I. A FLUOROMETRIC COUPLED ENZYMATIC METHOD FOR THE DETERMINATION OF ADENOSINE TRIPHOSPHATE IN PLATELETS. II. COLORIMETRIC DETERMINATION OF NON-ENZYMATICALLY GLYCATED ALBUMIN.

PATRICK STEPHEN MICHAEL. CAINES
University of Windsor

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ML 330 (f. 56/06)
I. A FLUOROMETRIC COUPLED ENZYMATIC METHOD FOR THE DETERMINATION OF ADENOSINE TRIPHOSPHATE IN PLATELETS

II. COLORIMETRIC DETERMINATION OF NON-ENZYMATICALLY GLYCATED ALBUMIN

by

PATRICK STEPHEN MICHAEL CAINES

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

Windsor, Ontario, Canada 1985
ABSTRACT

PART I

A FLUOROMETRIC COUPLED ENZYMATIC METHOD FOR THE
DETERMINATION OF ADENOSINE TRIPHOSPHATE IN PLATELETS

by

PATRICK STEPHEN MICHAEL CAINES

A new fluorometric, coupled, enzymatic method for the
determination of adenosine triphosphate is described. The
procedure uses the hexokinase reaction coupled with three
other enzymes in a system of phosphorylation and double
reduction to form NADPH. The NADPH is measured in a reaction
catalyzed by diaphorase in which the nonfluorescent material,
resazarin, is converted to the highly fluorescent compound,
resorufin.

This kinetic method can determine adenosine triphosphate
at the picomole level. Intra- and inter-assay CV's were both
less than 3% and recoveries were quantitative. No significant
interference was observed from EDTA or heparin. Correlation
with an established method was significant (r = 0.99).
Adenosine triphosphate was measured in platelets. Mean ATP
levels in the platelets of "normal" subjects were found to be
2.17 ± 0.76 nmol / 10⁶ platelets (14.22 ± 6.32 nmol / mg
protein).
PART II

COLORIMETRIC DETERMINATION OF NON-ENZYMATICALLY GLYCATED ALBUMIN

by

PATRICK STEPHEN MICHAEL CAINES

A new procedure was developed for the determination of non-enzymatically glycated albumin (GA). Albumin was separated from serum or plasma using Sepharose-blue dextran affinity chromatography. The "fructosamine assay" was improved and used to determine GA. The stable ketoamine linkage in GA reduced 3-(4,5-dimethylthiazol-2-y1)-2,4-diphenyltetrazolium bromide (MTT) to a colored formazan.

Optimum conditions for the assay were established using the simplex optimization technique. The reagent blank value was minimal and MTT had a 3-fold greater molar absorptivity than nitroblue tetrazolium used in the original "fructosamine assay". The assay was adapted for use in a centrifugal analyzer (Flexigem™) and GA was used as the standard. The within and between run CV's were 4.6% and 8.5%, respectively. Recovery of GA was quantitative.

The reference range for this method was 8.94 - 11.19% GA and normal and diabetic populations can be clearly
discriminated (p<0.005). Values obtained with this method correlate well with a thiobarbituric acid assay (r=0.974) but not with those for glycated hemoglobin (r=0.350).
ACKNOWLEDGEMENTS

I would like to express my sincerest thanks and appreciation to Dr. R.J. Thibert for his patient direction of this work as well as his guidance and encouragement throughout my entire program.

I thank Dr. T.F. Draisey for his comments during various phases of this work and for his generous assistance in obtaining the blood specimens. Thanks also to the other members of my committee, Dr. H.B. Fackrell, Dr. B. Mutus, and Dr. N.F. Taylor for their helpful comments and suggestions, and my external examiner Dr. W.H.C. Walker for his reading and critique of this dissertation.

My appreciation also extends to Mr. D. Cho and staff of the Hematology Laboratory, Windsor Western Hospital Centre, and Mr. M.R. Goodwin, Mr. E. Olivero, and staff of the Hematology and Chemistry Laboratories, Salvation Army Grace Hospital, Windsor, Ontario.

Finally, I would like to thank the staff and my colleagues at the Department of Chemistry, University of Windsor, all of whom through their genuine help and concern enabled the successful completion of this work and made my stay in Windsor an enjoyable and memorable one.
DEDICATION

to my family, whose love and understanding made all this possible.
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LIST OF ABBREVIATIONS

PART I

c
level of significance

λ
wavelength

ADP
adenosine diphosphate

ATP
adenosine triphosphate

ATPMGPRO
platelet levels of adenosine triphosphate in nmol per mg platelet protein

C.V.
coefficient of variation

EDTA
ethylenediaminetetraacetic acid

G6P-DH
glucose-6-phosphate dehydrogenase

ID
sample identification number

n
number of samples

NAD+
nicotinamide adenine dinucleotide

NADH
nicotinamide adenine dinucleotide reduced form

NADP+
nicotinamide adenine dinucleotide phosphate

NADPH
nicotinamide adenine dinucleotide phosphate reduced form

nm
nanometers

PLATATP
platelet adenosine triphosphate levels in nmol per 100 million platelets

PLATNO
number of platelets (x10^6) in an amount of platelet enriched plasma that was used to form 50 µL platelet lysate
PLATPROT platelet protein levels in mg per 50 μL platelet lysate

PERPC platelet enriched plasma count (x10^3 per mm^3)

PERPV volume of platelet enriched plasma used to form the platelet pellet (mL)

S.D: standard deviation

S.E.E. standard error of the estimate

S.E.M. standard error of the mean

Tris tris(hydroxymethyl)aminomethane

mean

PART II

DMF 1-deoxymorpholinofructose

GA glycated albumin

GHB glycated hemoglobin

HMF 5-hydroxymethylfurfural

HSA human serum albumin

NBT nitroblue tetrazolium

MTT 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide
PART I

A FLUOROMETRIC COUPLED ENZYMATIC METHOD FOR THE
DETERMINATION OF ADENOSINE TRIPHOSPHATE IN PLATELETS
CHAPTER I

INTRODUCTION

A. PHYSIOLOGICAL AND PATHOLOGICAL SIGNIFICANCE OF ADENOSINE TRIPHOSPHATE

Adenosine triphosphate (ATP) plays a central role in the transfer of chemical energy in cells. It occurs in all living cells and is present in concentrations of between 0.001 and 0.01 moles per liter of cell water (1). The presently accepted structure of ATP is shown in Fig. 1 (1).

Adenosine triphosphate is believed to be the main compound that links energy-yielding and energy-requiring functions in the cell. Chemical energy released by the degradation of fuel molecules is recovered by the coupled phosphorylation of adenosine diphosphate (ADP) to yield ATP. The energy-rich ATP then transfers its chemical energy, by hydrolysis of its terminal high energy phosphate group, to the energy requiring functions of the cell. These functions include biosynthetic processes, muscle contraction, and active transport against concentration gradients. The free energy of hydrolysis of ATP under physiological conditions is about 12 kcal/mol, significantly higher than that of many phosphorylated compounds. For this reason, ATP holds a central role in cellular metabolism and has been extensively
FIGURE 1

STRUCTURE OF ADENOSINE TRIPHOSPHATE

Legend

Shown is the presently accepted structure of adenosine triphosphate. This structure represents the ionized form at pH 7.0. The symbol $\sim$ represents the high energy bonds.
FIGURE 1

Adenine

D-ribose
investigated by the biochemist.

Adenosine triphosphate is, however, also of interest to the clinician and the clinical chemist. In certain diseases where nucleotide metabolism is altered the determination of ATP is often of great investigative and diagnostic value. A wide variety of diseases such as chronic renal failure (2), and malignant hyperthermia (3) are reported to result in alterations of intracellular ATP levels. Erythrocyte ATP levels are frequently used as a measure of red blood cell viability. The intrinsic ATP content of microorganisms is utilized as a measure of microbial numbers and forms the basis of rapid bacteriuria testing (4).

B. MEASUREMENT OF ADENOSINE TRIPHOSPHATE

Many assays for the measurement of ATP in cells and tissues have been previously described (5-13). Some of the earlier assays for ATP were laborious (13) and the more sensitive of these required pretreatment of samples or isolation of reaction products. One severe limitation in some of these assays is the relatively large sample sizes required in view of the usually small amount of material available for analysis. Other assays also require the handling of hazardous
radioactively labelled compounds (6, 7).

Although the bioluminescent procedures are the most sensitive for the determination of ATP (14, 15), they possess many methodological difficulties (16). In addition the need for specialized instrumentation and often expensive reagents limits their widespread usage.

C. THE STUDY

The primary aim of this study was to develop a method for the determination of ATP that has sensitivity comparable to the bioluminescent assays. The method developed is a fluorometric, coupled, enzymatic procedure. This assay utilizes the hexokinase reaction coupled with two other enzymes in a system of phosphorylation and double reduction to form NADPH (8). The complete sequence of reactions is shown in Fig. 2. The NADPH produced is measured in a reaction catalyzed by diaphorase in which the nonfluorescent compound, resazurin, is converted to the highly fluorescent resorufin (17), shown in Fig. 3.

The method described in this study was used to determine ATP in aqueous solutions and platelet lysates, and a reference interval was established.
FIGURE 2

PROPOSED ENZYMATIC SCHEME

Legend

Shown is the enzymatic scheme used in this study. For every mole of ATP two moles of the highly fluorescent compound resorufin were generated.

The following abbreviations are used: HK, hexokinase; G6P-DH, glucose-6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconic acid dehydrogenase.

The spectrophotometric assays (5) utilized steps 1-2 while the fluorometric assays (8,9) utilized steps 1-3.

The proposed method included step 4 in which the resorufin formed was measured kinetically.
FIGURE 2

1. ATP + Glucose $\rightarrow$ ADP + Glucose-6-phosphate

2. Glucose-6-phosphate $\rightarrow$ G6P-DH + 6-phosphogluconic acid

3. 6-phosphogluconic acid $\rightarrow$ NADP+ + NADPH + CO2 + ribulose-5-phosphate

4. Resazurin + NADPH $\rightarrow$ Resorufin + NADP+
FIGURE 3

STRUCTURES OF RESAZURIN AND RESORUFIN

Legend

Shown is resazurin, a nonfluorescent compound. This was used as the sodium salt. Its reduced product, resorufin, also shown, is highly fluorescent (λ excitation 560 nm, λ emission 580 nm).
Figure 3

RESAZURIN

RESORUFIN
CHAPTER II

EXPERIMENTAL

A. ANALYTICAL INVESTIGATION

1. Materials

   **Enzymes:** The following enzymes were purchased from Sigma Chemical Co., St. Louis MO 63178: hexokinase (HK) (from yeast) [ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1]; glucose-6-phosphate dehydrogenase (G-6-PDH) (from yeast) [D-glucose-6-phosphate: NADP+ oxidoreductase, EC 1.1.1.49]; 6-phosphogluconic acid dehydrogenase (6-PGDH) (from yeast) [6-phospho-D-gluconate: NADP+ 2-oxidoreductase, EC 1.1.1.44]; diaphorase (from *Clostridium kluveri*) [lipoyl dehydrogenase, EC 1.6.4.3].

   The enzyme activities quoted are those of the supplier. Unit definitions are as follows: HK, one unit is the amount of enzyme which will phosphorylate 1.0 μmol of glucose per min at pH 8.5 at 25°C; G6P-DH, one unit is the amount of enzyme which will oxidize 1.0 μmol of glucose-6-phosphate to 6-phosphogluconate per min at 25°C at pH 7.4 in the presence of NADP+; 6-PGDH, one unit is the amount of enzyme which will oxidize 1.0 μmol of 6-phosphogluconate to ribulose-5-phosphate and CO₂ per min at 37°C at pH 7.4; diaphorase, one unit is the amount of enzyme which will oxidize 1.0 μmol of
$\beta$-NADH with the corresponding reduction of 2,6-dichlorophenol
-indophenol per min at 25°C at pH 7.5.

