS

by

Narace Dyal Seudefal

A sensitive fluorometric assay for the determination of ATP is presented here. The method involved the production of NAD⁺ from a sequence of enzymatic reactions and its reaction with acetone in the presence of sodium hydroxide to form a very highly fluorescent product.

Conditions for the optimum formation of this product included a strong concentration of sodium hydroxide and boiling for 2 min. Once formed, the product was stable for 24 h or more at a pH of 3.5-4.0. A linear relationship was found for the determination of ATP in the range of 0.13-4.0 nmol, with a correlation coefficient of 0.9996.
Native fluorescence from NADH was eliminated with HCl, and metals and common anions did not present any problems.

The ATP determined in twenty samples of platelets showed a mean of 2.3 nmol/10^6 platelets. Precision studies for both within-run and between-run were less than 6%, and recovery studies showed approximately 98% of recovered ATP from platelets. A comparison study with a reference method gave a correlation coefficient of 0.97.

PART IV
THE SEQUENTIAL DETERMINATION OF ADENOSINE 5'-TRIPHOSPHATE AND PYROPHOSPHATE FROM ONE ALIQUOT OF PLATELETS

by

Narace Dyal Seudeal

A technique has been developed to determine ATP and pyrophosphate from one aliquot of platelets. The sample which contains both ATP and pyrophosphate was used to generate NAD^+ through a sequence of enzymatic reactions starting with ATP. The NAD^+ generated was condensed with acetone to form a fluorescent product. Prior to the formation of this product, pyrophosphate was separated from NAD^+ by passage of the reaction mixture through a Dowex-1 (Cl^-) resin and subsequent elution with HCl.

Recoveries for both assays ranged from 98-105%, and coefficients of variation for both within-run and between-run studies for both assays were below 7%.
ACKNOWLEDGEMENTS

I wish to thank my advisor, Dr. R. J. Thibert, for his financial and moral support throughout the entire period of this study. Moreover, I must thank him for his supervision and direction during my entire Ph.D program.

I shall also like to acknowledge the very helpful suggestions from my other committee members, Dr. L. R. Sabina, Dr. B. Mutus, Dr. N. F. Taylor, Dr. T. F. Draisey and Dr. P. Desjardins. I am especially grateful to Dr. T. F. Draisey for giving me the opportunity to train at the Salvation Army Grace Hospital, Windsor, Ontario. Likewise, I must sincerely thank Dr. P. Desjardins for taking time off his schedule to examine my dissertation.

A great deal of gratitude must be credited to my loved ones, Donna and Kyle Norris, family members and friends, for whose co-operation and affection have made this dissertation possible.

Special thanks must be credited to Donna for her patience and effort in typing this document.
DEDICATION

☆☆☆

To Donna and Kyle Norris

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## PART I

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LIST OF ABBREVIATIONS

Å  Angstrom
%  percent
V  volt
g  gram
mL milliliter
mg milligram
h  hour
DES dialysis encephalopathy syndrome
ppm parts per million
wt weight
L  liter
°C degree Celsius
v/v volume by volume
M  molarity
nm nanometer
nmol nanomole
N  normality
min minute
w/v weight by volume
mmol millimole
µmol micromole
CPPD calcium pyrophosphate deposition
PP_i pyrophosphate
\( P_i \) phosphate

\( \text{NAD}^+ \) nicotinamide adenine dinucleotide (oxidized form)

\( \text{NADH} \) nicotinamide adenine dinucleotide (reduced form)

\( \mu g \) microgram

\( r \) correlation coefficient

\( \text{S.D.} \) standard deviation

\( \text{S.E.M.} \) standard error of the mean

\( \text{C.V.} \) coefficient of variation

\( \text{EDTA} \) ethylenediaminetetraacetic acid

\( \text{ATP} \) adenosine 5' triphosphate

\( 3-\text{PGA} \) 3-phosphoglyceric acid

\( \text{PGK} \) phosphoglycerokinase

\( \text{ADP} \) adenosine 5' diphosphate

\( 1,3-\text{diPGA} \) 1,3-diphosphoglyceric acid

\( \text{GAP-DH} \) glyceraldehyde 3-phosphate dehydrogenase

\( \text{GP} \) glycerol phosphate

\( \text{TIM}^* \) triosephosphate isomerase

\( \text{DHAP} \) dihydroxyacetone phosphate

\( \text{GP-DH} \) glycerol phosphate dehydrogenase
PART I

THE DEVELOPMENT OF A FLUOROMETRIC TECHNIQUE TO
MEASURE ALUMINIUM IN BIOLOGICAL SPECIMENS
CHAPTER I

INTRODUCTION

A. GENERAL PROPERTIES OF ALUMINIUM

The earth’s crust is composed of about 8% aluminium and is widely distributed in igneous rocks, but the only workable ore is bauxite. The metal is odourless, tasteless and silvery white, but with increasing silicon and iron content, the appearance changes to a dull-bluish grey color. It is very malleable, ductile and quite soft.

The ionic radius of aluminium is 0.57 Å and it has a high oxidation potential of +1.66V and a +3 oxidation state (1). The metal appears unreactive because of the rapid formation, in air, of a tenacious oxide layer. Though its standard electrode potential is -1.66V, it does not dissolve in water, and even with dilute HCl reacts slowly until the oxide layer has been removed, after which dissolution is rapid.

Aluminium is found in vegetation and in all vertebrate species that have been studied. Its levels in plants and animals vary with the amount of the metal available in the environment. Aluminium may enter the human body through oral ingestion of medications, topical applications, cooking, water supplies, breathing, etc. Table I compares tomatoes cooked in porcelain iron and aluminium pots (2).
### TABLE I

**Aluminium Content of Tomatoes Cooked in Aluminium or Porcelain-Iron Pot**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (g/ml)</th>
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<th>mg Al/100 g dry weight</th>
<th>mg Al/60 ml serving</th>
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<td>Before cooking</td>
<td>0.059</td>
<td>3.8</td>
<td>1.3</td>
<td>0.046</td>
</tr>
<tr>
<td>p-i pot, 2 h</td>
<td>0.118</td>
<td>4.1</td>
<td>1.1</td>
<td>0.080</td>
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<tr>
<td>Al pot, 2 h</td>
<td>0.102</td>
<td>3.9</td>
<td>31.7</td>
<td>1.940</td>
</tr>
<tr>
<td>p-i: pot, overnight</td>
<td>0.130</td>
<td>3.7</td>
<td>1.0</td>
<td>0.079</td>
</tr>
<tr>
<td>Al pot, overnight</td>
<td>0.123</td>
<td>3.8</td>
<td>52.9</td>
<td>3.890</td>
</tr>
</tbody>
</table>

p-i: porcelained-iron.  
Al pot: aluminium pot.  
Table is taken from reference (2).
These results show a dramatic increase of aluminium in tomatoes cooked in an aluminium pot after 2 h, and a doubling of the aluminium level when the food was stored overnight in this pot.

Aluminium is now believed to be absorbed via the gut. Several reports showed a positive balance of plasma aluminium in patients given oral doses of aluminium gels (3-5). The absorption process may be either active or passive, though one report suggested the process to be active (6). Aluminium excretion in the urine increases during uptake (3), whether it is from gastrointestinal absorption or dialysis.

The major binding protein for aluminium is transferrin, though albumin has been suggested to bind very weakly to this metal (7,8). It forms various complexes with citrate, fluoride and phosphate once absorbed or in the gut depending on the pH. Indeed, it has been shown that dietary citrate enhances the absorption of aluminium from the gut (9).
B. CLINICAL SIGNIFICANCE OF ALUMINIUM

Ingestion of moderate doses of aluminium compounds deliberately or non-deliberately has for many years been assumed to be non-toxic. As a matter of fact, it was generally believed and still by some today that this trace metal has no biological role in man or animals. Current literature disproves this notion. Harrison et al. (10) showed that aluminium is a potent inhibitor of mammalian hexokinase. It has also been shown to enhance the non-enzymatic transamination of glutamic acid by 20-fold (11). Guirard and Snell (12) reported that aluminium binds to and stabilizes the pyridoxine complex that is presumably the intermediate in glutamic acid decarboxylase catalyzed decarboxylation, a major step in γ-aminobutyric acid production.

The pathological significance of aluminium is shown mainly in dialysis encephalopathy syndrome (DES) or dialysis dementia, Alzheimer's disease, and renal osteodystrophy.

Patients on long term hemodialysis for chronic renal failure tend to retain phosphate which can cause both ectopic calcifications and osteitis fibrosa cystica (4). These patients are given aluminium hydroxide orally to control their hyperphosphatemia (4,13,14). These antacids work in the following way to decrease phosphorus levels:
\[
\text{Al(OH)}_3 + \text{HCl} \rightarrow \text{AlCl}_3 + 3\text{H}_2\text{O} \\
\text{AlCl}_3 + \text{PO}_4^{2-} \rightarrow \text{AlPO}_4 + 3\text{Cl}^{-}
\]

The aluminium phosphate formed is insoluble and is excreted in the feces (15). Overtreatment with aluminium hydroxide gels may result in hypophosphatemia and this consequence of oral aluminium treatment can interfere with bone mineralization and result in osteomalacia (a form of osteodystrophy) (4,13,14,16). Osteomalacia, in the chronic renal failure patient, may also result from the aluminium absorbed during hemodialysis and deposited in the bone (17).

Dialysis encephalopathy syndrome (DES) was described by Alfrey et al. in patients on chronic hemodialysis (18). The features of this syndrome are illustrated in Table II. Some theories have been proposed to explain the etiological nature of this syndrome (19). Practically, all of these theories have been discounted over the years for a lack of experimental evidence. The current and experimentally documented theory is based on aluminium as the possible etiological agent in DES (20). Aluminium levels in muscle, bone and brain were determined by these workers and the results are shown below:

**Muscle**

- Normal: 1.2 ppm
- DES: 14.8 ppm

**Bone**

- Normal: 2.4 ppm
TABLE II

DIALYSIS ENCEPHALOPATHY SYNDROME (DES)

Clinical features:

- Altered mood
- Depression
- Diminished alertness
- Lethargy
- Coma

Speech difficulties:

- Dysphagia - difficulty in swallowing
- Dyspraxia - partial loss of coordinated movement
- Stuttering

Motor abnormalities:

- Myoclonus - muscle spasms
- Asterixis - tremors
- Seizures
  - Focal
  - Grand mal

Electroencephalogram (EEG)

- Diffuse slowing
- Burst of slow and high voltage activity

Table is taken from reference (18).
DES: 98.5 ppm
Brain (Gray Matter)
Normal: 2.2 ppm
DES : 25.0 ppm

Since this study was published, many reports have appeared around the world implicating aluminium with this syndrome (21-24). Though most patients have been on oral aluminium hydroxide gel therapy for 2 1/2 years or more before the DES developed, many patients still did not show symptoms of DES after prolonged dialysis. However, this observation should be regarded with caution since dialysis patients usually have abnormal levels of anions, cations, pH, hormones, etc., all of which vary from patient to patient, and which in totality may play some role in the availability of aluminium circulating to the brain. Apart from aluminium intoxication from oral therapy, the untreated tap-water of the dialyzate used for hemodialysis has been implicated as the source of aluminium in these patients (17,25). Aluminium sulfate is used as a flocculating agent to purify municipal water supplies. Again some patients do not show symptoms of DES after prolonged hemodialysis. Again these results must be interpreted with care since each water supply around the world will vary with pH. The amphoteric nature of aluminium causes a highly water-insoluble aluminium hydroxide to be formed near neutral pH. Small changes in pH, either to a more acid or
alkaline value, can make a large difference in the amount of aluminium in the dialyzable form. The final pH of the dialyzate can be affected by the pH of the water used to reconstitute the dialyzate (26).

Aluminium has been implicated in Alzheimer's disease. The etiology of this disease is not well understood, though some theories have been proposed to explain it (20). These include an incomplete viral infection, a disorder of the immune system, and a neurotoxic agent, possibly aluminium. The actual cause of the disease may be a combination of one or more of these three mechanisms and/or the effect of some agent yet to be found. The content of aluminium in the control brains have been studied by several groups. The data is shown below:

Crapper et al. (27): 0.1–3.9 \( \mu g/g \) dry wt

\[ \bar{x} = 1.9 \pm 0.7 \mu g/g \] dry wt

Tipton et al. (28): 1.25 \( \mu g/g \) dry wt

McLaughlin et al. (29): 3.0 \( \mu g/g \) dry wt

Alfrey et al. (20): 2.2 \( \mu g/g \) dry wt

In Alzheimer's disease patients, the concentration of aluminium varied from 0.4–107 \( \mu g/g \) dry wt for different regions of the brain (27).
C. METHODS USED TO DETERMINE ALUMINIUM

Several methods have been proposed to determine aluminium and these include gravimetric, titrimetric, photometric and fluorometric techniques. Spectrophotometric techniques for the determination of aluminium are based on binary complexes with triphenylmethane reagents, such as Chrome Azurol S, Eriochrome Cyanine R and Pyrocatechol Violet (30). These reagents exhibit moderate sensitivity, however, upon the addition of a third component (a long-chain quaternary base-cationic surfactant) to the binary system, a ternary complex is formed with higher molar absorptivities (usually greater than $1 \times 10^5 \text{ Lmol}^{-1} \text{ cm}^{-1}$). Sampson and Fleck (31) used Chromazurol S and cetyl pyridinium chloride to measure aluminium in dialysis fluid and water. A molar absorptivity of $1.25 \times 10^5 \text{ Lmol}^{-1} \text{ cm}^{-1}$, with a detection limit of $5 \mu\text{gL}^{-1}$ was obtained. Other colorimetric methods not utilizing the formation of the ternary complex, such as 8-hydroxyquinoline, are quite insensitive (32).

A large number of reagents are known which form fluorescent compounds with aluminium. Many of these reagents are characterized by the presence of a conjugated system of bonds incorporating the enolic hydroxy group and a carbonyl group or a bond with a nitrogen atom. Several hydroxyflavones containing the atomic groupings
have been examined for aluminium and their sensitivities are shown in Table III (33). As shown in this Table, the Al-morin reaction is quite sensitive and several groups have tried to develop an assay for aluminium with morin, in analytical aqueous systems (34-36). As yet, this system has never been applied to a biological matrix. Other fluorometric reagents that have been used to determine aluminium in aqueous systems include 2,4,2'-trihydroxyazobenzene 5'-sulfonic acid (Alizarin Garnet R), 2,2'-dihydroxyazo-naphthalene, salicylidene ortho-aminophenol, Pontachrome Blue Black, 3-hydroxy 2-napthoic acid (37).

X-ray fluorescence (19) and neutron activation analysis (38) have also been used to measure aluminium but these are not currently available in most clinical laboratories.

The most common method used to determine aluminium in a biological matrix is atomic absorption spectroscopy (39-43). Flame techniques, even with the hotter nitrous oxide-acetylene flame, do not perform as well as the flameless methods, using the graphite furnace. Though this technique is the method of choice, it is not commonly available in most clinical laboratories and the
TABLE III

SENSITIVITY AND FLUORESCENCE DETECTION OF Al BY FLAVONOIDS

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>pH 3-4</th>
<th>pH 5-6</th>
<th>pH 10-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morin</td>
<td>0.005</td>
<td>0.005</td>
<td>0.05</td>
</tr>
<tr>
<td>Datsiscetin</td>
<td>0.01-0.02</td>
<td>0.01-0.02</td>
<td>0.10</td>
</tr>
<tr>
<td>Datiscin</td>
<td>0.02</td>
<td>0.02</td>
<td>0.10</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.03-0.04</td>
<td>0.03-0.04</td>
<td>-</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.05</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.10</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Hesperitin</td>
<td>0.10</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>Hesperidine</td>
<td>Weak fluorescence</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Blank solution</td>
<td>No fluorescence</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table is taken from reference (33).
disadvantages associated with this technique include untreated graphite tubes, matrix interferences, and standardization procedures (44, 45).

D. PURPOSE OF THIS STUDY

As mentioned earlier, X-ray fluorescence and neutron activation analysis techniques are not readily available in the clinical laboratory. Atomic absorption spectrophotometry, though more available is yet to be implemented in smaller laboratories due to its cost. The colorimetric reagents are unsuitable to determine aluminium because of their relative insensitivities and although the fluorometric reagents are more sensitive, both groups of reagents suffer from major interferences from biological matrices, such as cations, anions and proteins.

A method is proposed to determine aluminium in serum using the following reaction:

\[ \text{Al} + \text{Morin} \rightarrow \text{Al-Morin} \]

Though this reaction has been studied before, the previous workers neglected to investigate the optimum conditions for this reaction as they would relate to a clinical situation (34-36). Thus, with the view of developing a technique for aluminium in biological specimens, several conditions for its reaction with morin are studied.
Furthermore, due to the aqueous environment used by others, interferences were not attempted to be eliminated. It is proposed here to use a serum sample to prepare an acid filtrate (46) that is free of cationic, anionic and protein interferences so that such a sample could be used for the determination of aluminium with morin. The method proposed to remove these interferences involve an ion exchange chromatographic technique.
CHAPTER II

EXPERIMENTAL

ANALYTICAL AND CLINICAL STUDIES

1. Equipment

Balance: A Mettler PC 1200N balance supplied by Fisher Scientific Co. (Canada), Don Mills, Toronto, Ontario, M3A 1A9, was used for measuring values above 1 g and a Mettler Type H15 balance from the same Company was used for weights less than 1 g.

Plastic and Glassware: Test tubes (13' x 100 mm), volumetric flasks and pipets, Pyrex® beakers and other glass material used in this study were obtained from Fisher Scientific Co. (Canada).

All plastic containers were obtained from Sarstedt, Inc., Montreal, Quebec.

The following procedure was used to prepare aluminium-free glass and plasticware. The container was filled with dilute nitric acid (100 mL/L) and allowed to stand for 48 h. The caps of the containers were placed in a beaker containing the acid solution. The containers and their caps were then rinsed with distilled water and placed in a metal-free water bath for 48 h. After rinsing (10x) with water, the glass containers were filled with metal-free water and the plasticware was allowed to air-dry upside down.
overnight.

The following procedure was used to produce siliconized glassware. The cleaned glassware was filled with a 1% (v/v) solution of Siliclud® and allowed to stand for 1 min. The solution was then poured into another container to be siliconized and the container rinsed with metal-free water. The glassware was then gently warmed (45-50°C) in the oven until dry.

All glassware and plasticware, though cleaned earlier, were rinsed with metal-free water just prior to use in order to eliminate any minute traces of dust particles that might have settled on the apparatus during closed storage.

Pipet tips used by the Pipetman® Pipettors were also cleaned and stored by the above procedure.

Micropipettors: An Oxford pipettor, (200 μL) available from Canadian Laboratory Supplies, Ltd., Toronto, Ontario, M8Z 2H4 and Gilson Pipetman Models P-200D and P-1000D with disposable pipet tips C20 and C200 purchased from Mandel Scientific Co., Ltd., Ville St. Pierre, P.Q., H8R 1A3, were used in this study.

pH Meter: A Fisher Accumet® pH meter supplied by Fisher Scientific Co. (Canada) was used to measure pH's. It was equipped with a glass electrode from Graphic Controls, Buffalo, NY 14240.
Fluorometer: Fluorescent intensity readings were taken from a Perkin-Elmer Model 204 Fluorescence Spectrophotometer equipped with a Perkin Elmer Model 150 Xenon Power Supply and a Model 56 Recorder, obtained from Perkin-Elmer Corp., Downsview, Ontario, M3N 1Y4. The fluorometer was calibrated with serial dilutions of a 10 mg/L quinine sulfate in 0.05 M sulphuric acid. These solutions when excited at 355 nm, emitted fluorescence at 450 nm.

Spectrophotometer: A Shimadzu UV-Visible Recording Spectrophotometer UV-240 connected to a Shimadzu Graphic Printer PR-1, distributed by Teckscience Ltd., Toronto, Ontario, MBH ST4, was used to measure absorbances.

Vortex: A Vortex-Genie obtained through Scientific Industries, Inc., Bohemia, NY 11719, was used to mix solutions in test tubes.

Cuvets: Quartz cuvets of 1.00-cm pathlength each and available from Beckman Instruments, Inc., Toronto, Ontario, MBZ ST2, were used to measure fluorescence and absorbances.

2. Materials

Chemicals: The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO 63178: morin (2',3,4',5,7-pentahydroxyflavone), chelating resin (Chelex-100, dry mesh 200-400, sodium form), Dowex-1(Cl−) (1 x 8-400; dry mesh, 50-100).

The following chemicals were obtained from Aldrich
Chemical Co., Milwaukee, WI 53201: aluminium atomic absorption standard (1020 ppm in 2% HCl) and chloroacetic acid.

The following chemicals were purchased from Fisher Scientific Co. (Canada): reference buffer, pH 6.0 (monobasic potassium phosphate/NaOH and quinine sulfate-dihydrate).

A pH reference buffer solution of 4.01 was purchased from Canlab, Toronto, Ontario, M8Z 2H4.

