Fluorometric method for determination of hydrogen peroxide and its applications to clinical chemistry.

George. Brotea
University of Windsor

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FLUOROMETRIC METHOD FOR DETERMINATION OF HYDROGEN PEROXIDE
AND ITS APPLICATIONS TO CLINICAL CHEMISTRY

by

GEORGE BROTEA

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

Windsor, Ontario, Canada
1988
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ISBN 0-315-48135-8
ABSTRACT

FLUOROMETRIC METHOD FOR DETERMINATION OF HYDROGEN PEROXIDE AND ITS APPLICATIONS TO CLINICAL CHEMISTRY

by

GEORGE BOTEAN

A fluorometric method for the determination of hydrogen peroxide using resorufin and peroxidase was developed using a one-step and a two-step procedure. For the one-step procedure, the within-run CVs at concentrations of hydrogen peroxide of 200 nmol/L and 40 nmol/L were 1.7 and 7.6%, respectively. The two-step procedure has a limit of quantitation of 4.4 nmol/L and a within-run CV of 6.5% at 20 nmol/L hydrogen peroxide concentration.

The determination of hydrogen peroxide with resorufin as the indicator reaction was applied to the measurement of glucose using glucose oxidase. This procedure was optimized for determination of glucose in serum at levels between 1.35-24 mmol/L. The within-run and between-run CVs determined for sera containing 4.50 ± 0.07 and 14.26 ± 0.2 mmol/L glucose were 0.5 and 1.7%, respectively, and 1.43 and 2.64%, respectively. Interferences from triglycerides, bilirubin, and hemoglobin up to 10.25 mmol/L, 150 μmol/L and 1.5 g/L, respectively, are
insignificant. Recoveries from glucose standard additions between 1.82 and 9.09 mmol/L to pooled sera containing 6.90 mmol/L glucose were between 97 and 102.8%. A correlation coefficient, $r = 0.993$ (regression equation $Y = 1.02X + 0.06$, $N = 60$), was obtained from the comparison studies with a Trinder method.

Determination of cholesterol using cholesterol oxidase, resorufin and peroxidase was also studied. This method permitted determination of cholesterol concentrations between 0.8 and 10 μmol/L. The within-run and between-run CVs for two control sera containing 3.19 and 5.62 mmol/L cholesterol were 1.9 and 2.5%, and 0.88 and 1.7%, respectively. Interferences from triglycerides, bilirubin, and hemoglobin up to 10.25 mmol/L, 115 μmol/L, and 5.6 g/L, respectively, were insignificant. Recoveries from standard additions between 2 and 6 mmol/L to a pool of sera containing 5.87 mmol/L cholesterol were between 94.5 and 104.3%. Comparison studies with a Trinder method showed a correlation coefficient, $r = 0.98$ ($N = 56$, regression equation $Y = 1.02X - 0.43$).

It was found that uric acid reacts with resorufin in the presence of peroxidase. This reaction was used for the determination of uric acid in aqueous solutions, but could not be applied to serum samples.
DEDICATION

Dedicated to my parents, my sister, and my daughter.
Their understanding and help made all this possible.
ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Roger J. Thibert for the encouragement, understanding, and kindness throughout my training in Clinical Chemistry, as well as for his direction of this work.

I would like also to thank Dr. Thomas F. Draisey for his helpful comments and suggestions during the completion of this work, and also for his guidance throughout my entire program.

I sincerely thank Dr. Bulent Mutus for his critique of this dissertation, and for his guidance and financial support during a part of my doctoral studies.

I thank Dr. Norman F. Taylor and Dr. Leslie R. Sabina for their helpful advice and comments, and also my external examiner, Dr. Roger R. Calam for his review and critique of my dissertation.

Many thanks to Dr. Harold S. Asselstine, for his direction and financial support during a part of my studies, and all the staff at Medical Laboratories of Windsor for their contribution to my training.

Many thanks also to Mr. Edwin Olivero and Mr. Michael R. Goodwin and the staff at the Pathology Department, Salvation Army Grace Hospital, Windsor, for their kind contribution to my training and for providing and analyzing the blood samples used in this work.
Special thanks to Mr. Herman Victorov, President, Technophar Equipment Service, Ltd., Windsor, Ontario, for his friendly support and encouragement to pursue my career goals.

Finally, I would like to express my thanks to all faculty, staff and colleagues at the Department of Chemistry and Biochemistry, University of Windsor. Their friendly and helpful approach made my years in the Clinical Chemistry program very fruitful and enjoyable.