**Chemicals** $\beta$-NADP$^+$ (monosodium salt, synthetic from
yeast $\beta$-NAD$^+$), $\beta$-NADPH (tetrasodium salt, by chemical
reduction of $\beta$-NADP$^+$), ATP (dipotassium salt),
6-phosphogluconic acid (trisodium salt), glucose-6-phosphate,
bovine serum albumin, and 2-methoxyethanol methyl cellulose
were purchased from Sigma Chemical Co.

Resorufin and resazurin were purchased from Eastman
Organic Chemicals, Rochester NY, 14650.

The following compounds were purchased from Fisher
Scientific Co., Fair Lawn NJ, 07410: tris(hydroxymethyl)-
aminomethane; magnesium chloride; zinc chloride; calcium
chloride; disodium ethylenediaminetetraacetate; hydrochloric
acid; sodium hydroxide; sodium carbonate; sodium potassium
tartrate; copper sulfate; Folin and Ciocalteu's phenol
reagent. These chemicals were reagent grade.

Siliclad® was obtained from Clay Adams, Division of
Becton Dickinson and Co., Parsippany NJ, 07054.

Ammonium heparin was purchased from Lancer, Sherwood
Medical Industries, St. Louis MO, 63103.

Centriflow® membrane cones, type CF25 were purchased
from Amicon Canada Ltd., Oakville ON, L6H 2B9.

**Glassware:** Pyrex glassware and borosilicate test tubes
were used throughout the study. It was observed that if
detergents are used to clean the glassware, a high background fluorescence of the reagents was experienced. With the subsequent soaking in a hydrochloric acid bath for 24 h followed by a detergent-free rinse, this problem disappeared.

Water: Deionized distilled water obtained using a Barnstead mixed bed ion-exchange column from Barnstead, Boston MA, 02132, was used throughout the study.

2. Apparatus

All fluorescence measurements and spectral scans were made using 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 ON, M3N 1Y4. A camera polarizing filter, F. Wansborough's Camera Shop Ltd., Windsor ON, was placed over the emission aperture and this helped to reduce the noise experienced at the very high sensitivity settings.

For weights above one gram, a Mettler PC 4400 Delta Range electronic balance was used. For weights below one gram, a Mettler H16 semiautomatic balance, Fisher Scientific Co., was used.

The micropipettors used in this study were Gilson Pipetman Models P-200 D and P-1000 D with disposable pipette tips C 20 and C 200 available from Mandel Scientific Co.
L Ltd., Ville St. Pierre PQ, HBR 1A3.

A Sorvall superspeed RC2-B automatic refrigerated centrifuge with a type SS-34 60 head (radius = 10.63 cm), Ivan Sorvall Inc., Newton CT, 06470, was used for all the centrifugation steps associated with the Amicon cone separations.

3. Reagents

Tris-HCl buffer: This solution was prepared to be 0.1 M, pH 8.0. This reagent was stable for several weeks when stored at room temperature.

Adenosine triphosphate standard: A stock solution of ATP, 1.0 mM in Tris-HCl buffer was prepared from dipotassium adenosine triphosphate. This solution was stable for 8 h at 4°C. Just prior to use, working standards were prepared by serial dilution of the stock solution to give calculated final concentrations in the cuvet as required. These working standards were kept on ice until ready for use.

Resazurin solution: This solution was prepared to be 0.2 mM in cellosolve. This reagent was stable for several months when stored at room temperature in the dark. A slow conversion to resorufin was observed when this solution was left exposed to light and during the analysis this solution was kept in the dark until ready for use.

Hexokinase solution: This solution was prepared to
contain 250 U. mL\(^{-1}\) in Tris-HCl buffer and the reagent was stable for several weeks at 4°C.

Glucose-6-phosphate dehydrogenase solution: This reagent was prepared to contain 250 U. mL\(^{-1}\) in Tris-HCl buffer and was stable for several weeks at 4°C.

6-Phosphogluconic acid dehydrogenase solution: This reagent was used directly as supplied by the manufacturer as a suspension in ammonium sulfate, 27 U. mL\(^{-1}\).

Diaphorase solution: This solution was prepared to contain 2 U. mL\(^{-1}\) in a 5% (w/v) bovine serum albumin solution which was prepared in a Tris-HCl buffer. This reagent was stable for 8 h at 0°C.

4. Methods

Immediately prior to use the following solutions were prepared:

<table>
<thead>
<tr>
<th>Solution E</th>
<th>G6P-DH (250 U. mL(^{-1})) 200 μL (0.82 U. mL(^{-1}))</th>
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<tr>
<td></td>
<td>6-PGDH (27 U. mL(^{-1})) 407 μL (0.18 U. mL(^{-1}))</td>
</tr>
<tr>
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<td>Tris-HCl buffer 393 μL (0.10 M)</td>
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<table>
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<tr>
<th>Solution S</th>
<th>Glucose 55 mg (10 mM)</th>
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<td></td>
<td>NADP(^+) 10 mg (0.10 mM)</td>
</tr>
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<td></td>
<td>MgCl(_2) 62 mg (10 mM)</td>
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<tr>
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<td>Tris-HCl buffer 2 mL (0.10 mM)</td>
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<table>
<thead>
<tr>
<th>Solution HK</th>
<th>Hexokinase (250 U. mL(^{-1})) 244 μL (1.0 U. mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl buffer 756 μL (0.10 M)</td>
</tr>
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</table>

The amounts shown are sufficient for a 20-tube assay and the final cuvet concentrations of the various components are
shown in parentheses on the right. The solutions E, S, HK, and diaphorase were prepared fresh just prior to each analytical batch and kept in an ice-bath during the analysis.

The fluorometer was allowed to warm up for an hour before use. At routine intervals, checks on the linearity of the instrument were performed using quinine sulfate (0.04 μg mL⁻¹ in 0.05 M H₂SO₄) at an excitation wavelength of 365 nm and an emission wavelength of 448 nm.

The following represent instrument settings for a typical run:

<table>
<thead>
<tr>
<th>Setting</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% adjustment knob</td>
<td>variable</td>
</tr>
<tr>
<td>100% adjustment knob</td>
<td>fully clockwise</td>
</tr>
<tr>
<td>sensitivity control</td>
<td>x 7</td>
</tr>
<tr>
<td>selector switch</td>
<td>x 10</td>
</tr>
<tr>
<td>analyzer wavelength control</td>
<td>580 nm</td>
</tr>
<tr>
<td>exciter wavelength control</td>
<td>560 nm</td>
</tr>
</tbody>
</table>

It was necessary to construct scales for the 0% and the 100% adjustment knobs to allow the exact duplication of instrument settings from run to run.

The protocol for the determination of ATP was as follows: Into a 1-cm by 1-cm quartz cuvet, 2.5 mL of Tris-HCl buffer were pipetted. To this solution, 200 μL of solution S, 50 μL of solution E, 100 μL of diaphorase solution, and 100 μL of resazurin solution were added. (The order of addition was found to be unimportant). Fifty microlitres of the sample or standard were added and to initiate the reaction, 50 μL of the hexokinase solution were
added. The mixture was then rapidly mixed by inversion, transferred to the fluorescence spectrophotometer and the increase in fluorescence recorded for 5 min at \( \lambda \) excitation 560 nm, \( \lambda \) emission 580 nm. From the recorder tracing, the slope of the initial part of the curve between 2 min and 4 min was used to calculate the initial rate of production of resorufin. The recorder was started at the moment the hexokinase was added so as to provide a zero time mark.

Blanking was performed as follows: The reagent blank consisted of resazurin, all the substrates, and enzymes including HK. In this blank the standard was substituted by using distilled deionized water. For the sample blank, the sample was added to the reagents but HK was substituted by using distilled deionized water. In a separate determination in which the HK was added, the total reading for the sample was obtained. The initial rate of production of resorufin for the sample blank or reagent blank was subtracted from the total reading of the sample or standard, respectively.

B. CLINICAL INVESTIGATION

1. Materials

All materials were those used for the ANALYTICAL INVESTIGATION described in CHAPTER IIA, p. 10.
2. Apparatus

Spectrophotometric measurements were performed using a Shimadzu model UV 240 Recording Spectrophotometer, Tekscience, Oakville ON, L6J 5E9.

All platelet counts were performed using a Coulter S-Plus II counter, Coulter Electronics Inc., Hialeah FL, 33101.

Vacutainers (EDTA), Becton Dickinson #4739, available from Fisher Scientific Company were used for the collection of blood samples. Plastic test tubes (10 x 75 mm) were obtained from Fisher Scientific Company. Other apparatus and glassware were as described for the ANALYTICAL INVESTIGATION in CHAPTER IIA, p. 12.

3. Reagents

All reagents used were those described for the ANALYTICAL INVESTIGATION in CHAPTER IIA, p. 13.

4. Specimen Collection

The "normal" samples used in the clinical study were split specimens obtained from blood samples drawn for routine analytical purposes. This obviated the need for patient authorization. Patient confidentiality was assured by the use of a sample identification system that was different from that used by the hospital laboratory.

For the study of "normal" individuals, EDTA whole blood was obtained from 50 subjects. These samples were obtained
within 3 h of collection, during which time the specimens were kept at 4°C. They were obtained from the hematology Laboratory, Windsor Western Hospital Centre, Windsor, Ontario. The "normal" individuals were both hospitalized and ambulatory patients, and were all believed to be free of any disease that might alter platelet ATP levels. Comparison studies with a reference method were performed on 25 of these subjects.

5. Methods

During the determination of ATP in "normal" samples, the Centriflo membrane cones were used exactly according to the manufacturer's instructions. The cones were preconditioned by soaking in distilled deionized water for at least 1 h. In order to avoid possible dilution of the sample, any water present inside the cones was removed by aspiration followed by centrifugation at 900 x g for 15 min. After use the cones were reconditioned by allowing them to soak in 5% (w/v) NaCl for at least 1 h followed by at least 3 centrifugations with distilled deionized water. The cones were stored in 10% (v/v) ethanol to avoid bacterial decomposition of the membrane. Prior to reuse they were treated as above.

Platelet protein was estimated using the method of Lowry et al. (18). All transfers of platelet containing material were done using siliconized Pasteur pipets or plastic pipets,
and into siliconized glass or plastic tubes. This was to avoid any possible destruction of the platelets.

The evaluation of ATP levels in platelets from "normals" is outlined in Fig. 4.
FIGURE 4

OUTLINE OF THE PROCEDURE USED IN THE "NORMAL" STUDY

Legend

Shown is a scheme illustrating the steps involved in preparing a sample for analysis.