The following chemicals were obtained from BDH Chemicals, Toronto, Ontario, M8Z 1K5: hydrochloric acid (A.C.S.); nitric acid (A.C.S.); acetic acid; sodium hydroxide, ethanol; sulphuric acid (A.C.S).

Siliclad® was purchased from Clay Adams, Division of Becton Dickinson Co., Parippany, NJ 07054.

Water: Water was purified by the following procedure: Water obtained from a distillation apparatus was passed through a reverse osmosis Xenopure® system and collected. This water was then passed through a mixed ion-exchange resin containing Chelex-100 (Na⁺) and Dowex-1(Cl⁻). The water collected was neutralized and stored in plastic containers with 0.5% chloroform to prevent bacterial growth. This is referred to as metal-free water.
3. Reagents

**Buffer (1M):** A solution was made by adding 94.48 g monochloroacetic acid, 57.5 mL acetic acid and 40.5 g sodium hydroxide to a 500-mL volumetric flask and diluting it to the mark with metal-free water. To eliminate interferences from reagents used to make this buffer, the solution was passed through a Chelex-100(Na\(^+\)) column, collected and re-adjusted to pH, 4.1. The buffer was stored in a plastic container and was stable for months at 4°C.

**Ethanol:** Ethanol (A.C.S.) was purified by doubly distilling 200 mL in a 500-mL round bottomed flask. A 65% (v/v) solution was prepared by adding 65 mL of purified ethanol to a 100-mL volumetric flask and diluting to the mark with metal-free water.

**Aluminium Standards:** The atomic absorption stock solution (1020 μg/mL in 2% HCl, was diluted to prepare a working stock solution of 3.78 x 10\(^{-5}\) M). This was then diluted to give final cuvet concentrations of 0-1500 nmol/L. Metal-free water was used for this purpose. The standards were stored in plastic containers and fresh standards were made every two weeks.

**Mixed Resin:** Equal aliquots of Chelex-100(Na\(^+\)) and Dowex-1(Cl\(^-\)) were mixed and slurried with water. After settling, the cloudy supernatant was discarded and the procedure repeated several times. The final pH of the mixed resin was adjusted to 7.6-8.0 with NaOH or HCl as required.
Stock Morin: A solution was prepared by adding 30.2 mg morin to a 100-mL volumetric flask and diluting it to the mark with absolute ethanol. This resulted in a concentration of $10^{-3}$M.

Working Morin Solution: Prior to the assay, 1 mL of morin stock solution was diluted to 10 mL with absolute ethanol to give a concentration of $10^{-4}$M.

Quinine Sulfate Calibration Standards: Quinine Sulfate (10 mg) was dissolved in 1.0 L 0.05M sulphuric acid to give a 10 ppm solution. This was serially diluted and used to calibrate the fluorometer.

4. Procedure

The procedure used to precipitate proteins (40,46) is as follows: an aliquot of 1.0 mL serum was added to a clean polypropylene test-tube, followed by 50 μL concentrated nitric acid. The mixture was vortex-mixed and heated in a boiling bath for 5 min. Subsequently, it was centrifuged at 1500 x g for 10 min. An aliquot of the clear supernate (0.80 mL) was pipetted into a cleaned polypropylene graduated tube and the pH adjusted to 7.6-8.0 (400 μL NaOH (1N) was required). Then 0.30 mL of the mixed resin was pipetted into the filtrate and mixed for 5 min. The mixture was maintained at a pH of 7.6-8.0, and the total volume adjusted to 1.5 mL. Subsequently, it was centrifuged and the supernatant was used for the analysis. There is
enough supernatant for duplicate analyses.

To a cleaned polypropylene tube were added 2.0 mL ethanol (65%, v/v), followed by 0.60 mL buffer, 0.60 mL supernatant and 0.40 mL working morin solution. The contents were mixed and incubated for 15 min at room temperature (22-25°C). Fluorescence readings were taken in quartz cuvets. The excitation wavelength was 435 nm and the emission wavelength was 510 nm. A blank was prepared in the same way except that serum was substituted with water. In order to relate the values obtained from clinical specimens from this procedure to calibration curves using pure aqueous solutions, all standards were treated in the identical way as outlined above, though these standards were theoretically pure.
CHAPTER III

RESULTS AND DISCUSSION

ANALYTICAL AND CLINICAL STUDIES

1. Control of Contamination

In the determination of trace metals, the most serious problem encountered is contamination. This is especially true for aluminium analysis, for its ubiquitous nature will allow for its presence in the chemicals, water, glassware, plasticware, etc., used in the analysis of this element. Distilled deionized water is unsuitable for this purpose. In this study, deionized water purified by reverse osmosis and passed through a mixed-bed (Chelex-100(\(\text{Na}^+\)) and Dowex-1(\(\text{Cl}^-\)) ion-exchange column produced water of good quality. This was used for the preparation of standards, reagent solutions, and final rinsings of plastic and glassware.

Solutions were not stored in glassware since the leaching of contaminants (including aluminium) would gradually contaminate these solutions. It was better to coat all glass surfaces with a 1% Siliclud \(^\text{®}\) solution which formed a thin film on the surfaces. This might not eliminate but certainly minimized leaching from exposed acid-washed glass surfaces.

Contamination with aluminium arising during blood
sampling and from handling devices is suggested to be the most important factor in the wide variation of values as noted by others (47). Indeed, mean reference values from different studies ranged from 3.77 to 1460 μg/L, and 3.72 to 240 μg/L using the same technique. Though each population’s reference range may be different, this wide variation is certainly attributable in part to experimental techniques and improper control of contamination. Thus, values reported in the literature for sensitivities of various methods must be viewed with caution.

2. Principle

The principle proposed to determine aluminium is simple and involves the formation of a highly fluorescent chelate with morin as shown below.

\[ \text{Al} + \text{Morin} \rightarrow \text{Al-Morin (Fluorescent)} \]

The excitation and emission spectra for both morin and its chelate with aluminium are shown in Fig. 1. The reagent, morin, essentially did not absorb in the range 400-580 nm, as shown by the emission spectrum, and was practically non-fluorescent in this region although fluorescent bands have been observed in the region of 320-380 nm and 240-270 nm (48). The chelate absorbed strongly at 435 nm and emitted intensely at 510 nm, thereby forming the basis for a very sensitive reaction. This allowed for the measurement of minute quantities of aluminium.
FIGURE 1

EMISSION AND EXCITATION SPECTRA OF MORIN
AND ITS CHELATE WITH ALUMINIUM

Legend

A: Excitation Spectra for Morin and Al-Morin.
B: Emission Spectra for Morin and Al-Morin.
a: Spectrum for chelate.
b: Spectrum for morin.

Absorption from morin in the region 300-478 nm is minimal.

Wavelength of maximum excitation for chelate = 435 nm.
Wavelength of maximum emission for chelate = 510 nm.
The reaction between aluminium and morin was found to follow a 1:1 stoichiometric relationship \( K = 2.96 \times 10^6 \) at \( 25^\circ C \) \( (33) \). Possibilities for such chelate formation are shown in Fig. 2. It is likely that the structures [1] and [2] are the structures predominantly found for this chelate in solution and an equilibrium may exist between these two structures when in solution. Though [3] has been suggested to be another way whereby this chelate could be formed, it is not as stable as the other two structures due to longer co-ordinate bond lengths required to form this chelate \( (33,48) \) and less aromatic character as shown in the structures [1] and [2].

3. Effect of pH on the Fluorescence of the Aluminium-Morin Chelate

A study was carried out to check the variation of pH on the fluorescence of the aluminium-morin chelate. The results are illustrated in Fig. 3. Two peaks corresponding to maximum fluorescence intensities were observed at pH's of 3.5 and about 5.0. The optimum pH of 3.5 was analytically not very useful, though a more sensitive reaction was noted here. This is due to the fact that the peak is quite sharp, thus, stringent buffer requirements may be necessary to control the pH here since minor changes in pH will change the response significantly. On the other hand, the broad peak at 5.0, though slightly less sensitive, is much easier to maintain with an appropriate buffer. It should be noted
FIGURE 2

THE STRUCTURAL CHEMICAL FORMS OF THE
ALUMINIUM-MORIN CHELATE

Legend

A: Structure of morin.

[1]: Structure of a 1:1 chelate involving the carbonyl
group and the hydroxy group at carbon-3.

[2]: Structure of a 1:1 chelate involving the hydroxy
group at carbon-5.

[3]: Structure of a 1:1 chelate involving the two
hydroxy groups at 2 and 4 carbons.
FIGURE 2

[A]

[1]

[2]

[3]
FIGURE 3

VARIATION OF pH ON THE FLUORESCENCE OF Al-MORIN

Legend

Two pH optima at 3.5 and 5.0 are shown for the biphasic response of this chelate.

The chelate mixture was adjusted to the appropriate pH's with HCl or NaOH.

[Al] = 1.33 \times 10^{-6} \text{ M (cuvet)}.

[Morin] = 1.33 \times 10^{-5} \text{ M (cuvet)}. 
here that the fluorescence of the chelate was essentially abolished at a pH greater than 7.0.

The biphasic response of this curve indicating two optima may suggest a mixture of chelates in solution. Indeed, Fletcher (49) varied the pH of morin and calculated the percentage of each morin specie. These results are shown in Table IV. As noted here, morin has the capability of forming six species, i.e., $H_5M$, $H_4M^-$, $H_3M^{2-}$, $H_2M^{3-}$, $HM^{4-}$ and $M^{5-}$. Thus, at a pH of 2.0 and 5.0, the predominant species would be $H_4M^-$ and $H_3M^{2-}$, respectively, thereby forming the respective predominant chelate at these pH's.

The effect of various buffers was studied on the maximum fluorescence of this chelate. The date are shown in Table V. Indeed, buffers such as citrate, phthalate, oxalacetate and phosphate almost completely abolished the fluorescence of the chelate. This was most likely due to the reaction of the anion in the buffer with the Al of the chelate, thereby displacing morin and rendering a non-fluorescent mixture. Succinate buffer provided appreciable fluorescence but the maximum fluorescence was obtained with sodium acetate-acetic acid and sodium acetate-monochloroacetic acid. The latter was used for this study and at a pH of 4.1 was stable for months. This buffer, when used along with all the other reagents as indicated in ANALYTICAL AND CLINICAL STUDIES (CHAPTER II, 4, p. 19) consistently gave a final pH of approximately 5.0.
TABLE IV

VARIATION OF pH ON MORIN

<table>
<thead>
<tr>
<th>pH (Calc.)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.92</td>
<td>90% $H_5M$</td>
</tr>
<tr>
<td>2.0</td>
<td>100% $H_4M^-$</td>
</tr>
<tr>
<td>5.84</td>
<td>88% $H_3M^{2-}$</td>
</tr>
<tr>
<td>8.0</td>
<td>80% $H_2M^{3-}$</td>
</tr>
<tr>
<td>11.0</td>
<td>98% $HM^{4-}$</td>
</tr>
<tr>
<td>13.7</td>
<td>84% $M^{5-}$</td>
</tr>
</tbody>
</table>

Table is taken from reference (49).
TABLE V

VARIATION OF BUFFERS ON THE ALUMINIUM-MORIN REACTION\(^a\)

<table>
<thead>
<tr>
<th>Buffers(^{b,c})</th>
<th>Fluorescence(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Phthalate</td>
<td>4.0</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>6.5</td>
</tr>
<tr>
<td>Succinate</td>
<td>56.8</td>
</tr>
<tr>
<td>Acetate-monochloroacetic acid</td>
<td>86.3</td>
</tr>
<tr>
<td>Acetate-acetic acid</td>
<td>80.5</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^a\) [Al] = 1.33 \times 10^{-6} \text{ M (cuvet)}; [Morin] = 1.33 \times 10^{-5} \text{ M}.

\(^b\) [Buffer] = 1.0 \text{ M (adjusted to a pH of 4.0}).

\(^c\) Sodium salts were used to make the buffer solutions.

\(^d\) Readings were averages of triplicate measurements.
4. Effect of Alcohols on the Fluorescence of the Aluminium-Morin Chelate

Several alcohols were tested to check their effects on the fluorescence of the chelate. These results are shown in Table VI. At concentrations of 50% (v/v), ethanol produced maximum fluorescence followed by methanol. Marked decrease in fluorescence was noted for isopropanol and butanol.

Several concentrations of ethanol were also tested. As the concentration of ethanol was increased, the fluorescence increased. However, when triplicate samples were tested using higher concentrations of ethanol, very poor reproducibility in the readings were observed. This may be due to changes in pH and difficulty in maintaining the optimum pH or, slight (but unnoticeable) colloidal suspension of salts in the solution, since the buffer salts, etc., may be insoluble at higher concentrations of ethanol. Thus, a 50% (v/v), ethanol solution was used for the assay.

5. Aluminium:Morin Ratio for Maximum Fluorescence

Table VII shows various ratios of morin to Al and their consequences on the fluorescence of the chelate. At a ratio of 1:1, maximum fluorescence was not produced within the 15 to 20-min incubation period, though the stoichiometric relationship is 1:1. If the solution were allowed to incubate for a longer period of time, it is quite likely that all the aluminium would have reacted with the morin thereby forming the maximum amount of chelate possible. At


**TABLE VI**

**VARIATION OF ALCOHOLS ON THE FLUORESCENCE OF AL-MORIN$^a$**

<table>
<thead>
<tr>
<th>Alcohols$^b$</th>
<th>Fluorescence$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>50.5</td>
</tr>
<tr>
<td>Butanol</td>
<td>20.3</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>26.5</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>50% (v/v)</td>
<td>79.5</td>
</tr>
<tr>
<td>60% (v/v)</td>
<td>86.0</td>
</tr>
<tr>
<td>70% (v/v)</td>
<td>92.5</td>
</tr>
<tr>
<td>80% (v/v)</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

$^a$[Al] = 1.33 x 10$^{-6}$ M; [Morin] = 1.33 x 10$^{-5}$ M.

$^b$Methanol, isopropanol and butanol were 50% (v/v).

All alcohols were of spectrophotometric grade; ethanol was re-distilled with potassium permanganate.

$^c$Readings were averages of triplicate measurements.


<table>
<thead>
<tr>
<th>Al:morin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fluorescence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>70.5</td>
</tr>
<tr>
<td>1:4</td>
<td>86.0</td>
</tr>
<tr>
<td>1:10</td>
<td>89.5</td>
</tr>
<tr>
<td>1:20</td>
<td>88.0</td>
</tr>
<tr>
<td>1:50</td>
<td>69.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Al:morin refers to the ratio of concentration of aluminium to concentration of morin in the cuvet.

<sup>b</sup> Readings taken within 15-20 min and were averages of triplicate measurements.

The aluminium concentration is kept constant while the morin concentration is varied.
higher concentrations of morin, maximum fluorescence of the chelate was reached within 15 to 20-min. This is not unusual since a larger number of molecules of morin are available for the reaction and thus, equilibrium to the right is favored. It should be noted here that at very high concentrations of morin (1:50), a decrease in fluorescence was observed. Thus, a ratio of approximately 1:8-1:20 was used for these studies.

6. Time Required to Produce Maximum Fluorescence of the Chelate and its Stability Once Formed

A study was carried out to determine the maximum time that was necessary for the complete formation of the Al-morin chelate as determined by its fluorescence. As shown in Table VIII, the maximum fluorescence of the chelate was formed within 15-20 min after the addition of the reagents. Once formed it was stable for about 15 min or so and then gradually decreased from 88.5 to approximately 69.8 in about an hour when incubated in light. Thus, the chelate was sensitive to light when left exposed for a prolonged period of time. Therefore, readings must be taken within the 15 to 30 min period after the addition of reagents.
## TABLE VIII

TIME REQUIRED TO PRODUCE MAXIMUM FLUORESCENCE
AND THE STABILITY OF THE CHELATE$^a$

<table>
<thead>
<tr>
<th>Time $^b$</th>
<th>Fluorescence $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>50.5</td>
</tr>
<tr>
<td>2.0</td>
<td>60.5</td>
</tr>
<tr>
<td>3.0</td>
<td>68.3</td>
</tr>
<tr>
<td>5.0</td>
<td>74.5</td>
</tr>
<tr>
<td>8.0</td>
<td>82.3</td>
</tr>
<tr>
<td>10.0</td>
<td>86.0</td>
</tr>
<tr>
<td>15.0</td>
<td>88.5</td>
</tr>
<tr>
<td>20.0</td>
<td>88.0</td>
</tr>
<tr>
<td>25.0</td>
<td>87.5</td>
</tr>
<tr>
<td>30.0</td>
<td>87.8</td>
</tr>
<tr>
<td>40.0</td>
<td>80.5</td>
</tr>
<tr>
<td>50.0</td>
<td>74.0</td>
</tr>
<tr>
<td>60.0</td>
<td>69.8</td>
</tr>
</tbody>
</table>

$^a$ $[\text{Al}] = 1.33 \times 10^{-6}$ M, $[\text{Morin}] = 1.33 \times 10^{-5}$ M; incubated in light.

$^b$ Time taken after addition of morin.

$^c$ Readings were averages of triplicate measurements.
7. Effect of Temperature on the Fluorescence of the Al-Morin Chelate

The effect of temperature on the fluorescence of the aluminium-morin chelate was very pronounced, as shown in Table IX. An average of approximately 1 unit of fluorescent intensity/°C was noted as the temperature was changed. At higher temperatures, the fluorescent readings were noticeably lower and the opposite was true at lower temperatures. Though the fluorescent readings at room temperature (22-25°C) did not result in maximum intensities, this temperature was selected for this assay since it was inconvenient to maintain temperatures at 15°C and 20°C and are indeed more time-consuming, and the determination at room temperature did not outweigh the little loss in sensitivity at this temperature. However, it is important to maintain the same temperature when the assay is performed on different days, otherwise a correction of 1 unit of fluorescent intensity/°C must be made.

8. Generation of Standard Curve for the Determination of Aluminium

A calibration curve for the determination of aluminium is illustrated in Fig. 4. The curve was linear for the range of 0 to 1500 mmol/L. The equation for the line of regression is \( y = 0.06x + 4.01 \) with a correlation coefficient of 0.998. The procedure was followed as outlined in ANALYTICAL AND CLINICAL STUDIES (CHAPTER II, 4,
TABLE IX

EFFECT OF TEMPERATURE ON THE FLUORESCENCE
OF THE Al-MORIN CHELATE

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Fluorescence b</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>88.5</td>
</tr>
<tr>
<td>15</td>
<td>85.0</td>
</tr>
<tr>
<td>25</td>
<td>75.0</td>
</tr>
<tr>
<td>37</td>
<td>63.5</td>
</tr>
<tr>
<td>45</td>
<td>52.8</td>
</tr>
</tbody>
</table>

\[ [\text{Al}] = 1.00 \times 10^{-6} \text{ M; [Morin]} = 1.33 \times 10^{-5} \text{ M.} \]

b Readings were averages of triplicate measurements.
FIGURE 4

STANDARD CURVE FOR THE DETERMINATION OF WITH MORIN

Legend

Equation for line of regression: \( y = 0.06x + 4.01 \).
Correlation coefficient: \( r = 0.998 \).
Concentrations are for final cuvet concentrations.
Readings were averages of triplicate measurements.
The procedure was carried out at a pH of 5.5 and a temperature of 23-25°C.
p. 19). It must be emphasized that reproducible results can only be obtained by careful and methodical elimination of aluminium and other external/internal interferences. Thus, section (1) of this Chapter and the recommendations thereof should be followed for the reproducible determination of this metal.

9. Study of Interferences

Several metals were tested to see whether they would interfere with the determination of aluminium with the reagent, morin. The results are shown in Table X. The concentrations shown beside each metal are the concentrations of the metal in the cuvet. A positive interference was noted for iron and a negative interference for copper at all the concentrations tested. Probably, iron reacted with excess morin to form a chelate that was either more fluorescent or, to form insoluble colloidal particles suspended in the solution. Copper, on the other hand, must have bound morin to form a different chelate with different absorption and emission characteristics but features analogous to those of the aluminium-morin reaction. Furthermore, it is likely that the chelate from the copper-morin reaction is much less fluorescent. The other metals, zinc, cobalt, chromium and molybdenum, at the concentrations tested, did not produce any changes in fluorescence, except for cobalt, chromium and molybdenum at
TABLE X

EFFECT OF VARIOUS CATIONS ON THE FLUORESCENCE OF
THE A1-MORIN CHELATE

<table>
<thead>
<tr>
<th>Metal added</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>No metal</td>
<td>36.0</td>
</tr>
<tr>
<td>Iron, 22 μmol/L</td>
<td>77.0</td>
</tr>
<tr>
<td>88</td>
<td>91.0</td>
</tr>
<tr>
<td>196</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Copper, 20 μmol/L</td>
<td>18.0</td>
</tr>
<tr>
<td>80</td>
<td>8.7</td>
</tr>
<tr>
<td>160</td>
<td>8.3</td>
</tr>
<tr>
<td>Zinc, 19 μmol/L</td>
<td>35.3</td>
</tr>
<tr>
<td>76</td>
<td>37.2</td>
</tr>
<tr>
<td>152</td>
<td>37.5</td>
</tr>
<tr>
<td>Cobalt, 0.5 μmol/L</td>
<td>34.2</td>
</tr>
<tr>
<td>3.3</td>
<td>36.5</td>
</tr>
<tr>
<td>6.6</td>
<td>54.5</td>
</tr>
<tr>
<td>Chromium, 0.008 μmol/L</td>
<td>39.5</td>
</tr>
<tr>
<td>0.026</td>
<td>37.5</td>
</tr>
<tr>
<td>0.024</td>
<td>43.5</td>
</tr>
<tr>
<td>Molybdenum, 0.0006 μmol/L</td>
<td>38.0</td>
</tr>
<tr>
<td>0.0012</td>
<td>39.5</td>
</tr>
<tr>
<td>0.002</td>
<td>45.5</td>
</tr>
</tbody>
</table>

\[^a\] [Al] = 5.0 \times 10^{-7}; [Morin] = 1.33 \times 10^{-5} M.

\[^b\] Concentrations of metal shown are concentrations of metal in cuvet.

\[^c\] Readings were averages of triplicate measurements.
the highest of the three concentrations used.

