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FORM 1

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952
APPLICATION FOR A STANDARD PATENT

Miles Inc., incorporated in Indiana, of 1127 Myrtle Street, Elkhart, Indiana, 46514, UNITED STATES OF AMERICA, hereby apply for the grant of a standard patent for an invention entitled:

Latex Agglutination Immunoassay in the Presence of Hemoglobin

which is described in the accompanying complete specification.

Details of basic application(s):-

<u>Basic Applic. No:</u>	<u>Country:</u>	<u>Application Date:</u>
118469	US	9 November 1987

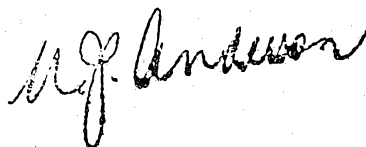
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DATED this SECOND day of NOVEMBER 1988

Miles Inc.

By:



Registered Patent Attorney

TO: THE COMMISSIONER OF PATENTS
OUR REF: 69994
S&F CODE: 58320

AMENDMENT ACCEPTED AND AMENDMENTS

5003388 03/11/88

ALLOWED 3.1.91.

5845/3

Spruson & Ferguson

DECLARATION IN SUPPORT OF A
CONVENTION APPLICATION FOR A PATENTIn support of the Convention Application made for a
patent for an invention entitled:

Title of Invention

LATEX AGGLUTINATION IMMUNOASSAY IN THE PRESENCE OF HEMOGLOBIN

Full name(s) and
address(es) of
Declarant(s)

I/We Adrien L. Ringuette
of 17445 County Road 10
Elkhart, Indiana 46514
USA

do solemnly and sincerely declare as follows:-

Full name(s) of
Applicant(s)1. ~~I am/We are the applicant(s) for the patent~~

(or, in the case of an application by a body corporate)

1. I am/We are authorised by
MILES INC.

the applicant(s) for the patent to make this declaration on
its/their behalf.

2. The basic application(s) as defined by Section 141 of the
Act was/were made

in United States of America

on November 9, 1987

by Lynette A. Lewis
Lowry Messenger
Frances M. Yeager
Kin Fai Yip

3. ~~I am/We are the actual inventor(s) of the invention referred
to in the basic application(s)~~

(or where a person other than the inventor is the applicant)

3. Lynette A. Lewis, Lowry Messenger, Frances M. Yeager
and Kin Fai Yip

of 57417 O'Sage Dr., Goshen, IN 46526 USA;
17453 Green Oaks, Ct., Granger, IN 46530 USA;
306 Eastern Star Dr., Middlebury, IN 46540 USA;
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(respectively)

~~is/are~~ the actual inventor(s) of the invention and the facts upon
which the applicant(s) ~~is/are~~ entitled to make the application are
as follows:

The said applicant is the assignee of the
actual inventors.

Set out how Applicant(s)
derive title from actual
inventor(s) e.g. The
Applicant(s) ~~is/are~~ the
assignee(s) of the
invention from the
inventor(s)

4. The basic application(s) referred to in paragraph 2 of this
Declaration was/were the first application(s) made in a Convention
country in respect of the invention(s) the subject of the application.

Declared at Elkhart, IN this 17th day of August, 1988.

ATTEST:

MILES INC.

Franklin E. Breckenridge
To: The Commissioner of Patents

A L Ringuette
Signature of Declarant(s)

Franklin E. Breckenridge
Assistant Secretary

A. L. Ringuette
Secretary

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(54) Title
LATEX AGGLUTINATION IMMUNOASSAY IN THE PRESENCE OF HEMOGLOBIN

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(56) Prior Art Documents
GB 2024829
US 4247533

(57) A latex agglutination immunoassay method for determining an analyte in a blood sample in which the pH of the reaction mixture is maintained at about 8.5 or greater in order to overcome nonspecific agglutination of latex particles by hemoglobin present in the sample. The method is particularly applicable to the determination of glycated hemoglobin, e.g., Hb Alc. In another embodiment, native hemoglobin (Ao) can be determined based on its ability to cause agglutination of latex particles suspended in an aqueous solution having a pH of about 8 or below.

CLAIM

1. In an analytical method for ^{the determination of} ~~determining~~ an analyte in a blood sample by latex agglutination immunoassay, wherein (a) an aqueous reaction mixture is formed by combining the blood sample with a multivalent latex antibody reagent comprising an anti-analyte antibody, or a fragment

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thereof, bound to a water suspensible latex particle, and additionally when the analyte is monovalent, with an agglutinator reagent comprising a plurality of epitopic binding sites for the anti-analyte antibody or fragment thereof, and (b) the resulting agglutination in the aqueous reaction mixture is measured as a function of analyte in the blood sample,

the improvement which comprises forming the aqueous reaction mixture to have a pH of about 8.5 or greater, whereby nonspecific agglutination of the latex reagent due to the presence of hemoglobin in the blood sample is substantially overcome.

7. An analytical method for ^{the determination of} ~~determining~~ hemoglobin Alc in a test sample comprising blood lysate by particle agglutination inhibition immunoassay, which method comprises the steps of:

(a) forming an aqueous reaction mixture comprising the blood sample and assay reagents comprising (1) a multivalent antibody reagent comprising an antibody, or a fragment thereof, to hemoglobin Alc bound to a water suspensible polystyrene latex particle, and (2) an agglutinator reagent comprising a plurality of glycosylated peptides corresponding to the glycosylated peptide sequence of hemoglobin Alc, said reaction mixture formed to have a pH of about 8.5 or greater, and

(b) measuring the resulting agglutination as a function of hemoglobin Alc in the blood sample.

23. An analytical method for ^{the determination of} ~~determining~~ hemoglobin Ao in a blood sample, comprising the steps of:

(a) forming an aqueous reaction mixture comprising the blood sample and suspended latex particles and maintaining the pH of the reaction mixture at about 8 or below, and

(b) measuring the agglutination of said latex particles as a function of hemoglobin Ao in the blood sample.

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S & F Ref: 69994

FORM 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

Class Int Class

Complete Specification Lodged:
Accepted:
Published:

Priority:

Related Art:

This document contains the
amendments made under
Section 49 and is correct for
printing.

Name and Address
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Complete Specification for the invention entitled:

Latex Agglutination Immunoassay in the Presence of
Hemoglobin

The following statement is a full description of this invention, including the
best method of performing it known to me/us

ABSTRACT OF THE DISCLOSURE

A latex agglutination immunoassay method for determining an analyte in a blood sample in which the pH of the reaction mixture is maintained at about 8.5 or greater in order to overcome nonspecific agglutination of latex particles by hemoglobin present in the sample. The method is particularly applicable to the determination of glycated hemoglobin, e.g., Hb Alc. In another embodiment, native hemoglobin (Ao) can be determined based on its ability to cause agglutination of latex particles suspended in an aqueous solution having a pH of about 8 or below.

ALK/mc

LATEX AGGLUTINATION IMMUNOASSAY IN
THE PRESENCE OF HEMOGLOBIN

BACKGROUND OF THE INVENTION

5 This invention relates to methods for
determining an analyte in a blood sample based on
latex agglutination immunoassay. More
particularly, the invention concerns a method for
overcoming nonspecific agglutination from
hemoglobin present in a test sample. The invention
10 is particularly directed to the determination of
glycated hemoglobin, e.g., hemoglobin Alc, in
samples of whole blood.