I would also like to acknowledge Natural Sciences and Engineering Research Council of Canada for the scholarship which was essential in providing the financial help to accomplish this work.
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<td>CE</td>
<td>Cholesterol esterase</td>
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<td>COX</td>
<td>Cholesterol oxidase</td>
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<td>CV</td>
<td>Coefficient of variation</td>
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<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<tr>
<td>GOX</td>
<td>Glucose oxidase</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>λex</td>
<td>Excitation wavelength</td>
</tr>
<tr>
<td>λem</td>
<td>Emission wavelength</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidized)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
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<td>PHPAA</td>
<td>p-hydroxyphenylacetic acid</td>
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<tr>
<td>SAGH</td>
<td>Salvation Army Grace Hospital, Windsor, Ontario</td>
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<td>SD</td>
<td>Standard deviation</td>
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CHAPTER I
INTRODUCTION

Hydrogen peroxide, discovered at the beginning of the last century, is a weak acid, probably dissociating according to the scheme (1,2):

\[ \text{H}_2\text{O}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{O}^+ + \text{HO}_2^- \]

It is stable in both, acidic and basic solutions, but it is decomposed under the influence of heat, light, inorganic ions (such as Fe$^{3+}$, Al$^{3+}$), metals in the colloidal state (such as Pt, Au, Ag), and organic catalysts (such as catalase, peroxidase) (2). Hydrogen peroxide is a relatively strong oxidizing agent.

The development of techniques for measuring hydrogen peroxide at very low concentrations has been the focus of substantial research effort (3) in the last few years. There have been many reasons for this as can be seen from the following.

It was found that hydrogen peroxide could rapidly oxidize dissolved S(IV) compounds to sulfuric acid throughout the normal pH range of rain, cloud and fog waters (pH 2-7), thus being involved in the formation of acid rain (3-11).

Environmental scientists involved in investigations of the fate of dissolved chemical contaminants in surface
and ground waters have found that the presence of hydrogen peroxide in such waters influences the speciation of other chemicals and the establishment of a redox condition (12,13).

The determination of peroxidic species is a sensitive technique to measure yields of the \( \gamma \)-radiolysis of aqueous systems (14).

It has been recognized that the hydrogen peroxide arising from the oxidation of NADPH by a membrane bound NADPH oxidase is a functional end-product of the stimulated metabolic events associated with phagocytizing cells (15,16).

There has been increased interest in direct determination of lipid hydroperoxides (17) as a better alternative to the existing methods for quantitation of lipid peroxidation (18). Lipid peroxidation has been associated with a variety of pathological conditions, including atherosclerosis, aging, rheumatic diseases, cardiac and cerebral ischemia, respiratory distress syndrome, various liver disorders, irradiation, thermal injury, and toxicity induced by certain metals, solvents, pesticides, and drugs (18).

There has been wide-spread use of oxidases in enzyme-coupled assays for the determination of an increasing number of analytes in biological matrices, especially in clinical chemistry (19-42).
Although many selective spectrophotometric and
electrochemical methods have been described, the
determination of hydrogen peroxide at very low
concentrations requires fluorescence or chemiluminescence
methods (5). Chemiluminescence, although very sensitive is
not very selective (5), and the catalysts and pH
conditions required (41,42) are not very appropriate when
oxidases are used to produce hydrogen peroxide (42).
Fluorescence procedures, when used under optimum
conditions, are capable of yielding detection limits
similar to those of chemiluminescence methods (5).

There are two approaches to the enzymatic
determination of hydrogen peroxide at very low
concentrations: the measurement of formation of a
fluorescent compound from a nonfluorescent substrate and
hydrogen peroxide under the action of peroxidase (usually
horseradish peroxidase, HRP); and the transformation of a
fluorescent compound into a nonfluorescent one when it
reacts with hydrogen peroxide in the presence of
HRP (Fig. 1).

The most common nonfluorescent substrate of HRP for
the determination of hydrogen peroxide is \( p \)-hydroxy-
phenylacetic acid (PHPAA) (9,43). The fluorescent dimeric
product has a peak excitation wavelength (\( \lambda_{\text{ex}} \)) of 320
nm, and a peak emission wavelength (\( \lambda_{\text{em}} \)) of 400 nm
(10). PHPAA and HRP have been used lately for
FIGURE 1

FLUOROMETRIC APPROACHES TO THE ENZYMATIC DETERMINATION
OF HYDROGEN PEROXIDE

Legend

A. Direct fluorometry. The nonfluorescent
p-hydroxyphenylacetic acid (PHPAA) is transformed into
a fluorescent dimer, 6,6'-dihydroxy-7,7'-biphenyldiacetic
acid.