Several anions were tested as shown in Table XI. The results indicated that fluoride, citrate, oxalate and phosphate interfered very strongly. As a matter of fact, the fluorescence was dramatically reduced to approximately zero at physiological (serum) concentrations of these anions, when tested individually. Similar findings were observed when aluminium was determined with Chromazurol-S and cetylpyridinium chloride (31) (See Fig. 5). Physiologic concentrations of chlorides, acetates, sulfates and nitrates showed no effect on the fluorescence of the chelate.

Based on these results, it is quite obvious that the determination of aluminium in serum is only practical if anionic and cationic interferences can be removed. Thus, a study was undertaken to remove such interferences with ion-exchange resins. Riley and Taylor tested the effect of Chelex-100(H⁺) on various cations in sea-water (50). This data is shown in Table XII. At a pH of 7.6, the retention of aluminium to the resin is 0%, whereas, at this pH interfering copper, cobalt, lead, manganese, nickel and zinc, amongst others are 100% retained on the resin. Thus, the interfering cations can easily be removed from aluminium at a pH of 7.6 with Chelex-100. The results are shown in Table XIII, where individual and a mixture of various cations (iron, copper, zinc, cobalt and molybdenum) were added to an aluminium standard solution and the aluminium
TABLE XI

ANIONIC INTERFERENCES ON THE ALUMINIUM-MORIN CHELATE

<table>
<thead>
<tr>
<th>Anions $^{b,c}$</th>
<th>Fluorescence $^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate (4.0 mmol/L)</td>
<td>0.0</td>
</tr>
<tr>
<td>Fluoride (400 μmol/L)</td>
<td>10.5</td>
</tr>
<tr>
<td>Oxalate (400 μmol/L)</td>
<td>6.3</td>
</tr>
<tr>
<td>Citrate (400 μmol/L)</td>
<td>3.0</td>
</tr>
<tr>
<td>Chloride (200 mmol/L)</td>
<td>50.5</td>
</tr>
<tr>
<td>Acetate (200 mmol/L)</td>
<td>53.2</td>
</tr>
<tr>
<td>Sulfate (200 mmol/L)</td>
<td>50.8</td>
</tr>
<tr>
<td>Nitrate (200 mmol/L)</td>
<td>51.5</td>
</tr>
</tbody>
</table>

$^{a} [\text{Al}^{3+}] = 7.5 \times 10^{-7} \text{ M}; [\text{Morin}] = 1.33 \times 10^{-5} \text{ M}$.

$^{b}$ Concentrations of anions shown are concentrations of anions in cuvet.

$^{c}$ Sodium salts were used.

$^{d}$ Readings were averages of triplicate determinations.
FIGURE 5

EFFECT OF VARIOUS ANIONS ON THE ABSORBANCE OF Al–CHROMAZUROL S COMPLEX

Legend

Final concentration of Al in cuvet = 6.2 μmol L⁻¹.

Phosphate did not interfere with this reaction, but strong interferences from fluoride, citrate and oxalate were noted.

A: Phosphate (mmol/L).
B: Fluoride (μmol/L).
C: Oxalate (μmol/L).
D: Citrate (μmol/L).

Figure is taken from reference (31).
### TABLE XII

**Adsorption and Elution of Trace Elements from Sea Water with Chelex-100**

<table>
<thead>
<tr>
<th>Metals</th>
<th>pH for adsorption</th>
<th>Retention (%)</th>
<th>Eluent</th>
<th>Total % recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium (Al³⁺)</td>
<td>7.6</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Arsenic (AsO₄³⁻)</td>
<td>7.6</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Barium</td>
<td>5.0</td>
<td>25</td>
<td>2N HNO₃</td>
<td>25</td>
</tr>
<tr>
<td>Bismuth</td>
<td>9.0</td>
<td>100</td>
<td>2N HClO₄</td>
<td>100</td>
</tr>
<tr>
<td>Cadmium</td>
<td>7.6</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Caesium (Ce⁺³)</td>
<td>7.6</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Cerium (Ce³⁺)</td>
<td>9.0</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Chromium (Cr⁴⁺)</td>
<td>5.0</td>
<td>25</td>
<td>2N HNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Cobalt</td>
<td>7.6</td>
<td>100</td>
<td>2N HCl</td>
<td>100</td>
</tr>
<tr>
<td>Copper</td>
<td>7.6</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Indium</td>
<td>9.0</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Lead</td>
<td>7.6</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Manganese (Mn²⁺)</td>
<td>9.0</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Mercury (Hg²⁺)</td>
<td>7.6</td>
<td>85</td>
<td>2N HNO₃</td>
<td>40</td>
</tr>
<tr>
<td>Molybdenum (MoO₄²⁻)</td>
<td>5.0</td>
<td>100</td>
<td>4N NH₄OH</td>
<td>100</td>
</tr>
<tr>
<td>Nickel</td>
<td>7.6</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Phosphorus (PO₄³⁻)</td>
<td>7.6</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Rhenium (ReO₄²⁻)</td>
<td>7.6</td>
<td>90</td>
<td>4N NH₄OH</td>
<td>90</td>
</tr>
<tr>
<td>Scandium</td>
<td>7.6</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Selenium (SeO₄²⁻)</td>
<td>7.6</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Silver</td>
<td>7.6</td>
<td>100</td>
<td>2N HNO₃</td>
<td>90</td>
</tr>
<tr>
<td>Thallium (Tl⁺)</td>
<td>7.6</td>
<td>50</td>
<td>2N HNO₃</td>
<td>50</td>
</tr>
<tr>
<td>Thorium</td>
<td>7.6</td>
<td>100</td>
<td>2N H₂SO₄</td>
<td>100</td>
</tr>
<tr>
<td>Tin (Sn²⁺)</td>
<td>7.6</td>
<td>85</td>
<td>2N HNO₃</td>
<td>60</td>
</tr>
<tr>
<td>Tungsten (WO₄³⁻)</td>
<td>6.0</td>
<td>100</td>
<td>4N NH₄OH</td>
<td>100</td>
</tr>
<tr>
<td>Uranium (UO₂³⁻)</td>
<td>7.6</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Vanadium (VO₃⁻)</td>
<td>6.0</td>
<td>100</td>
<td>4N NH₄OH</td>
<td>100</td>
</tr>
<tr>
<td>Yttrium</td>
<td>9.0</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
<tr>
<td>Zinc</td>
<td>7.6</td>
<td>100</td>
<td>2N HNO₃</td>
<td>100</td>
</tr>
</tbody>
</table>

Table is taken from reference (50).
| Metals added\(^a\) | Aluminium (nmol/L)\(^b\) | | | |
|---|---|---|---|
| | Expected | Recovered | \% recovery\(^c\,d\) |
| Iron (Fe\(^{3+}\)) | 1500 | 1555 | 103.6 |
| Copper (Cu\(^{2+}\)) | 1500 | 1475 | 98.3 |
| Zinc (Zn\(^{2+}\)) | 1500 | 1525 | 101.6 |
| Cobalt (Co\(^{2+}\)) | 1500 | 1575 | 105.0 |
| Molybdenum (Mo\(^{2+}\)) | 1500 | 1585 | 105.7 |
| Mixture of metals | 1500 | 1565 | 104.3 |

\(^a\)Cuvet concentration of each metal in the cuvet was equivalent to physiologic concentration.

\(^b\)Concentration in cuvet.

\(^c\)Average recovery = 103.1%.

\(^d\)Readings were averages of triplicate measurements.
determined before and after addition. Recovery of aluminium using a standard solution of aluminium was approximately 103.1%. These results indeed confirm those of Riley and Taylor (50) that aluminium is not retained on Chelex-100 resin at a pH of 7.6, whereas most other metals are. Similarly, when a mixture of various amounts of phosphate, fluoride, citrate and oxalate were added to a standard solution of aluminium and the aluminium determined before and after spiking, the recovery was approximately 99% (Table XIV). From these results it is clear that aluminium can be separated from interfering cations and anions. Mixed batch chromatography was then tried to remove these interfering ions since the pH for removal of either cations or anions is similar (7.6 as opposed to 8.0, respectively). After mixing and centrifugation at high speed, the supernatant which contained aluminium free of interfering substances was determined with morin. Using batch chromatography, an average of 95% recovery was obtained for three levels of aluminium. It is important to maintain the pH for the chromatography at 7.6-8.0, since failure to do so will result in lower recoveries of aluminium.

10. Comparison with a Reference Method

The aluminium levels in ten serum samples were determined by the proposed method and compared with the flameless emission with graphite furnace (Fig. 6), (41).
TABLE XIV

REMOVAL OF ANIONIC INTERFERENCES WITH DOWEX-1(Cl-)

<table>
<thead>
<tr>
<th>Anions added</th>
<th>Aluminium (nmol/L)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected b</td>
<td>Recovered</td>
<td>% recovery c,d</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>1500</td>
<td>1475</td>
<td>98.4</td>
<td></td>
</tr>
<tr>
<td>Fluoride</td>
<td>1500</td>
<td>1463</td>
<td>97.5</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>1500</td>
<td>1532</td>
<td>102.1</td>
<td></td>
</tr>
<tr>
<td>Oxalate</td>
<td>1500</td>
<td>1478</td>
<td>98.5</td>
<td></td>
</tr>
</tbody>
</table>

aCuvet concentration of each anion in the cuvet was equivalent to physiologic concentration.
bConcentration in cuvet.
cAverage recovery = 99.1%.
dReadings were averages of triplicate measurements.
Sodium salts were used for this experiment.
FIGURE 6

COMPARATIVE STUDY FOR THE DETERMINATION OF ALUMINIUM WITH A REFERENCE METHOD

Legend

Equation for line of regression: \( y = 0.98x + 250 \).

Correlation coefficient: \( r = 0.93 \).

\( n = 10 \).

Readings were averages of triplicate measurements.
The equation for the line of regression was $y = 0.98x + 250$

with a correlation coefficient of 0.93, thus indicating good correlation between the two methods.
CHAPTER IV

SUMMARY AND CONCLUSIONS

Aluminium is an ubiquitous element and thus; it is found in all vegetation, vertebrate species, etc. In plasma, it is mainly bound to transferrin; though albumin may bind it very weakly. The pathological significance of aluminium is shown mainly in dialysis encephalopathy syndrome (DES), Alzheimer's disease and renal osteodystrophy. Colorimetric, fluorometric, and spectroscopic techniques have been proposed to determine aluminium. Of these flameless emission spectroscopy with a graphite furnace seems to be the method of choice.

A new fluorometric method was developed to determine aluminium in serum. The technique involved the reaction of aluminium with morin to form a highly fluorescent aluminium-morin chelate. The chelate has an excitation maximum wavelength of 435 nm and an emission wavelength of 510 nm. It fluoresced very strongly at a pH of 5.0 ± 0.75. A buffer of monochloroacetic acid-sodium acetate (pH, 4.1) was used to produce maximum fluorescence. A ratio of not greater than 1:20 aluminium:morin was used to produce maximum fluorescence in about 15-20 min. A plot of fluorescence versus aluminium concentrations of 200-1500 nmol/L yielded a regression line of \( y = 0.06x + 4.01 \), with a
correlation coefficient of 0.998. Cationic and anionic interferences were removed with Chelex-100(Na⁺) and Dowex-1(Cl⁻) resins, respectively.

Serum was deproteinized with nitric acid and heating. This also released aluminium from transferrin and albumin. Ten samples of serum were deproteinized and their levels of aluminium were determined by this procedure. The values were correlated with those obtained from the flameless emission spectroscopic technique with graphite furnace at the laboratory of University Hospital, London, Ontario. A correlation coefficient of 0.93 was obtained, thus, indicating significant correlation between the two methods.
REFERENCES


PART II

AN INVERSE FLUOROMETRIC TECHNIQUE TO DETERMINE
PYROPHOSPHATE IN PLATELETS USING
THE ALUMINIUM-MORIN CHELATE
CHAPTER I

INTRODUCTION

A. GENERAL

The structure of pyrophosphate (PP$_4$) is illustrated in Fig. 1. It has three negative charges at the physiological pH of 7.4. The four H's of pyrophosphate have pKa's of 0.85, 1.96, 6.54 and 8.44, respectively (1).

Fukami et al. investigated the localization of human platelet pyrophosphate by secretion and subcellular fractionation studies (2). These studies showed that pyrophosphate, nucleotides, calcium and 5-Hydroxytryptamine originate from the same subcellular localization. Others claimed that pyrophosphate and adenine nucleotides have a common storage site—different from that of calcium and hydroxytryptamine (3). It is now accepted that pyrophosphate is stored in the dense granules of platelets well shielded from inorganic pyrophosphatase (2). Like the storage pool of ATP and ADP, pyrophosphate is probably packaged into the organelles at some early stage of platelet formation and is relatively metabolically inert (2,3).

The main role of pyrophosphate metabolism, especially the rapid removal of pyrophosphate by inorganic pyrophosphatase, was long thought to be that of ensuring the irreversibility of certain biosynthetic reactions (4). More
FIGURE 1

STRUCTURE OF PYROPHOSPHATE

Legend

pKₐ's of pyrophosphate = 0.85, 1.96, 6.54 and 8.44.
At physiological pH it has 3 negative charges.
FIGURE 1

[Chemical structure diagram]
recently it has been shown that pyrophosphate can serve as an energy source in microorganisms (5).

Pyrophosphate plays a very important role in many biosynthetic reactions. These were reviewed and are shown in Table I (6).

B. CLINICAL SIGNIFICANCE OF PYROPHOSPHATE

Pyrophosphate is produced in large amounts (100 g to 1 Kg per day) in all tissues and plays a very important role in bone metabolism (7-9). Apatite crystals to which pyrophosphate has adsorbed, grow and dissolve more slowly than non-treated crystals (10). This suggests that pyrophosphate known to be present in bone, may be able to control the rates at which bone crystals grow and dissolve, and may be important in calcium homeostasis (8).

Pyrophosphate is rapidly broken down by pyrophosphatases and increased synthesis, diminished enzymatic degradation, and lower solubility of pyrophosphate or a combination of these factors may be responsible for calcium pyrophosphate dihydrate deposition (CPDD). In hypophosphatasia, there is a deficiency of alkaline phosphatase, thereby leading to a pyrophosphate arthropathy (11). McClure et al. have suggested that chronic arthropathy is in part at least a chronic pyrophosphate arthropathy and that the osseous organization of CPPD deposits in the invertebral disc provides a mechanism of
# TABLE I

**EXAMPLES OF ENZYMATIC REACTIONS RELEASING PYROPHOSPHATE**

<table>
<thead>
<tr>
<th>Biosynthetic Pathway</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coenzyme A</td>
<td>$4'\text{-dephosphopantetheine} + \text{ATP} \rightarrow \text{dephospho-CoA} + \text{PP}_i$</td>
</tr>
<tr>
<td>Amino acids, e.g., arginine</td>
<td>$\text{citrulline} + \text{aspartate} + \text{ATP} \rightarrow \text{arginosuccinate} + \text{AMP} + \text{PP}_i$</td>
</tr>
<tr>
<td>Urea synthesis</td>
<td>$\text{xanthosine} 5'\text{-phosphate} + \text{glutamate} + \text{ATP} \rightarrow \text{guanosine} 5'\text{-phosphate} + \text{AMP} + \text{PP}_i$</td>
</tr>
<tr>
<td></td>
<td>$\text{orotate} + \text{xanthosine} 5'\text{-phosphate} \rightarrow \text{orotidine} 5'\text{-phosphate} + \text{PP}_i$</td>
</tr>
<tr>
<td>Sulfur fixation</td>
<td>$\text{SO}_4^{2-} + \text{ATP} \rightarrow \text{adenyl sulfate} + \text{PP}_i$</td>
</tr>
<tr>
<td>DNA</td>
<td>$n \text{deoxynucleoside triphosphate} + \text{DNA}_n \rightarrow 2 \text{DNA}_n + n \cdot \text{PP}_i$</td>
</tr>
<tr>
<td>RNA</td>
<td>$n \text{nucleoside triphosphate} + \text{RNA}_n \rightarrow 2 \text{RNA}_n + n \cdot \text{PP}_i$</td>
</tr>
<tr>
<td>Glycogen</td>
<td>$\alpha-D\text{-glucose} 1\text{-phosphate} + \text{UTP} \rightarrow \text{UDP-glucose} + \text{PP}_i$</td>
</tr>
<tr>
<td>Proteins</td>
<td>$\text{acceptor RNA} + \text{amino acid} + \text{ATP} \rightarrow \text{aminoacyl-RNA} + \text{AMP} + \text{PP}_i$</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>$\text{deamido-NAD} + \text{glutamine} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{glutamate} + \text{NAD}^+ + \text{AMP} + \text{PP}_i$</td>
</tr>
<tr>
<td>FAD</td>
<td>$\text{riboflavin} 5'\text{-phosphate} + \text{ATP} \rightarrow \text{FAD} + \text{PP}_i$</td>
</tr>
<tr>
<td>Lipids</td>
<td>$\text{fatty acid} + \text{CoA} + \text{ATP} \rightarrow \text{acyl-CoA} + \text{AMP} + \text{PP}_i$</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>$\text{CTP} + \text{phosphocholine} \rightarrow \text{CDP-choline} + \text{PP}_i$</td>
</tr>
</tbody>
</table>
**TABLE I (Continued)**

<table>
<thead>
<tr>
<th>Process</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnosine</td>
<td>β-alanine + ATP → histidine → B-adenylhistidine + AMP + PP&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>Transmethylation</td>
<td>methionine + ATP → PP&lt;sub&gt;1&lt;/sub&gt; + P&lt;sub&gt;i&lt;/sub&gt; + S-adenosylmethionine</td>
</tr>
<tr>
<td>Steroids, terpenes</td>
<td>isopentenyl-PP&lt;sub&gt;1&lt;/sub&gt; → dimethylallyl-PP&lt;sub&gt;1&lt;/sub&gt; → geranyl-PP&lt;sub&gt;1&lt;/sub&gt; + PP&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>Structural polysaccharides</td>
<td>α-D-xylose-1-phosphate + UTP → UDP-xylose + PP&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>α-D-mannose 1-phosphate + GTP → GDP-mannose + PP&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>PEP + CO&lt;sub&gt;2&lt;/sub&gt; + P&lt;sub&gt;i&lt;/sub&gt; → oxaloacetate + PP&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>CoA activation</td>
<td>acetate + ATP + CoA → acetyl CoA + AMP + PP&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Table is taken from reference (6).
partial or complete ankylosis of the vertebral column (12). CPPD deposition has also been shown in a case of Wilson's disease (13). In chondrocalcinosis, crystals of CPPD are deposited in cartilage leading to severe arthritis (14). Pyrophosphate levels in plasma and platelets of patients on hemodialysis were found to be significantly lower than the reference population (15). Caines et al. suggested that this may explain the occurrence of metastatic calcification seen in this patient population (15).

Pyrophosphate levels in platelets may also play an important role in the disease malignant hyperthermia. Thus, it is expected that levels of this analyte will be increased in platelets of patients suffering from this disease.

C. METHODS USED TO DETERMINE PYROPHOSPHATE

Several methods have been described to determine pyrophosphate in tissue and body fluids. These mainly fall into three categories, viz., the molybdenum blue technique, radiisotopic and enzymatic techniques. In the molybdenum blue method, pyrophosphate and molybdate form a complex similar to that for orthophosphate. The reduction of this complex is achieved through the use of monothiol glycerol as the reducing agent. Phosphate also forms a complex with molybdate which is reduced under these conditions, but this reduced complex is different from the one formed by the pyrophosphate and molybdate (16). However, this technique
is only useful when low amounts of phosphate are present and it is quite insensitive.

The radioisotopic method uses labelled UDPG in a UDPG pyrophosphorylase reaction and the measurement of labelled UTP produced (17).