Latex agglutination immunoassay is based on
the formation of detectable agglutination by
15 binding between a multivalent latex antibody
reagent and a corresponding multivalent form of the
antigen or hapten. Where an analyte of interest is
itself multivalent relative to the latex antibody
reagent, a direct assay can be conducted. However,
20 where the analyte of interest is not multivalent,
such as in the case of a low molecular weight
hapten, a competitive assay is performed in order
to quantitate such haptenic analyte. An
agglutinator reagent is added to the system
25 comprised of a plurality of epitopic binding sites
for the anti-analyte antibody reagent. Binding

between the agglutinator reagent and the latex antibody reagent results in formation of detectable agglutination. With increasing amounts of analyte, which competes with the agglutination reagent for binding to the latex antibody reagent, the amount of resulting agglutination is reduced.

5 The prior art has applied the latex agglutination immunoassay method principally to serum or plasma samples in order to determine analytes of interest in blood. It has been found that the presence of whole blood components can give rise to nonspecific agglutination of the latex reagent. Therefore, in situation where it is desirable or necessary to
10 assay whole blood, the precision of the assay result is subject to possible large errors. This particularly applies to the determination of blood components associated with erythrocytes such as hemoglobin derivatives of diagnostic importance, e.g., the glycated hemoglobins.

SUMMARY OF THE INVENTION

15 According to a first embodiment of this invention there is provided an analytical method for the determination of an analyte in a blood sample by latex agglutination immunoassay, wherein (a) an aqueous reaction mixture is formed by combining the blood sample with a multivalent latex antibody reagent comprising an anti-analyte antibody, or a fragment thereof, bound
20 to a water suspensible latex particle, and additionally when the analyte is monovalent, with an agglutinator reagent comprising a plurality of epitopic binding sites for the anti-analyte antibody or fragment thereof, and (b) the resulting agglutination in the aqueous reaction mixture is measured as a function of analyte in the blood sample,

25 the improvement which comprises forming the aqueous reaction mixture to have a pH of about 8.5 or greater, whereby nonspecific agglutination of the latex reagent due to the presence of hemoglobin in the blood sample is substantially overcome.

30 According to a second embodiment of this invention there is provided an analytical method for the determination of hemoglobin Alc in a test sample comprising blood lysate by particle agglutination inhibition immunoassay, which method comprises the steps of:

35 (a) forming an aqueous reaction mixture comprising the blood sample and assay reagents comprising (1) a multivalent antibody reagent comprising an antibody, or a fragment thereof, to hemoglobin Alc bound to a water suspendible polystyrene latex particle, and (2) an agglutinator reagent comprising a plurality of glycated peptides corresponding to the glycated



peptide sequence of hemoglobin Alc, said reaction mixture formed to have a pH of 8.5 or greater, and

(b) measuring the resulting agglutination as a function of hemoglobin Alc in the blood sample.

5 According to a third embodiment of this invention there is provided a test system when used for the determination of an analyte in a blood sample by latex agglutination immunoassay, comprising:

(1) a multivalent latex antibody reagent comprising an anti-analyte antibody, or a fragment thereof, bound to a water suspensible latex particle,

10 (2) when the analyte is monovalent, an agglutinator reagent comprising a plurality of epitopic binding sites for the anti-analyte antibody or fragment thereof, and

(3) a buffer that maintains the pH of the reaction mixture formed by combination of the test system components with the blood sample at about 8.5 or above.

15 According to a fourth embodiment of this invention there is provided an analytical method for the determination of hemoglobin Ao in a blood sample, comprising the steps of:

20 (a) forming an aqueous reaction mixture comprising the blood sample and suspended latex particles and maintaining the pH of the reaction mixture at about 8 or below, and

(b) measuring the agglutination of said latex particles as a function of hemoglobin Ao in the blood sample.

25 According to a fifth embodiment of this invention there is provided a test system when used for the determination of hemoglobin Ao in a blood sample, comprising:

(1) latex particles, and

30 (2) a buffer that maintains the pH of an aqueous reaction mixture comprising the latex particles and the blood sample at about 8 or below.

It has now been found the nonspecific agglutination in latex agglutination immunoassays conducted on whole blood samples can be effectively overcome by maintaining the pH of the reaction mixture at about 8.5 or above. It is believed that nonspecific agglutination is due to the presence of native, unmodified hemoglobin, referred to as hemoglobin Ao, which, because of its net positively charged character, is attracted nonspecifically to negatively charged latex particles. By performing the assay at elevated pH, the hemoglobin Ao net



charge would be neutralized and agglutination by electrostatic attraction to the latex particles would not occur.

Accordingly, the present invention applies to
5 the determination of an analyte in a blood sample
by latex agglutination immunoassay wherein (a) an
aqueous reaction mixture is formed by combining the
blood sample with a multivalent latex antibody
10 reagent comprising an anti-analyte antibody, or a
fragment thereof, bound to a water suspensible
latex particle, and additionally when the analyte
is only monovalent, with an agglutinator reagent
comprising a plurality of epitopic binding sites
15 for the anti-analyte antibody or fragment thereof,
and (b) the resulting agglutination in the aqueous
reaction mixture is measured as a function of
analyte in the blood sample. The invention is
applicable to the use of latex particles, e.g.,
polystyrene latex, which have an effective net
20 negative surface charge when suspended in an
aqueous solution having a pH of about 7 or below.

The observed nonspecific agglutination of the
latex particles by hemoglobin also provides the
basis for determining hemoglobin Ao in a blood
25 sample. A reaction mixture is formed comprising
the blood sample and suspended latex particles and
the pH maintained at about 8 or below. The
resulting agglutination is a function of hemoglobin
Ao in the sample. At pH 8 or below, the latex
30 particles retain their net negative surface charge
and are agglutinated by electrostatic attraction of
hemoglobin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1-4 are graphical representations of data collected according to the experiments described in the Examples below.

5 Fig. 1 is a graph showing the effect of varying hemoglobin Ao (native, unmodified hemoglobin) concentrations on the agglutination of latex particles.

10 Fig. 2 is a graph showing the effect of pH on the ability of hemoglobin Ao to agglutinate latex particles.

15 Fig. 3 is a graph showing the correlation between HbA1c assay results obtained following the present invention and results obtained following a standard affinity chromatography method.

 Fig. 4 is a graph showing a hemoglobin Ao dose response curve following the method of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 In the course of developing a latex agglutination immunoassay for determining the hemoglobin derivative known as hemoglobin Alc, it was found that the dose response curve was essentially the same between assays run at pH 7.4
25 with the complete reagent system and those run in the absence of the agglutination reagent. Latex coated with bovine serum albumin rather than the hemoglobin Alc analog was found also to be agglutinated in the presence of a blood sample. It
30 was suspected that native hemoglobin in the blood

sample could be the culprit. Treatment of the blood sample with the proteolytic enzyme pepsin eliminated the nonspecific agglutination. The multivalency of hemoglobin Ao, native hemoglobin that is unmodified at the N-terminal amino group in the beta-chain, would be destroyed by pepsin digestion. Furthermore, in subsequent experiments it was found that hemoglobin Ao agglutinates the latex particles in proportion to its concentration in an aqueous latex suspension.