B. Inverse fluorometry. The fluorescent scopoletin is
transformed into a nonfluorescent compound. The structure
of the reaction product of scopoletin with hydrogen
peroxide is not known.
determination of trace amounts of hydrogen peroxide in environmental samples using automated methods (3, 5-11), and also for the determination of phosphatidylcholine lysophosphatidylcholine, and sphingomyelin in human serum (44).

Other nonfluorescent substrates which are transformed into fluorescent compounds by reaction with hydrogen peroxide under the action of HRP are homovanillic acid (4-hydroxy-3-methoxy-phenylacetic acid) (40, 45), dichlorofluorescin, acetyldichlorofluorescin, and a few other derivatives of dichlorofluorescin (15, 17, 46), but their applications are limited, mostly due to their instability in air.

The determination of hydrogen peroxide using inverse fluorometry, sometimes inappropriately called "fluorescence quenching", involves the transformation of scopoletin (7-hydroxy-6-methoxy-2H-benzopyran-2-one) into a nonfluorescent product when it reacts with hydrogen peroxide under the action of HRP (47, 48). For scopoletin λex and λem are 350 and 420-450 nm, respectively.

Esculetin (6, 7-dihydroxy-2H-benzopyran-2-one) was also found to be a substrate for HRP (47), but due to its weak fluorescence it was never used for analytical purposes.

The scopoletin method has been recently applied to the determination of hydrogen peroxide in environmental
samples (12). Inverse fluorescence using scopoletin has also been used for the determination of hydrogen peroxide in hepatic microsomes (49), and for the determination of glutathione peroxidase activity (by measuring the remaining hydrogen peroxide at fixed-time intervals, after the glutathione peroxidase was allowed to transform the reduced glutathione in oxidized glutathione using hydrogen peroxide) (50-52).

As a result of our laboratory's interest in the use of the resazurin-resorufin system in clinical chemistry (53-57), we have found that resorufin (7-hydroxy-3H-phenoxazin-3-one) is a substrate for HRP (58). Under the action of HRP, the highly fluorescent resorufin is rapidly transformed into a nonfluorescent compound (Fig. 2). Due to the fact that it is a highly fluorescent compound (59) and it can be easily transformed into nonfluorescent derivatives (53-57,60-65) which are substrates for various enzymes, resorufin already has many applications in bioanalytical chemistry (Fig. 3).

The above described fluorescence methods for the determination of hydrogen peroxide have some shortcomings due to long incubation times, large amounts of HRP required, and necessity to change the pH during the assay in order to get good sensitivities. Because the extinction coefficient of resorufin is almost three times higher than that of scopoletin, we considered resorufin as an
Figure 2

Reaction of Resorufin with Hydrogen Peroxide

Legend

Disappearance, with time, of the absorption peak of resorufin in the presence of HRP and excess hydrogen peroxide. Low concentration of HRP (0.3 U/cuvette) were used in order to have a slow reaction and to allow spectra to be taken before all the resorufin was used.

A: 0 min;
B: 0.5 min;
C: 2 min;
D: 3 min.

Distilled water was used as a blank.
FIGURE 3
APPLICATIONS OF RESORUFIN TO BIOANALYTICAL CHEMISTRY

Legend
A: $\beta$-Galactosidase;
B: Reductases;
C: Cytochrome P-450;
D: Choline esterase;
E: HRP;
R: Alkyl.
alternate substrate for HRP.

The primary aim of this study was to develop a method for the determination of hydrogen peroxide using resorufin and peroxidase, and to apply this method to the determination of analytes in body fluids using oxidase-peroxidase-coupled assays. Some of the advantages foreseen are indicated below.

The sensitivity and precision for the determination of hydrogen peroxide in water and biological matrices is increased, due to the fact that the extinction coefficient of resorufin is quite high, 40,000 (59).

The measurement of many analytes in body fluids can require smaller amounts of sample such as from pediatric patients and for mass screening applications.

A smaller amount of sample is used in many cases (66) to reduce the interferences known to exist when HRP is used for the determination of hydrogen peroxide (67-72).

Resorufin, as a reagent, presents the advantage that its excitation and emission spectra overlap only minimally with the natural fluorescence of serum, whereas scopoletin, PHPAA and fluorescein absorb and emit either in the ultraviolet or visible regions where some serum constituents also absorb and emit light (73) (Fig. 4).

Shorter reaction times may be required, since the reaction of hydrogen peroxide with resorufin in the
FIGURE 4

ABSORPTION SPECTRUM OF RESORUFIN AND EMISSION SPECTRA OF SERUM AND RESORUFIN

Legend

The spectra for serum and resorufin were taken in different cuvettes, and their intensities are arbitrarily illustrated.