The enzymatic technique revolves around the production of pyridine nucleotides. The following sequence of reactions gives the principle of one such procedure that has been used to determine PP$_i$ (18).

\[
\text{Fructose 6-phosphate + PP}_i \xrightarrow{\text{PFK}} \text{Fructose 1,6-diphosphate + P}_i
\]

\[
\text{Fructose 1,6-diphosphate } \xrightarrow{\text{Aldolase}} \text{Glyceraldehyde phosphate (GAP) + DHAP}
\]

\[
\text{GAP } \xrightarrow{\text{TIM}} \text{DHAP}
\]

\[
\text{NADH + DHAP } \xrightarrow{\text{GP-DH}} \text{Glycerol 3-phosphate + NAD}^+
\]

PFK: Phosphofructokinase.

TIM: Triosephosphate dehydrogenase.

GP-DH: Glycerol 3-phosphate dehydrogenase.

The reduction in absorbance due to the production of 2 moles of NAD$^+$ correlates with the amount of pyrophosphate present. This procedure is not sensitive enough to measure ATP in the nmol range. A more sensitive procedure has been developed by reacting pyrophosphate and UDPG in the presence of UDPG pyrophosphorylase (15,19), as shown below:

\[
\text{PP}_i + \text{UDPG } \xrightarrow{\text{UDPG pyrophosphorylase}} \text{UTP + Glucose 1-phosphate}
\]

\[
\text{Glucose 1-phosphate } \xrightarrow{\text{PGIuM}} \text{Glucose 6-phosphate}
\]
Glucose 6-phosphate + NADP$^{+} \xrightarrow{G6P-DH} \text{NADPH} +$ 6-Phosphogluconic acid
6-Phosphogluconic acid + NADP$^{+} \xrightarrow{6-PGDH} \text{NADPH} + \text{CO}_2 +$ Ribulose 5-phosphate
Resazurin + NADPH $\xrightarrow{\text{Diaphorase}}$ Resorufin + NAD$^{+}$
UDPG: Uridine 5'-diphosphoglucose.
UDPGP: UDPG pyrophosphorylase.
PGluM: Phosphoglucomutase.
G6P-DH: Glucose 6-phosphate dehydrogenase.
However, this assay utilizes many expensive reagents, some of the enzymes (especially diaphorase) are not stable for more than a few hours and may not necessarily be pure (18). Drake et al. reported a method whereby pyrophosphate and adenosine phosphosulfate (APS) in the presence of ATP sulfurylase, react to produce 1 mol NADPH through a series of coupled reactions (20). The overall equation is shown here:

$$ \text{PP}_i + \text{APS} + \text{glucose} + \text{ADP} \rightarrow \text{SO}_4^{2-} + \text{ADP} + \text{gluconate 6-P} + \text{NADPH} $$

Another enzymatic method is based on the inactivation of pyrophosphatase in the presence of fluoride (21). Activation of this enzyme has an absolute requirement for Mg$^{2+}$. The inactivation is accompanied by extensive conversion of pyrophosphate by the still active enzyme. The inactivation stops after all the pyrophosphate is hydrolyzed.
D. PURPOSE OF THIS STUDY

As noted in the previous section, the colorimetric assays for pyrophosphate are relatively insensitive and not useful to measure this analyte in the nmol range. The enzymatic techniques are also insensitive unless they are coupled to a fluorescent system. However, expense, purity and stability of reagents make these procedures difficult to implement in a routine clinical laboratory.

A new method is proposed here to determine pyrophosphate in platelets. It does not involve any enzymes and is quite sensitive. The principle is outlined in Fig. 2. A preformed aluminium-morin chelate, which is highly fluorescent, is reacted with phosphate from pyrophosphate hydrolysis to produce aluminium phosphate and morin, neither of which is fluorescent. Thus, the decrease in fluorescence is related to the amount of phosphate present and subsequently, to the amount of pyrophosphate present. Interferences from anions and cations are removed by using an anion exchange resin. The method was applied to platelets as a model for its usage in a clinical situation, especially for the disease, malignant hyperthermia.
FIGURE 2

PRINCIPLE USED TO DETERMINE PYROPHOSPHATE

Legend

The fluorescent aluminium-morin chelate was pre-formed and stored in a dark plastic bottle at $4^\circ$C. Pyrophosphate was hydrolyzed to phosphate with 1N HCl for 7 min. The phosphate generated was reacted with the chelate to form aluminium phosphate. Thus, the fluorescence was decreased according to the amount of pyrophosphate, and subsequently, phosphate from hydrolysis present.

Morin = $2',3,4',5,7$-pentahydroxyflavone.
Al + MORIN $\rightarrow$ Al-MORIN

$\text{PP}_i + \text{HCl} \rightarrow 2 \text{P}_i$

Al-MORIN + PO$_4$ $\rightarrow$ AlPO$_4$ + MORIN
CHAPTER II

EXPERIMENTAL

ANALYTICAL AND CLINICAL STUDIES

1. Equipment

All equipment used in this study were as described in ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER II, p. 14).

2. Materials

Water: Metal-free water was obtained as described in ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER II, p. 17).

Chemicals: The chemicals used in this part were obtained from the same suppliers as described in ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER II, p. 16). In addition, potassium phosphate (monobasic) was obtained from Fisher Scientific Co. (Canada). Aluminium potassium sulfate (alum) was obtained from Aldrich Chemical Co.

3. Reagents

Buffer: A sodium acetate-acetic acid buffer (0.1M) was prepared by mixing 5.77 mL acetic acid with 8.2 g sodium acetate (anhydrous) and diluting to 1000 mL with metal-free water in a volumetric flask. The pH recorded was 4.4.

Morin Stock Solution: 30.2 mg morin was dissolved in 100 mL absolute ethanol to give a concentration of $10^{-3}$ M.
**Aluminium Stock Solution**: 47.42 mg aluminium potassium sulfate (AlK(SO₄)₂·12H₂O) was dissolved in 100 mL metal-free water in a volumetric flask to give a concentration of 10⁻³ M Al.

**Ethanol (50%, v/v)**: Ethanol (A.C.S.) was purified by distillation. A 50% solution was prepared by adding 50 mL purified ethanol to a 100-mL volumetric flask and diluting it to 100 mL.

**Stock Solution of Al-Morin Chelate**: An aliquot of 10 mL of morin stock solution was mixed with a 10-mL aliquot of aluminium stock solution in a 100-mL volumetric flask. The contents were diluted to the mark with a 50% ethanol solution. This solution was left overnight before usage. It was stored in a dark plastic bottle at 4°C.

**Stock Phosphate Solution**: 136.1 mg KH₂PO₄ was dissolved in 100 mL metal-free water and stored at 4°C in a plastic bottle. This resulted in a phosphate concentration of 10⁻² M.

**Working Phosphate Standard Solutions**: Dilution of the stock phosphate solution to 10⁻⁴ M was made just prior to the assay then various aliquots were diluted to 10 mL which when used in the assay gave final cuvet concentrations of 0-15 nmol.

**Working Pyrophosphate Solution**: A solution containing 5 x 10⁻⁵ M pyrophosphate was prepared from sodium pyrophosphate just prior to the analysis. Then various
 aliquots were diluted to 10 mL which when used in the assay gave final concentrations of 0-15 nmol.

4. Procedure

Analytical: The Dowex-1(Cl−) resin was washed with water and allowed to settle. The cloudy supernatant was discarded and the procedure repeated several times. An aliquot (0.50 mL) of the resin was loaded onto a mini-LipoSep column to give a bed volume of approximately 0.3 mL. Several bed volumes of water at a pH of 8.0 (adjusted with NH₄OH) were allowed to flow through the column.

An aliquot of 1.0-mL standard containing pyrophosphate (at a pH of 7.6-8.0) was loaded onto the bed and allowed to drain to the bed level. About 5.0 mL water (pH, 8.0) was used to wash the sides of the column to get all the sample onto the column. The water eluate was discarded and 3 mL HCl (0.025N) were applied to the column and again the eluate discarded. Then 1.0 mL 0.10N HCl was applied to the column and the eluate was collected and kept for analysis. The column was fitted with a disc on top of the resin bed to prevent it from running dry.

The eluate was neutralized with 6N NaOH and a few (2-5) milligrams of charcoal were added to it. After mixing for 5 min, the mixture was centrifuged and 1.00 mL of the supernatant was pipetted into a large polypropylene tube. An aliquot of 0.20-mL 6N HCl was added to the supernatant
and it was placed in a water bath at 95-100 °C for 7 min. The hydrolysate was then neutralized with 6N NaOH and brought to a total volume of 1.50 mL with sodium acetate-acetic acid buffer. An aliquot of 1.4-mL absolute ethanol was added followed by 0.25 mL of working aluminium-morin solution. The mixture was incubated for about 10 min in the dark. The contents were read in a quartz cuvet in the fluorometer at an excitation wavelength of 435 nm and an emission wavelength of 510 nm. A blank was prepared in the same way as the above procedure except that the pyrophosphate standard was replaced with water that was used to make up the standards.

**Clinical:** Platelets were isolated by a centrifugation technique (15) with minor modification (Fig. 3). The platelets were counted prior to final pelletization. The pellet was lysed in 0.5 mL water and allowed to pass through the resin. The procedure henceforth was followed exactly as shown in the previous section.
FIGURE 3

ISOLATION OF PLATELETS

Legend

The platelet pellet was counted just prior to final pelletization to ensure an accurate number of platelets used for the analysis.

The pellet was lysed in 0.5 mL water just prior to the analysis.
FIGURE 3

WHOLE BLOOD
Count Platelets

↓

150 x g
20 min

EDTA PLASMA
Enriched, Count

↓

1000 x g
20 min

SUPERNATANT

PLATELET PELLET
Resuspend in
2 ml 0.15M NaCl
1000 x g; 20 min

SUPERNATANT

PLATELET PELLET

SUPERNATANT

PLATELET PELLET
Count

Repeat
CHAPTER III

RESULTS AND DISCUSSION

A. ANALYTICAL STUDIES

1. Principle

The principle used to determine pyrophosphate was illustrated in Fig. 2. The pre-formed aluminium-morin chelate is intensely fluorescent (ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER III, p. 24)) and emitted at 510 nm when excited at 435 nm. When phosphate (obtained from the hydrolysis of pyrophosphate) was added to the chelate, a displacement reaction occurred in which the morin of the chelate was replaced with phosphate, thereby forming aluminium phosphate. The fluorescence of the chelate was therefore reduced accordingly since neither aluminium phosphate nor morin can be excited at 435 nm.

2. Stability of Al-Morin Chelate

The fluorescence intensity readings of the same aluminium chelate solution were measured over a period of several days to check the stability of the chelate (Table II). These readings did not change significantly over a period of 30 days, thus, storage of the chelate in a 50% (v/v) ethanolic solution at 4°C allowed for the usage of the same stock solution for a month or more. For each day readings were taken, the fluorometer was calibrated.
TABLE II

STABILITY OF Al-MORIN CHELATE$^a$

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Fluorescent intensity$^b$ of Al-morin chelate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98.5</td>
</tr>
<tr>
<td>3</td>
<td>98.0</td>
</tr>
<tr>
<td>5</td>
<td>95.0</td>
</tr>
<tr>
<td>7</td>
<td>97.0</td>
</tr>
<tr>
<td>10</td>
<td>96.0</td>
</tr>
<tr>
<td>15</td>
<td>99.0</td>
</tr>
<tr>
<td>20</td>
<td>97.5</td>
</tr>
<tr>
<td>25</td>
<td>96.0</td>
</tr>
<tr>
<td>30</td>
<td>94.0</td>
</tr>
</tbody>
</table>

$^a$[Al-morin chelate] = 10$^{-5}$ M.

$^b$Readings were averages of triplicate determinations.

Excitation wavelength = 435 nm.

Emission wavelength = 510 nm.

The fluorometer was calibrated with quinine sulfate (10 mg/L) in 0.05M H$_2$SO$_4$.
with quinine sulfate.

3. Variation of pH on the Fluorescence of Al-Morin and its Suppression with Phosphate

The variation of pH on the fluorescence of both the Al-morin chelate and its suppression with phosphate are shown in Fig. 4. Two optima peaks of fluorescence intensities corresponding to pH's of 3.5 and 5.0 were observed, Fig. 4A. This was discussed in ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER III, p. 25).

The variation of pH on the suppression of fluorescence of the aluminium chelate with phosphate is shown in Fig. 4B. A very rapid suppression of fluorescence was noted as the pH was increased from 3.0. The fluorescence was completely suppressed at a pH of 5.5 and onwards. Thus, the reaction of phosphate with the aluminium-morin chelate to produce aluminium phosphate and morin occurred optimally at a pH of 5.5 or greater.

The fluorescence and suppression data as a function of pH suggested two different pH optima; one for maximum fluorescence of the chelate (pH, 5.0) and another for suppression by phosphate (pH, 5.5). Thus, a compromise in the selection of the pH for the reaction of phosphate with aluminium-morin chelate must be made. This was achieved by looking at some work done on the aluminium-morin reaction with phosphate by previous workers (21). The data is illustrated in Fig. 5. Analysis of this data showed that
FIGURE 4

VARIATION OF pH ON THE FLUORESCENCE OF Al-MORIN
AND ITS SUPPRESSION WITH PHOSPHATE

Legend

A. Variation of pH on the fluorescence of Al-morin.

[Chelate] = 7.9 \times 10^{-6} \text{ M}.

B. Variation of pH on the suppression of fluorescence using phosphate.

Chelate + 5.0 \text{ nmol phosphate}.
FIGURE 5

SELECTION OF pH FOR THE REACTION OF PHOSPHATE WITH AL-MORIN

Legend

X: Suppression of aluminium-morin with phosphate at a pH of 5.5.

Y: Suppression of aluminium-morin with phosphate at a pH of 4.5:

Al-morin$^1$ = $10^{-5}$ M.

For X: $\Delta F = 45$ for 1.0 $\mu$g (10.5 nmol) phosphate.

For Y: $\Delta F = 10$ for 1.0 $\mu$g (10.5 nmol) phosphate.
FIGURE 5

FLUORESCENCE

0 1 2 4 6 8 10
$P_i$ ($\mu$g), pH 5.5

$X$

100
50
0

$Y$

100
50
0

$P_i$ ($\mu$g), pH 4.5
the aluminium-morin phosphate reaction was essentially non-linear for the range of 0-10 µg (0-105 nmol) phosphate at a pH of 5.5 (corresponding to a pH of maximum suppression). However, non-linearity did not occur until concentrations of 4.0 nmol or greater of phosphate were used. Furthermore, when the log fluorescence intensity readings for 0-1 µg (0-10.5 nmol) phosphate were plotted, a linear relationship was obtained. At the pH of 4.5 (corresponding to a pH of maximum fluorescence), a linear relationship was observed for 0-60 nmol phosphate with no log linearization necessary for this range. However, the reaction is less sensitive than at pH 5.5, i.e., the change in fluorescence for 10.5 nmol phosphate at a pH of 5.5 and 4.5 were 45 and 10, respectively. Thus, the pH of 5.5 was selected for the phosphate and aluminium-morin chelate reaction because of higher sensitivity and a log linear relationship for 0-10.5 nmol phosphate.

4. Effect of Buffers

Buffers were tested to show their effects on the suppression of fluorescence of the aluminium-morin chelate with phosphate. As shown earlier, acetate buffer had no effect on the aluminium-morin chelate fluorescence. Citrate and oxalacetate competed for the aluminium and thereby decreased the fluorescence. Thus, acetate buffer produced a maximum change in fluorescence when a specified amount of
phosphate was present. However, when citrate, phthalate, and oxalacetate buffers were used, the change in fluorescence was practically zero due to the reaction of these anions with aluminium (Table III). Thus, the buffer of acetic acid–sodium acetate was chosen. It was made to a pH of 4.4, and upon the addition of all the other reagents, the final pH of the reaction mixture was consistently recorded at 5.5.

5. Effect of Various Alcohols on the Suppression of Fluorescence of the Al-Morin Chelate with Phosphate

A study was carried out to check the effect of various alcohols on the suppression of fluorescence of the Al-Morin chelate with phosphate. As shown in Table IV, methanol and ethanol (50%, v/v) produced maximum change in fluorescence, whereas isopropanol and butanol produced smaller changes in fluorescence. As shown earlier (ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER III, p. 33)), the aluminium-morin chelate was much more fluorescent in ethanol and methanol as compared to the other alcohols, thereby providing a more sensitive reaction in the presence of phosphate.

Several concentrations of ethanol were used to determine which produced maximum sensitivity for the determination of phosphate. As shown in Table IV, as the concentration of ethanol was increased, a more sensitive reaction was observed. However, when triplicate readings were taken for these concentrations, the reproducibility of
TABLE III

EFFECT OF BUFFERS ON THE ALUMINIUM-MORIN PHOSPHATE REACTION

<table>
<thead>
<tr>
<th>Buffers(^b)</th>
<th>Fluorescence(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>2.5</td>
</tr>
<tr>
<td>Acetate</td>
<td>45.0</td>
</tr>
<tr>
<td>Phthalate</td>
<td>6.3</td>
</tr>
</tbody>
</table>

\(^a\) [Phosphate] = 6 nmol.
\(^b\) [Buffer] = 0.1M.
\(^c\) Readings were averages of triplicate measurements.

Sodium salts were used to make up the buffers.
# TABLE IV

**EFFECT OF ALCOHOLS ON THE ALUMINIUM-MORIN PHOSPHATE\(^a\) REACTION**

<table>
<thead>
<tr>
<th>Alcohols(^b)</th>
<th>(\Delta)Fluorescence(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>20.3</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>15.5</td>
</tr>
<tr>
<td>Butanol</td>
<td>15.0</td>
</tr>
<tr>
<td>Ethanol (50%)</td>
<td>35.5</td>
</tr>
<tr>
<td>(60%)</td>
<td>40.3</td>
</tr>
<tr>
<td>(70%)</td>
<td>44.5</td>
</tr>
<tr>
<td>(80%)</td>
<td>47.3</td>
</tr>
</tbody>
</table>

\(^a\) [Phosphate] = 8 nmol.

\(^b\) Alcohols were 50% (v/v).

\(^c\) Readings were averages of triplicate measurements.
the change in fluorescence was poor. This probably was due to the difficulty in maintaining a pH at 5.5 when the alcohol concentration was increased. Thus, a 50% (v/v) ethanol solution was used for all future studies. At this concentration, the readings were quite reproducible.

6. Time Required for Maximum Suppression of Fluorescence of Al-Morin Chelate with Phosphate

A study was initiated to determine the maximum time that was necessary for maximum change in fluorescence in the presence of phosphate. The results are shown in Table V. These results indicated a decreasing blank and test solution. However, when the change in fluorescence readings were taken, a constant value was obtained after 8 min of incubation at room temperature. Apparently, there was some external/internal factor, such as oxygen, light, etc., that might be involved in reducing the fluorescence in both the blank and standard by the same amount. Because a constant change in fluorescence was observed, readings were taken after 8-10 min of incubation.

7. Effect of Temperature on the Suppression of the Fluorescence of the Al-Morin Chelate with Phosphate

The effect of temperature on the suppression of the fluorescence of aluminium-morin with phosphate is shown in Table VI. Indeed, the effect of temperature was extremely pronounced and averaged about a change of 1 unit of fluorescence intensity/°C. At higher temperatures, the
TABLE V

TIME REQUIREMENT FOR THE SUPPRESSION OF FLUORESCENCE
OF ALUMINIUM-MORIN WITH PHOSPHATE

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>FLUORESCENCE (F) (Std., 6.0 nmol)</th>
<th>Blank phosphate</th>
<th>ΔF = Blank - F</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>68.0</td>
<td>100</td>
<td>32.5</td>
</tr>
<tr>
<td>8</td>
<td>51.5</td>
<td>95.0</td>
<td>43.5</td>
</tr>
<tr>
<td>13</td>
<td>48.5</td>
<td>91.3</td>
<td>42.8</td>
</tr>
<tr>
<td>18</td>
<td>45.5</td>
<td>87.5</td>
<td>42.0</td>
</tr>
<tr>
<td>23</td>
<td>41.5</td>
<td>83.5</td>
<td>42.0</td>
</tr>
<tr>
<td>28</td>
<td>40.0</td>
<td>82.0</td>
<td>42.0</td>
</tr>
<tr>
<td>33</td>
<td>39.0</td>
<td>81.0</td>
<td>42.0</td>
</tr>
<tr>
<td>38</td>
<td>37.0</td>
<td>78.0</td>
<td>41.0</td>
</tr>
</tbody>
</table>
TABLE VI

EFFECT OF TEMPERATURE ON THE SUPPRESSION OF FLUORESCENCE OF AL-MORIN WITH PHOSPHATE

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>ΔFluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>70.3</td>
</tr>
<tr>
<td>15</td>
<td>65.5</td>
</tr>
<tr>
<td>25</td>
<td>58.0</td>
</tr>
<tr>
<td>37</td>
<td>45.0</td>
</tr>
<tr>
<td>45</td>
<td>37.5</td>
</tr>
</tbody>
</table>

\( [\text{Phosphate}] = 4 \text{ nmol} \).