It is therefore theorized, but is not considered critical to the present invention, that hemoglobin Ao interacts with the latex by ionic attraction between negatively charged groups on the surface of the latex, e.g., sulfate groups on polystyrene, and the positively charged amino groups on hemoglobin, e.g., the N-terminal amino groups on the beta-chain of hemoglobin. The tetrameric form of hemoglobin thereby affords sufficient multiple positive charges to cause agglutination by the bridging of latex particles. Further, it is believed that hemoglobin derivatives such as glycated hemoglobins are modified in a way that the positive charges are diminished or removed, so that a differential capacity to agglutinate latex particles exists between hemoglobin Ao and particular hemoglobin derivatives. Thus, it is possible to perform an assay for a particular hemoglobin derivative, e.g., hemoglobin Alc, which does not substantially cause nonspecific latex agglutination by conducting the assay under pH conditions that neutralize the nonspecific agglutination effect of hemoglobin Ao.

The present invention is applicable to agglutination immunoassays based on a variety of latex particles. Most latexes are composed of particles having a net negative surface charge at neutral pH. The charge repulsion between the negative surface charges on the latex is important in maintaining the particles in suspension. As used herein, the term latex is intended to mean the property of suspension of discrete microparticles in an aqueous liquid.

Latex particles useful in the present invention will be evident to the worker familiar with the field of latex agglutination immunoassay. In general, such particles require the properties necessary to serve as a stable support for the desired antibody reagent for the assay and to undergo agglutination in the presence of an agglutinator reagent sufficient for analytical purposes. Latex particles are prepared generally by emulsion polymerization or suspension polymerization [Bangs, L.B. (1984) Uniform Latex Particles, Seragen Diagnostics Inc., Indianapolis, IN, USA]. Swollen emulsion polymerization can also be used [Ugelstad, J. et al (1980) Adv. Colloid and Interface Sci. 13:101-140]. A good selection of latex particles are commercially available. Polystyrene particles are particularly useful.

The density of the latex particles, without limitation, will vary generally between about 0.1 mg/mL and about 0.1 g/mL. Most latexes are composed of particles that are roughly microspherical, having diameters, without limitation, that vary between about 0.04 and about 1.2 microns.

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The attachment of the antibody reagent to the latex particles is a matter of applying conventional techniques. In general, the attachment can be covalent or noncovalent. The antibody reagent can consist of whole antibodies, antibody fragments, polyfunctional antibody aggregates, and the like. Normally, whole antibody or IgG fragments such as Fab, Fab', or F(ab')₂ are employed. The antibody reagent can be derived by any available technique such as conventional antiserum and monoclonal techniques.

Normally, conducting the immunoassay reaction at a pH of about 8.5 or greater will be sufficient to neutralize the positive character of hemoglobin Ao and thereby eliminate the nonspecific agglutination of the latex reagent in the presence of the blood sample. The pH of the reaction mixture will, of course, not be allowed to reach the point at which the immunoreactivity between the analyte and the latex reagent is substantially diminished, that is, to the point that a useful assay is no longer obtainable. Preferably, the pH of the aqueous reaction mixture will have a pH of between about 8.75 and about 10, preferably less than about 9.5, with a pH around 9.0 being particularly useful. Suitable buffers for this purpose can be selected on the basis of convenience and assay performance. Glycine and bicine buffers are preferred.

The agglutinator compound will be prepared according to techniques familiar to the field of agglutination immunoassays. This reagent will, in general terms, comprise a plurality of epitopic binding sites for the anti-analyte antibody

reagent. Such sites can be provided by using the analyte itself or a suitable analog that retains sufficient capacity to be bound by the antibody for purposes of an assay. Such analog can, in the case
5 of a protein analyte, comprise a suitable fragment, prepared synthetically or by digestion, comprising the epitope for the antibody reagent, e.g., glycated peptide residues of hemoglobin Alc as described in the examples below.

10 The present invention is applicable to the assay of test samples which comprise hemoglobin as a potential nonspecific interferant. Such test samples will generally comprise a lysate of a blood sample such as whole blood or separated
15 erythrocytes.

The ability of hemoglobin Ao to agglutinate latex particles also provides a method for Ao determinations. In this embodiment, the latex particles need not carry an antibody reagent, nor
20 is an agglutinator compound required. Simply by maintaining the pH of an aqueous mixture of hemoglobin and latex particles at about 8 or below, one can obtain agglutination correlatable with hemoglobin Ao concentration. The lower point of
25 the useful pH range for this aspect of the invention will be the pH at which hemoglobin Ao is found empirically to lose its ability to agglutinate the latex particles. Normally, this will require a pH above about 4.

30 The present invention will now be illustrated, but is not intended to be limited, by the following examples.

EXAMPLES

1. PREPARATION OF REAGENTS

A. Antibody-Latex Reagent:

Materials Required:

- 5 2% latex suspension [0.085 μ diameter
 polystyrene latex, Seragen,
 Indianapolis, IN, U.S.A.]
- Antibody solution [Monoclonal antibody
 prepared as described in U.S. Pat. No.
10 4,647,654, purified from ascites fluid
 by protein A affinity chromatography
 (BioRad Laboratories, Richmond, CA,
 U.S.A.)]
- 10 mM glycine buffer, 0.02% azide, pH 9.3
- 15 100 mM NaCl

 The antibody coating is done at a latex
 concentration of 0.5% and the antibody is normally
 used in a final concentration of 1 mg/mL in the
 coating reaction. An antibody solution at 2x
20 concentration is prepared by diluting the required
 amount of antibody into a 10 mM glycine buffer with
 added NaCl to give the final conductivity desired
 (between 0.5 and 1.8 mohm). The 2% latex is
 diluted to 2x concentration (or 1%) by mixing with
25 an equal volume of the 10 mM glycine buffer. The
 reaction is initiated by pouring the latex
 suspension into a vessel containing the antibody
 solution. The antibody solution is mixed with a
 magnetic stir bar when the latex is added. All
30 solutions are at room temperature. The mixing is
 continued overnight (at least 15 hours) taking care

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to insulate the vessel so that heating from the magnetic stir plate does not occur. This can be accomplished by suspending the vessel above the stir plate leaving about an inch air space for insulation.

After the 15 hours mixing, the resulting suspension is divided equally into polypropylene centrifuge tubes (approximately 10 mL per tube) for a Sorvall SS-34 rotor [Dupont, Wilmington, DE, U.S.A.]. The suspension is centrifuged at 15,000 rpm (2700 x g) for 60 minutes. The supernatant is decanted. The pellet is washed two times with 10 mM glycine buffer containing the desired overcoating protein [typically 1% protease free bovine serum albumin (BSA-pf) obtained from Miles Inc., Elkhart, IN, U.S.A.]. To wash the pellet, a volume of wash solution equal to the original volume in the tube is added. The pellet is resuspended by vigorous vortexing and short-term sonication (10-15 seconds at a time). After the initial resuspension, the Ab-latex is allowed to stand at room temperature for one hour before recentrifuging. After the initial resuspension and centrifuging, subsequent resuspensions are centrifuged immediately once the pellet is completely dispersed. After the second wash, the pellets are resuspended in a volume equal to the initial reaction volume. The suspension is filtered through a 0.8 μ filter and stored at 5°C.

The concentration is determined by measuring the absorbance at 546 nm of the original supernatant, the supernatant from the first and second washings, and a 100x dilution of the final sample. The sum of these absorbances is assumed to be 100% or equal to 0.5% latex. The absorbance of

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the final sample is divided by the sum of the absorbances used to calculate 100%. The absorbance of the 100x dilution of the final sample is multiplied by 100 to generate an absorbance for the
5 final sample.