See APPENDIX A for a sample calculation of reported ATP levels in platelets.
FIGURE 4

Whole Blood

EDTA Plasma

Centrifugation at 150 x g for 20 min

Platelet Enriched Plasma

Platelet Free Plasma Platelet Pellet

2 mL of 0.85% saline added and lightly mixed
Centrifugation at 12000 x g for 10 min

Discarded

Supernatant Platelet Pellet

2 mL of 0.85% saline added and lightly mixed
Centrifugation at 12000 x g for 10 min

Discarded

Supernatant Platelet Pellet

Frozen at -20°C until use

Discarded

Platelet Pellet

Pellet thawed and 0.5 mL of water added to lyse

Platelet Lysate

Vigorously vortex mixed

Platelet Protein Measured

Lysate ultrafiltered

ATP in Platelet Lysate
Ultrafiltrate measured
CHAPTER III

RESULTS AND DISCUSSION

A. ANALYTICAL INVESTIGATION

1. Preliminary Studies

Figures 5 and 6 show the excitation and emission spectra of resazurin and resorufin. The wavelengths of maximum excitation and emission are in agreement with the literature values of \( \lambda_{\text{emission}} \) 580 nm, \( \lambda_{\text{excitation}} \) 560 nm for resorufin. At these wavelengths it can be seen that there is very little resazurin contribution to the resorufin signal leading to a low reagent blank fluorescence arising from this source. The fluorometer has a spectral bandwidth of 10 nm so that even though there is only a 20 nm difference between the excitation and emission wavelengths, very little of the exciting energy should be passing to the detector. Further, a polarizing filter placed over the emission aperture, helped reduce the possibility of any light reaching the detector other than the emitted fluorescence.

Studies were undertaken to check the linearity of the fluorometer. A quinine sulfate reference material (19) was used and the instrument was linear in the concentration range of 20 to 100 mg.L\(^{-1}\) quinine sulfate (Fig. 7).

The facility of a constant temperature cuvet holder was
FIGURE 5

EXCITATION SPECTRA OF RESAZURIN AND RESORUFIN

Legend

Shown are the excitation spectra of resazurin and resorufin. The solvent used was Tris-HCl (0.1 M, pH 8.0). The cuvet concentrations of both resazurin and resorufin are 6.7 μM. Sensitivity setting = 6. Selector setting = 10.

The λ maximum excitation for resorufin (560 nm) is in good agreement with the literature value.

■■■■ = resazurin

--- = resorufin
FIGURE 6

EMISSION SPECTRA OF RESAZURIN AND RESORUFIN

Legend

Shown are the emission spectra of resorufin and resazurin. The solvent used was Tris-HCl (0.1 M, pH 8.0). The cuvet concentrations of both resazurin and resorufin are 6.7μM. Sensitivity setting = 6. Selector setting = 10.

The λ max emission for resorufin (580 nm) is in agreement with the literature value.

- - - - - = resazurin

- - - = resorufin
FIGURE 7

QUININE SULFATE CALIBRATION

Legend

Shown is a linearity study of the fluorometer using quinine sulfate as a calibration standard. The emission spectra of quinine sulfate standards (20 - 100 mg.L\(^{-1}\)) are shown. \(\lambda_{\text{excitation}} = 350\) nm. Sensitivity setting = 7. Selector setting = 1.

The fluorometer was linear in response over the concentration range 20 - 100 mg/L of quinine sulfate in 0.05 M H\(_2\)SO\(_4\).

The regression line for the calibration curve is \(y = 1.0 \times \).
not available during this study; however, temperature measurements made inside the cuvet compartment indicated that after a 1 h warmup period the temperature inside the cuvet compartment remained relatively constant. Since there was only a small temperature differential (2°C) between the temperature of the cuvet compartment and room temperature, very little heat transfer would be expected to occur during the 5-min time period the reaction is monitored. In addition, any effect on the performance of the method due to temperature would be minimal since the blank, standards, and sample were run under identical conditions.

The initial phase of this study consisted of the reconstruction of the entire assay system, in particular the diaphorase step starting from NADPH (Scheme 1).

1. ATP + Glucose $\rightarrow$ ADP + glucose-6-phosphate
2. Glucose-6-phosphate + NADP$^+$ $\rightarrow$ 6-phosphogluconic acid + NADPH
3. 6-Phosphogluconic acid + NADP$^+$ $\rightarrow$ NADPH + ribulose-5-phosphate + CO$_2$
4. NADPH + resazurin $\rightarrow$ NADP$^+$ + resorufin

SCHEME 1

Solutions of diaphorase in buffer or water are remark-
ably unstable, a feature previously reported (20, 21). A rapid loss of activity was experienced with such solutions at room temperature within 3 h of preparation. It was found that the stability was greatly enhanced if the diaphorase solution was prepared in a 5% (w/v) bovine serum albumin solution and indeed, when kept on ice, these solution were stable for at least 8 h. During an experimental run, however, standards were run at frequent intervals as an additional check on the diaphorase stability.

During the development of the assay system, it became apparent that the sensitivity of the assay was limited by a relatively large increase in fluorescence of the reagents with time. This prompted an investigation into the nature of this increase in fluorescence. The increase in fluorescence of the reagents appeared to originate in the early stages of the assay system. A series of experiments were performed in which substrates, enzymes, and cofactors were sequentially added and the change in fluorescence with time monitored. These experiments indicated that adenosine triphosphate was responsible for the increase in fluorescence. This occurs not only as a result of the fact that the reagents contain endogenous ATP but also the glassware, pipette tips, and cuvets may also be contaminated by ATP. For this reason, special care was taken to clean the equipment before use. The technique used was to soak the equipment in 2 M hydrochloric
acid and then thoroughly wash with previously boiled deionized distilled water. Care was taken also in the performance of the assay not to contaminate the equipment with somatic cells from the fingertips or the breath since these cells contain relatively large amounts of ATP, and may increase the interference problem of background ATP.

As previously demonstrated (22), one important factor that determines the performance of high sensitivity ATP assays is the quality of the water used in preparing the reagents. It was observed that the use of sterile water significantly reduced the blank value. During routine work, however, the use of previously boiled deionized distilled water that was kept in glass containers, together with the precautions mentioned above, kept the endogenous ATP interference problem to within tolerable limits.

The reconstructive experiments were designed to examine the rate of the reaction for each step in the coupled system with respect to varying concentrations of enzymes and substrates. It was verified that for all the enzymes in the coupled assay, the pH optimum is in the range 7.5 - 8.0, indicating that Tris-HCl buffer (pH 8.0) was appropriate. The overall system was constructed so that only the concentration of ATP would be rate determining. This necessitated adjusting the concentration of substrates to approximately 100 times the Km of the respective enzymes. The activity of the enzymes
in steps 1-3 (Scheme 1), were adjusted so as to achieve maximum rates, while at the same time, for economy purposes, a large excess of enzyme was avoided. All of these steps were so evaluated starting with their respective reactants at concentrations equivalent to the range of ATP standards used. In step 4 (Scheme 1) the diaphorase activity was chosen so as to provide rates that enabled their recording over a reasonable time interval (5 min), and that were not so fast as to prohibit their calculation from the recording. With the enzymes in steps 1-4 operating at maximum rates, and all the substrates at roughly 100 times their Km, the rate limiting factor becomes the concentration of ATP, and the rate determining step, becomes step 1. Table I shows a list of optimum concentrations for all the enzymes and substrates used in the coupled assay.

In the development of the assay it became necessary to be able to predict the magnitude of the signal for varying concentrations of resazurin and of the other components in the assay. A study was undertaken to devise a means of doing this and use was made of the constant referred to as the fluorescence coefficient. The fluorescence coefficient is defined as the ratio of the fluorescence in units observed on the fluorometer to the molar concentration (23). Two aspects became apparent. The first is that a comparison showed the fluorescent coefficient value for resorufin to be $2.51 \times 10^6$
**TABLE I**

OPTIMUM CONCENTRATIONS FOR THE REAGENTS USED IN THE COUPLED ASSAY

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10 mM</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>10 mM</td>
</tr>
<tr>
<td>TRIS-HCl buffer, pH 8.0</td>
<td>0.1 M</td>
</tr>
<tr>
<td>G6P-DH</td>
<td>0.02 U.mL$^{-1}$</td>
</tr>
<tr>
<td>6-PGDH</td>
<td>0.18 U.mL$^{-1}$</td>
</tr>
<tr>
<td>HK</td>
<td>1.0 U.mL$^{-1}$</td>
</tr>
<tr>
<td>Diaphorase</td>
<td>0.066 U.mL$^{-1}$</td>
</tr>
<tr>
<td>Resazurin</td>
<td>0.0065 mM</td>
</tr>
</tbody>
</table>

$^a$ Final concentration in cuvet after addition of all reagents. The enzyme unit definitions were as described in CHAPTER IIA, p.10.
versus $3.4 \times 10^3$ for NADPH and $7.6 \times 10^4$ for quinine.

Therefore, there is an enhancement in the signal by a factor of 1000-fold if resorufin is measured compared to the native fluorescence of NADPH. This factor determined the theoretical limits for the increase in sensitivity that is possible with this method. Table II shows a comparison between previous methods and the proposed method for the lower limits of determination of ATP.

Secondly, this study enabled one to predict within reasonable limits the approximate signal intensity for various concentrations of resorufin producing substances. This facilitated the choosing of instrument settings so as to obtain the highest possible signal to noise ratio.

2. Blanking

Blanking in the method was achieved using both a reagent blank and a sample blank. Each specimen had a unique sample blank in which water was substituted for the HK. Since the sample blank had its own rate of increase of fluorescence, it was essential that the time interval over which the measurement was made be the same for the sample blank and the sample. It was found that the optimum time interval was between 2 min and 4 min. At times earlier than 2 min, the sample blank was still very large and this tended to decrease the precision with which the differences between the blank value and the sample value could be obtained. Zero time was
# TABLE II

**LIMITS FOR THE DETERMINATION OF ADENOSINE TRIPHOSPHATE**

<table>
<thead>
<tr>
<th>Method principle</th>
<th>Lower limit pmol</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>250</td>
<td>11</td>
</tr>
<tr>
<td>Isotachophoresis</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Radioenzymatic</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>$10^a$</td>
<td>6</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>$10^a$</td>
<td>8</td>
</tr>
<tr>
<td>Chemical</td>
<td>$10^a$</td>
<td>12</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>$10^a$</td>
<td>10</td>
</tr>
<tr>
<td>Bioluminescent</td>
<td>1.0</td>
<td>14</td>
</tr>
<tr>
<td>Proposed method</td>
<td>3.1</td>
<td></td>
</tr>
</tbody>
</table>
taken to be the time of mixing the HK or water with the reagent and sample.

Figure 8 shows how actual data is obtained from recorder tracings while Fig. 9 shows a typical calibration curve.

3. Interferences

The effects of EDTA and heparin as anticoagulants were also evaluated. EDTA was found to have only minimal effect on the diaphorase step of the assay with a less than 1% lowering of the reaction rate (Table III). Heparin was found to have no effect on the assay when present in concentrations approaching that required for its use as an anticoagulant.

The assay setup included a sample requirement of 50 µL. To accommodate smaller sample sizes that could be used in the assay protocol, predilutions would be required. Dilution experiments indicated that the platelet lysate could be diluted up to 5-fold without any significant change in the determined value. Furthermore, the recovery experiments indicated that platelet lysate matrix interferences in the assay are minimal.

4. Recovery

Recovery studies were performed by supplementing pooled platelet lysates with various concentrations of ATP and assaying replicates of 3. These studies differed from the usual type of investigation in that the efficiency of the ultrafiltration process was also evaluated. Three levels
FIGURE 8

RECORER TRACINGS FOR ADENOSINE TRIPHOSPHATE
CALIBRATION CURVE

Legend

Shown are the recorder tracings used to plot an ATP calibration curve in the range 10 - 100 pmol. Sensitivity setting = 9. Selector setting = 10.

A: 10.0 pmol
B: 32.5 pmol
C: 55.0 pmol
D: 77.5 pmol
E: 100.0 pmol
F: 0 pmol

The tracings were made using the standard assay protocol (CHAPTER IIA, p. 15).
FIGURE 9

ADENOSINE TRIPHOSPHATE CALIBRATION CURVE

Legend

Shown is a calibration curve for adenosine triphosphate for the entire range over which the method is applicable. In routine use only a part of this range (0.31 - 3.1 nmol) was used. The fluorometer setting for this part of the curve was sensitivity = 9, selector = 10.