\( ^b \) Lower temperatures (10 and 15 °C) were obtained by using ice.

\( ^c \) Readings were averages of triplicate determinations.
fluorescence was markedly decreased as opposed to lower temperatures. For convenience, a temperature of 23-25°C or (room temperature) was used for this study. The effect of temperature on the fluorescence of the aluminium chelate followed the same pattern (ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER III, p. 38)). Thus, the change in fluorescence for the determination of phosphate as a function of temperature must be attributable to the effect of temperature on the aluminium chelate itself.  

8. Generation of Calibration Curve  

The procedure used to generate this curve was outlined in ANALYTICAL AND CLINICAL STUDIES (CHAPTER II, p. 74). The fluorescent intensity as a function of concentration of phosphate generated from pyrophosphate hydrolysis is shown in Fig. 6. Though the response was essentially non-linear over the range 0-15 nmol phosphate, it appeared that at very low concentration (0-4 nmol) of phosphate, a linear response was observed.  

An alternative form of the curve showed that a linearized version was obtained for the range 0-12 nmol when the log fluorescence was plotted against the concentration (Fig. 7). The equation for the line of regression was \( \hat{y} = -0.05x + 1.93 \) with a correlation coefficient of 0.992.  

To explain this exponential decay, it may be necessary to look at the reaction under investigation as a simple
FIGURE 6

STANDARD CURVE FOR THE DETERMINATION OF PYROPHOSPHATE

Legend

From 0-4 nmol phosphate or 0-2 nmol pyrophosphate, the curve was essentially linear, with a line of regression of
\[ y = -7.41x + 85.6 \]
and a correlation coefficient of 0.940.

Concentrations given were those for final cuvet concentrations.

Each point was an average of triplicate readings.
FIGURE 7

A LINEARIZED VERSION OF THE STANDARD CURVE
FOR THE DETERMINATION OF PYROPHOSPHATE

Legend

Equation for line of regression: $y = -0.05x + 1.93$.
Correlation coefficient: $r = 0.992$.
Each point was an average of triplicate readings.
FIGURE 7

LOG. FLUORESCENCE

$[P_i]$, nmols-cuvet

0 4 8 12 15

0 1.2 1.4 1.6 1.8 2.0
competitive first order reaction. That is, the phosphate and morin will compete for the aluminium, and thereby setting up an equilibrium. It is interesting to note that at very low concentrations of phosphate, the reaction was essentially linear, indicating that the reaction was completed quickly. However, as the concentration of phosphate was increased, more competition for aluminium takes place thereby resulting in a longer time to reach equilibrium.

The method developed here is sensitive and can measure pyrophosphate in the nmol range. Though, some enzymatic procedures are more sensitive, Table VII, this procedure will allow one to measure pyrophosphate in platelets inexpensively when compared to enzymatic procedures. The limit of sensitivity tested here was 0.13 nmol of pyrophosphate. Indeed, with scale expansion, the instrumental sensitivity can be increased by a factor of 10 or so, if necessary.

9. Recovery of Phosphate from Pyrophosphate Hydrolysis

A study was carried out to determine the completeness of pyrophosphate hydrolysis as determined by the recovery of phosphate. Table VIII shows the results for the hydrolysis of pyrophosphate in 1.0N HCl for 7 min. The recoveries at three levels of pyrophosphate tested ranged from 95-105%, with an average of 101%. It was important to perform the
### TABLE VII

**SENSITIVITY OF VARIOUS METHODS**

<table>
<thead>
<tr>
<th>Method</th>
<th>Limit of detection (pmol)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorimetric</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>500</td>
<td>20</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>500</td>
<td>19</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>250</td>
<td>18</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>Proposed</td>
<td>125</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE VIII

**RECOVERY OF PHOSPHATE FROM PYROPHOSPHATE HYDROLYSIS**

<table>
<thead>
<tr>
<th>[PP$_i$] nmol$^b$</th>
<th>[P$_i$] expected$^c$</th>
<th>[P$_i$] found$^c$</th>
<th>% recovery$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>8.0</td>
<td>8.1</td>
<td>101.3</td>
</tr>
<tr>
<td>2.0</td>
<td>4.0</td>
<td>4.2</td>
<td>105.0</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>1.9</td>
<td>95.0</td>
</tr>
</tbody>
</table>

$^a$Phosphate was determined by the aluminium-morin chelate.

1.0N HCl --- 7 min.

$^b$Standard pyrophosphate solutions were used.

$^c$Concentration of phosphate is in nmol.

$^d$Readings were averages of triplicate measurements.

Average = 101%.
hydrolysis in large polypropylene tubes to avoid extraneous contamination and to avoid any loss of sample during the hydrolysis step.

10. Removal of Interferences

As was noted in ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER III, p.43), metals such as iron and copper interfered with the aluminium-morin reaction. Also, anions such as fluorides, citrate, oxalate and phosphate showed a negative interference. Thus, it was important to remove these endogenous interferences from the sample before reacting the phosphate generated from pyrophosphate hydrolysis with the aluminium-morin chelate. Towards this end the Dowex-1(Cl⁻) resin was used to selectively remove both cationic and anionic interferences from pyrophosphate. The sample was adjusted to a pH of 8.0 and applied to a pre-equilibrated column of Dowex-1(Cl⁻) (with water at a pH of 8.0). In order to obtain good recoveries, all the sample must be washed with H₂O, pH 8.0, onto the column. Subsequent elution with about 5 bed volumes of 0.025N HCl removed the less binding anions such as oxalate, fluoride, sugar phosphates, creatine phosphate, etc, (Table IX) (22-25). Pyrophosphate and nucleotides remained on the column due to their stronger binding affinities for the column. Thus, when 0.10N HCl was used as the eluent, pyrophosphate and these nucleotides were eluted (25).
TABLE IX

SELECTIVITY OF ANIONS FOR DOWEX-1(CI⁻) RESIN

<table>
<thead>
<tr>
<th>Counterion</th>
<th>Relative selectivity for AG 1-X8</th>
<th>Relative selectivity for AG 2-X8</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH⁻</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Benzene</td>
<td>500</td>
<td>75</td>
</tr>
<tr>
<td>Sulfonate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I⁻</td>
<td>175</td>
<td>17</td>
</tr>
<tr>
<td>Phenate</td>
<td>110</td>
<td>27</td>
</tr>
<tr>
<td>HSO₄⁻</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>ClO₃⁻</td>
<td>74</td>
<td>12</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>65</td>
<td>8</td>
</tr>
<tr>
<td>Br⁻</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>CN⁻</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>HSO₃⁻</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>BrO₃⁻</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>22</td>
<td>2.3</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>6.0</td>
<td>1.2</td>
</tr>
<tr>
<td>IO₃⁻</td>
<td>5.5</td>
<td>0.5</td>
</tr>
<tr>
<td>HPO₄⁻</td>
<td>5.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Formate</td>
<td>4.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Acetate</td>
<td>3.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Propionate</td>
<td>2.6</td>
<td>0.3</td>
</tr>
<tr>
<td>F⁻</td>
<td>1.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Taken from reference (22).
Neutralization of this eluate with NaOH and the subsequent addition of a few milligrams of charcoal essentially allowed for the nucleotides to be adsorbed onto the charcoal and leaving the pyrophosphate in the supernatant (24,25).

In order to check whether phosphate was completely removed by this procedure, various levels of phosphate were used to spike samples of pyrophosphate and the recovered pyrophosphate determined by the aluminium-morin reaction before and after spiking. The results are shown in Table X. As can be seen from these results, the recovery of pyrophosphate was essentially quantitative. Thus, phosphate (endogenous or exogenous) was completely removed from pyrophosphate by elution with 0.025N HCl. It can be safely concluded here that sugar phosphates, fluorides, oxalate, etc., that have similar selectivities for the resin as phosphate (Table X), were similarly eluted from the resin with 0.025N HCl, thereby leaving pyrophosphate on the column.

It must be noted here that the interfering trace metals posed absolutely no problem since they did not bind onto the Dowex-1(Cl⁻) resin and were washed off the column with the 0.025N HCl used to elute phosphates, and other interfering anions. Proteins, cell debris, etc., were also washed away during this step as well. Thus, there was no need for an ultracentrifugation step in this procedure.
**TABLE X**

**RECOVERY OF PYROPHOSPHATE FROM COLUMN BEFORE AND AFTER SPIKING WITH PHOSPHATE**

<table>
<thead>
<tr>
<th>Phosphate (nmol)</th>
<th>PP$_i$ expected (nmol)</th>
<th>PP$_i$ recovered (nmol)</th>
<th>% recovery$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>5.0</td>
<td>4.8</td>
<td>96.0</td>
</tr>
<tr>
<td>1.0</td>
<td>5.0</td>
<td>4.7</td>
<td>94.0</td>
</tr>
<tr>
<td>2.0</td>
<td>5.0</td>
<td>5.1</td>
<td>102.0</td>
</tr>
<tr>
<td>4.0</td>
<td>5.0</td>
<td>5.3</td>
<td>94.0</td>
</tr>
</tbody>
</table>

$^a$Average recovery from spiking with phosphate = 96.5%

Readings were averages of triplicate measurements and were done using standard solutions.
B. CLINICAL STUDIES

1. Platelet Isolation

A complete discussion on the isolation of platelets will be given in RESULTS AND DISCUSSION (PART IV, CHAPTER III, pp. 183-188).

2. Recovery Studies with Platelets

Pooled platelet lysates were spiked with different concentrations of pyrophosphate and the concentrations of the latter determined before and after spiking. The results are shown in Table XI. At a lower concentration of pyrophosphate, the recovery was 94% as opposed to a higher concentration spike with a recovery of 98%. Thus, the pyrophosphate added was not affected by the matrix components of the sample. It should be noted here that most of the sample matrix, such as cell debris, proteins, etc., were washed away during the chromatography step and pyrophosphate eluted later was practically free of such components. For this reason, this method is easily applicable to serum samples as well.

3. Precision Studies with Platelets

Within-run and between-run studies for precision were carried out for three pools of platelet lysates (Table XII). The within-run coefficients of variation ranged from 2.31 to 6.27% with an average of 4.6%. At lower concentrations of pyrophosphate the precision was greater. The between-run
### TABLE XI

**RECOVERY STUDIES FOR THE DETERMINATION OF PYROPHOSPHATE IN PLATELETS**

<table>
<thead>
<tr>
<th>Platelet pool&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Expected&lt;sup&gt;b&lt;/sup&gt; or added</th>
<th>Recovered&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% recovery&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>3.06</td>
<td>3.04</td>
<td>98.1 ± 2.0</td>
</tr>
<tr>
<td>Low</td>
<td>0.306</td>
<td>0.296</td>
<td>94.0 ± 1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>High and low refer to platelet lysate pools containing high and low amounts of base level pyrophosphate.

<sup>b</sup>Values are given in nmol/10<sup>8</sup> platelets.

<sup>c</sup>All data were averages of triplicate measurements.
### TABLE XII

**PRECISION STUDIES FOR THE DETERMINATION OF PYROPHOSPHATE IN PLATELETS**

<table>
<thead>
<tr>
<th>Platelet Pool</th>
<th>Mean&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S.D.&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S.E.M.&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.51</td>
<td>0.032</td>
<td>0.008</td>
<td>6.27</td>
</tr>
<tr>
<td>B</td>
<td>1.55</td>
<td>0.035</td>
<td>0.009</td>
<td>2.31</td>
</tr>
<tr>
<td>C</td>
<td>3.13</td>
<td>0.113</td>
<td>0.029</td>
<td>3.61</td>
</tr>
<tr>
<td><strong>Between-run&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.53</td>
<td>0.036</td>
<td>0.015</td>
<td>6.79</td>
</tr>
<tr>
<td>B</td>
<td>1.54</td>
<td>0.090</td>
<td>0.037</td>
<td>5.84</td>
</tr>
<tr>
<td>C</td>
<td>3.15</td>
<td>0.176</td>
<td>0.070</td>
<td>5.59</td>
</tr>
</tbody>
</table>

<sup>a</sup>nmol/10<sup>8</sup> platelets.

<sup>b</sup>Each platelet pool was assayed 15 times.

<sup>c</sup>Each platelet pool was assayed in duplicate in six batches.

S.E.M. = standard error of the mean.
precision study showed coefficients of variation from 5.59 to 6.79%, with an average of 6.07%. Indeed, the precision for the between-run study was less than that of the within-run study. This was due to varying conditions in terms of reagents, technique, contamination, etc., from day-to-day.

4. Reference Range for Pyrophosphate in Platelets

Twenty samples of platelets were assayed and the values for pyrophosphate/10^8 platelets are shown in Table XIII. The values ranged from 0.86 to 4.96 nmol/10^8 platelets with a mean of 2.79 nmol/10^8 platelets. Other workers found 1.4–3.1 and 2.14–2.56 nmol/10^8 platelets (3,15).

5. Comparison with a Reference Method

A comparison study between the proposed method and that of Caines et al. was performed (15). The results are illustrated in Fig. 8. The line of regression was y = 0.96x + 0.16, with a correlation coefficient of 0.98, thus, indicating a significant correlation between the two methods.
### TABLE XIII

**REFERENCE RANGE FOR PYROPHOSPHATE IN PLATELETS**

<table>
<thead>
<tr>
<th>Patient #</th>
<th>([\text{PP}_i]) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.60</td>
</tr>
<tr>
<td>2</td>
<td>3.10</td>
</tr>
<tr>
<td>3</td>
<td>4.13</td>
</tr>
<tr>
<td>4</td>
<td>2.68</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
</tr>
<tr>
<td>6</td>
<td>3.11</td>
</tr>
<tr>
<td>7</td>
<td>3.01</td>
</tr>
<tr>
<td>8</td>
<td>0.86</td>
</tr>
<tr>
<td>9</td>
<td>1.90</td>
</tr>
<tr>
<td>10</td>
<td>2.68</td>
</tr>
<tr>
<td>11</td>
<td>3.89</td>
</tr>
<tr>
<td>12</td>
<td>2.61</td>
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<tr>
<td>13</td>
<td>2.51</td>
</tr>
<tr>
<td>14</td>
<td>3.26</td>
</tr>
<tr>
<td>15</td>
<td>3.11</td>
</tr>
<tr>
<td>16</td>
<td>4.01</td>
</tr>
<tr>
<td>17</td>
<td>2.00</td>
</tr>
<tr>
<td>18</td>
<td>2.36</td>
</tr>
<tr>
<td>19</td>
<td>4.96</td>
</tr>
<tr>
<td>20</td>
<td>3.81</td>
</tr>
</tbody>
</table>

\(^a\) nmol \(\text{PP}_i\)/10\(^8\) platelets.

Mean \([\text{PP}_i]\) is 2.79 nmol/10\(^8\) platelets.

Readings were averages of triplicate measurements.
FIGURE 8

COMPARISON STUDY WITH A REFERENCE METHOD

Legend

Line of regression: \( y = 0.96x + 0.16 \).

Correlation coefficient: \( r = 0.98 \).

Reference method is that of Caines et al. (15).

Readings were averages of triplicate measurements.

Values are given in nmols.
CHAPTER IV

SUMMARY AND CONCLUSIONS

Pyrophosphate (PP$_4$) is released in a number of biochemical reactions and plays a very important role in calcium and bone metabolism. Significant decrease of pyrophosphate in platelets may be of diagnostic significance in the disease, malignant hyperthermia. Some of the methods that have been used to determine PP$_4$ include radioisotopic, formation of molybdenum blue complex and coupled enzymatic assays.

The method proposed in this dissertation to determine PP$_4$ involved the suppression of the fluorescence of the aluminium-morin chelate by phosphate which was obtained from the hydrolysis of PP$_4$ with HCl. The determination was carried out in 50% ethanol at a pH of 5.5. Other alcohols, such as isopropanol, butanol and methanol (50%, v/v) did not give superior results. The excitation and emission wavelengths for the aluminium-morin phosphate reaction were 435 and 510 nm, respectively. The time required for suppression was about 5 min and the buffer used was sodium acetate-acetic acid.

The platelets were isolated from EDTA blood by a centrifugation technique and lysed in water. The log fluorescence versus concentration resulted in a straight
line for 0.13-6.0 nmol of PP\textsubscript{i} (the mean per 10\textsuperscript{8} platelets is 2.5 nmol) with a correlation coefficient of 0.992. Cationic/anionic interferences were removed by passage of the sample through an anionic resin (Dowex-1(Cl\textsuperscript{-})) and then by selective elution with HCl. Interfering nucleotides eluted with PP\textsubscript{i} were removed by the addition of a few milligrams of charcoal to the neutralized eluate. Two concentrations of PP\textsubscript{i} were used to spike platelet lysates. Recovery of the PP\textsubscript{i} ranged from 94-98%, with an average of 98%. The precision studies for within-run and between-run had coefficients of variation of 4.0 and 6.1%, respectively. An analysis of 20 samples of platelets from patients showed an average of 2.9 nmol/10\textsuperscript{8} platelets which compared favorably with published results. A comparison of this assay with an enzymatic technique gave a correlation coefficient of 0.98.
REFERENCES


PART III

THE DEVELOPMENT OF A SIMPLE AND SENSITIVE ASSAY TO MEASURE
ADENOSINE 5'-TRIPHOSPHATE IN PLATELETS
CHAPTER I

INTRODUCTION

A. GENERAL AND CLINICAL SIGNIFICANCE

Adenosine 5'-triphosphate (ATP) is a nucleotide containing adenine, ribose and three phosphate groups (Fig. 1). The two high-energy pyrophosphate linkages in the molecule have roughly equivalent energies of hydrolysis. Most reactions, however, utilize only the terminal phosphate anhydride bond. In its reactions in the cell, it functions as the Mg\(^{2+}\) complex.

ATP is the keystone of all cellular activity. It is an essential ingredient in the initial biochemical steps of substrate utilization and in new cell synthesis (1). This high-energy compound in every living cell is synthesized from intermediate and final reactions of substrate oxidation and is also utilized intracellulary for osmotic and mechanical work (Table I) (2,3).

The measurement of ATP has proven to be quite useful in a clinical situation. Its measurement in red blood cells has been used as an indication of cell viability (4,5). As cells become older, they have decreased levels of ATP. This is especially important for blood that has been stored for a period of time or is suspected of being exposed to harsh conditions (6). ATP determinations have been proven useful
FIGURE 1

STRUCTURE OF ADENOSINE 5'-TRIPHOSPHATE

Legend

A: Adenine group.
B: Ribose group.
C: Pyrophosphate group.
Y: Adenine-ribose bond.

Figure is taken from reference (1).
<table>
<thead>
<tr>
<th>Processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle contraction</td>
</tr>
<tr>
<td>Brain and nerve action</td>
</tr>
<tr>
<td>Sperm motility</td>
</tr>
<tr>
<td>Absorption of sugars from intestine</td>
</tr>
<tr>
<td>Reabsorption of glucose from kidney tubules</td>
</tr>
<tr>
<td>Synthesis of sugar phosphates</td>
</tr>
<tr>
<td>Synthesis of proteins from amino acids</td>
</tr>
<tr>
<td>Acetylation of choline and amines</td>
</tr>
<tr>
<td>Synthesis of thiamine pyrophosphate</td>
</tr>
<tr>
<td>Synthesis of urea in liver</td>
</tr>
<tr>
<td>Formation of ammonia in kidney</td>
</tr>
</tbody>
</table>
in assessing red blood cell abnormalities (7,8). Thus, cells with low levels of ATP may not function properly or may have shortened life spans. These types of irregular cells may manifest themselves as a diseased condition.

ATP has been used as a measure of viable bacteria in a number of situations since this high energy phosphate compound is present only in living cells. Hamilton and Holm-Hansen measured ATP content in sea-water as an indication of its heterotrophic population (9). Lee et al. used its determination as an index of microbial biomass in sediments (10). Several published reports used the measurement of ATP in urine as a screening test for detecting low-level bacteriuria (11-13). The general consensus is that such a measurement is quite useful for this purpose. The assay has also been used for the rapid estimation of microbial contamination of raw meat (14) and as a measure of metabolic activity and/or biomass in activated sludge (15).

Platelet ATP measurement has been suggested as a means of screening for the disease, malignant hyperthermia (16). This assay gave a 100% correlation with the muscle-biopsy findings in patients with a proven episode of malignant hyperthermia. However, these results could not be reproduced by others (17).
B. METHODS

Several methods have been developed to measure ATP. The most common method is the bioluminescent reaction (18-21) as shown below:

\[
\begin{align*}
\text{Mg}^{2+} & \quad \text{E + LH}_2 + \text{ATP} \quad \rightarrow \quad \text{E.LH}_2-\text{AMP} + \text{PP}_1 \\
\text{E.LH}_2-\text{AMP} + \text{O}_2 & \quad \rightarrow \quad \text{oxyluciferin} + \text{CO}_2 + \text{AMP} + \text{light} \\
\text{E} & = \text{Luciferase}; \quad \text{LH}_2 = \text{Luciferin}
\end{align*}
\]

This method is extremely sensitive and can detect picogram levels of ATP. Several disadvantages of this technique render it inaccessible to most clinical laboratories. Apart from being expensive, this assay requires specialized instrumentation. Patterson et al. reported that Ca\(^{2+}\), K\(^+\), Na\(^+\) and Li\(^+\) interfered with this assay (15). Others have reported that the anions SCN\(^-\), I\(^-\), NO\(_3\)\(^-\), Br\(^-\) and Cl\(^-\) inhibit firefly luciferase (22). Moreover, the response of the light emission is variable and the light pulse may take 3-20 sec and up to 2.5 min to obtain peak emission (23). It has also been reported that contaminating transphosphorylase activity was present in commercial preparations of luciferase (15). Thus, CTP and GTP also produce light.