Example:

	Sample	A_{546}	
	Supernatant	0.044	
	First Wash	0.034	
10	Second Wash	0.021	
	Final (100 x dil)	0.051	x 100 = 5.1

$$100\% \text{ (or } 0.5\% \text{ latex)} = 5.10 + 0.044 + 0.034 + 0.021 = 5.199$$

15 Latex concentration of final sample =
 $(5.1/5.199) \times 0.5\% = 0.49\%$

B. Agglutinator Reagent:

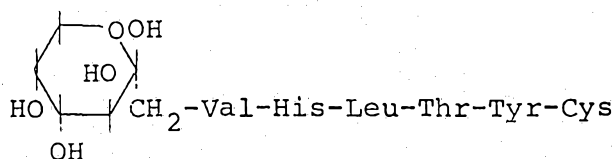
Poly(aspartic acid) was prepared according to the procedure of Alpert J. Chromatography 266:23(1983).

20 Aminoethanol (80 mmols) and 4,9-dioxa-1,12-dodecanediamine (20 mmols) were dissolved in dimethylformamide (DMF) under argon. The solution was treated with a solution of poly(aspartic acid) (10 mmols) and DMF. The reaction was stirred at
25 room temperature for 1 hour and then 70°C for 2 hours. The mixture was then cooled and most of the liquid was removed by evaporation under reduced pressure. The oily residue was washed repeatedly with ether and then warm tetrahydrofuran. The
30 product was solidified and recovered by
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filtration. The crude product was dissolved in water and the pH was adjusted to neutral. The solution was then purified with a BioRad P6-DG desalting gel column (BioRad Laboratories,
5 Richmond, CA, U.S.A.). Fractions containing the amino-functionalized polymer were pooled and lyophilized.

The number of amino groups on the polymer was determined by Habeeb's TNBS assay [Anal. Biochem.
10 14:328-336(1966)] and found to be 22 per mg polymer.

Amino functionalized poly(aspartic acid) (10.7 mg) and SMCC (30 mg) were dissolved in DMF. The reaction was allowed to stir at room
15 temperature for 2 hours. Ice water was added to the mixture and the activated polymer was separated from the mixture with a BioRad 6P-DG gel column. The activated polymer was then allowed to react at room temperature for 3 minutes with the glycated
20 peptide (20 mg)



prepared according to the methods described in European Patent Publication 185,870. After the reaction, the product was again purified with a
25 6P-DG gel column and lyophilized.

The number of maleimido groups on the activated polymers was determined by the PDS assay [Grasseti and Murray, Arch. Biochem. Biophys.
119:44-49(1967)] and found to be 0.46 μ mole per mg
30 polymer. The amount of Glc-peptide on the polymers was determined by UV/Vis absorption measurement MS-1513

using the molar extinction coefficient at 275 nm for tyrosine and found also to be 0.46 μ mole per mg polymer.

C. Bovine Serum Albumin-Coated Latex:

5 A 2% latex solution (4 ml) was mixed with the 10 mM glycine buffer containing 1% protease-free BSA (12 ml). The mixture was briefly sonicated.

2. INTERACTION OF THE BSA-COATED LATEX WITH HEMOGLOBIN Ao

10 I. At pH=7.4

 The BSA-coated latex was diluted to a concentration of 0.036% in a 50 mM sodium phosphate buffer, 0.05% BSA, 0.02% sodium azide (pH=7.4). The latex was mixed with denatured blood samples having various known Ao concentrations. The change in absorbance at 550 nm at room temperature was recorded over time and is shown in Fig. 1 of the drawings [Hemoglobin Ao concentrations: A=99.5%, B=96%, C=90%, D=86%, E=78%, and F=71%].

20 II. At pH=9.0

 The experiment was repeated using BSA-coated latex diluted to 0.05% in 50 mM glycine, 80 mM sodium chloride, 0.05% BSA, 0.02% sodium azide (pH=9.0). No agglutination was observed upon addition of any of the blood samples.

3. EFFECT OF pH ON HEMOGLOBIN Ao AGGLUTINATION

BSA-coated latex was diluted to about 0.03% with the assay buffer. A 0.5 ml volume of the diluted latex was mixed with denatured Ao and the reactions incubated at room temperature for 20 minutes. The absorption at 540 nm for each mixture was then measured. The results are shown in Fig. 2 of the drawings.

Hemoglobin Ao was present at about 16 g/dl before dilution with the denaturant solution. For pH=6.75 and 7.40, the buffer was phosphate at 50 mM and 80 mM sodium chloride. For pH=8.5, 9.0 and 9.5, the buffer was glycine at 50 mM and 80 mM sodium chloride.

4. ASSAY PROCEDURE FOR DETERMINATION OF % HbA1c

A. Reagents:

Ab-Latex

The concentrated Ab-Latex is diluted to the appropriate concentration with 200 mM glycine buffer, pH containing 0.05% BSA-pf and 0.1% sodium azide. Mannitol at a 4% concentration may also be present in the buffer. Following dilution, the Ab-latex solution is sonicated briefly (5 to 10 sec.)

Agglutinator

A working solution of the agglutinator reagent is prepared from 1.0 mg/mL water stock solution. A

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10 $\mu\text{g/mL}$ solution is prepared by diluting the stock with 20 mM phosphate buffer, pH 6, containing 0.1% BSA-pf and 0.1% sodium azide.

Blood-based Calibrators and Clinical Samples

5 Blood based calibrator and blood samples must be denatured before use. For use in the assay, blood samples are well mixed by a rocker mixer such as the Ames Aliquot mixer (Miles Inc., Elkhart, IN, U.S.A.). These samples are then diluted 1:31 with
10 denaturant/oxidant (3 M NH_4SCN , 2 mg/mL $\text{K}_3\text{Fe}(\text{CN})_6$, 10 mM Tris, pH 7.5). The presence of the ferricyanide in the denaturant allows the diluted sample or calibrator to be used for the determination of the hemoglobin concentration. The
15 samples are allowed to sit at least 15 seconds before assaying on the OPTIMATETM Instrument (Miles Inc., Elkhart, IN, U.S.A.) to allow for complete denaturation of the protein and oxidation of the heme.

20 B. Turbidimetric Assay for HbA1c

Three different procedures for the OPTIMATE instrument have been used for determination of HbA1c. Two are endpoint formats; one requiring the manual addition of the agglutinator solution and
25 the other completely automating the assay. The third assay format is a rate assay. The following description briefly explains the general format of the three assays.