The calibration curve was constructed using aqueous standards that were run according to the standard assay protocol (CHAPTER IIA, p. 15).

The equation of the line of best fit is $y = 0.15x + 0.27$. 
TABLE III

EFFECT OF EDTA ON THE DIAPHORASE REACTION

<table>
<thead>
<tr>
<th>NADPH&lt;sup&gt;a&lt;/sup&gt; x 10&lt;sup&gt;-9&lt;/sup&gt; M</th>
<th>EDTA present&lt;sup&gt;b&lt;/sup&gt; ΔF.min&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>EDTA absent ΔF.min&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>10.3</td>
<td>10.5</td>
</tr>
<tr>
<td>5.0</td>
<td>13.5</td>
<td>13.7</td>
</tr>
<tr>
<td>7.5</td>
<td>16.6</td>
<td>16.9</td>
</tr>
<tr>
<td>10.0</td>
<td>19.8</td>
<td>20.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentrations are those in the cuvet.

<sup>b</sup> EDTA concentration = 3.18 x 10<sup>-3</sup> M.
of ATP supplement were added both before and after the ultrafiltration of the platelet lysates. The results are summarized in Table IV and the recoveries were found to be quantitative with no detectable loss of ATP occurring during the ultrafiltration procedure.

5. Precision

To evaluate the within-run and day-to-day reproducibility of the assay, three platelet lysate pools were obtained and ultrafiltrates prepared from these. To minimize the possibility of hydrolysis leading to a change in ATP levels from batch to batch during the between-run studies, the entire evaluation was done over 3 days and the ultrafiltrates were frozen at -20°C until required. Table V summarizes the results. For each pool, both the within-run and between-run precision were found to have coefficients of variation of less than 3%.

B. CLINICAL INVESTIGATION

For this study, ATP levels in platelets of "normal" individuals were determined (CHAPTER IIB, p. 16). These individuals were chosen at random and were believed to be free of any condition that might alter platelet ATP levels. No attempt was made to ascertain circadian status nor was the sex of the individual noted.

Table VI shows a list of the raw data for the "normal" individuals. Figures 10 and 11 show histogram plots of this
<table>
<thead>
<tr>
<th>Expected (nmol)</th>
<th>Recovered (nmol)</th>
<th>% recovery</th>
<th>Expected (nmol)</th>
<th>Recovered (nmol)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.0</td>
<td>24.8</td>
<td>99.1 ± 0.23</td>
<td>25.0</td>
<td>24.8</td>
<td>99.1 ± 0.23</td>
</tr>
<tr>
<td>2.50</td>
<td>2.46</td>
<td>98.4 ± 0.36</td>
<td>2.50</td>
<td>2.47</td>
<td>98.8 ± 0.30</td>
</tr>
<tr>
<td>0.250</td>
<td>0.243</td>
<td>97.2 ± 0.56</td>
<td>0.250</td>
<td>0.245</td>
<td>98.0 ± 0.51</td>
</tr>
</tbody>
</table>

a All analytical data reported are the mean of three replicate analyses.

b % recovery given as the mean ± S.D.
TABLE V

PRECISION STUDY

<table>
<thead>
<tr>
<th>Platelet pool</th>
<th>Mean (nmol/10⁶ pltts)</th>
<th>S.D. (nmol/10⁶ pltts)</th>
<th>S.E.M. (nmol/10⁶ pltts)</th>
<th>C.V. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.36</td>
<td>0.059</td>
<td>0.015</td>
<td>2.50</td>
</tr>
<tr>
<td>B</td>
<td>1.71</td>
<td>0.031</td>
<td>0.008</td>
<td>1.80</td>
</tr>
<tr>
<td>C</td>
<td>2.06</td>
<td>0.050</td>
<td>0.013</td>
<td>2.40</td>
</tr>
<tr>
<td>Between-run</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.35</td>
<td>0.070</td>
<td>0.029</td>
<td>2.98</td>
</tr>
<tr>
<td>B</td>
<td>1.72</td>
<td>0.032</td>
<td>0.013</td>
<td>2.40</td>
</tr>
<tr>
<td>C</td>
<td>2.06</td>
<td>0.056</td>
<td>0.023</td>
<td>2.72</td>
</tr>
</tbody>
</table>

a Each platelet pool was assayed fifteen times.

b Each platelet pool was assayed in duplicate in six analytical batches.
### TABLE VI

STUDY OF "NORMAL" PLATELET LEVELS OF
ADENOSINE TRIPHOSPHATE

<table>
<thead>
<tr>
<th>ID</th>
<th>PERPC</th>
<th>PERPV</th>
<th>PLATNO</th>
<th>PLATPROT</th>
<th>PLATATP</th>
<th>ATPNGPRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>591</td>
<td>3.65</td>
<td>2.16</td>
<td>0.286</td>
<td>2.49</td>
<td>18.15</td>
</tr>
<tr>
<td>2</td>
<td>699</td>
<td>3.20</td>
<td>2.64</td>
<td>0.283</td>
<td>4.38</td>
<td>18.46</td>
</tr>
<tr>
<td>3</td>
<td>550</td>
<td>3.75</td>
<td>1.53</td>
<td>0.395</td>
<td>1.99</td>
<td>7.72</td>
</tr>
<tr>
<td>4</td>
<td>350</td>
<td>3.55</td>
<td>1.17</td>
<td>0.160</td>
<td>2.61</td>
<td>19.06</td>
</tr>
<tr>
<td>5</td>
<td>146</td>
<td>3.50</td>
<td>0.51</td>
<td>0.266</td>
<td>2.39</td>
<td>4.59</td>
</tr>
<tr>
<td>6</td>
<td>433</td>
<td>3.05</td>
<td>1.32</td>
<td>0.128</td>
<td>2.55</td>
<td>26.25</td>
</tr>
<tr>
<td>7</td>
<td>487</td>
<td>3.00</td>
<td>1.46</td>
<td>0.183</td>
<td>2.95</td>
<td>23.53</td>
</tr>
<tr>
<td>8</td>
<td>238</td>
<td>3.30</td>
<td>0.79</td>
<td>0.119</td>
<td>2.54</td>
<td>26.25</td>
</tr>
<tr>
<td>9</td>
<td>190</td>
<td>2.00</td>
<td>0.76</td>
<td>0.140</td>
<td>2.01</td>
<td>12.63</td>
</tr>
<tr>
<td>10</td>
<td>635</td>
<td>3.30</td>
<td>2.22</td>
<td>0.265</td>
<td>2.51</td>
<td>21.48</td>
</tr>
<tr>
<td>11</td>
<td>498</td>
<td>3.50</td>
<td>1.46</td>
<td>0.142</td>
<td>2.09</td>
<td>27.59</td>
</tr>
<tr>
<td>12</td>
<td>289</td>
<td>3.30</td>
<td>1.24</td>
<td>0.145</td>
<td>3.23</td>
<td>8.01</td>
</tr>
<tr>
<td>13</td>
<td>562</td>
<td>2.80</td>
<td>1.57</td>
<td>0.191</td>
<td>0.98</td>
<td>12.67</td>
</tr>
<tr>
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</table>
FIGURE 10

HISTOGRAM OF PLATELET ADENOSINE TRIPHOSPHATE LEVELS

Legend

Shown is a histogram for the data obtained for platelet ATP levels in nmol per 10⁶ platelets in "normal" individuals. Values on the horizontal axis represent the midpoints of the intervals. (x̄ = 2.17; S.D. = 0.76; S.E.M. = 0.11; minimum value = 0.94; maximum value = 3.64)
FIGURE 10

PLATELET ATP LEVELS n mol / 10^8 platelets

FREQUENCY

1.5 2.0 2.5 3.0
0.5 1.0 1.5 2.0
FIGURE 11

HISTOGRAM OF ADENOSINE TRIPHOSPHATE LEVELS PER MILLIGRAM PLATELET PROTEIN

Legend

Shown is a histogram of the data obtained for levels of ATP in nmol per milligram platelet protein in "normal" individuals. Values on the horizontal axis represent the midpoints of the intervals. ($\bar{x} = 14.22$; S.D. = 6.32; S.E.M. = 0.89; minimum value = 2.84; maximum value = 27.97)
The mean ± S.D. values of platelet ATP were found to be 2.17 ± 0.70 nmol per 10^9 platelets or 14.22 ± 6.32 nmol per milligram platelet protein. Previously reported studies on platelet ATP levels in "normals" found levels of 2.5 nmol per 10^9 platelets and 1.34 – 2.09 nmol per 10^9 platelets, respectively, (2,24). These values are comparable with those found in this study.

Further examination of the data revealed that for plots of PLATATP (platelet ATP levels in nanomol / 100 million platelets) and ATPMSPROT (platelet ATP levels in nanomol / milligram platelet protein), the data approach that of Gaussian distributions. However, it was decided that a more definitive estimate as to whether or not the data are adequately described by Gaussian or log-Gaussian curves should be made. Some workers believe that even for data that are not Gaussian distributed, the bias introduced by parametric tests is small and that these tests are still valid (25). Despite this, however, in this study the data were first examined with respect to distribution and the statistical methods used to estimate the normal range were selected on this basis.

One goodness of fit test is the Kolmogorov-Smirnov (K-S) test (26). Both the variables PLATATP and ATPMSPROT failed the K-S test indicating the existence of frequency
distributions that deviate significantly from Gaussian. The conclusion, therefore, is that the use of nonparametric methods which do not require that the data are Gaussian distributed is indicated. It should be noted that even the nonparametric methods, while being superior in that they do not require any assumptions about the distributions of the data, give normal range estimates that are lower in precision if the sample sizes are small. However, in the estimation of the normal range both parametric and nonparametric methods were used.

One method of estimating the normal range is the method of parametric or nonparametric tolerance intervals (27). The tolerance interval is an interval that includes a specified proportion, \( P \), of the population with a specified probability, \( \gamma \). The constant, \( k \), depends on the sample size and is related to the tolerance interval. The probability that 95\% or more test results in the normal population are between \( x + k \) S.D. and \( x - k \) S.D. is \( \gamma \). Tables that give an estimate of \( k \) for various sample sizes and various values of can be found in the literature (28).

When the \( k \)-factor method was applied to the data and a Gaussian distribution was assumed, the normal range estimated was 0.44 - 3.91 nmol / 10^9 platelets and 0.22 - 28.66 nmol / mg platelet protein for PLATATP and ATPGPROT, respectively. The 95\% nonparametric tolerance interval gave an estimate of
the normal range of 0.94 - 3.64 nmol / 10⁶ platelets and 2.84 - 27.97 nmol / mg platelet protein, for PLATATP and ATPMGPROT, respectively.

The proposed method was compared with the method of Lamprecht and Trautschold (8). Figure 12 shows a plot relating the two methods of determining ATP. The correlation coefficient, r, was 0.99 indicating a significant correlation between the two methods. The data passed both the t-test and the F-test.

The data obtained for this study were checked for homogeneity and found to contain no outliers. Nonparametric tests are especially vulnerable to distortion if the data are contaminated. A nonparametric test of comparison of data is the sign test (29). Using this test with a 5% level of significance, no difference between the two methods was found.
FIGURE 12

CORRELATION PLOT BETWEEN THE PROPOSED METHOD
AND THE LAMPRECHT AND TRAUTSCHOLD METHOD

Legend

Shown is a plot of the proposed method against the Lamprecht and Trautschold method. The equation of the line of best fit is \( y = 1.053x - 0.059 \). Standard Error of the Estimate = 0.48. \( n = 40 \).
FIGURE 12.
CHAPTER IV

SUMMARY AND CONCLUSIONS

The determination of adenosine triphosphate in tissues such as platelets require the use of methods that provide very high sensitivity. Those methods that do provide such sensitivity are often time consuming. The radioenzymatic methods, for example, are tedious and are of limited usefulness in a clinical laboratory.