A: Resorufin absorption;
B: Resorufin fluorescence;
C: Serum fluorescence.

Fluorescence and absorption spectra of resorufin overlap between 500 and 575 nm.
presence of HRP is very fast. This, again, may reduce some interferences.

The fluorescence methods are not as expensive as the chemiluminescence methods, but their use in oxidase-peroxidase coupled assays for determination of analytes in body fluids is yet very limited, possibly due to the lack of appropriate substrates for HRP. Resorufin may prove to be a better substrate than the existing ones.

HRP is, besides the appropriate fluorophor (or chromophor), the other reagent necessary in the enzymatic determination of hydrogen peroxide. HRP is a globular glycoprotein consisting of 308 amino acid residues, a hemin group, and eight neutral carbohydrate side chains, and has a molecular weight of 40,000 Daltons. HRP catalyzes the oxidation of organic substrates with hydrogen peroxide as the ultimate electron acceptor:

\[ \text{RH}_2 + \text{H}_2\text{O}_2 \rightarrow \text{R} + 2\text{H}_2\text{O} \]

The mechanism of the reaction involves the addition of hydrogen peroxide to the Fe(III) resting state to form compound I (Fig. 5), a high-valent iron-oxo derivative (74) (formally two oxidation equivalents above the Fe(III) state), which is then reduced to the Fe(III) resting state through compound II (75). HRP catalyzes some reactions in which the source of the incorporated oxygen is not hydrogen peroxide (74), suggesting that the oxo transfer
FIGURE 5
CATALYTIC CYCLE OF HRP

Legend

The overall charge on the Fe(III) state (top left) and on compound I (top right) is +1, whereas compound II (bottom center) is neutral. Reproduced with modifications from reference 74.
from compound I is not involved. These reactions are the N-dealkylation of alkylamines, the hydroxylation of a benzylic methyl group, and the conversion of sulfides to sulfoxides (74, 76-80). HRP has also been reported to catalyze epoxidation of styrene (81).

We investigated the resorufin method for determination of hydrogen peroxide in oxidase-peroxidase coupled assays for measuring glucose, uric acid, and cholesterol in serum.

D-Glucose exists in aqueous solutions in two configurational modifications, α-D-glucose (36%) and β-D-glucose (64%) (82). D-Glucose is the most important carbohydrate which occurs naturally in large amounts as a component of di- and polysaccharides. It is found in free form in sweet fruits and in the blood of animals and human beings, serving as one of their sources of energy (83). Plasma glucose concentration is regulated by the interaction of a number of hormones with insulin being one of the most important. The main clinical use of glucose measurement is the detection and management of diabetes mellitus.

The most employed assays for glucose in biological fluids are: the hexokinase and glucose-6-phosphate dehydrogenase method (84), colorimetric methods using glucose oxidase (GOX) and HRP (19,25,29,30,35,36,85), and the glucose dehydrogenase method (86).
The determination of the glucose level in blood samples is one of the assays most frequently carried out in a clinical chemistry laboratory (84).

Glucose oxidase is a globular protein formed by two identical subunits linked by disulfide bonds. One mole of iron and one mole of FAD (87-89) are found in each subunit. This enzyme is specific for β-D-glucose, and catalyzes the reaction:

\[ \text{D-GLUCOSE} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{GOX} \rightarrow \text{D-GLUCONIC ACID} + \text{H}_2\text{O}_2 \]

Glucose oxidase is widely used for the determination of glucose in biological fluids based on the fact that the hydrogen peroxide produced can be measured using fluororometric (90), polarographic (31,33,91), or colorimetric (19,25,29,30,37) methods. In the last few years, efforts were made to improve the sensitivity of the colorimetric methods (25,29) and also some chemiluminescence methods were proposed (32,42).

Uric acid is the main product (92) of the catabolism of purines (adenosine and guanosine). The purines can be either of dietary source or from degradation of endogenous nucleic acids. Man is the only mammal that does not extend purine metabolism up to the formation of allantoin. Hyperuricemia, as in gout, (serum or plasma uric acid concentrations higher that 0.420 mmol/L in men, and 0.360 mmol/L in women) is the major pathological
manifestation related to abnormal levels of uric acid and is the result of either overproduction or underexcretion. Hypouricemia is rare and can be generated either by reduced purine synthesis due to hepatocellular disease or by defective renal tubular reabsorption (92).