Endpoint Assay With Manual Addition of
Agglutinator

1. Using Chemistry 37 - Program New Test,
the following assay parameters are programmed as
5 USER CHEMISTRY #25.

Units - None
Test Type - Endpoint
Decimal Places - 2
Dispenser Usage - A
10 Lag Time A - 1200 sec. (the time between
dispensing of reagents and
absorbance read)
Equil Time - 6 sec. (the time sample is
held in the cuvette before
15 absorbance read)
Read Time - 5 sec. (time over which data
is collected, absorbance is read 7.7
times/sec.)
Cuvette Temp - 25°C
20 Standard Conc. - None
Factor - 1000 (factor by which result is
multiplied, converts to
milliabsorbance units)
25 Low I/A (Abs) - 2.000 (setting both the
low and high
absorbance limits
to 2.00 forces the
High I/A (Abs) - 2.000 OPTIMATE to print
the actual
30 absorbance
readings for each
sample)

Low Normal - None
High Normal - None
Absorbance Filter - 540 nm
Transport Temp - Room Temperature
5 Sample Volume - 10 μ L
Reagent Volume - 0.5 mL

2. Prior to starting the assay, verify that the pipetter/diluter is equipped with a 100 μ L sample syringe and a 1.0 mL reagent syringe.
10 The turrets of the pipetter/diluter are set to 10% (10 μ L) for the sample syringe and 50% (0.5 mL) for the reagent syringe.
3. Pipette at least 50 μ L of sample into the OPTIMATE sample cups.
- 15 4. Prime the pipetter/diluter with the Ab-latex solution.
5. Manually dispense 25 μ L of the agglutinator solution in the OPTIMATE reaction cups using an Eppendorf Repeater Pipette (1.25 mL capacity tip, setting = 1). Leave blank cup #60 and any other cups in which the turbidimetric reaction is not to take place. Pipette 25 μ L of buffer (20 mM phosphate, pH 6, 0.1% BSA-pf, 0.1% sodium azide) into these cups.
20
6. Start Chemistry #25 - USER CHEMISTRY #25 following all printed instructions. The OPTIMATE then automatically pipettes and dispenses sample and Ab-latex into the reaction cups and reads the absorbance after 20 minutes.
25
30

Endpoint Assay With Automatic Addition of
Agglutinator

1. Equip the OPTIMATE with a Gilford Automatic Dispenser with 1.0 mL syringe. The communication cable is connected to port J4 on the back of the OPTIMATE. The use of this external dispenser is enabled by:
- a. Enter Utility 15 followed by the security code 980456.
- b. Enter Utility 50, Check = 0, Option 20
- The turret of this dispenser is set to 50% (0.5mL).
2. Using Chemistry 37 - Program New Test, the following assay parameters are programmed as USER CHEMISTRY #24.
- Units - None
- Test Type - Endpoint
- Decimal places - 2
- Dispenser Usage - A and B
- Dispense B from Tower - Yes
- Time from Dispense A to Dispense B - 5 sec
- Lag Time A - 1200 sec
- Equil Time - 6 sec
- Read Time - 5 sec
- Cuvette Temp - 25°C
- Standard Conc. - None
- Factor - 1000
- Low I/A (Abs) - 2.000
- High I/A (Abs) - 2.000
- Low Normal - None
- High Normal - None

Absorbance Filter - 540 nm

Transport Temp - room temperature

Sample Volume - 10 μ L

Reagent Volume - 0.1 mL

- 5 3. Prior to starting the assay, verify that the pipetter/diluter is equipped with a 100 μ L sample syringe and a 250 μ L reagent syringe. The turrets of the pipetter/diluter are set to 10% (10 μ L) for the sample syringe and 10% (25 μ L) for the reagent syringe. Also verify that an immunoassay probe is used on the pipetter/diluter.
- 10 4. Pipette at least 50 μ L of sample into the OPTIMATE sample cups.
- 15 5. Prime the pipetter/diluter with the agglutinator solution.
6. Prime the external dispenser with Ab-latex solution.
7. Start Chemistry #24 - USER CHEMISTRY #24 following all printed instructions. The OPTIMATE then automatically pipettes and dispenses sample and agglutinator into the reaction cups, followed by the addition of Ab-latex five seconds later. The absorbance of the reaction is read after 20 minutes.
- 20 25

Rate Assay

1. Equip the OPTIMATE with a Gilford Automatic Dispenser with a 1.0 mL syringe. The communication cable is connected to port J4 on the back of the OPTIMATE. The use of this external dispenser is enabled by:
- 30

a. Enter Utility 15 followed by the security code 980456.

b. Enter Utility 50, Check = 0, Option 20.
The turret of the dispenser is set to 50% (0.5 mL).

2. Using Chemistry 37 - Program New Test, the following assay parameters are programmed as USER CHEMISTRY #23.

Units - None

Test Type - kinetic enzyme

Decimal Places - 2

Dispenser Usage - A and B

Dispense B from Tower - Yes

Time from Disp A to Disp B - 5 sec

Lag Time A - 20 sec

Equil Time - 5 sec

Read Time - 30 sec

Cuvette Temp. - 30°C

Standard Conc - None

Factor - 1000

Low I/A (Abs) - 2.000

High I/A (Abs) - 2.000

Absorbance Filter - 540 nm

Transport Temp - 30°C

Sample Volume - 10 µL

Reagent Volume - 0.1 mL

3. Prior to starting the assay, verify that the pipetter/diluter is equipped with a 100 µL sample syringe and a 250 µL reagent syringe.

The turrets of the pipetter/diluter are set to 10% (10 µL) for the sample syringe and 10% (25 µL) for the reagent syringe. Also verify that

an immunoassay probe is used on the pipetter/diluter.

4. Pipette at least 50 μ L of sample into the OPTIMATE sample cups.

5 5. Prime the pipetter/diluter with the agglutinator solution.

6. Prime the external dispenser with Ab-latex solution.

10 7. Start Chemistry #23 - USER CHEMISTRY #23 following all printer instructions. The

OPTIMATE then automatically pipettes and dispenses sample and agglutinator into the reaction cups, followed by the addition of

15 of this reaction is read after the 14-second lag period. The absorbance is read for a total of 30 seconds. The OPTIMATE determines the linear regression line through the data it has collected and presents the data in terms
20 of the change in absorbance per minute.

Hemoglobin Determination

For all blood samples, the concentration of hemoglobin in the sample must be determined in order to calculate the percent of HbA1c in the
25 sample. The same denatured sample used for the determination of HbA1c is used for the hemoglobin determination using the following protocol.

30 1. Using Chemistry 37 - Program New Test, the following assay parameters are programmed as USER CHEMISTRY #26.

Units - None
Test Type - Endpoint
Decimal Places - 2
Dispenser Usage - A
5 Lag Time A - 300 sec
Equil Time - 6 sec
Read Time - 5 sec
Cuvette Temp. - 25°C
Standard Conc - None
10 Factor - 1000
Low I/A (Abs) - 2.000
High I/A (Abs) - 2.000
Low Normal - None
High Normal - None
15 Absorbance Filter - 540 nm
Transport Temp - Room Temperature
Sample Volume - 70 µL
Reagent Volume - 0.5 mL
2. Prior to starting the assay, verify that
20 the pipetter/diluter is equipped with a 100 µL
sample syringe and a 1.0 mL reagent syringe.
The turrets of the pipetter/diluter are set to
70% (70 µL) for the sample syringe and 50%
(0.5 mL) for the reagent syringe.
25 3. Pipette at least 120 µL of sample into
the OPTIMATE sample cups.
4. Prime the pipetter/diluter with 200 mM
glycine, pH 9 buffer containing 0.05% BSA-pf
and 0.1% sodium azide.
30 5. Start Chemistry #26 - USER CHEMISTRY #26
following all printed instructions. The
OPTIMATE then automatically pipettes and
dispenses sample and buffer into the reaction

cups and reads the absorbance after five minutes.

Calculations

5 A number of calculations must be performed in order to transform the information provided by the OPTIMATE into a % HbA1c result.

10 1. A latex blank reaction (500 μ L latex + 35 μ L buffer) is included in every assay run. The absorbance of this reaction must be subtracted from every other result.

15 2. For all blood samples, the contribution of the absorbance of hemoglobin to the reaction is calculated using the information collected from USER CHEMISTRY #26. The absorbance result obtained there is divided by seven to calculate the absorbance of 10 μ L of blood. This value is then subtracted from the absorbance result obtained above.