The sensitivity of the bioluminescent methods for the determination of ATP, using specialized instrumentation has been unrivalled. The fluorometric coupled enzymatic assay presented in the text can, however, yield comparable sensitivity. The proposed assay combines high sensitivity with a low coefficient of variation. The method is also considerably less expensive than the bioluminescent assay.

The high sensitivity attainable obviates the need for amplification techniques used in previous enzymatic assays (5). This high sensitivity allows not only the use of reduced platelet sample sizes, but also a possible dilution of the sample so as to reduce the effect of any interferences that may exist.

Further studies are warranted to assess the applicability of the proposed method to other tissues and
body fluids. The use of this method to determine ATP levels in urine as a rapid screen for bacteriuria is one such example.
APPENDIX

SAMPLE CALCUALTION OF REPORTED ADENOSINE TRIPHOSPHATE LEVELS IN PLATELETS

Data:

PERP platelet count (PERPC) = $7.55 \times 10^5$ / mm$^3$

Volume of PERP used for making the pellet (PERPV) = 0.80 mL

ATP found in 50 µL of platelet lysate (ATP) = $2.09 \times 10^{-9}$ mol

Protein found in 50 µL of platelet lysate (PLATPROT) = 0.296 mg

Calculation:

(1) PERPC x $10^3$ = PERPC in units / mL.

(2) PERPC x PERPV = number of platelets in pellet.

(3) Since pellet lysed in 0.5 mL water, number of platelets in 50 µL lysate =

$$\frac{\text{number of platelets in pellet} \times 1}{0.5} = 20$$

(4) Using the above data, number of platelets in 50 µL lysate (PLATNO) =

$$\frac{7.55 \times 10^5 \times 10^3 \times 0.8 \times 1}{0.5} = 0.60 \times 10^9 \frac{0.5}{20}$$

(5) Since 50 µL lysate contained $2.09 \times 10^{-9}$ mol ATP, then $0.60 \times 10^9$ platelets contained $2.09 \times 10^{-9}$ mol ATP, and $10^9$ platelets contained $2.09 \times 10^{-9}$

or $3.48 \times 10^{-9}$ mol ATP.

(6) Since 0.296 mg platelet protein was found in 50 µL lysate, and $2.09 \times 10^{-9}$ mol ATP was found in 50 µL lysate then 0.296 mg platelet protein contained $2.09 \times 10^{-9}$ or $7.1 \times 10^{-9}$ mol ATP.
REFERENCES


PART II

COLORIMETRIC DETERMINATION OF NON-ENZYMATICALLY GLYCATED ALBUMIN
CHAPTER I

INTRODUCTION

A. PATHOPHYSIOLOGY OF GLYCATED PROTEINS

The main mechanisms of blood glucose control exist in the pancreas where specialized islet cells secrete the polypeptide hormones insulin and glucagon. The hormone insulin, secreted by the islet cells, acts to lower blood glucose levels by promoting the entry of glucose from plasma to inside the cells. It also suppresses the secretion of glucagon by the islet cells. Glucagon acts in an opposite fashion by elevating blood glucose and is, therefore, called a counterregulatory hormone. Two other counterregulatory hormones, cortisol and epinephrine also elevate blood glucose levels.

In the normal individual, the concerted actions of insulin and the counterregulatory hormones maintain blood glucose levels within narrow limits (3.6 – 8.1 mM) despite wide fluctuations in carbohydrate intake and assimilation. In the disease diabetes mellitus, this careful balance is impaired and excessively high glucose levels circulate in the blood.

Diabetes mellitus is a serious incurable disease with
many different causes. It is of unknown origin with perhaps hereditary as well as environmental characteristics. It usually occurs as a result of the body being unable to produce enough insulin or not utilizing the insulin that is produced. Abnormally high concentrations of plasma glucose result with subsequent excretion in the urine (1).

Prior to the discovery of insulin and its subsequent use in the management of diabetes, death usually resulted from the acute symptoms of the disease, notably ketoacidosis. With the use of insulin therapy the long-term survival of diabetics was permitted. However, chronic complications of diabetes soon became apparent and today these complications are responsible for the major morbidity and mortality associated with the disease. Diabetes is a major cause of blindness, and diabetics are at increased risk of heart disease, stroke, kidney failure, circulatory and immune disorders (1).

In recent years it has become apparent that these chronic complications of diabetes are a result of chronic hyperglycemia even in a "well" controlled diabetic. As blood glucose control is made more precise approaching the physiological tight control in normal individuals, the secondary complications of diabetes cease to progress and in some cases may even reverse (2). Two main theories exist to account for the correlation between the chronic complications
of diabetes and chronic hyperglycemia. The first is that the tissues most likely to be damaged are those that are freely permeable to glucose unlike muscle and fat which require insulin. Inside these cells, the high levels of glucose participate in two reactions that are of little consequence at normal concentrations of glucose:

\[
\text{Glucose} \xrightarrow{\text{aldose reductase}} \text{sorbitol} \xrightarrow{\text{sorbitol dehydrogenase}} \text{fructose}
\]

Both sorbitol and fructose are impermeable to the cell membrane and accumulate inside the cell, while glucose continues to diffuse inward because its concentration gradient is unchanged. The buildup of sorbitol and fructose acts osmotically to draw water into the cell which suffers damage as it swells.

The second theory holds that a direct addition of aldose sugars to protein amino groups alters protein structure significantly to adversely affect cell physiology (2). This process is termed non-enzymatic glycation, and a simplified reaction sequence involving glucose is shown in Fig. 1. Both theories are believed to be operative. However, the protein glycation mechanism is of greater significance to clinical chemists because it forms the basis of several laboratory tests that are finding increased use in the management of diabetes mellitus.

A wide variety of proteins owe many of their functional
FIGURE 1

OUTLINE OF THE NON-ENZYMATIC GLYcation PROCESS

Legend

Shown is an outline of the sequence of reactions involved in the process of non-enzymatic glycation. The example shown involves glucose. Other sugars can undergo this reaction as well.

The initial step involves the condensation of an amino moiety with the aldehyde form of a particular sugar to form a Schiff base. This Schiff base can subsequently rearrange to form a more stable ketoamine.
properties to the covalent attachment of carbohydrates to certain residues in the polypeptide chain. These specific modifications are generally under precise enzymatic control. In contrast, the non-enzymatically catalyzed attachment of carbohydrates to protein amino groups can also occur (3).

The Joint Commission on Biochemical Nomenclature defines glycation as any reaction that links a sugar to a protein whether it is enzymatically catalyzed or not (4). The process shown in Fig. 1 involves non-enzymatically catalyzed reactions (3). This process in which a Schiff base is first formed followed by an Amadori rearrangement to an amino-linked 1-deoxy-fructose derivative is a specific kind of glycation. Although this is correctly termed non-enzymatic glycation, it has been commonly referred to as glycosylation and in the case of glucose, glucosylation (5). It differs from enzyme-catalyzed glycoprotein formation not only in that the process is not enzyme-catalyzed but also in other ways. The groups on the protein to which the sugar is attached are usually the ε-amino group of lysine or the N-terminal amino acid. In glycoside formation, the covalent glycosidic linkages are formed via O or N and the groups involved are never the ε-amino group of lysine or the N-terminal amino acid. In diabetes it is the level of non-enzymatically glycated proteins that is increased rather than that formed by the enzymatically mediated glycation process.
Some of the main characteristics of this process are summarized in Table I. The extent of non-enzymatic glycation is determined by the following factors: length of exposure to the sugar; the sugar concentration; temperature; and pH. With increasing incubation time, temperature, pH, and sugar concentration increasing amounts of non-enzymatically glycated albumin are formed (6). Different sugars are also incorporated at different rates. Under similar conditions, galactose, fucose, and glucose are incorporated in decreasing rates.

Glycation of proteins can, therefore, occur as a non-enzymatic post-translational modification directly dependent upon the prevailing glucose concentration. Glycated protein levels reflect the average serum glucose concentration over the biological half-life of that particular protein and provide a means of monitoring diabetic control.

Table II shows the wide variety of proteins that have been identified as undergoing post-synthetic modification by the non-enzymatic addition of glucose or a glucose derived product. In most instances, the non-enzymatic glycation leads to deleterious structural and functional changes in the protein. These are summarized in Table III.

The most widely studied glycated protein is hemoglobin and its determination is extensively used in the management
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<td><strong>CHARACTERISTICS OF GLYCATED PROTEINS</strong></td>
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<tr>
<td>1.</td>
<td>Formation is post-translational by means of an Amadori reaction.</td>
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<td>2.</td>
<td>The number of exposed valine, lysine, or tyrosine groups determine the ability of a given protein to be glycated.</td>
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<td>3.</td>
<td>The relative concentration of glycated protein is dependent upon the sugar type and concentration versus the exposure time.</td>
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<td>4.</td>
<td>The absolute concentration of glycated protein is dependent on the exposure time versus sugar type and concentration.</td>
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<td>5.</td>
<td>The half-life of the protein determines the time exposed to the sugar. The half-life of the protein is modified by disease states.</td>
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<td>6.</td>
<td>Other factors determining the extent of glycation are: a) pKa of reactive amino groups; b) equilibrium between the open and ring form of the sugar; c) rate of conversion of the initial aldimine linkage to the ketoamine.</td>
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**TABLE II**

PROTEINS UNDERGOING NON-ENZYMATIC GLYCATION

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### TABLE III

**POSSIBLE EFFECTS OF NON-ENZYMATIC GLYCATION OF PROTEINS**

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<td>Stability</td>
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<td>resistance to heat</td>
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<td>resistance to enzyme attack</td>
<td>Cellular uptake</td>
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<td>Size, shape, and viscosity</td>
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<td>Intra- and intermolecular cross-linking</td>
<td>Interaction with other molecules</td>
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of diabetes mellitus (7,8). The N-terminal valine of the chain is the predominant site of sugar attachment in the hemoglobin molecule. The ε-lysyl amino groups are also reported to be possible sites of sugar attachment. Albumin is known not to be a glycoprotein yet, carbohydrate can be detected on the molecule suggesting the existence of the non-enzymatic glycation process (9).

Albumin is quantitatively the most important plasma component and recently there has been a great deal of interest in the determination of glycated albumin (GA).

Because of the slow rate of glycation of hemoglobin and the 120-day lifespan of erythrocytes, levels of glycated hemoglobin are indicative of the time-averaged blood glucose concentrations over a period of months. Glycated hemoglobin, therefore, is useful as an index of long-term (one to two months) glycemic control. At the other extreme, individual plasma glucose concentrations reflect minute-to-minute changes in glucose homeostasis. The half-life of serum albumin is less than that of hemoglobin and changes in the levels of GA should be obtained more rapidly in response to changes in diabetes control. The determination of GA is useful as an index of short-term (two to three weeks) glycemic control (10 - 12).

It has been shown that only the ε-amino groups of lysine residues in serum albumin become non-enzymatically glycated
**in vivo** and **in vitro**. **In vivo**, approximately half of this glycation occurs at the lysine residue 525 which is the second or carboxyl lysine in a lysine-lysine sequence (13). **In vitro**, however, lysine 199 seemed to be the predominant site of glycation.