The determination of uric acid in biological fluids is performed in most cases using either the reduction of alkaline phosphotungstate (a method modified with many variations in order to increase its selectivity) (93,94) or the oxidation of uric acid to allantoin and hydrogen peroxide by uric acid oxidase (95,96).

Besides being a major analyte in serum or plasma, uric acid is also an important interference in peroxidase indicator reactions (97).

Uric acid oxidase (uricase) is an enzyme which catalyzes the reaction:

\[
\text{URIC ACID} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{URICASE}} \text{ALLANTOIN} + \text{H}_2\text{O}_2
\]

Uricase is found in bacteria, mammals, but not in humans. Uricase obtained from pig liver contains one atom of copper per molecule (98). Uricase isolated from other sources such as bovine liver, Candida utilis and Arthrobacter pasceus does not contain copper. However, some of their properties, such as inhibition and pH dependence are similar to those of the pig liver enzyme (98).
Cholesterol is an essential constituent of all types of cellular membranes in higher organisms. It is the precursor of the numerous steroid hormones of the adrenal cortex and sexual glands. Cholesterol is degraded by the liver, mainly into the bile acids, which in turn serve as emulsifiers in the intestinal tract. In blood, cholesterol is mainly esterified with fatty acids and associated with lipoproteins. High levels (above 5.2 mmol/L) of "total" (sum of free and esterified) cholesterol in serum are a major risk factor for the development of atherosclerosis and myocardial infarction (99,100). Increased levels of cholesterol may also be found in the presence of various liver and renal diseases. Decreased cholesterol levels are usually indicative of genetic lipid disorders or inadequate liver function.

Precise and accurate cholesterol measurements are required to identify and treat individuals with high blood cholesterol (100). Most of the currently used methods for the determination of total cholesterol use cholesterol esterase (CE) and cholesterol oxidase (COX) (21,26,99) with photometric quantitation of 4-cholesten-3-one (101), or the measurement of generated hydrogen peroxide by colorimetric (21,26), fluorometric (102), electrochemical (103), or chemiluminescence (104) methods. Direct chemical methods for determination of cholesterol (105) are no longer appealing to today's automated clinical
chemistry laboratories.

Cholesterol esterases with broad ranges of specificity towards various fatty acid residues are necessary in order to achieve complete hydrolysis of cholesterol esters (about 70-80% of serum cholesterol is esterified with a large variety of fatty acids) within a minimum incubation period. A CE isolated from a strain of Pseudomonas (106) has been shown to be very appropriate for the determination of total cholesterol.

Cholesterol oxidases isolated from microbial sources (107) catalyze the following reaction:

\[
\text{CHOLESSTEROL} + O_2 + H_2O \xrightarrow{\text{COX}} 4\text{-CHOLESTEN-3-ONE} + H_2O_2
\]

Their structural properties are not well known yet, but the enzymatic properties have been investigated in detail in certain cases (108). The COX isolated from Streptomyces species show a high substrate specificity for 3β-hydroxysteroids, with the highest oxidation rates when cholesterol is the substrate (108).

A study of the current status of blood cholesterol measurement in clinical laboratories in the United States (100) recommends that the bias (deviation from the true value) and CVs of cholesterol methodologies currently in use should not exceed 5% (3% as a target within five years), and modifications in reagent and instrument systems have to be made to achieve adequate specificity.
and to minimize the effect of interfering substances.

Studies performed in order to assess the reliability of blood cholesterol measurements have documented (100,109) the difficulty in obtaining reliable results, and inaccuracy appears to be the major problem.

It is also recommended (100) that all adults should know their cholesterol level, and indeed, there is lately a trend toward population screening for cholesterol. Portable chemistry analyzers for cholesterol measurement are available (100), but their performance and cost per test are still in need of improvement.

In the light of the above considerations, the purpose of this study was to investigate the possible use of the resorufin reaction with hydrogen peroxide for the determination of hydrogen peroxide in aqueous solutions, and as indicator for oxidase-peroxidase coupled assays.
CHAPTER II
MATERIALS AND METHODS

II.1. Materials

Peroxidase (donor:hydrogen-peroxide oxidoreductase; E.C. 1.11.1.7) was obtained from Boehringer Mannheim Canada, Dorval, PQ H9P 1A9, and from Sigma Chemical Company, St. Louis, MO 63176.

Glucose oxidase (β-D-glucose:oxygen oxidoreductase; E.C. 1.1.3.4) type V-S from Aspergillus niger, uricase (urate oxidase; urate:oxygen oxidoreductase; E.C. 1.7.3.3) type V from porcine liver, cholesterol oxidase (cholesterol:oxygen oxidoreductase; E.C. 1.1.3.6) from Streptomyces species, cholesterol esterase (sterol-ester acylhydrolase; E.C. 3.1.1.13) from Pseudomonas fluorescens, α-D-glucose, cholesterol (99%), and bilirubin, were from Sigma Chemical Company. Uricase, from Aspergillus flavus, was purchased from Boehringer Mannheim Canada.