20 3. In order to calculate a standard curve, a dummy immunoassay is programmed using Immunoassay #37 - Program New Test. The following parameters are programmed as USER IMMUNOASSAY #27.

Protocol - #1

25 Calibrator Values - enter the assigned calibrator values for the Glc-peptide calibrators (in terms of μ M HbA1c). These are dependent on the lot of Ab-latex used. The values of all other parameters are unimportant but must be

entered in order to store the
calibrator values.

4. A four parameter logit standard curve is
generated using Immunoassay #33 - Immunoassay
5 Calculations.

Test # - #27

Calc Scheme - 1. 4 Param Logit

Enter Absorbance results (minus latex blank)
for the Glc-peptide calibrators.

10 The OPTIMATE then generates the standard
curve, calculating the standard deviation of
the curve and the four parameters. Once
completed, calculate HbA1c of the unknown
samples by entering the absorbance (-ltx
15 blank, -Hb contrib) for all samples. The
result printed is the μ M HbA1c concentration.

5. The concentration of hemoglobin in each of the
blood samples is calculated using the information
from USER CHEMISTRY #26. The concentration of
20 hemoglobin is first calculated in terms of g/dL and
then this information is used to determine the mM
concentration of hemoglobin β chains present.

$$\text{Hb (g/dL)} = \frac{\text{Abs. from CHEM \#26} \times 16114.5 \times 252}{9.79 \times 1.0 \times 10,000}$$

25 where 16114.5 = MW of one Hb subunit
252 = dilution factor
9.79 = quarter millimolar
extinction coefficient
10,000 = correction for unit
conversion

Conc of hemoglobin in mM beta subunits:

$$\text{mM beta-Hb Subunits} = \frac{\text{g/dL Hb} \times 10}{64,456} \times 1000 \times 2$$

where 10 = conversion factor to g/L
64,456 = MW of hemoglobin (four
subunits)
1000 = conversion factor to mM
2 = conversion factor for Hb to
beta-Hb

6. Now that the $\mu\text{M HbA}_{1\text{C}}$ concentration and the
hemoglobin concentration (both in terms of the beta
subunits) are known, the % $\text{HbA}_{1\text{C}}$ can be determined.

Clinical Study

Clinical samples (71) were obtained from a
local clinical laboratory which had assayed the
samples by the conventional $\text{HbA}_{1\text{C}}$ affinity
chromatography method (Isolab, Akron, OH, U.S.A.).
The samples were assayed as above using the present
invention. The correlation is presented as Fig. 3
of the drawing.

5. HEMOGLOBIN Ao ASSAY

Using a buffer of 50 mM phosphate, pH 7.4,
containing 80 mM sodium chloride, 0.05% BSA and
0.02% sodium azide, the % Ao of a sample was
determined using the procedure described above for
% Alc. A typical dose response curve is shown in
Fig. 4 of the drawing.

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The present invention has been particularly described and exemplified above. Obviously, many other variations and modifications of the invention can be made without departing from the spirit and scope thereof.

5

The claims covering the invention are as follows:

~~WHAT IS CLAIMED IS:~~

1. In an analytical method for ^{the determination of} ~~determining~~ an analyte in a blood sample by latex agglutination immunoassay, wherein (a) an aqueous reaction mixture is formed by combining the blood sample with a multivalent latex antibody reagent comprising an anti-analyte antibody, or a fragment thereof, bound to a water suspensible latex particle, and additionally when the analyte is monovalent, with an agglutinator reagent comprising a plurality of epitopic binding sites for the anti-analyte antibody or fragment thereof, and (b) the resulting agglutination in the aqueous reaction mixture is measured as a function of analyte in the blood sample,

the improvement which comprises forming the aqueous reaction mixture to have a pH of about 8.5 or greater, whereby nonspecific agglutination of the latex reagent due to the presence of hemoglobin in the blood sample is substantially overcome.

2. The method of claim 1 wherein said latex particle has an effective net negative surface charge when suspended in a aqueous solution having a pH of about 7 or below.

3. The method of claim 1 wherein said latex particle is polystyrene.

4. The method of claim 1 wherein the aqueous reaction mixture is formed to have a pH of from about 8.5 up to the pH at which the

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immunoreactivity between the analyte and the latex reagent is substantially diminished.

5. The method of claim 1 wherein the aqueous reaction mixture is formed to have a pH of between about 8.75 and about 10.

6. The method of claim 1 wherein the aqueous reaction mixture is formed to have a pH of about 9.

7. An analytical method for ^{the determination of} ~~determining~~ hemoglobin Alc in a test sample comprising blood lysate by particle agglutination inhibition immunoassay, which method comprises the steps of:

- 5 (a) forming an aqueous reaction mixture comprising the blood sample and assay reagents comprising (1) a multivalent antibody reagent comprising an antibody, or a fragment thereof, to hemoglobin Alc bound to a water suspensible
10 polystyrene latex particle, and (2) an agglutinator reagent comprising a plurality of glycosylated peptides corresponding to the glycosylated peptide sequence of hemoglobin Alc, said reaction mixture formed to have a pH of about 8.5 or greater, and
15 (b) measuring the resulting agglutination as a function of hemoglobin Alc in the blood sample.

8. The method of claim 7 wherein the pH of the reaction mixture is less than about 10.

9. The method of claim 7 wherein the pH of
20 the reaction mixture is between about 8.75 and about 9.5.

10. The method of claim 7 wherein the pH of the reaction mixture is about 9.

11. The method of claim 7 wherein the blood
25 sample has been pretreated with a hemoglobin denaturant and the antibody reagent comprises a monoclonal antibody, or a fragment thereof, that binds specifically to denatured hemoglobin Alc.

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12. A test system when used for the determination of an analyte in a blood sample by latex agglutination immunoassay, comprising:

(1) a multivalent latex antibody reagent comprising an anti-analyte antibody, or a fragment thereof, bound to a water suspensible latex particle,

(2) when the analyte is monovalent, an agglutinator reagent comprising a plurality of epitopic binding sites for the anti-analyte antibody or fragment thereof, and

(3) a buffer that maintains the pH of the reaction mixture formed by combination of the test system components with the blood sample at about 8.5 or above.

13. The test system when used according to claim 12 wherein said latex particle has an effective net negative surface charge when suspended in an aqueous solution having a pH of about 7 or below.

14. The test system when used according to claim 12 or 13 wherein said latex particle is polystyrene.

15. The test system when used according to any one of claims 12 to 14 wherein the aqueous reaction mixture is formed to have a pH of from about 8.5 up to the pH at which the immuno-reactivity between the analyte and the latex reagent is substantially diminished.

16. The test system when used according to any one of claims 12 to 14 wherein the aqueous reaction mixture is formed to have a pH of between about 8.75 and about 10.

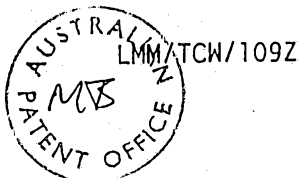
17. The test system when used according to claim 12 wherein the aqueous reaction mixture is formed to have a pH of about 9.

18. The test system when used according to claim 12 wherein the analyte is hemoglobin Alc and the agglutinator reagent comprises a plurality of glycated peptide residues corresponding to the glycated peptide sequence of hemoglobin Alc.

19. The test system when used according to claim 18 wherein the latex particle is polystyrene.

20. The test system when used according to claim 19 wherein the buffer is capable of maintaining the pH of the reaction mixture at between about 8.75 and about 10.