B. MEASUREMENT OF GLYCATED PROTEINS

Many methods have been developed to determine glycated proteins. These procedures fall into three main groups: chromatographic; electrophoretic; and chemical (14 - 19). Figure 2 summarizes some of the main methodological approaches taken to determine glycated proteins. As previously reported (20), of the many techniques available for the determination of glycated hemoglobin, only two are readily amenable to the routine determination of glycated albumin in the clinical laboratory. In addition, there is no established reference method for determining the extent of non-enzymatic glycation of a protein.

One widely used procedure is the thiobarbituric acid assay (15). In this assay, the stable ketoamine adduct is hydrolyzed under mild acidic conditions with heating for an extended period of time. This hydrolysis releases hydroxymethylfurfural (HMF) which reacts with thiobarbituric acid to yield a colored product, λ maximum 443 nm. This method has some serious drawbacks in that depending on the conditions of hydrolysis used a variable and incomplete
FIGURE 2

METHODS USED TO DETERMINE GLYCATED PROTEINS

Legend

Shown is a summary of some of the principal methods used to determine glycated proteins. The most widely used procedures to determine glycated hemoglobin are affinity chromatography (17, 18) and ion-exchange chromatography (16).

Only aminophenylboronate affinity chromatography (18) and the thiobarbituric acid assay (15) have been applied successfully to the determination of glycated albumin.
**FIGURE 2**

- Affinity Chromatography
- Electrophoresis
- IRC-50
- Bio-Rex 70
- 1-Deoxy-fructosyl protein adduct
- 0.5 M Oxalic acid
  - 6 h 100°C
- S-Hydroxymethyl furfuraldehyde
conversion to HMF is experienced. In addition, it is influenced by the sample matrix and free glucose is also a serious interference. Dialysis of the sample is, therefore, necessary. Nevertheless, experimental conditions for this assay have been standardized and the assay was optimized to determine GA (14, 15).

The newer aminophenylboronate affinity chromatographic procedure (17) is not affected by the presence of the labile glycated fraction, is much simpler to perform, and has been used in the determination of GA (18). This gel has an affinity for all glycated proteins because it reversibly binds glycoproteins and simple molecules containing cis-diol groups. Like the thiobarbituric acid assay, this procedure is affected by glucose which must be removed prior to analysis.

C. THE STUDY

A novel approach to the determination of glycated protein relied on the ability of ketoamines (fructosamines) in alkaline solution to act as reducing agents (21). This simple procedure utilized a tetrazolium salt (nitroblue tetrazolium) as an electron acceptor to form a colored formazan (22 - 24) (Fig. 3). The original "fructosamine assay" possessed many difficulties (22). A synthetic Amadori rearrangement product, 1-deoxy-1-morpholinofructose (DMF), was used as the standard but it did not adequately represent
FIGURE 3

THE "FRUCTOSAMINE ASSAY"

Legend

Shown is an outline of the original "fructosamine assay" (22). The standard used was a synthetic Amadori rearrangement product, 1-deoxy-1-morpholinofructose (DMF).

Under alkaline conditions, DMF was converted to a glycosone with the concomitant reduction of 2,2-di-(p-nitrophenyl)-5,5-diphenyl-3,3-(3,3-dimethoxy-4,4-diphenylene)-dibenzobistetrazolium chloride (NBT) to a colored formazan, λ maximum = 530 nm.
the glycated proteins the assay attempted to measure. In addition, serious interferences from albumin, glucose, and reducing substances present in the serum were experienced (22).

The primary aim of this study was to develop a simple colorimetric method to determine GA. This was accomplished by optimizing the analytical conditions of the "fructosamine assay" for the determination of GA. Sepharose-blue dextran affinity chromatography was used to separate albumin from serum or plasma. The fraction containing only albumin was used for the determination of GA. The assay system developed used the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) which was converted to its formazan by reacting with glycated albumin in alkaline solution (Fig. 4). Optimum conditions for the separation of albumin from serum and the automated colorimetric assay of GA were established.

The clinical applicability of the method developed was demonstrated by determining GA in both a "normal" and a diabetic population.
OUTLINE OF THE PROPOSED METHOD USED TO DETERMINE GLYCATED ALBUMIN

Legend

Shown is an outline of the proposed method used to determine glycated albumin:

I Conversion of the ketoamine to a glycosone:
   Under alkaline conditions, the ketoamine formed by an Amadori rearrangement during glycation is converted to a 1,2-ene-diol. This ene-diol adduct is further rearranged to a glycosone with the liberation of two protons.

II Reduction of the tetrazolium salt to a formazan:
   The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) can be reduced to a colored formazan, $\lambda_{\text{maximum}} = 557\text{nm}$.

   In the proposed method, glycated human serum albumin was used as the standard.
\[
\begin{align*}
\text{H}_2\text{C-} & \text{NH} \beta\text{A} \\
\text{C=O} & \\
\text{HO-CH} & \\
\text{HC-OH} & \\
\text{HC-OH} & \\
\text{CH}_2\text{OH} & \\
\text{ketoamine} & \\
\text{H}_2\text{C-} & \text{NH-} \beta\text{A} \\
\text{C=O} & \\
\text{HO-CH} & \\
\text{HC-OH} & \\
\text{HC-OH} & \\
\text{CH}_2\text{OH} & \\
\text{1,2-ene-diol} & \\
\text{H}_2\text{C-} & \text{OH} \\
\text{HC-OH} & \\
\text{HC-OH} & \\
\text{CH}_2\text{OH} & \\
\text{glycosone} & \\
\end{align*}
\]
CHAPTER II

EXPERIMENTAL

1. Materials

Chemicals:  Human serum albumin, cyanogen bromide activated Sepharose-4B blue dextran, 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT), 2-methoxyethanol methyl cellosolve, bilirubin, hemoglobin, and nitroblue tetrazolium were obtained from Sigma Chemical Co., St. Louis MO, 63178.

Ammonium heparin was obtained from Lancer, Sherwood Medical Industries, St. Louis MO, 63103.

Sepharose-blue dextran affinity medium was prepared exactly as previously described (25). The Affi-gel was then packed into microcolumns (Isolab Inc., Akron OH, 44321) with support discs above and below the gel. Each microcolumn was packed with 1.5 mL of Affi-gel Blue. The columns were reused up to five times, with regeneration between use. For regenerating the columns, the gel was washed with a 6 M urea solution followed by two washings with the equilibrating phosphate buffer.

Agarose gel protein electrophoresis was performed using the Corning ACA I Cassette Electrophoresis System, Corning Medical, Palo Alto CA, 94306.
Radial immunodiffusion was performed using the NOR-Partigen® plate system available from Hoechst Canada Inc., Behring Diagnostics, Montreal PQ, H4R 1R6.

Aminophenylboronate affinity chromatography was performed using a kit obtained from Isolab Inc., Akron OH, 44321.

Glycated Hemoglobin assays were performed by aminophenylboronate affinity chromatography as previously described (17). The kit was obtained from Isolab Inc.

Glucose assays were performed using an o-toluidine method in kit form purchased from Sigma Chemical Co.

All other chemicals were purchased from Fisher Scientific Co., Don Mills ON, M3A 1A9 and were reagent grade.

2. Apparatus

Reagent verifications and manual assays were performed with a Model HP8451A Diode Array Spectrophotometer from Hewlett-Packard Scientific Instruments Division, Palo Alto CA, 94304.

Automated analysis was performed using a Flexigem™ centrifugal analyzer and Autoloader from Electro-Nucleonics Inc., Fairfield NJ, 07006.

All other apparatus used were as described in PART I (CHAPTER IIA, p.10)

3. Reagents and Standards
Phosphate equilibrating buffer: This solution was prepared to be 0.02 M, pH 7.1. This reagent was stable for several days when stored at 4°C.

High-salt buffer: This solution was prepared to contain 1.5 M sodium chloride in the phosphate equilibrating buffer. This reagent was stable for one week when stored at 4°C.

Carbonate buffer: This reagent was prepared to be 0.5 M, pH 12.4 at 37°C. The pH of this reagent was stable for one week when stored at 4°C.

MTT stock solution: A stock solution of MTT was prepared to be 2.5 M in methyl cellosolve. This reagent was stable for at least one week.

Glycated albumin standards: These standards were prepared by incubating human serum albumin and glucose in Delbecco's phosphate buffered saline at 37°C for 4 days (14). Following the incubation, the standards were dialyzed against distilled water for 48 h at 4°C. The level of GA present in these standards were determined using aminophenylboronate affinity chromatography (18).

4. Samples

Blood specimens from "normal" and diabetic subjects were collected in Vacutainers containing either EDTA or no anticoagulant and, if necessary, were stored at 4°C for no more than 6 h before the glycated albumin level was
determined. These blood specimens were split samples taken from blood used for routine laboratory investigation. The "normal" individuals were both hospitalized and ambulatory patients and were all believed to be free of any condition that might alter their glycated protein levels. The diabetic population was comprised of mixed groups of insulin and non-insulin dependent individuals whose status was being assessed by routine monitoring of their glycated hemoglobin levels.

5. Methods

The Affi-gel Blue columns were equilibrated with a phosphate equilibrating buffer and standard or patient's sample (150 μL) was applied to the upper disc of the gel and the column was allowed to drain. Equilibrating buffer (2 mL) was then added and the eluate discarded. The fraction containing albumin was eluted using 1.5 mL of high-salt buffer and used for the determination of glycated albumin.

Just prior to performing the analysis, the MTT reagent was prepared by adding 2.24 mL of stock MTT solution to 18.0 mL of the carbonate buffer.

Glycated albumin was determined by using a microcentrifugal analyzer (Flexigem™). The autoloader was programmed to deliver 100 μL of sample and 900 μL of reagent. The rotor disc was placed in the analyzer and sample and reagent mixed. After the reaction period (300 s), the net absorbance for
each cuvet was determined. Glycated albumin levels were automatically calculated by the multi-standard routine and math model (log-logit) built into the Flexigem™ microprocessor. Instrument settings for the autoloader and the analyzer are summarized in Table IV.
**TABLE IV**

PARAMETERS FOR GLYCATED ALBUMIN DETERMINATION USING THE FLEXIGEM® CENTRIFUGAL ANALYZER

<table>
<thead>
<tr>
<th>Loader settings</th>
<th>Analyzer settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Samples</td>
<td>Chemistry number 83</td>
</tr>
<tr>
<td>Blank 1</td>
<td>Data type 7</td>
</tr>
<tr>
<td>Sample I a 0 μL</td>
<td>Multi-standard Yes</td>
</tr>
<tr>
<td>Sample O b 100 μL</td>
<td>Temperature 37°C</td>
</tr>
<tr>
<td>Sample 0</td>
<td>Direction increasing Yes</td>
</tr>
<tr>
<td>Flush 0</td>
<td>Units 0</td>
</tr>
<tr>
<td>Reagent I 900 μL</td>
<td>Decimal places 2</td>
</tr>
<tr>
<td>Reagent O 0</td>
<td>Min. absorbance 0.0</td>
</tr>
<tr>
<td>Reagent 0</td>
<td>Max. absorbance 2.0</td>
</tr>
<tr>
<td>Incubation time 0</td>
<td>Wavelength 546 nm</td>
</tr>
<tr>
<td>1st read time 6 s</td>
<td>2nd read time 306 s</td>
</tr>
<tr>
<td>Math model 1</td>
<td></td>
</tr>
</tbody>
</table>

a Refers to the inner well of the sample disk.

b Refers to the outer well of the sample disk.
CHAPTER III

RESULTS AND DISCUSSION

1. Optimization of the Color Reaction

Figure 4 showed an outline of the reaction sequence involved in the formation of the colored formazan. The original fructosamine assay (22) utilized nitroblue tetrazolium as the tetrazolium salt in the color reaction. Preliminary studies indicated, however, that under similar conditions a 3-fold increase in absorbance could be obtained if MTT is used as the tetrazolium salt in the color reaction (Fig. 5). For the remainder of the work, the reagent used was MTT.