The enzyme activities quoted are those indicated by the supplier. The unit definitions are: HRP, the amount of enzyme that forms 1.0 μmol purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C; GOX, the amount of enzyme that oxidizes 1.0 μmol of β-D-glucose to D-gluconic acid and hydrogen peroxide per min at pH 5.1 at 35°C; uricase, the amount of enzyme that converts 1.0 μmol of uric acid to allantoin per min at pH 8.5

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at 25°C; CE, the amount of enzyme that hydrolyzes 1.0 
μmol of cholesteryl oleate to cholesterol and oleic 
acid per min at pH 7.0 at 37°C, in the presence of 
taurocholate; COX, the amount of enzyme that converts 1.0 
μmol of cholesterol to 4-cholesten-3-one per min at pH 
7.5 at 25°C.

Uric acid (99.7%) was obtained from the National 
Bureau of Standards, Gaithersburg, MD 20899.

Lyposin II, 10% solution (intravenous fat emulsion 
10%) was from Abbott Laboratories, Mississauga, 
ON L5N 3R7.

Reagent kits for the determination of glucose, uric 
acid, and cholesterol on the Perspective Analyzer, 
calibrators for glucose, uric acid and cholesterol (Omni 
Cal I, and II) were purchased from American Monitor 
Corporation, Indianapolis, IN 46268.

Control sera, Serachem Clinical Chemistry Control 
Serum (bovine), unassayed, Level I and II were from Fisher 
Scientific Co., (Canada), Nepean, ON K2L 7L6.

Resorufin (7-hydroxy-3H-phenoxazin-3-one, sodium 
salt), was purchased from Aldrich Chemical Co., Milwaukee, 
WI 53233.

Triton X-100 was obtained from United States 
Biochemical Corporation, Cleveland, OH 44128.

Hydrogen peroxide solution (about 30 % w/w) and other 
reagents of analytical grade mentioned below were obtained
from BDH Chemicals Canada, Toronto, ON M82 1K5.

The reagent water was distilled and passed through a Zenopure Laboratory Water System, Mega-90, purchased from Canlab, Mississauga, ON L5N 3P1.

Blood and serum samples used in the clinical studies were split specimens obtained from blood samples drawn for routine analytical purposes at The Salvation Army Grace Hospital, Windsor, Ontario (SAGH). Since they were collected after the ordered tests were performed, these samples were qualified as leftovers, and thus there was no need to obtain patient authorization. When collected, the samples were assigned numbers, rather than the patients' names, in order to assure patient confidentiality.

II.2. Instrumentation

**Spectrofluorometer**: Fluorescence measurements were performed 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 Corporation, Downsview, ON M3N 1X3.

**Spectrophotometer**: Absorbance spectra were obtained using a Hewlett Packard Diode Array Spectrophotometer Model 8451A, equipped with a Hewlett Packard Plotter Model 7470A, purchased from Hewlett Packard (Canada) Ltd., London, ON N6E 2S5.

Spectrophotometric determination of the glucose, uric acid, and cholesterol were performed at SAGH on a
Perspective Analyzer purchased from American Monitor Corporation.

**Balances:** A Mettler PC 4400 Delta Range electronic balance was used for weights above 1.0g, and a Mettler H16 semiautomatic balance was used for weights below one gram. Both balances were from Fisher Scientific Co.

**pH Meter:** The pH was measured using a Fisher Accumet pH meter obtained from Fisher Scientific Co. It was equipped with a glass electrode purchased from Graphic Controls, Buffalo, NY 14240.

**Micropipettors:** The micropipettors used were Gilson Pipetman Models P-200D and P-1000D, and SMI (10 μL) with disposable pipet tips available from Mandel Scientific Company Ltd, Rockwood, ON NOB 2K0.

**Cuvettes:** Quartz fluorometer cuvettes (dimension: 1.00 x 0.20 x 4 cm) were used to measure fluorescence. The cuvettes were obtained from Hellma (Canada) Ltd., Concord, ON L4K 2C8. The cuvettes were positioned in the fluorometer in such a way that the emitted light had the smaller pathlength (maximum 0.20 cm) through the solution. A plastic spacer was placed at the bottom of the cuvette holder to elevate the cuvette and to allow for small volume measurements (0.5 mL).