21. The test system when used according to claim 19 wherein the buffer is capable of maintaining the pH of the reaction mixture at about 9.



22. The test system when used according to claim 19 which additionally comprises a hemoglobin denaturant and wherein the antibody reagent comprises a monoclonal antibody, or a fragment thereof, that binds specifically to denatured hemoglobin Alc.

LMM/TCW/109Z



23. An analytical method for ^{the determination of} ~~determining~~ hemoglobin Ao in a blood sample, comprising the steps of:

5 (a) forming an aqueous reaction mixture comprising the blood sample and suspended latex particles and maintaining the pH of the reaction mixture at about 8 or below, and

10 (b) measuring the agglutination of said latex particles as a function of hemoglobin Ao in the blood sample.

24. The method of claim 23 wherein said latex particles have an effective net negative surface charge when suspended in an aqueous solution having a pH of about 7 or below.

15 25. The method of claim 24 wherein said latex particles are made of polystyrene.

20 26. The method of claim 23 wherein the pH of the reaction mixture is maintained from about 8 down to the pH at which the ability of hemoglobin Ao to agglutinate the polystyrene latex particles is substantially diminished.

27. The method of claim 23 wherein the pH of the reaction mixture is maintained between about 8 and about 4.

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28. A test system when used for the determination of hemoglobin Ao in a blood sample, comprising:

- (1) latex particles, and
- (2) a buffer that maintains the pH of an aqueous reaction mixture comprising the latex particles and the blood sample at about 8 or below.

29. The test system when used according to claim 28 wherein said latex particles have an effective net negative surface charge when suspended in an aqueous solution having a pH of about 7 or below.

30. The test system when used according to claim 29 wherein said latex particles are made of polystyrene.

31. The test system when used according to claim 28 wherein the buffer is capable of maintaining the pH of the reaction mixture at between about 8 and about 4.

32. An analytical method for the determination of hemoglobin Alc in a test sample comprising blood lysate by particle agglutination inhibition immunoassay, substantially as hereinbefore described with reference to Example 4 and Fig. 3.

33. An analytical method for the determination of hemoglobin Ao in a blood sample, substantially as hereinbefore described with reference to Example 5 and Fig. 4.

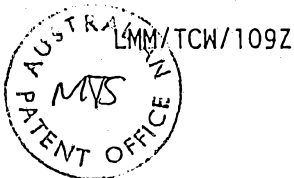
34. A test system when used for the determination of hemoglobin Ao in a blood sample, substantially as hereinbefore described with reference to Example 5.

35. A test system when used for the determination of hemoglobin Alc in a blood sample, substantially as hereinbefore described with reference to Example 4.

DATED this TWENTY-FOURTH day of DECEMBER 1990

Miles Inc.