Figure 5 also shows that the λ maximum for MTT was 557 nm and this value is in good agreement with a previously reported value (26). One of the goals of this study was to automate the color reaction using the Flexigem™ centrifugal analyzer. The Flexigem™ is a filter photometer and it was not possible to use the λ maximum of 557 nm. A wavelength of 546 nm was used for the analysis. This choice of wavelength represents a decrease in absorbance of <1% than if the measurements were made at the λ maximum.

As previously demonstrated, several factors affected the intensity of color produced (22). The color produced was more
Figure 5

Absorption Spectra of MTT and NBT

Legend

Shown are the absorption spectra of MTT and NBT.

The λ max of MTT was 530 nm and 557 nm for NBT.

The reaction mixture contained 2.5 mL carbonate buffer (0.5 M, pH 12.4), 100 μL human serum albumin solution (40% w/v), and 100 μL MTT or NBT solution (2.5 mM). The blank was prepared by using distilled water instead of the albumin solution. The incubation time was 5 min at 37°C.
intense with increasing pH, length of incubation, temperature of incubation, MTT concentration, and level of glycated protein. Instrumental constraints on the Flexigem™ resulted in a maximum possible temperature of 37°C. It was considered disadvantageous to utilize more than 5 min Flexigem™ time per batch so the length of incubation was set at 300 s. The two remaining variables that influence the assay, pH and MTT concentration, were then adjusted to produce the maximum color possible.

The traditional approach to optimizing experimental variables has been to vary one factor at a time while holding the other factors constant. In this way the optimum for each variable can be found sequentially. This approach may not necessarily provide the unique combined optimum of all the variables particularly if the variables are interdependent. Of the several optimization techniques currently available, the design based on a class of figures called the simplex is probably the most useful (27,28). Further, since it was only necessary to consider two variables, the simplex technique was readily applicable. A simplex is a geometric figure having a number of vertices equal to one more than the number of dimensions of the space it occupies. Each dimension is an experimental variable such as pH or temperature. When two variables are being examined simultaneously, the simplex is a triangle. The response to be optimized e.g., absorbance, is
first defined and starting vertices established. The simplex method forces the simplex to move in the region of optimum response utilizing fixed "rules" of the simplex procedure. Each simplex enables the assignment of the variables to be used in the next experiment.

Figure 6 shows a study in which the simplex method was used to optimize the pH and MTT concentration to produce the maximum color within the incubation period. It was readily apparent that under these conditions the reagent blank value increased significantly. This prompted an investigation into the effect of pH and MTT concentration on the reagent blank value (Figs. 7 and 8). From these data, it was decided that boundaries should be placed on the simplex. These boundaries corresponded to the pH and MTT concentration at which the reagent blank value became unacceptably high. The simplex study was then repeated with boundary conditions in place and the results are shown in Fig. 9. The optimum pH and MTT concentration in the cuvet were found to be 12.4 (37°C) and 0.25 M, respectively. These parameters gave the maximum color with the reagent blank value being minimal.

2. Efficiency of Affi-Gel Blue Columns

Since the object of this study was to determine the level of glycated albumin, it was essential that the separation of albumin from plasma or serum be as clean as possible. Several studies were undertaken to assess how
FIGURE 6

UNCONSTRAINED SIMPLEX OPTIMIZATION STUDY

Legend

Shown is the simplex obtained in an attempt to optimize 
pH and MTT concentration to produce the largest absorbance 
change (response).

Carbonate buffers (0.5 M) were prepared to give the 
appropriate pH values at 37°C. An MTT stock solution (2.5 mM) 
was prepared in cellosolve. The reaction mixture contained 
2.5 mL carbonate buffer, 100 μL MTT solution, and 100 μL 
human serum albumin solution (40% w/v). The incubation time 
was 5 min at 37°C.

Initial vertices, 1 - 3, were selected. These 
represented pH and MTT concentration coordinate pairs, and 
experimental runs were performed in duplicate for each pair 
of coordinates. The pair that gave the lowest response, 1, 
was reflected across an axis that joined the other two pairs, 
2 - 3. The coordinates of the reflected vertex established 
the parameters for the next experimental run, 4.

An optimum was reached at vertex 8, and this 
corresponded to pH 13.0, MTT concentration 0.52 mM. A new 
simplex started at vertices 16 - 18, returned to vertex 8.
FIGURE 7

EFFECT OF pH ON THE BLANK REACTION

Legend

Shown is the pH dependence of the reagent blank. This experiment was performed using 0.5 M carbonate buffers adjusted to give the appropriate pH values at 37°C. The reaction mixture contained 2.5 mL carbonate buffer, 100 μL human serum albumin solution (40% w/v), and 100 μL MTT solution (2.5 mM). The incubation time was 5 min at 37°C.

The values on the x axis represent the pH of the final reaction mixture.
FIGURE 7

Graph showing the relationship between absorbance and pH.
FIGURE 8

EFFECT OF MTT CONCENTRATION ON THE BLANK REACTION

Legend

Shown is the dependence of the reagent blank on the concentration of MTT. This experiment was performed using reaction mixtures that contained increasing concentrations of MTT. The reaction mixture contained 2.5 mL carbonate buffer (pH 12.4), 100 μL human serum albumin solution (40% w/v), and 100 μL MTT solution. The incubation time was 5 min at 37°C.

The values on the x axis represent final cuvet concentrations of MTT.
Figure 9

Constrained Simplex Optimization Study

Legend

Shown is the simplex obtained when boundaries were set for a maximum pH and MTT concentration of 12.4, and 2.5 mM, respectively.

This experiment was performed using reaction mixtures that contained 2.5 mL carbonate buffer, 100 μL MTT solution, and 100 μL human serum albumin (40% w/v).
efficiently the Affi-gel Blue columns separated albumin from serum or plasma. Recovery experiments were performed in which varying amounts of protein were loaded onto the column. The results indicated that when 2.5, 5.0, 10.0, and 15.0 mg human serum albumin were loaded per mL of gel, the high-salt fraction contained 2.43±0.11, 4.95±0.06, 9.85±0.09, and 7.98±0.39 mg albumin, respectively. These results were the mean±S.D. of four determinations. The studies were repeated with pooled plasma supplemented with HSA and similar results were obtained. It would appear that a maximum of 10.0 mg albumin/mL of gel could be bound and this represents a significantly lower binding capacity than that reported previously (29). Albumin loading greater than 10 mg/mL of gel was found to elute in a non-discriminatory fashion with the albumin appearing in both the low- and high-salt fractions. The sample sizes applied to the column, therefore, were calculated so as to not exceed this level of loading.

It was necessary to adjust the volume of high-salt buffer used so that most of the albumin is eluted in a small volume as possible to avoid excessive dilution of the glycated fraction. The elution profile of the Affi-gel showed that if up to 10 mg of albumin were loaded onto the column, 98.5% of the protein eluted in the first 1.5 mL of high-salt buffer.

The high-salt eluates from the Affi-gel columns should
contain only albumin, and this was verified by protein electrophoresis. Agarose gel electrophoresis carried out on the high-salt eluates showed only one band corresponding to albumin, (Fig. 10). Further, immunodiffusion studies carried out on both the low- and high-salt eluates indicated that the high-salt eluates contain only albumin. No IgG, IgM or IgA was detected in the high-salt eluates.

The above studies on the efficiency of the Affi-gel Blue columns in separating albumin from plasma were repeated after the columns were regenerated and reused for five times and similar results were obtained.

3. Linearity

Glycated albumin standards were analyzed using the assay protocol developed in this study, and described in CHAPTER II, p. 81. These standards were first calibrated using aminophenylboronate affinity chromatography to separate the glycated from the non-glycated fraction and determining the albumin present in each fraction by the assay of total protein. Figure 11 shows a good linear relation with absorbance for glycated albumin levels between 4 and 18%.

4. Interference Studies

Hemolysis up to 10 g.L⁻¹ hemoglobin, lipemia up to 60 mM triglycerides, or bilirubin up to 20 mg.L⁻¹ had no significant effect (p>0.05) on the values determined for glycated albumin. These studies were performed by adding the
FIGURE 10

PROTEIN ELECTROPHORESIS OF HIGH-SALT ELUATES

Legend

Shown is an agarose gel protein electrophoresis pattern illustrating that the high-salt eluates from the Affi-gel Blue columns contain only albumin.

This study was performed applying 150 μL of sample to Affi-gel Blue columns as per the standard assay protocol described in CHAPTER II, p. 84. The high-salt eluates were dialyzed against distilled water for 24 h and then lyophilized. The solid material obtained was dissolved in 300 μL distilled water and used as the high-salt samples applied to the agarose gel.

The following samples were applied to the agarose gel:

A  plasma from a normal subject.
B  human serum albumin standard (40 g.L\(^{-1}\)).
C  high-salt eluate from Affi-gel Blue column.
D  high-salt eluate from Affi-gel Blue column.
FIGURE 11

GLYCATED ALBUMIN CALIBRATION CURVE

Legend

Shown is a standard curve for the determination of glycated albumin. The curve was constructed using glycated human serum albumin standards that were calibrated with aminophenylboronate affinity chromatography. These standards were run as per the standard assay protocol described in CHAPTER II, p. 84.

The equation of the regression line is \( y = 0.011 x + 0.111 \).
appropriate level of the compound studied to pooled plasma in which the level of glycated albumin had been previously determined. There was no significant difference between glycated albumin determined in serum or plasma or with EDTA or heparin used as anticoagulants. Ascorbate and glutathione are two additional potential interferences. At levels up to 0.5 mM, they also did not affect the results.

Free glucose is also a potential interference in the MTT color reaction, and any possible effect on the assay was examined. Pooled serum was supplemented with glucose to final concentrations of 7.1, 15.0, 40.0, and 60.0 mM. Glycated albumin levels in these samples were $10.82 \pm 0.21$, $10.79 \pm 0.18$, $11.17 \pm 0.28$, and $11.13 \pm 0.26\%$, respectively. These results were the mean $\pm$ S.D. of four determinations and were compared with the baseline value using analysis of variance. Free glucose at the levels studied in this method does not interfere.

The observation that the assay system is unaffected by many potentially interfering substances probably results from the separation of albumin from plasma prior to the quantification of the glycated fraction. These interferences are presumably eluted in the non-albumin fraction.

If the results obtained with this assay were to be valid and to indicate correctly the diabetic status of the patient, it was necessary to demonstrate that only the stable
(ketoamine) glycated albumin adduct was being measured. This was especially important since many of the previous assays to determine glycated proteins were subject to interference from the labile (aldimine) fraction (15,22). The formation of the labile fraction is a short term, relatively rapid process and was approximated by the following study. Glucose was added to a pooled serum sample and incubated from 1 to 24 h at 37 °C. Increasing either the concentration of glucose or the duration of incubation did not increase the level of glycated albumin (TABLE V). The absence of interference from this labile (aldimine) fraction is significant in that the results obtained represent only the stable (ketoamine) glycated fraction. Removal of this labile fraction before analysis is thus obviated.