**Glassware:** Disposable borosilicate tubes and all other glassware were obtained from Fisher Scientific Co.
II.3. Reagents

II.3.1. Determination of hydrogen peroxide

Phosphate and citrate buffers were prepared to be 0.2 mol/L and then diluted to 0.1 mol/L with water containing the appropriate amount of Triton X-100 so that the working buffers were 0.1 mol/L and contained 0.2% v/v Triton X-100. Sodium tetraborate buffer was made to be 0.1 mol/L. The buffers were stable for several weeks when stored at room temperature.

Resorufin solution (42.5 μmol/L) was prepared by dissolving 2.5 mg resorufin in 250 mL water. This solution should be prepared fresh before the assay. Stored in the dark, this solution is stable for several hours.

Hydrogen peroxide stock solution was prepared by diluting 5 mL hydrogen peroxide 30% w/w to 500 mL with water. The concentration of the stock solution was determined using a permanganate titration method (110). The hydrogen peroxide solution is stable for several weeks at 4°C, in the dark, as determined by weekly titration. Hydrogen peroxide working solutions were prepared by dilution of the stock solution before the assay. These solutions were kept on ice, in the dark, and used within one hour.

HRP solutions were prepared by dilution of the original suspension (about 250 U/mg as defined by the supplier) with reagent water. Dilute solutions of the
enzyme, stored in the dark at 4°C, were stable for several weeks.

II.3.2. Determination of glucose

Glucose oxidase was diluted with distilled water to contain 100,000 U/L. HRP solution was made to contain 250,000 U/L, by diluting the original solution with distilled water. These solutions were kept in the dark at 4°C, and were stable for several weeks.

Glucose was dissolved in distilled water to obtain a 0.1 mol/L solution (1.82 g/100 mL). The solution was left at room temperature for at least 24 hours to allow for mutarotation to occur. All glucose concentrations are expressed in this text as total amount of glucose present in solution.

Resorufin solutions, 425 μmol/L, were obtained by dissolving 2.5 mg resorufin in 25 mL water. This solution should be prepared before the assay. Stored in the dark, this solution is stable for several hours.

II.3.3. Determination of uric acid

Uricase from porcine liver or from Aspergillus flavus was dissolved in distilled water to contain 10,000 U/L. These solutions, kept at 4°C, in the dark, are stable for several weeks.

Glycine buffer was prepared to contain 0.7 mol/L and the pH was adjusted to 9.5.

Working urate standards (0.1 mmol/L to 1 mmol/L) were
prepared by dissolving uric acid in glycine buffer (iii).

HRP and resorufin solutions were prepared as described in part II.3.2.

II.3.4. Determination of cholesterol

Cholesterol esterase was dissolved in distilled water to contain 10,000 U/L. Cholesterol oxidase was dissolved in distilled water to contain 100,000 U/L. Both solutions were stable for several weeks when kept in the dark at 4°C.

Cholesterol solutions were obtained by dissolving 0.0386 g cholesterol in 1 mL Triton X-100 using stirring and slight heating (40°C). This solution was diluted with distilled water to 10 mL to obtain a concentration of 10 mmol/L cholesterol. This solution was stable for several weeks in the dark at room temperature.

HRP was diluted with distilled water to contain 75,000 U/L.

Resorufin was prepared as described in part II.3.2.

II.3.5. Interference and standard addition studies

Saline solution was prepared to contain 150 mmol/L sodium chloride.

Glucose solution, 1 mol/L, was prepared as described in part II.3.2.

Cholesterol solution, 60 mmol/L, was prepared as described in part II.3.4.

Bilirubin solution was obtained by dissolving 37 mg
bilirubin in 10 mL 0.1 mol/L sodium carbonate. This solution was prepared immediately before use and kept in the dark (110).

Hemoglobin solution was obtained by hemolysis of red blood cells. A lavender top tube (Vacutainer Brand, Becton-Dickinson no. 6455, available from Fisher Scientific Co.) was centrifuged, and the plasma removed and replaced with distilled water. After about 30 min the tube was centrifuged again and the supernatant was analyzed at SAGH using a Coulter counter (Automated Hematology analyzer, model S 880, purchased from Coulter Electronics Inc., Burlington, ON L7L 5J6). The hemoglobin content was found to be 62 g/L, and cells were absent.

Lyposin II solution, 10%, was used for the preparation of lipemic sera.