Patent Attorneys for the Applicant
SPRUSON & FERGUSON



A₀ INTERFERENCE

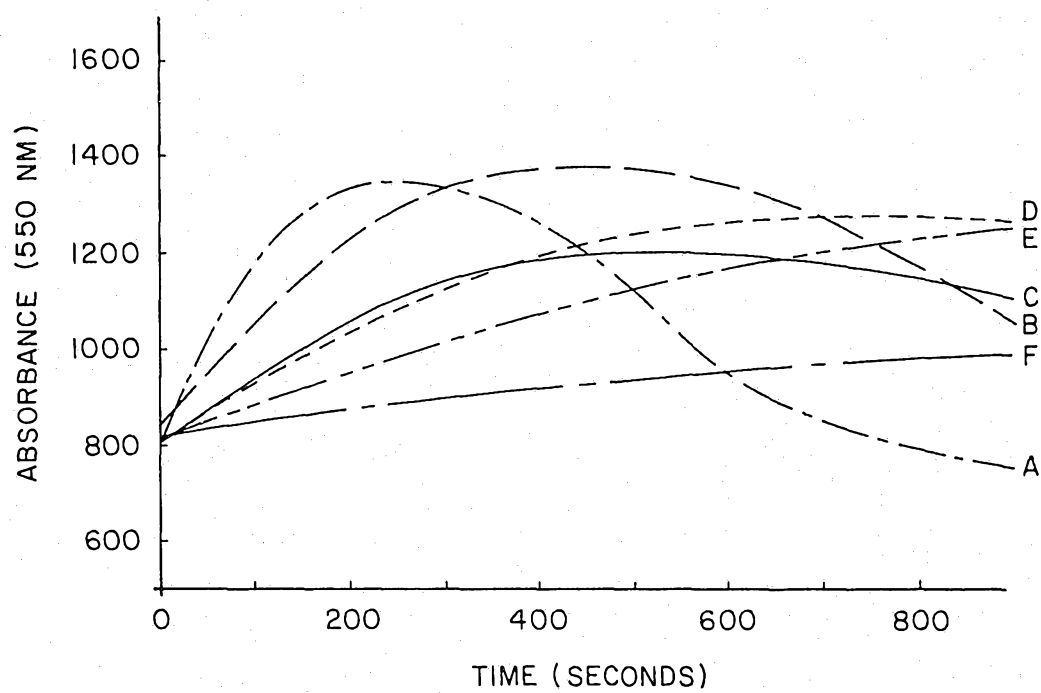


FIG. I

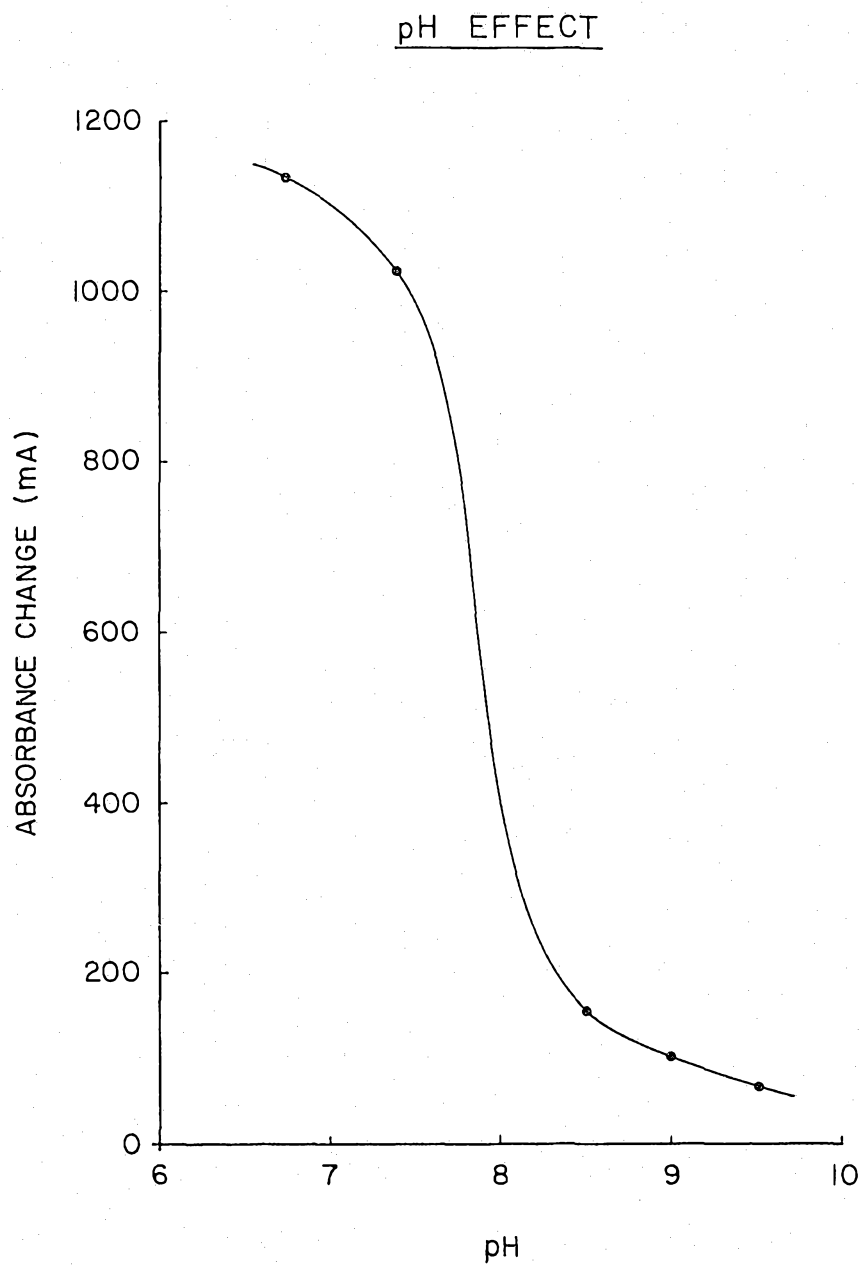


FIG. 2

Hb A1c IMMUNOASSAY

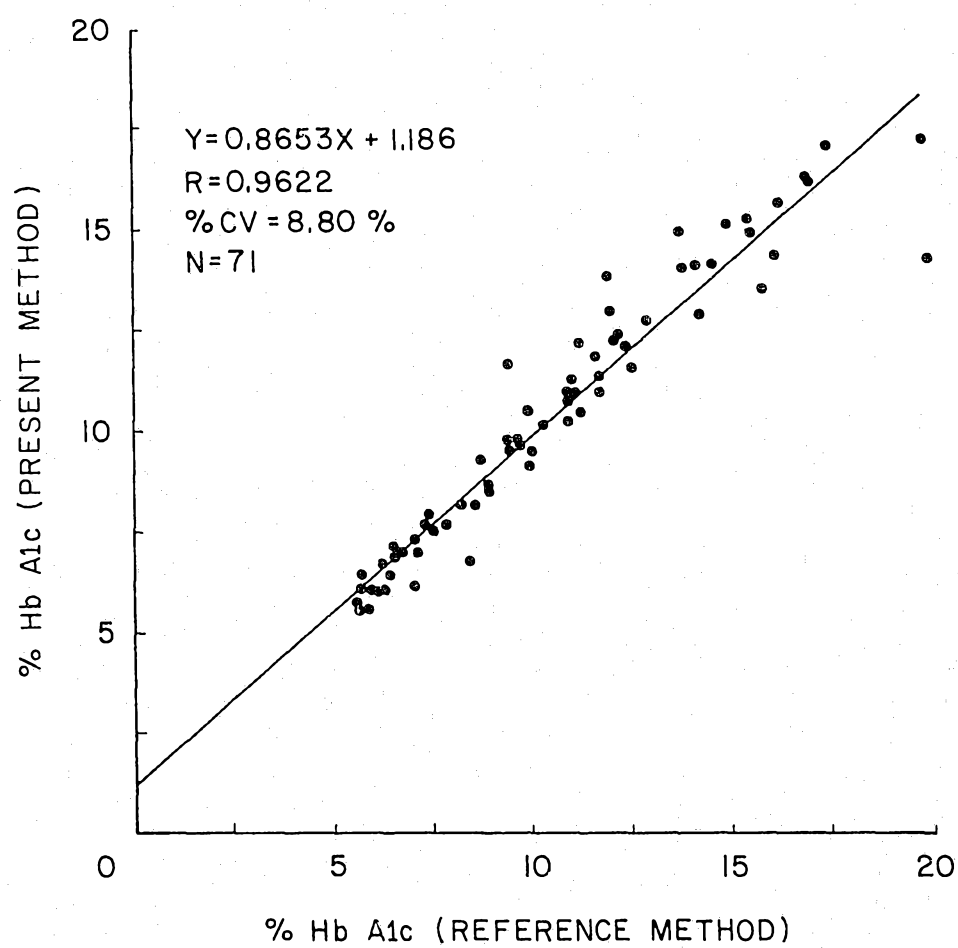


FIG. 3

Hb A₀ ASSAY

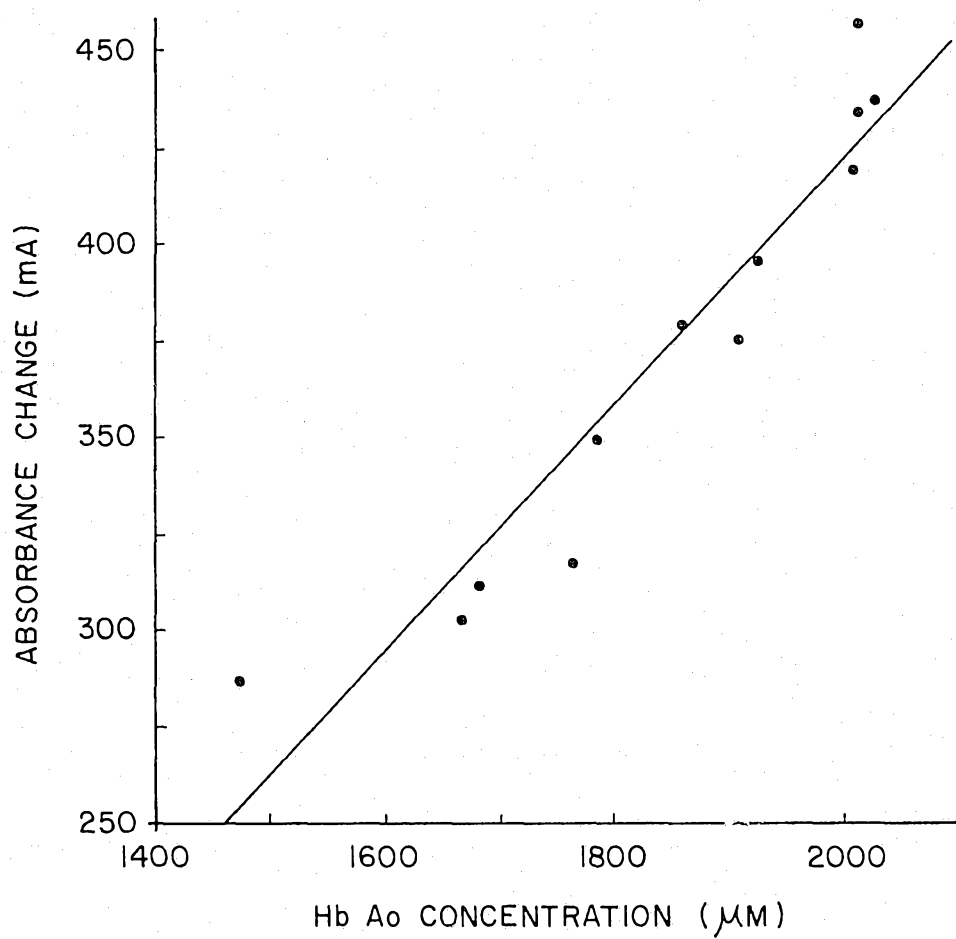


FIG. 4