The majority of the enzymatic techniques for ATP revolve around the production of pyridine nucleotides. One procedure used ATP and 3-phosphoglycerate in the presence of phosphoglycerokinase to produce NAD\(^+\) through a sequence of reactions (24). However, this assay is quite insensitive
and is not applicable to the measurement of nmol concentrations of ATP. Another enzymatic procedure used glucose and ATP in the presence of hexokinase to produce NADPH, through a sequence of reactions (25,26). In order to increase the sensitivity of this technique, the NADPH was coupled to resazurin in the presence of diaphorase to form resorufin which is highly fluorescent (26). Again, this entire coupled sequence of reactions provide for an expensive procedure and the instability problem with diaphorase is of some concern (26,27).

Pruneau et al. used high-performance liquid chromatography to measure ATP with a strong anion-exchange column (28). However, most clinical laboratories cannot afford this luxury. A radioenzymatic method has been described for the assay of ATP in biological materials (29). This assay is based on the phosphorylation of a radioactively labelled sugar in the presence of the appropriate kinase. The labelled sugar phosphate is then separated from the unreacted sugar using an anion-exchange resin and counted for radioactivity. A modification of this procedure produces glucose 6-phosphate by the phosphorylation of excess tritiated glucose with hexokinase (30). The product is selectively precipitated with ethanolic barium acetate.
C. THE STUDY

As mentioned in the previous section, several methods have been used to determine ATP. However, most of these suffer from distinct disadvantages as discussed above. Thus, a new method for the measurement of ATP is proposed here. The principle for its determination is illustrated in Fig. 2 and is based on a relatively inexpensive coupled assay system (24) and the development of a very fluorescent product with NAD⁺, acetone and NaOH (31-34).

Platelets are used as a model to measure ATP levels since this analyte in platelets may be of some significance when used in conjunction with other tests to diagnose the disease, malignant hyperthermia.
FIGURE 2

SCHEME USED TO DETERMINE ATP IN PLATELETS

Legend

3-PGA: 3-phosphoglyceric acid.
PGK: phosphoglycerokinase.
GAP-DH: glyceraldehyde 3-phosphate dehydrogenase.
DHAP: dihydroxyacetone phosphate.
TIM: triosephosphate isomerase.
GP: glycerol phosphate.
GP-DH: glycerol phosphate dehydrogenase.

The NAD generated from the sequence of reactions is condensed with acetone in the presence of NaOH. Prior to condensation, excess NADH is destroyed with HCl. The condensation product is stable at a pH of 2.5.
ATP + 3-PGA $\xrightarrow{\text{PGK}}$ ADP + 1,3-diPGA

$\text{NAD}^+$ $\xrightarrow{\text{GP-DH}}$ GP $\xrightarrow{\text{GP-DH}}$ DHAP $\xrightarrow{\text{TIM}}$ GAP + Pi

2 $\text{NAD}^+$

1. HCl
2. Acetone
3. NaOH
4. pH, 2.5
CHAPTER II

EXPERIMENTAL

ANALYTICAL AND CLINICAL STUDIES

1. Equipment

All equipment used in this study were described in
ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER II, 1, p. 14).

2. Materials

Water: Purified water was obtained by the procedure
described in ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER II, 1, p. 17).

Enzymes and coenzymes: The following enzymes and
coenzymes were obtained from Sigma Chemical Co., St. Louis,
MO 63178: β-nicotinamide adenine dinucleotide, reduced form
(β-NADH), disodium salt; β-nicotinamide adenine dinucleotide
(β-NAD+), L-glycerophosphate dehydrogenase (Type X, rabbit
muscle, 242 Units/mg protein) [E.C. 1.1.1.8]; triosephos-
phate isomerase (rabbit muscle, 4600 Units/mg protein)
[E.C.5.3.1.1].

Chemicals: Adenosine 5′-triphosphate (equine muscle,
disodium salt) was also obtained from Sigma Chemical Co.
Sodium hydroxide, hydrochloric acid (A.C.S.), potassium
phosphate (monobasic) and acetone (A.C.S.) were obtained
from B.D.H. Chemicals, Toronto, Ontario, M8Z 1K5. Acetone
was purified by distillation in potassium permanganate.

3. Reagents

The following reagents were obtained from
Sigma Chemical Co.

3-Phosphoglyceric Acid Stock (3-PGA) Solution: This
solution contained 18 mmol/L 3-phosphoglyceric acid,
magnesium ions and EDTA. Chloroform was added as a
preservative.

Glyceraldehyde 3-Phosphate Dehydrogenase/3-Phospho-
glyceric Phosphokinase Stock Mixture: A suspension in
ammonium sulfate of glyceraldehyde 3-phosphate dehydrogenase
(rabbit muscle), 800 Units/mL [E.C.1.2.1.12] and 3-phospho-
glyceric phosphokinase (yeast), 450 Units/mL [E.C.2.7.2.3].

The above reagents are stable for one year or more at
0-5°C.

Working Enzyme Mixture: Aliquots of 0.05 mL each of
glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglyceric
phosphokinase stock mixture, triosephosphate isomerase
stock, and glycero phosphate dehydrogenase were added to 0.5
mL water and gently mixed in a plastic (1.5 mL) vial. This
was placed in ice and used as required.

PGA/NADH Mixture: An aliquot of 2.0-mL 3-PGA stock
solution was added to the NADH vial containing 0.3 mg NADH
and then diluted with 12 mL deionized distilled water and
gently mixed. This was stored in ice and used as required.
Adenosine 5'-Triphosphate (ATP) Stock Solution: 2.2 mg ATP was weighed into a plastic vial and 1.0 mL deionized, distilled water was used to dissolve it. Then a 0.2-mL aliquot was diluted to 10 mL and this was used to prepare diluted standards with a range of $8.0-0.25 \times 10^{-6}$ M ATP.

HCl (6N): Stock HCl (A.C.S.) was diluted 1:1 with water to give a 6N solution.

NaOH (12N) Stock Solution: A solution was made by adding 48.0 g NaOH to a 100-mL volumetric flask and diluting it to the mark with water.

Working NaOH (6N) Solution: A 1:1 dilution of a 12N NaOH Stock Solution was made prior to the assay.

KH$_2$PO$_4$ (25%, w/v): This was prepared by adding 25.0 g potassium phosphate, monobasic, to a 100-mL volumetric flask and diluting it to the mark with water.

4. Methods

Analytical: To a clean 15-mL graduated centrifuge glass tube were added 1.0 mL PGA/NADH mixture, 0.5 mL ATP standard, and 0.04 mL working enzyme mixture. The mixture was gently mixed and incubated at room temperature for 5 min. To this mixture were added 0.06 mL HCl (6N) and 0.50 mL re-distilled acetone. The solution was vortex mixed vigorously for 30 sec then 0.30 mL NaOH (6N) was added to the mixture and vortex mixed again. The contents were incubated at room temperature for 5-10 min. Subsequently,
0.40 mL HCl (6.0N) was added and the contents mixed, and placed in a water bath at 90-100°C for 5 min. The solution was then cooled to room temperature and 1.0 mL KH₂PO₄ (25%) was added to it and diluted to 5.0 mL with water. The solution was read in the fluorometer at an excitation wavelength of 365 nm and an emission wavelength of 460 nm, after centrifugation at 14,500 xg for 5-10 min. A blank was prepared by omitting ATP and adding water instead, in the analysis.

Clinical: Platelets were isolated by an centrifugation technique (26), (ANALYTICAL AND CLINICAL STUDIES (Part II, CHAPTER II, p. 77)). The platelets were counted prior to final pelletization and lysed with 0.5 mL water. The procedure for ATP analysis was followed as shown above except that the standard was replaced with platelet lysate. A blank was prepared by omitting PGK in the enzyme mixture and using water instead.
CHAPTER III

RESULTS AND DISCUSSION

A. ANALYTICAL STUDIES

1. Production of NAD⁺

The absorption spectra of NADH and NAD⁺ are shown in Fig. 3. An intense peak at 340 nm, corresponding to a molar absorptivity of 6.36x10³ Lmol⁻¹cm⁻¹, was noted for NADH. NAD⁺ did not absorb in this region. Thus, the oxidation of NADH to NAD⁺ resulted in a decrease in absorbance which can be directly correlated with the amount of ATP coupled in a sequence of reactions to generate NAD⁺ (see Fig. 2). The coupled enzymatic procedure (kit) for ATP (Sigma Chemical Co.) produces one mole of NAD⁺.

By using the above kit, a decrease in absorbance at 340 nm (due to the consumption of NADH) produced a change in absorbance of 0.228 for 44.6 μmol/dL of ATP (Fig. 4A). The addition of the two enzymes, triosephosphate isomerase and glyceraldehyde phosphate dehydrogenase, produced a total change in absorbance of 0.458 for the same amount of ATP (Fig. 4B). Thus, the amount of NAD⁺ produced was doubled, thereby increasing the sensitivity by 100% with two enzymes that are relatively inexpensive, extensively characterized, obtained in pure forms, and stable for months.
FIGURE 3

ABSORPTION SPECTRA OF NAD⁺ AND NADH

Legend

A: Absorption spectrum of NADH.

B: Absorption spectrum of NAD⁺.

\[ [\text{NADH}] = 1.25 \times 10^{-4} \text{ M.} \]

\[ [\text{NAD}^+] = 1.25 \times 10^{-4} \text{ M.} \]

The spectra were recorded at a pH of 7.6.

Wavelength of maximum absorption for NADH = 340 nm.
FIGURE 4

PRODUCTION OF NAD⁺ AND ITS ENHANCEMENT
WITH TWO ADDITIONAL ENZYMES

Legend

A: Production of NAD⁺ from the kit.
B: Production of NAD⁺ from the kit + two additional enzymes.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Test</th>
<th>ΔA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit</td>
<td>0.811</td>
<td>0.583</td>
<td>0.228</td>
</tr>
<tr>
<td>Kit + 2 enzs.</td>
<td>1.320</td>
<td>0.862</td>
<td>0.458</td>
</tr>
</tbody>
</table>

Procedure was followed according to product manual information (See Ref. 24).

For the reaction involving the two additional enzymes, an extra 0.2 mg NADH was added to the NADH vial supplied by the kit.

All scans were taken against water at 340 nm.
One of the major problems with inverse spectrophotometry is a very high blank (NADH absorbance at 340 nm). Thus, small changes in absorbances (NADH oxidized to NAD\(^+\)) may not produce any detectable change in the spectrophotometer reading. This limits the sensitivity of any assay based on this principle.

2. Effect of pH on NADH and NAD\(^+\)

The sequence of reactions for the determination of ATP (see Fig. 2) produced 2 mol NAD\(^+\). Though the sensitivity was doubled, the procedure still could not measure nmol concentrations of ATP. In order to do this, a fluorescent product was generated with NAD\(^+\) and acetone in the presence of NaOH. As will be shown later, native fluorescence from NADH interfered with this product.

Table II shows the half-lives for the destruction of NADH in the presence of HCl and for NAD\(^+\) in the presence of NaOH. NADH only required 0.43 min at a pH of 2.3 (23°C) for half of it to be destroyed (34, 35). Indeed, it was shown that 99% NADH was completely destroyed in about 10 sec in 0.1N HCl and the destruction obeyed first order kinetics (35). On the other hand, NADH is very stable in alkali and no loss of NADH was observed after 30 min heating at 100°C in 0.1N NaOH (36).

Kaplan et al. showed that NAD\(^+\) was destroyed in weak (0.005-.02N) NaOH with little development of fluorescence (37). Thus, a half-life of 0.5 min was obtained for its
# TABLE II

**EFFECT OF pH ON NADH AND NAD⁺**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NADH</strong></td>
<td></td>
</tr>
<tr>
<td>pH, 4.4</td>
<td>49</td>
</tr>
<tr>
<td>pH, 2.3</td>
<td>0.43</td>
</tr>
<tr>
<td><strong>NAD⁺</strong></td>
<td></td>
</tr>
<tr>
<td>0.005N NaOH</td>
<td>12</td>
</tr>
<tr>
<td>0.02 N NaOH</td>
<td>5</td>
</tr>
<tr>
<td>0.04 N NaOH</td>
<td>3.5</td>
</tr>
<tr>
<td>0.005N NaOH</td>
<td>2</td>
</tr>
<tr>
<td>0.02N NaOH</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*a Recorded at 23°C.*

*b Recorded at 38°C.*
destruction in 0.02N NaOH at 60°C (Table II). However, a very high concentration of NaOH (about 5N) produced a highly fluorescent product (31-34,37).

Destruction of NAD+ with weak alkali resulted in the release of adenylc acid and has been shown that the nicotinamide-ribose linkage of NAD+ is much more sensitive to alkali than the pyrophosphate bond (37) (Fig. 5).

Adenylc acid is not liberated from NADH, even by treatment with 1N NaOH for 30 min at 100°C. Since the reduced nicotinamide-ribose linkage is extremely stable in alkali, it seems, therefore, that the splitting of the pyrophosphate linkage of NAD+ in weak alkali may be dependent on the previous splitting of the molecule at the pyridinium riboside grouping.

It was further shown that strong alkali (5N) did not destroy free nicotinamide, and the loss of nicotinamide in strong alkali occurred only when the nicotinamide was in the bound form. Thus, it was suggested that a condensation occurred between the pyridinium ring and the adjacent ribose, without cleavage of the nicotinamide-ribose linkage. The NaOH added directly to the double bond in NAD+ to form a pseudobase which rearranged to form a fluorescent compound.

3. Generation of the Fluorescent Product with NAD+

Though the sensitivity of ATP determination by the sequence of reactions proposed in Fig. 2 was doubled, a
FIGURE 5

STRUCTURES OF NAD$^+$ AND NADH

Legend

A: Adenosine 5'-monophosphate.
B: Nicotinamide mononucleotide.
C: Ribose.
Y: Nicotinamide-ribose linkage.
K: Oxidized Nicotinamide.
W: Reduced Nicotinamide.
greater sensitivity was obtained by forming the condensation product of NAD$^+$ and acetone in the presence of NaOH. The mechanism for the formation of this product is illustrated in Fig. 6. NAD$^+$ is relatively unreactive, however, in the presence of strong NaOH, a very reactive α-carbinol of the nicotinamide group is formed. Thus, with the addition of acetone it rapidly condenses to form an intermediate which subsequently loses water and cyclizes to form a highly fluorescent compound. The condensation reaction must be done with acetone in the mixture prior to the addition of NaOH, since the extremely reactive carbinol is destroyed in the absence of acetone, and condensation does not occur. For this reason, upon the addition of NaOH, the mixture must be vortexed immediately so that condensation with acetone is effected.

The fluorescence spectra for the condensation product, the product formed with alkali and NAD$^+$ alone, and that of NADH are shown in Fig. 7. The former two products exhibited a wavelength of maximum excitation at 365 nm, whereas, NADH exhibited one at 340 nm. All three emission spectra were obtained at an excitation wavelength of 365 nm. As shown here, NADH has a slight fluorescence at 365 nm when compared to the other two products. The fluorescence of the NAD$^+$ produced with alkali is about ten times that of NADH. The fluorescence of the NAD$^+$ condensed with acetone in the presence of NaOH is approximately four times that of the NAD$^+$.
FIGURE 6
MECHANISM FOR THE FORMATION OF THE NAD$^+$
CONDENSATION PRODUCT WITH ACETONE

Legend

A: Nicotinamide group of NAD$^+$.
B: Reactive α-carbinol.
C: Condensation product of carbinol and acetone.
D: Stabilization of condensation product to form a two-ring structure.
FIGURE 7

EMISSION SPECTRA FOR NADH, THE ALKALI PRODUCT WITH NAD$^+$
AND THE CONDENSATION PRODUCT WITH ACETONE

Legend

A: Emission spectrum of NADH.
B: Emission spectrum of the alkali product with NAD$^+$.
C: Emission spectrum of the condensation product formed with acetone.

All three spectra were recorded at an excitation wavelength of 365 nm.

Wavelength of maximum emission for [B] and [C] = 460 nm.
[NAD$^+$] in cuvet = 8.0 nmol.
product with alkali. Thus, the fluorescence of the condensation product is almost forty times that of the native fluorescence of NADH. It has been shown that the native fluorescence of NADH is at least 1000-fold more sensitive than its spectrophotometric measurement (34). Thus, the fluorescence of the NAD$^+$-condensation product should be at least 4000 times more sensitive than the spectrophotometric measurement of NAD$^+$/NADH. The practical sensitivity obtained by this assay will be discussed in CHAPTER III, p. 157.

4. Effect of NaOH Concentrations on the Formation of the Condensation Product

Figure 8 shows the effect of various concentrations of NaOH on the fluorescence of the condensation product. At a concentration of 1.0N NaOH, some fluorescent product was formed and it required 60 min or more for a constant amount of fluorescence to be obtained. At higher concentrations of NaOH, more fluorescent product was formed as shown by the measurement of the fluorescence at an excitation wavelength of 365 nm and an emission wavelength of 430 nm. Also, less time was required to produce a constant amount of fluorescence. Thus, at 6.0N NaOH, maximum fluorescence was obtained in about 10 min. This concentration of NaOH was used in the development of the assay for ATP. Higher, NaOH concentrations were not tested, but it is quite conceivable that they may not produce any significant increases in
FIGURE 8

EFFECT OF CONCENTRATION OF NaOH ON THE FLUORESCENCE
OF THE CONDENSATION PRODUCT

Legend

A: 1.0N NaOH.
B: 2.0N NaOH.
C: 4.0N NaOH.
D: 6.0N NaOH.

[ATP] in cuvet = 2.5 nmol, thus, [NAD⁺] in cuvet = 5.0 nmol.
FIGURE 8

FLUORESCENCE

TIME (min)

A  B  C  D
fluorescence, since the change in fluorescence from 4.0N NaOH to 6.0N NaOH was only 8.0, i.e., it appears to be levelling off.

In order to obtain 6.0N NaOH, it was best to prepare a 12N NaOH stock solution and then make the appropriate dilution prior to the assay. The stock solution was allowed to stand for a few days before dilution. This caused impurities such as carbonates, etc., to precipitate and form a solid cake at the bottom of the stock solution of NaOH. A 12N NaOH solution is quite viscous, thus the pipet tips were rinsed with water into the solution to be diluted so as to ensure that all the NaOH was delivered from the pipet tip.

5. Effect of Boiling on the Formation of the Condensation Product

Boiling was effected in order to enhance the time required to produce the condensation product of NAD⁺ with acetone. As shown in Table III, after heating for 1 min, the fluorescence intensity reached 46.0. Continued heating changed the intensity to 50.0 and this remained practically constant for approximately 20 min. Thus, it was necessary to heat for only 2 min in order to completely form this product. Probably, most of it was formed prior to the heating process.
TABLE III

EFFECT OF BOILING\(^a\) ON THE FORMATION OF THE CONDENSATION PRODUCT\(^b\)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Fluorescence(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46.0</td>
</tr>
<tr>
<td>2</td>
<td>50.0</td>
</tr>
<tr>
<td>3</td>
<td>49.0</td>
</tr>
<tr>
<td>4</td>
<td>51.3</td>
</tr>
<tr>
<td>6</td>
<td>50.5</td>
</tr>
<tr>
<td>8</td>
<td>48.3</td>
</tr>
<tr>
<td>10</td>
<td>49.5</td>
</tr>
<tr>
<td>15</td>
<td>48.0</td>
</tr>
<tr>
<td>20</td>
<td>51.5</td>
</tr>
</tbody>
</table>

\(^a\) A water bath at 90-100°C was used for this purpose.

\(^b\) [ATP] = 2.0 nmol.

\(^c\) All reaction mixtures were cooled to 22-25°C before KH\(_2\)PO\(_4\) (25%) was added.

All readings were averages of triplicate measurements.
6. The Effect of Ketones and Alcohols on the Formation, of the Condensation Product

Several ketones and alcohols were tested to see which produced maximum fluorescence of the condensation product. The results are shown in Table IV. When no acetone or alcohol was present, the fluorescence reading was 21.5, i.e., only the product of NAD⁺ in strong base gave this fluorescence. Upon the addition of acetone, the fluorescence was increased approximately four-fold and even further for methyl ethyl ketone. However, the latter had a strong odour and therefore its usage was discontinued. Because benzophenone produced a turbid solution, probably due to its insolubility in an aqueous environment, further investigations were not done.