It is interesting to speculate why the aldimine fraction which itself can produce a color with MTT, does not interfere in the assay. The formation of the aldimine from glucose and albumin is a rapid, freely reversible reaction. It is possible that the conditions used for the separation of albumin from plasma facilitate the decomposition of the aldimine fraction on the Affi-gel column thereby resulting in its inability to participate in the MTT color reaction. The glucose formed from the aldimine would then be eluted in the non-albumin fraction.

The original fructosamine assay (22) as well as a
TABLE V

EFFECT OF THE LABILE FRACTION ON THE DETERMINATION OF GLYCATED ALBUMIN

<table>
<thead>
<tr>
<th>Glucose mM</th>
<th>Glycated albumin %</th>
<th>Incubation time h</th>
<th>Glycated albumin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.82 ± 0.21</td>
<td>1</td>
<td>14.43 ± 0.10</td>
</tr>
<tr>
<td>1</td>
<td>14.32 ± 0.37</td>
<td>2</td>
<td>14.48 ± 1.23</td>
</tr>
<tr>
<td>5</td>
<td>14.98 ± 0.67</td>
<td>4</td>
<td>14.68 ± 0.50</td>
</tr>
<tr>
<td>10</td>
<td>14.77 ± 0.31</td>
<td>8</td>
<td>14.60 ± 0.83</td>
</tr>
<tr>
<td>15</td>
<td>14.27 ± 0.12</td>
<td>12</td>
<td>14.86 ± 0.49</td>
</tr>
<tr>
<td>20</td>
<td>14.52 ± 0.71</td>
<td>24</td>
<td>14.16 ± 0.71</td>
</tr>
<tr>
<td>25</td>
<td>14.36 ± 0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>14.51 ± 0.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>14.76 ± 0.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>14.56 ± 0.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Results are the mean ± S.D. of four determinations.

Statistical significance of all results compared with their respective baseline values was determined by analysis of variance.

b Serum samples were supplemented with glucose in concentrations shown, then incubated for 4 h at 37°C.

c Serum sample supplemented with glucose (30 mM), then incubated for the times shown.
subsequent modification (24) were both subject to interference from albumin. Albumin at levels greater than the normal range gave a significant blank reaction. This problem was pronounced since both these assays were standardized by dissolving a synthetic Amadori rearrangement product in human serum albumin (40 g.L⁻¹)(22). This led to speculation that the type of albumin preparation used, i.e., a liquid preparation versus lyophilized, may affect the results (24). Our studies have shown that commercial lyophilized human serum albumin preparations contain significant amounts (>4.5%) of glycated albumin. If this glycated fraction is removed by aminophenylboronate affinity chromatography, the interference problem may be circumvented. The proposed assay system was calibrated using a series of glycated human serum albumin standards and not a synthetic Amadori rearrangement product. The levels of glycated albumin determined are, therefore, not affected by the serum albumin concentration.

5. Precision

To evaluate the within-run and the day-to-day reproducibility of the assay, two plasma pools were obtained and the levels of glycated albumin determined. For the within-run study, each pool was assayed 10 times in the same analytical batch. For the between-run study, each pool was assayed in duplicate over six different analytical batches (TABLE VI). For each pool, both the within-run and
### TABLE VI

**PRECISION STUDY**

<table>
<thead>
<tr>
<th>Plasma pool</th>
<th>Mean(^a)</th>
<th>S.D.(^a)</th>
<th>S.E.M.(^a)</th>
<th>C.V.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td><em>Within-run</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>11.67</td>
<td>0.53</td>
<td>0.19</td>
<td>4.57</td>
</tr>
<tr>
<td>B</td>
<td>11.16</td>
<td>0.48</td>
<td>0.17</td>
<td>4.30</td>
</tr>
<tr>
<td><em>Between-run</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>11.75</td>
<td>0.94</td>
<td>0.39</td>
<td>8.03</td>
</tr>
<tr>
<td>B</td>
<td>11.38</td>
<td>0.69</td>
<td>0.28</td>
<td>6.08</td>
</tr>
</tbody>
</table>

\(^a\) Reported as % glycated albumin.

\(^b\) Each plasma pool was assayed ten times in the same analytical batch.

\(^c\) Each plasma pool was assayed in duplicate in six different analytical batches.
between-run C.V. were less than 8%.

6. Recovery

The analytical recovery of glycated albumin is summarized in TABLE VII. This study was performed by supplementing pooled serum with various levels of glycated albumin standards and non-glycated albumin human serum albumin to calculated final concentrations. The non-glycated HSA used was prepared from commercially available lyophilized HSA (Sigma). This preparation was first passed through aminophenylboronate affinity columns (Isolab) and the non-glycated fraction utilized for this study.

7. Correlation Studies

The proposed method was compared with the thiobarbituric acid assay (15). Figure 12 shows a plot correlating the two methods of determining glycated albumin. The correlation coefficient, r, was 0.97 indicating a significant correlation between the two methods. Additionally, the data passed the F-test (there is no significant difference between the variance of the two methods).

Glycated hemoglobin and glycated albumin were measured concurrently in a group of "normal" and diabetic subjects (Fig. 13). The correlation coefficient, r, was 0.35. This poor correlation between glycated albumin and glycated hemoglobin suggests that the two analytes reflect different
TABLE VII

RECOVERY STUDY\textsuperscript{a}

<table>
<thead>
<tr>
<th>Expected\textsuperscript{b}</th>
<th>Recovered\textsuperscript{b}</th>
<th>Recovery\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>11.50</td>
<td>11.40</td>
<td>99.1\pm 0.11</td>
</tr>
<tr>
<td>9.56</td>
<td>9.20</td>
<td>96.2\pm 0.14</td>
</tr>
<tr>
<td>5.64</td>
<td>5.30</td>
<td>93.9\pm 0.16</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All data reported are the mean of three replicate assays.

\textsuperscript{b} Reported as % glycated albumin.

\textsuperscript{c} Recovery (%) reported as the mean \pm S.D.
CORRELATION PLOT BETWEEN THE PROPOSED METHOD AND THE THIOBARBITURIC ACID ASSAY

Legend

Shown is a correlation plot between the proposed method and a presently accepted method, the thiobarbituric acid assay (15), for determining glycated albumin.

The equation of the regression line is $y = 5.35x + 8.9$. The sample size, $n = 25$. Standard error of the estimate = 0.18. The correlation coefficient, $r = 0.97$. 
FIGURE 13

CORRELATION PLOT BETWEEN LEVELS OF GLYCATED ALBUMIN AND GLYCATED HEMOGLOBIN

Legend

Shown is a correlation plot between glycated albumin and glycated hemoglobin levels in a mixed group of diabetic and non-diabetic subjects. The sample size, n = 25. The equation of the regression line is $y = 0.076x + 10.93$. Standard error of the estimate = 0.30. The correlation coefficient, $r = 0.35$. 
aspects of glycemic control (6). Diabetic patients with a recent improvement in glycemic control could have low levels of glycated albumin but not necessarily of glycated hemoglobin. Some workers have reported good correlation between glycated albumin and glycated hemoglobin (11). However, it should be noted that in these studies the diabetic populations consisted largely of Type II individuals in whom blood glucose levels are usually fairly stable.

8. Reference Interval

Normal ranges reported for glycated albumin and glycated proteins vary widely (9,18,30). This is largely a result of the many different methods used to measure glycated proteins. In addition, some of these methods give results in units that are not easily compared with other studies.

The reference interval for glycated albumin was determined by assaying samples from 30 "normal" and 30 diabetic subjects. Figure 14 shows a histogram of the data for the "normal" subjects, while Fig. 15 shows a scattergram of the data for "normal" and diabetic subjects.

The 95% confidence interval for "normal" subjects was 8.94 - 11.19% glycated albumin. In contrast, the levels in diabetic subjects were statistically significantly higher (p < 0.005).
FIGURE 14

HISTOGRAM OF GLYCATED ALBUMIN LEVELS IN "NORMAL" SUBJECTS

Legend

Shown is a histogram of the data obtained for glycated albumin levels in "normal" subjects. Values on the horizontal axis represent the midpoints of the intervals. ($\bar{x} = 10.06$; S.D. = 0.56; S.E.M. = 0.10; minimum value = 8.73; maximum value = 11.02)
FIGURE 15

SCATTERGRAM OF GLYCATED ALBUMIN LEVELS IN "NORMAL" AND
DIABETIC SUBJECTS

Legend

Shown is a scattergram of glycated albumin levels in
"normal" and diabetic subjects (n=30). The bars represent ±
S.D.
CHAPTER IV

SUMMARY AND CONCLUSIONS

The method proposed to determine glycated albumin (GA) is a simple procedure capable of good precision. It is also rapid and up to 75 analyses per hour can be performed. On a reagent cost per test basis it is also less expensive than the aminophenylboronate affinity chromatography procedure (18). This fact together with its better precision than the affinity chromatography procedure makes the proposed method an attractive alternative for the routine determination of GA in the clinical laboratory.

Several improvements over the original "fructosamine assay" (22) resulted from the proposed method. The original "fructosamine assay" was developed to measure total glycated proteins and its proposed use was limited as a screening test for diabetes mellitus. The method proposed in this study specifically measures glycated albumin and is potentially useful as an index of diabetic control. Additionally, separating the albumin from plasma or serum prior to the color reaction, resulted in many of the interferences that affected the original "fructosamine assay" not affecting the proposed method. The analytical conditions were optimized to produce the maximum color with the lowest reagent blank value.
possible. Instead of using a synthetic Amadori rearrangement product (DMF) and expressing the results in non-physiological units as in the original "fructosamine assay", a glycated human serum albumin standard was used. Not only were the results expressed as percent glycated albumin, thus allowing easy comparison with previous work as well as with glycated hemoglobin levels, but this standard more closely approximated the sample in matrix composition.

Semi-automating the procedure allowed a greater throughput and concomitant savings in labor costs. The good precision obtained is in part attributable to this factor as well.

The advantages of determining GA over glycated hemoglobin (GHb) have been documented (10,20), and there is increasing use of GA levels as an adjunct in the assessment of diabetic control. In patients with severe diabetic ketoacidosis and non-acidotic coma, within 1 week of therapy, GA levels decreased by 21% and by 40% during a 17-day period (31). In contrast, the GHb levels remained unchanged during this time.

It has been suggested that the determination of GHb levels is more useful for assessing the degree of control in insulin-dependent diabetics whose day-to-day fluctuations in plasma glucose concentrations are large (11). In the pregnant diabetic GHb determinations are useful in establishing a baseline degree of blood glucose control. However, in newly
diagnosed or poorly stabilized diabetics as well as certain disease states such as anemia where GHB levels may be misleading, the determination of GA may provide the physician with objective evidence of the glycemic status of the diabetic patient. When tight glucose control is desired, e.g., in the pregnant diabetic, the more responsive GA levels may provide objective evidence as to the glycemic status of the patient earlier than is possible with GHB. The possibility of earlier therapeutic intervention is, thus, permitted.

The elucidation of the role of chronic hyperglycemia in the pathogenesis of diabetes is still incomplete and one of the factors hindering such study is the lack of reliable indices of glycemic control as well as simple methodology to determine these indices. Glycated albumin is of possible value in such studies and methodology such as the one proposed to determine GA may be of useful in this regard.

The inability to achieve normal physiological control with insulin therapy, the long-term evolutionary nature of the chronic complications associated with diabetes mellitus, and the wide variety of proteins affected by the glycation process has led to speculation as to whether glycation might be a mechanism of molecular aging (32,33). This hypothesis is presently under investigation and as other glycated proteins are studied methodology for their determination will be
required. If the method proposed for GA is viewed as a model system, it may be worthwhile to investigate whether the procedure is applicable to the determination of other glycated proteins.
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