Of the four alcohols tested, none seemed to condense with the product of NAD⁺ in strong base. As a matter of fact, the fluorescence was decreased below the fluorescence of the alkali product for the case of ethanol. The reason for such readings may be attributable to two causes. The condensation product might have been formed, but was not stable under the experimental conditions used for this assay, or the alcohol did not condense at all with the alkali product of NAD⁺. Evidence seems to support the former notion (32). Thus, for all studies, acetone was used to condense with the alkali product of NAD⁺.
**TABLE IV**

**KETONES AND ALCOHOLS<sup>a</sup> TESTED FOR MAXIMUM FORMATION OF THE CONDENSATION PRODUCT<sup>b</sup>**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fluorescence&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21.5</td>
</tr>
<tr>
<td>Acetone</td>
<td>88.0</td>
</tr>
<tr>
<td>Methyl ethyl ketone</td>
<td>95.0 (smelly)</td>
</tr>
<tr>
<td>Benzophenone</td>
<td>turbid</td>
</tr>
<tr>
<td>Methanol</td>
<td>20.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10.0</td>
</tr>
<tr>
<td>Propanol</td>
<td>17.5</td>
</tr>
<tr>
<td>Butanol</td>
<td>25.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> These were spectrophotometric grade reagents.

<sup>b</sup> [ATP] in cuvet = 3.5 nmol.

<sup>c</sup> All readings were averages of triplicate measurements.
7. Effect of pH on the Fluorescence of the Condensation Product

After the condensation product was formed, its fluorescence at various pH's was measured. The results are illustrated in Fig. 9. The product was stable in an acidic media and practically the same fluorescent intensity was observed for the pH range of 2.0-6.0. This was easily maintained by the addition of HCl to the final condensation mixture followed by monobasic potassium phosphate. Repeated measurements of the final pH, recorded a pH in the range of 3.5-4.0. At a pH of approximately 8.0, there was a sudden drop in the fluorescence which probably indicated some type of ionized specie being formed. As the pH was increased from 8.0, a maximum amount of fluorescence was observed at a pH of 10.0. This peak, however, was too sharp and therefore, analytically too difficult to maintain. Thus, this was not used, though it was more sensitive. The production of a fluorescent peak at a pH of 10.0, probably indicates the formation of some resonance stabilized aromatic structure (see Fig. 6) in the presence of OH⁻.

8. Stability of the Condensation Product

One of the most interesting features of this product is its stability. As shown in Table V, the product when left in its final reaction mixture (pH, 3.5-4.0), was stable for 24 h or more. This indeed is a very beneficial aspect of this assay for readings need not be taken immediately after
FIGURE 9

EFFECT OF pH ON THE FLUORESCENCE
OF THE CONDENSATION PRODUCT

Legend

The pH's of the reaction mixtures in the absence of
KH$_2$PO$_4$ were adjusted with 3N HCl and/or 3N NaOH to give the
pH's shown:

[ATP] in cuvet = 2.0 nmol.
## TABLE V

**STABILITY OF THE CONDENSATION PRODUCT**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Fluorescence (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0 (^c)</td>
</tr>
<tr>
<td>0.5</td>
<td>90.5</td>
</tr>
<tr>
<td>1.0</td>
<td>95.2</td>
</tr>
<tr>
<td>2.0</td>
<td>96.5</td>
</tr>
<tr>
<td>3.0</td>
<td>95.6</td>
</tr>
<tr>
<td>4.0</td>
<td>94.0</td>
</tr>
<tr>
<td>12.0</td>
<td>94.5</td>
</tr>
<tr>
<td>24.0</td>
<td>96.5</td>
</tr>
</tbody>
</table>

\(^a\) Product was left in the dark for the first 4 h and then in the light for the next 20 h.

\(^b\) All readings were averages of triplicate measurements.

\(^c\) Concentration of ATP in the cuvet is in nmol.
the assay. The product, whether stored in the light or dark, gave the same results as shown in Table V. The majority of reactions which produce chromophores or fluorophores do not possess this unusual stability in the final product. Most are stable for minutes only.

9. Effect of UV-Irradiation on the Alkali and Condensation Products of NAD⁺

The wavelength of maximum excitation for both the alkali product of NAD⁺ and its condensation product with acetone was 365 nm, i.e., in the UV-region. Thus, a study was undertaken to see whether excitation at this wavelength had any effect on these two products. The results are shown in Table VI. Indeed, the alkali product of NAD⁺ was extremely unstable under UV-irradiation. Within 4 min after irradiation, the fluorescence was decreased by almost 50%, and was practically negligible by 50 min. Thus, the usage of this product as a basis for measuring ATP was not attempted, in addition to its 4-fold less sensitivity than the condensation product.

The excitation of the condensation product at 365 nm presented a more stable situation. After 4 min of excitation, the fluorescence was practically the same. However, as the time was increased, the fluorescence gradually decreased. Thus, by 92 min the fluorescence decreased from 86.0 to 67.0 or by 22.0%. Thus, it is important to take fluorescence readings during the first
### TABLE VI

**EFFECT OF UV-LIGHT\(^a\) ON THE FLUORESCENCE OF THE ALKALI AND CONDENSATION PRODUCTS\(^b\) OF NAD\(^+\)**

<table>
<thead>
<tr>
<th>Time irradiated (min)</th>
<th>Fluorescence(^c)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkali product</td>
<td>Condensation product</td>
</tr>
<tr>
<td>2</td>
<td>80.0</td>
<td>86.0</td>
</tr>
<tr>
<td>4</td>
<td>45.0</td>
<td>85.3</td>
</tr>
<tr>
<td>8</td>
<td>25.3</td>
<td>80.5</td>
</tr>
<tr>
<td>12</td>
<td>16.5</td>
<td>76.0</td>
</tr>
<tr>
<td>16</td>
<td>8.3</td>
<td>77.0</td>
</tr>
<tr>
<td>32</td>
<td>5.0</td>
<td>75.5</td>
</tr>
<tr>
<td>42</td>
<td>5.5</td>
<td>72.5</td>
</tr>
<tr>
<td>52</td>
<td>3.0</td>
<td>71.0</td>
</tr>
<tr>
<td>62</td>
<td>3.0</td>
<td>70.5</td>
</tr>
<tr>
<td>72</td>
<td>2.5</td>
<td>70.5</td>
</tr>
<tr>
<td>82</td>
<td>3.0</td>
<td>68.5</td>
</tr>
<tr>
<td>92</td>
<td>3.0</td>
<td>67.0</td>
</tr>
</tbody>
</table>

\(^a\)Excitation at 365 nm.

\(^b\)[ATP] = 3.5 nmol.

\(^c\)Readings were averages of triplicate measurements.
4 min after excitation, though the product is stable for 24 h or more when not excited.

10. Effect of Temperature on the Fluorescence of the Condensation Product

Table VII shows the variation of temperature on the fluorescence of the condensation product. Again, as observed previously, lower temperatures produced maximum fluorescence as opposed to higher temperatures. A temperature of 23-25°C was maintained for this assay. It was important to maintain the same temperature throughout the studies in order to obtain reproducible readings.

11. Generation of the Standard Curve for ATP

Several concentrations of ATP were determined and the fluorescence generated from these assays were plotted as a function of concentration (Fig. 10). The procedure was followed as outlined in ANALYTICAL AND CLINICAL STUDIES (PART III, CHAPTER II, 4, p. 127). The line of regression was represented by the equation $y = 23.52x + 5.33$, with a correlation coefficient of 0.9996.

Several methods have been proposed to measure ATP in biological specimens, and their sensitivities are shown in Table VIII. The proposed method has a limit of sensitivity of about 0.13 nmol of ATP. This compares quite favorably with other methods as shown in Table VIII. Indeed, there are only 4 methods with better sensitivities. However, each
TABLE VII

EFFECT OF TEMPERATURE\(^a\) ON THE FLUORESCENCE
OF THE CONDENSATION PRODUCT

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Fluorescence (^b) 2.0 nmol ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>66</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>45</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\)The temperature of 10°C was obtained by cooling with ice as necessary to maintain 10°C.

\(^b\)The temperature of 25°C was that of room temperature.

The temperatures of 37 and 45°C were obtained from a heating block.

\(^b\)Readings were averages of triplicate measurements.
FIGURE 10

STANDARD CURVE FOR THE DETERMINATION OF ATP

Legend

Equation for line of regression:  \( y = 23.52x + 5.33 \).
Correlation coefficient:  \( r = 0.9996 \).
Range tested:  0.25-4.0 nmol (cuvet).
Linearity was observed for this entire range.
All readings were averages of triplicate measurements.
<table>
<thead>
<tr>
<th>Method</th>
<th>Lower limit (pmol)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic</td>
<td>$10^9$</td>
<td>38</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>$10^6$</td>
<td>30</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>$10^4$</td>
<td>25</td>
</tr>
<tr>
<td>Chemical</td>
<td>$10^4$</td>
<td>39</td>
</tr>
<tr>
<td>HPLC</td>
<td>250</td>
<td>28</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>3.1</td>
<td>26</td>
</tr>
<tr>
<td>Isotachophoresis</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Radioenzymatic</td>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>Bioluminescent</td>
<td>1.0</td>
<td>19</td>
</tr>
<tr>
<td>Proposed</td>
<td>130</td>
<td></td>
</tr>
</tbody>
</table>

*a* Refers to the limit of detection of ATP.
of these has its unique disadvantages as outlined in METHODS (PART III, CHAPTER I, B, p. 120).

B. CLINICAL STUDIES

1. Isolation of Platelets

Platelets were used as a model for the assay of ATP in a biological system. These were isolated by a centrifugation technique (Fig. 11), and will be discussed in Part IV.

2. Interferences

The effects of iron, copper and zinc on the determination of ATP were studied to see whether they interfered with the reaction. Aliquots of these metals were added to platelet lysates to give final physiologic concentrations, and the fluorescence determined before and after addition. The results are shown in Table IX. No significant change in fluorescence was observed, indicating that these metals did not interfere with the determination of ATP. Additions of chloride, nitrate, sulfate and phosphate, as shown in this Table, did not produce any significant changes in fluorescence, and thus these common anions did not interfere with the determination of ATP.

3. Recovery Studies for ATP in Platelets

High and low levels of platelet lysates were spiked with ATP and their respective concentrations determined before and after spiking (Table X). Average recoveries of
FIGURE 11

ISOLATION OF PLATELETS

Legend

Freshly collected EDTA blood was used for the isolation. All procedures were carried out with siliconized glassware.

Platelets were counted prior to final pelletization to ensure accuracy in reporting values of ATP/10^8 platelets.

Platelet pellet was lysed with 0.5 mL water just prior to analysis.
FIGURE 11

WHOLE BLOOD
Count Platelets

150 x g
20 min

EDTA PLASMA
Enriched, Count

1000 x g
20 min

SUPERNATANT

PLATELET PELLET
Resuspend in 2 ml 0.15 M NaCl
1000 x g; 20 min

SUPERNATANT

PLATELET PELLET
Repeat

SUPERNATANT

PLATELET PELLET
Count
TABLE IX

EFFECT OF METALS AND ANIONS ON THE DETERMINATION OF ATP

<table>
<thead>
<tr>
<th>Interferent</th>
<th>Fluorescence</th>
<th>Before addition</th>
<th>After addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>52.0</td>
<td>50.3</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>51.0</td>
<td>51.0</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>50.5</td>
<td>51.5</td>
<td></td>
</tr>
<tr>
<td>Anions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>53.5</td>
<td>53.0</td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>52.3</td>
<td>50.2</td>
<td></td>
</tr>
<tr>
<td>Sulfate</td>
<td>53.6</td>
<td>50.5</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>50.5</td>
<td>50.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ATP in cuvet = 2.0 nmol.

\(^b\) Concentration in cuvet was equivalent to physiologic concentrations.

\(^c\) Readings were averages of triplicate determinations.

\(^d\) Atomic absorption standards (1000 μg/mL as Fe\(^{3+}\), Cu\(^{2+}\) and Zn\(^{2+}\)) were used to spike samples.

\(^e\) Sodium salts were used to spike samples.
### TABLE X

**RECOVERY \(^a\) STUDIES FOR ATP IN PLATELETS**

<table>
<thead>
<tr>
<th>Platelet lysate pool (^b)</th>
<th>Expected or added</th>
<th>Recovered</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>4.20</td>
<td>3.99</td>
<td>95.0 ± 2.1</td>
</tr>
<tr>
<td>Low</td>
<td>0.420</td>
<td>0.445</td>
<td>106 ± 0.03</td>
</tr>
</tbody>
</table>

\(^a\)All data were averages of triplicate determinations.

\(^b\)High and low refer to platelet lysate pools containing high and low amounts of base level ATP.

\(^c\)% recovery was calculated as the difference between expected and recovered ATP divided by the expected and the answer multiplied by 100.

Units are given in nmol/10^8 platelets.
95 and 106% were obtained, and therefore, the assay was not interfered with by matrix components. The recoveries for this method are comparable to the assays shown in Table VIII.

4. Precision Studies for ATP in Platelets

Within-run and between-run precision studies were carried out on three levels of ATP in platelet lysate pools. As shown in Table XI, the coefficients of variation for the within-run study varied from 0.99 to 6.71% with an average of 3.39%. The between-run precision study varied from 4.21 to 6.21%, with an average of 5.3%. Platelet lysate pools were not stored for more than 24 h and only at -20°C.

5. Reference Values for ATP in Platelets

Twenty samples of platelets were used to determine the levels of ATP. These are shown in Table XII. The values ranged from 0.50 to 5.02 nmol/10^8 platelets, with an average of 2.36 nmol/10^8 platelets. Literature values reported for ATP in platelets are 2.17 nmol/10^8 platelets (26), 2.5 nmol/10^8 platelets (41), and 1.34-2.09 nmol/10^8 platelets (42).

6. Comparison with a Reference Method

The assay developed here was compared with that of Caines, et al. (26). The equation for the line of regression was \( y = 0.92x + 0.14 \) with a correlation
<table>
<thead>
<tr>
<th>Platelet lysate pool</th>
<th>Mean&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S.D.&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S.E.M.&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C.V.&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.59</td>
<td>0.041</td>
<td>0.010</td>
<td>6.71</td>
</tr>
<tr>
<td>B</td>
<td>1.64</td>
<td>0.040</td>
<td>0.011</td>
<td>2.48</td>
</tr>
<tr>
<td>C</td>
<td>3.85</td>
<td>0.038</td>
<td>0.010</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Between-run</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.62</td>
<td>0.039</td>
<td>0.016</td>
<td>6.21</td>
</tr>
<tr>
<td>B</td>
<td>1.69</td>
<td>0.093</td>
<td>0.038</td>
<td>5.48</td>
</tr>
<tr>
<td>C</td>
<td>3.90</td>
<td>0.164</td>
<td>0.003</td>
<td>4.21</td>
</tr>
</tbody>
</table>

<sup>a</sup> mmol/10<sup>8</sup> platelets.
<sup>b</sup> Each platelet pool was assayed 15 times.
<sup>c</sup> Each platelet pool was assayed in duplicate in six batches over a period of 24 h.

S.E.M. = standard error of the mean.
<table>
<thead>
<tr>
<th>Patient #</th>
<th>ATP[^a][^b][^c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.12</td>
</tr>
<tr>
<td>2</td>
<td>2.11</td>
</tr>
<tr>
<td>3</td>
<td>1.71</td>
</tr>
<tr>
<td>4</td>
<td>2.98</td>
</tr>
<tr>
<td>5</td>
<td>3.10</td>
</tr>
<tr>
<td>6</td>
<td>2.21</td>
</tr>
<tr>
<td>7</td>
<td>2.33</td>
</tr>
<tr>
<td>8</td>
<td>2.61</td>
</tr>
<tr>
<td>9</td>
<td>3.12</td>
</tr>
<tr>
<td>10</td>
<td>0.50</td>
</tr>
<tr>
<td>11</td>
<td>5.02</td>
</tr>
<tr>
<td>12</td>
<td>3.21</td>
</tr>
<tr>
<td>13</td>
<td>0.63</td>
</tr>
<tr>
<td>14</td>
<td>4.10</td>
</tr>
<tr>
<td>15</td>
<td>2.61</td>
</tr>
<tr>
<td>16</td>
<td>0.58</td>
</tr>
<tr>
<td>17</td>
<td>2.22</td>
</tr>
<tr>
<td>18</td>
<td>3.02</td>
</tr>
<tr>
<td>19</td>
<td>1.21</td>
</tr>
<tr>
<td>20</td>
<td>1.66</td>
</tr>
</tbody>
</table>

[^a]: nmols ATP/10^8 platelets.
[^b]: Mean [ATP] is 2.36 nmol/10^8 platelets.
[^c]: Each sample was done in duplicate and the values averaged.
FIGURE 12

COMPARISON STUDY WITH A REFERENCE METHOD

Legend

Regression line equation: \( y = 0.92x + 0.14 \).

Correlation coefficient: 0.97.

\( n = 15 \).

Values are given in Amols.

Readings were averages of duplicate measurements.
coefficient of 0.97 (Fig. 12). Thus, there was a significant correlation between the two methods. The data passed both the t-test and F-test.
CHAPTER IV

SUMMARY AND CONCLUSIONS

ATP measurements have been used to check for RBC viability and to determine microbial numbers. Recently, decreased platelet ATP levels have shown a 100% correlation with a muscle biopsy test for the disease, malignant hyperthermia, though other evidence indicates otherwise.

Some of the methods used to determine ATP include radioactive, bioluminescent, and coupled enzymatic assays. The method proposed to determine ATP levels in platelets involved a coupled enzymatic system. 3-Phosphoglycerate (3-PGA) was converted to 1,3-diPGA in the presence of phosphoglycerokinase. The 1,3-diPGA was then converted to glyceraldehyde 3-phosphate (GAP) in the presence of GAP dehydrogenase. This sequence of reactions produced 1 mol of NAD+ and constituted the assay for ATP as provided by a commercially available kit. This assay, however, was not sensitive enough to measure ATP levels in platelets. Increased sensitivity was obtained by converting GAP to dihydroxyacetone phosphate (DHAP) in the presence of triosephosphate isomerase and DHAP to glycerol 3-phosphate (GP) in the presence of GP dehydrogenase. The entire sequence of reactions produced 2 mol of NAD+.
The NAD$^+$ was reacted with acetone in the presence of NaOH to form a highly fluorescent condensation product. The excitation and emission wavelengths for maximum fluorescence of this product were 365 and 460 nm, respectively. A pH optimum of 2.0-6.0 was obtained for the formation of this product and once formed it was stable for at least 24 h. However, upon irradiation (365 nm), the product started to decompose after 4 min and therefore, readings must be taken within 4 min after excitation. A plot of fluorescence versus concentration of ATP showed a linear relationship between 0.13-4.0 nmol with a correlation coefficient of 0.9997. Native fluorescence from interfering NADH was removed with HCl prior to the condensation reaction.

Platelets were isolated by a centrifugation technique and lysed in water. All final reaction mixtures were centrifuged prior to measuring the fluorescence. Two concentrations of ATP were used to spike the platelets. Recoveries of ATP ranged from 95-106%, with an average of 98%. The precision studies for within-run and between-run produced C.V.'s of 3.4 and 5.3%, respectively. An analysis of 20 platelet samples showed a range of 0.5 to 3.8 nmol ATP/10$^8$ platelets with an average of 2.3 nmol/10$^8$ platelets. This correlated quite well with literature values. The proposed method was correlated with a reference method and the coefficient of correlation was 0.97.
REFERENCES


PART IV

THE SEQUENTIAL DETERMINATION OF ADENOSINE 5'-TRIPHOSPHATE AND PYROPHOSPHATE FROM ONE ALIQUOT OF PLATELETS
CHAPTER I

INTRODUCTION

A. GENERAL AND CLINICAL SIGNIFICANCE OF ATP AND PP\textsubscript{i}

The general and clinical significance of measuring ATP was reviewed in GENERAL and CLINICAL SIGNIFICANCE (PART III, CHAPTER I, pp. 1-5). The general and clinical significance of measuring PP\textsubscript{i} was reviewed in GENERAL and CLINICAL SIGNIFICANCE OF PYROPHOSPHATE (PART II, CHAPTER I, pp. 60-66).

The measurement of ATP or PP\textsubscript{i} individually may not be of much diagnostic significance for the disease, malignant hyperthermia. However, a panel of biochemical tests which include ATP and PP\textsubscript{i} may be of some diagnostic importance for this disease (1).

Malignant hyperthermia is a hereditary disease and its most debilitating effect occurs upon the administration of gaseous anesthetics such as halothane and succinylcholine. Upon the administration of these anesthetics, the patient develops an uncontrollable rise in temperature which may lead to cardiac arrest, rhabdomyolysis, renal failure, brain damage and coma (2). If supportive measures are not taken immediately, the patient usually dies. A crisis episode can also be initiated from stress and anxiety. This disease is fairly prevalent (about 2000 cases in the Essex County
area) and affects mostly the French population (3). For a number of years the only diagnostic test for this disease was a muscle biopsy test. Recently, this has been replaced with a platelet bioassay which measures intracellular ionized calcium. Levels of ATP in this disease are expected to decrease whereas levels for PP$_4$ are expected to increase. Thus, these two analytes along with creatine kinase, and intracellular calcium may constitute a useful panel to diagnose this disease (1,4).

B. THE STUDY

The most limiting factor in the simultaneous assay of ATP and PP$_4$ (or any other analyte) in platelets is the availability of enough sample from one patient for the assay. Thus, a study is undertaken to use one sample (aliquot) of isolated platelets and to determine both ATP and PP$_4$ from this single aliquot. In order to do so, the ATP is converted to NAD$^+$, via a sequence of reactions and is separated from PP$_4$ by passage through a Dowex-1(Cl$^-$) resin and subsequent elution with HCl. The PP$_4$ is then hydrolyzed with HCl and the phosphate determined by the aluminium-moquin reaction as shown in ANALYTICAL AND CLINICAL STUDIES (PART II, CHAPTER II, p. 74). NAD$^+$ is reacted with acetone in the presence of NaOH to form a highly fluorescent product as shown in ANALYTICAL AND CLINICAL STUDIES, (PART III, CHAPTER II, p. 127).
As of the present moment, there is no study that uses one aliquot of platelets or serum to measure both of these analytes using any analytical method. In all cases, separate aliquots of the patient's specimen are required.
CHAPTER II

EXPERIMENTAL

ANALYTICAL AND CLINICAL STUDIES

1. Equipment

The equipment used here was described in "ANALYTICAL AND CLINICAL STUDIES (PART I, CHAPTER II, p. 14)."

2. Materials and Reagents

The materials and reagents used for the determination of pyrophosphate were described in "ANALYTICAL AND CLINICAL STUDIES (PART II, CHAPTER II, p. 72)." The materials and reagents used for the determination of ATP were described in "ANALYTICAL AND CLINICAL STUDIES (PART III, CHAPTER II, pp. 125-127)."

Each mixed standard was prepared to contain the same amounts of ATP and PP_{i} as indicated in "ANALYTICAL AND CLINICAL STUDIES (PART III, CHAPTER II, p. 127)" and PART II, CHAPTER II, p. 73).

3. Procedures

To a clean 1.5-mL centrifuge plastic vial were added 1.0 mL PGA/NADH mixture, 0.5 mL mixed standard (i.e., standard containing ATP and PP_{i}) or platelet lysate and 0.04 mL working enzyme mixture. The mixture was gently mixed and incubated at room temperature for 5 min. The
entire contents were loaded onto a Dowex-1(Cl-) resin column and the sides of the column washed with 0.5 mL H₂O (pH, 8.0). Then 1.5 mL 0.025N HCl was added to the column, and the combined eluate kept for NAD⁺ analysis. Henceforth, the procedure to determine NAD⁺ by formation of a fluorescent condensation product with acetone is outlined in ANALYTICAL AND CLINICAL STUDIES (PART III, CHAPTER II, p. 127).

Pyrophosphate was eluted from the column with 1.0 mL 0.10N HCl and the eluate was kept for pyrophosphate analysis, using the aluminium-morin reaction as outlined in ANALYTICAL AND CLINICAL STUDIES (PART II, CHAPTER II, p. 74).
CHAPTER III

RESULTS AND DISCUSSION

A. ANALYTICAL AND CLINICAL STUDIES

1. Isolation of Platelets

A centrifugation technique was used to isolate platelets (Fig. 1). Freshly collected blood (EDTA) was used for this purpose. A platelet pellet was isolated with two high-speed centrifugation steps as compared to four high-speed centrifugation steps in other procedures. An aliquot (0.1 mL) of the final platelet suspension was diluted to 0.60 mL with saline and counted for platelets. Thus, the actual platelet count in the final pellet was known, rather than calculating the amount of platelets present in the final pellet (from calculations based on the platelets in the platelet enriched plasma).

2. Calculation of % Recoveries

Most of the procedures used to isolate platelets revolved around centrifugation techniques. These procedures suffer from the distinct disadvantage of platelets being destroyed during the centrifugation steps. A study was carried out to find out the recovery of platelets after the centrifugation steps. The results are shown in Table 1. The recoveries were consistently lower and ranged from 90.1 to 95.1%, with an average of 92.4%. Thus, it was important to count the platelets prior to its final
FIGURE 1

ISOLATION OF PLATELETS

Legend

The platelet pellet was counted just prior to final pelletization to ensure an accurate number of platelets used for the analysis.

The pellet was lysed in 0.5 mL water just prior to the analysis.
FIGURE 1

WHOLE BLOOD
Count Platelets

150 × g
20 min

EDTA PLASMA
Enriched, Count

1000 × g
20 min

SUPERNATANT

PLATELET PELLET
Resuspend in
2 ml 0.15M NaCl
1000 × g, 20 min

SUPERNATANT

PLATELET PELLET

SUPERNATANT

PLATELET PELLET

Count
### Table I

**Recovery of Platelets**

<table>
<thead>
<tr>
<th>PERP $^{a}$ (x10$^8$)</th>
<th>After centrifugation $^{a}$ (x10$^8$)</th>
<th>% recovery $^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>398</td>
<td>358</td>
<td>90.1</td>
</tr>
<tr>
<td>406</td>
<td>373</td>
<td>91.9</td>
</tr>
<tr>
<td>328</td>
<td>306</td>
<td>93.3</td>
</tr>
<tr>
<td>597</td>
<td>561</td>
<td>94.0</td>
</tr>
<tr>
<td>366</td>
<td>348</td>
<td>95.1</td>
</tr>
<tr>
<td>427</td>
<td>387</td>
<td>89.9</td>
</tr>
<tr>
<td>501</td>
<td>466</td>
<td>93.0</td>
</tr>
<tr>
<td>209</td>
<td>190</td>
<td>90.9</td>
</tr>
<tr>
<td>701</td>
<td>645</td>
<td>92.0</td>
</tr>
<tr>
<td>326</td>
<td>306</td>
<td>93.9</td>
</tr>
</tbody>
</table>

$^{a}$ PERP = platelet enriched plasma.

$^{b}$ Recoveries were averages of triplicate determinations.

The average recovery = 92.4%.
pelletization step to give a more realistic count of the platelets in the final pellet. Counting platelets at the plasma enriched step and relating analyte determination to this count may not be very accurate. Some researchers based their results per mg protein of platelets, rather than platelet count. Indeed, this may be a more accurate way than counting the final pellet. However, it must be noted that platelets have the innocuous tendency to adhere onto things and most noticeably, albumin and other proteins. Centrifugation steps will not get rid of all these proteins and again results may be misleading.

A simple, less traumatic way to isolate platelets was attempted with Sepharose 2B. This procedure was used to isolate platelets from hamster blood with a 100% recovery (5). This chromatographic procedure was tried with very good results. A band of 2-3 mL eluate contained all the platelets. However, this procedure was quite tedious, and involved using too many columns when a large number of samples were needed for analysis. Thus, it was abandoned and the centrifugation procedure was used instead.
3. Temperature for Isolation of Platelets and Storage of Lysates

Platelets were isolated at room temperature and at 0-4°C (ice-bath). Platelets isolated at room temperature did not aggregate and thus, resuspension was very easy. At 0-4°C (ice-bath), the platelets were difficult to resuspend and thus additional gentle mixing was required with a siliconized pipet to ensure that the count from the Coulter Counter was not in error as a consequence of aggregation of the platelets. Since additional, gentle agitation was only required here, the platelets were isolated at 0-4°C.

The platelet pellets once isolated were tested to find out whether there were any changes in pyrophosphate and ATP levels under different storage conditions. At -4°C, the platelets showed approximately 10% loss in ATP activity and 15% loss in pyrophosphate activity for one day of storage. At -20°C, the platelets did not show any significant loss of either pyrophosphate or ATP levels for two weeks of storage. At room temperature (22-25°C), almost all activity of both analytes were lost when stored for one day. Thus, it was essential to store the platelets at -20°C in order to ensure that ATP and pyrophosphate levels were maintained when subsequent analyses were performed.
4. Determination of ATP and PP$_i$

The scheme used to separate these two analytes is shown in Fig. 2. A lysed sample was used to determine ATP as outlined by the sequence of reactions in THE STUDY (PART III, CHAPTER I, p. 122). The NAD$^+$ generated was not condensed yet, but the entire contents were applied to a Dowex-1(Cl$^-$) resin and washed with H$_2$O (pH, 8.0) to ensure that the anions were adsorbed onto the column. Elution with 0.025N HCl released phosphates, sugar phosphates and other labile phosphates and NAD$^+$ as well. Pyrophosphate and nucleotides (GTP, UTP, etc.) still remained bound onto the resin. Thus, the combined eluates of water and 0.025N HCl were used for the condensation reaction of NAD$^+$ and acetone in the presence of NaOH.

Table II shows the recovery of pure NAD$^+$ from a Dowex-1(Cl$^-$) resin. Several concentrations of NAD$^+$ were added to a mixture of enzymes and buffer without any ATP. The eluate collected (A and B combined in Fig. 2) was neutralized and NAD$^+$ determined by the condensation reaction. The recoveries for pure NAD$^+$ from the column were quantitative and ranged from 95-108%, Table II.

Pyrophosphate and other nucleotides were released from the resin with 0.1N HCl. After neutralization and the addition of a few milligrams of charcoal, the supernatant was used to determine the pyrophosphate.
FIGURE 2

THE DETERMINATION OF ATP AND PYROPHOSPHATE IN ONE SAMPLE

Legend

Lysed sample contains ATP and PP$_i$.

A series of sequential reactions with ATP produces NAD$^+$.

Reaction mixture now contains NAD$^+$ and PP$_i$.

A. Apply reaction mixture to column.
B. Wash with 0.5 mL H$_2$O (pH 8.0)
C. Elute with 0.25N HCl (1.5 mL). Collect, neutralize and determine NAD$^+$.
D. Elute with 0.10N HCl (1.0 mL). Add a few milligrams charcoal. Centrifuge, and determine PP$_i$ in supernatant.
FIGURE 2

Lysed sample

→ Determine ATP

Reaction mixture

→ NAD$^+$ + PP$_i$

\{ ABCD \}

Dowex-1(Cl$^-$)
TABLE II.

RECOVERY OF PURE NAD$^+$ FROM COLUMN

<table>
<thead>
<tr>
<th>NAD$^+$ added to column</th>
<th>NAD$^+$ expected or added</th>
<th>NAD$^+$ recovered</th>
<th>% recovery$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>1.9</td>
<td>95.0$^a$</td>
</tr>
<tr>
<td>4.0</td>
<td>4.0</td>
<td>4.3</td>
<td>107.5</td>
</tr>
<tr>
<td>6.0</td>
<td>6.0</td>
<td>5.8</td>
<td>96.7</td>
</tr>
<tr>
<td>8.0</td>
<td>8.0</td>
<td>7.6</td>
<td>97.5</td>
</tr>
</tbody>
</table>

$^a$ Concentration of NAD$^+$ in cuvet.

$^b$ Average recovery = 99.1%.

All values were averages of triplicate determinations.

Concentrations are given in nmol.
5. Generation of Calibration Curve for ATP

Figure 3 shows the calibration curve for the determination of ATP as outlined in ANALYTICAL AND CLINICAL STUDIES (PART III, CHAPTER II, p. 169). A linear relationship was observed from 0-4.0 nmol ATP. The equation for the line of regression was \( y = 24.7x + 2.5 \), with a correlation coefficient of 0.997.

6. Recovery Studies for ATP Determinations in Platelets

The recovery study for the determination of ATP in high and low platelet lysate pools, are shown in Table III. The platelet lysates were spiked with ATP and the ATP levels determined before and after the spiking. The recoveries ranged from 98.3-106% with an average of 102.2%.

7. Precision Studies for ATP Determinations in Platelets

The results for the precision studies for the determination of ATP are shown in Table IV. The within-run study for the three pools of platelets showed coefficients of variation ranging from 2.68 to 4.96%, with an average of 4.12%. The between-run study resulted in coefficients of variation of 5.81 to 6.76%, with an average of 6.24%. It is noted here that the C.V. at lower levels of ATP was not as reproducible as that at higher levels. Also, the between-run C.V. was higher than the within-run C.V. This was due to the greater variation between each run, in reagents, water supplies, pipetting, etc.
FIGURE 3
CALIBRATION CURVE FOR ATP FROM SEQUENTIAL SEPARATION

Legend

Equation for regression line: \( y = 24.7x + 2.5 \).
Correlation coefficient = 0.997.
All points were averages of triplicate determinations.
### TABLE III

**RECOVERY STUDIES FOR THE SEQUENTIAL DETERMINATION OF ATP IN PLATELETS**

<table>
<thead>
<tr>
<th>Platelet lysate pool&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Expected or added</th>
<th>Recovered</th>
<th>% recovery&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>4.01</td>
<td>3.94</td>
<td>98.3 ± 2.2</td>
</tr>
<tr>
<td>Low</td>
<td>0.401</td>
<td>0.425</td>
<td>106.0 ± 1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>High and low refer to platelet lysate pools with high and low base levels of ATP.

<sup>b</sup>All data were averages of triplicate measurements.

Values are given in nmol/10<sup>8</sup> platelets.
TABLE IV

PRECISION STUDIES FOR THE SEQUENTIAL DETERMINATION OF ATP IN PLATELETS

<table>
<thead>
<tr>
<th>Platelet pool</th>
<th>Mean a</th>
<th>S.D. a</th>
<th>S.E.M. a</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-run b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.56</td>
<td>0.028</td>
<td>0.007</td>
<td>4.96</td>
</tr>
<tr>
<td>B</td>
<td>2.12</td>
<td>0.100</td>
<td>0.025</td>
<td>4.72</td>
</tr>
<tr>
<td>C</td>
<td>4.08</td>
<td>0.109</td>
<td>0.028</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-run c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.59</td>
<td>0.040</td>
<td>0.016 b</td>
<td>6.76</td>
</tr>
<tr>
<td>B</td>
<td>2.16</td>
<td>0.133</td>
<td>0.054</td>
<td>6.15</td>
</tr>
<tr>
<td>C</td>
<td>4.01</td>
<td>0.232</td>
<td>0.095</td>
<td>5.81</td>
</tr>
</tbody>
</table>

a nmol/10^8 platelets.
b Each platelet pool was assayed 15 times.
c Each platelet pool was assayed in duplicate in six batches.

S.E.M. = standard error of the mean.
8. Calibration Curve for the Determination of $PP_i$

The calibration curve for the determination of pyrophosphate is shown in Fig. 4, and was established according to the procedure outlined in ANALYTICAL AND CLINICAL STUDIES (PART II, CHAPTER II, p. 74). The linearized version shown here, was obtained from a plot of log fluorescence versus concentration of phosphate. The plot was linear for concentrations varying from 0-12 nmol phosphate or 0-6.0 nmol pyrophosphate. The equation for the line of regression was $y = -5.0x - 1.94$, with a correlation coefficient of 0.991.

9. Recovery Studies for Pyrophosphate Determinations in Platelets

Low and high levels of $PP_i$ in platelet lysate pools were spiked with pyrophosphate and the concentrations of pyrophosphate determined before and after the spiking. The results are shown in Table V. Recoveries were quantitative and ranged from average of 96.1 and 103.2% with an average of 96.7%.

10. Precision Studies for Pyrophosphate Determinations in Platelets

The results for the precision studies for the determination of pyrophosphate in platelets are shown in Table VI. The within-run study showed coefficients of variation ranging from 4.26 to 5.83%, with an average of 5.10%. The between-run studies showed coefficients of
FIGURE 4

CALIBRATION CURVE FOR THE DETERMINATION OF PYROPHOSPHATE

Legend

Equation for line of regression: $y = -5.0x - 1.94$.
Correlation coefficient: $r = 0.991$.
Points were averages of triplicate determinations.
### TABLE V

**RECOVERY STUDIES FOR THE SEQUENTIAL DETERMINATION OF PP<sub>i</sub> IN PLATELETS**

<table>
<thead>
<tr>
<th>Platelet lysate pool&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Expected or added</th>
<th>Recovered</th>
<th>% recovery&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>3.15</td>
<td>2.98</td>
<td>96.1 ± 1.5</td>
</tr>
<tr>
<td>Low</td>
<td>0.315</td>
<td>0.320</td>
<td>103.2 ± 1.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>High and low refer to platelet lysate pools containing high and low amounts of base levels pyrophosphate.

<sup>b</sup>All data were averages of triplicate determinations. Values are given in nmol/10<sup>8</sup> platelets.
TABLE VI

PRECISION STUDIES FOR THE SEQUENTIAL DETERMINATION OF PP$_i$
IN PLATELETS

<table>
<thead>
<tr>
<th>Platelet pool</th>
<th>Mean$^a$</th>
<th>S.D.$^a$</th>
<th>S.E.M.$^b$</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.49</td>
<td>0.029</td>
<td>0.007</td>
<td>5.83</td>
</tr>
<tr>
<td>B</td>
<td>1.86</td>
<td>0.097</td>
<td>0.025</td>
<td>5.21</td>
</tr>
<tr>
<td>C</td>
<td>5.52</td>
<td>0.235</td>
<td>0.061</td>
<td>4.26</td>
</tr>
<tr>
<td>Between-run$^c$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.47</td>
<td>0.033</td>
<td>0.013</td>
<td>6.93</td>
</tr>
<tr>
<td>B</td>
<td>1.78</td>
<td>0.121</td>
<td>0.049</td>
<td>6.80</td>
</tr>
<tr>
<td>C</td>
<td>5.51</td>
<td>0.371</td>
<td>0.151</td>
<td>6.73</td>
</tr>
</tbody>
</table>

$^a$nmol/10$^8$ platelets.

$^b$Each platelet pool was assayed 15 times.

$^c$Each platelet pool was assayed in duplicate in six batches.

S.E.M. = standard error of the mean.
variation ranging from 6.73 to 6.93, with an average of 6.82%.
CHAPTER IV

SUMMARY AND CONCLUSIONS

The determination of more than one analyte from a patient's sample of platelets is not feasible in most instances due to small amount of platelets present for the analyses. Thus, a technique was developed to use the same aliquot of platelets to determine both ATP and PPi.

One aliquot of platelets was used to determine ATP in a vial. The entire contents containing the NAD⁺ produced from the ATP sequence of reactions and other substances such as PPi, Pi, Ca²⁺, etc., were loaded onto a Dowex-1(Cl⁻) resin. Washing with water (pH 8.0) followed by 0.025N HCl eluted NAD⁺, Ca²⁺, along with other substances. This combined eluate was then used to develop the acetone condensation product as a measure of the initial concentration of ATP in the original platelet sample.

The PPi, still on the column, was eluted with 0.1N HCl and the eluate neutralized with NaOH. A few milligrams of charcoal were added to adsorb any nucleotides present (if any) and the clear supernatant was used for PPi hydrolysis and its subsequent phosphate determination with the Al-morin reaction.

A standard curve was established for ATP and a working linear range of 0 to 4.0 nmol was obtained with a correla-
tion coefficient of 0.997. Recovery of two concentrations of ATP from platelet lysate pools yielded 98-106% with an average of 102%. A precision study for within-run assays produced an average C.V. of 4.1% and a between-run C.V. of 6.2%. A standard curve for PP$_i$ showed linearity in the working range of 0.13 to 6.0 nmol with a correlation coefficient of 0.991. Recovery of two concentrations of PP$_i$ from platelet lysate pools yielded 96-103% with an average of 97%. A precision study for within-run assays produced an average C.V. of 5.1% and a between-run C.V. of 6.8%.
REFERENCES


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(1) Castillo, G., Thibert, R.J., Zak, B., and
    Copper and Zinc in a Single Aliquot of Serum,
    Using 5-Bromo-2-pyridylazo Resorcinol.
    Microchem. J. (Accepted for Publication)

(2) Seudeal, N.D., Thibert, R.J., Zak, B. (1986)
    Sequential Determination of Iron, Copper and Zinc
    in a Single Aliquot Using 2-Amino-5-bromo-
    pyridylazo Resorcinol. Microchem. J. 34, 131-139
    (1986)

(3) Hayes, P.C., Osman, A., Seudeal, N.D.,
    Synthesis of 2,2'-Bipyridine Adducts of
    Organomagnesium Halides and, of Organometallic
Abstracts:


Presentations:
(1) Presented a Poster Session at the National Meeting of the CSCC in Ottawa, June 8, 1987 (See ABSTRACT # 1)

(2) Presented a Poster Session at the National Meeting of the CSCC in Ottawa, June 9, 1987 (See ABSTRACT # 2)

(3) Presented a Poster Session at the National Meeting of the AACC in Atlanta, Georgia, July 25, 1985 (See ABSTRACT # 3)

(4) Presented a Seminar to the Michigan Section of the AACC at Children's Hospital, Detroit, MI, October 24, 1984 (See ABSTRACT # 3)

(5) Presented a Seminar to the Michigan Section of the AACC at Wayne State University, Dept. of Pathology, Detroit, MI, October 23, 1986 (See ABSTRACT # 2)

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