II.4. Procedure

II.4.1. Determination of hydrogen peroxide

One-step determinations. Unless otherwise specified in the text, 10 μL resorufin, 10 μL HRP (0.75 units) and 470 μL buffer were added to a 1-mL cuvette. The fluorescence was measured using a sensitivity control setting of 4 and a selector control setting of X10, at a λεx of 568 nm and a λεm of 583 nm. Next, 10 μL of hydrogen peroxide (standard or sample) solution was added and the end point of fluorescence measured (the reaction occurs almost instantly). The
decrease in fluorescence due to dilution (blank) was determined by a similar procedure, but replacing the hydrogen peroxide with water. The blank value was subtracted from the value obtained for the hydrogen peroxide determination to obtain the actual decrease in fluorescence due to the reaction of hydrogen peroxide with resorufin. Knowing that the decrease in fluorescence is proportional to the amount of hydrogen peroxide, the concentration of hydrogen peroxide in the sample can be calculated using either a standard curve or the value of a single standard hydrogen peroxide solution. The blank and the standard measurements were performed three times in a run and the averages were used for calculations.

Two-step determinations. Unless otherwise specified in the text, 10 µL resorufin, 10 µL HRP (0.75 units), 10 µL hydrogen peroxide solution and 100 µL citrate buffer were added to a 1-mL cuvette. The mixture was agitated slightly for about 1 min and then 360 µL borate solution was added and the fluorescence was determined. A blank was performed using a similar procedure, but replacing the hydrogen peroxide with water. The concentration of hydrogen peroxide in the sample was determined either from a standard curve or by running a single hydrogen peroxide standard. The measurements of the blank and standard were performed three times in each run and the averages were used for calculations.
II.4.2. Determination of glucose

Reagent A: 200 µL HRP solution, 200 µL resorufin solution and 9.2 mL phosphate buffer are mixed together and kept in the dark. This reagent is sufficient for 20 samples.

480 µL Reagent A, and 10 µL of sample (aqueous glucose solution or serum) were added to a 1-mL cuvette and the cuvette was placed in the spectrofluorometer. Using the 0% adjustment knob, the recorder pen was adjusted to a position very close to the upper edge of the recorder paper. The settings for the sensitivity control and selector control were 1 and X10, respectively. Using a SMI pipettor, 10 µL of GOX were added to the reaction mixture and the pipettor tip was used to mix the solution before the decrease in fluorescence with time was measured. The reaction rate is determined by measuring the linear portion of the curve usually occurring between 2 and 4 min from the starting time. Using standards and/or serum calibrators, a standard curve can be obtained and used for quantitation of unknown samples.

For correlation studies, glucose was determined in serum using the American Monitor Corporation method (modified Trinder) as applied on the Perspective Analyzer (66).
II.4.3. Determination of uric acid

Phosphate buffer, 470 μL, 10 μL of resorufin and 10 μL HRP were added to a 1-mL cuvette and the cuvette was placed in the spectrofluorometer. Using the 0% adjustment knob, the recorder pen was adjusted to a position very close to the upper edge of the recorder paper. The settings for the sensitivity control and selector control were 1, and X10, respectively. Using a SMI pipettor, 10 μL of aqueous uric acid sample were added and the decrease in fluorescence was measured. Using standard solutions, a calibration curve can be obtained and used for quantitation of unknown samples. A blank, in which uric acid was substituted for by distilled water, was used to make the appropriate corrections.

II.4.4. Determination of cholesterol

II.4.4.1. Determination of cholesterol in aqueous solution and serum

Reagent A: 200 μL HRP solution, 200 μL resorufin solution and 9.2 mL of phosphate buffer, pH 6.4 are mixed together. This reagent was kept in the dark and it was sufficient for measuring 20 samples.

Reagent A, 480 μL, and 10 μL of sample (aqueous cholesterol solution or serum already submitted to the action of CE) were added to a 1-mL cuvette and the cuvette was placed into the spectrofluorometer. The sensitivity control setting was 1, and the selector
control setting was X10. Using the 0% adjustment knob, the recorder pen was adjusted to a position very close to the upper edge of the recorder paper. COX (10 μL) was added with a SMI pipettor and the subsequent decrease in fluorescence was measured (the end-point of the reaction was reached in about 1-2 min from the start). A blank, in which the cholesterol sample was replaced with distilled water, was utilized to measure the decrease in fluorescence due to dilution. The value obtained was used for the necessary correction of the differences in fluorescence obtained for samples (the blank is usually 3-5 units). Employing standards and/or serum calibrators, a standard curve was obtained and used for quantitation of unknown samples.

The hydrolysis of cholesterol esters in serum samples was combined with the following dilution process. A 10-μL sample of serum was added to 80 μL of saline and 10 μL of CE. The mixture was slightly shaken and left for 10 min at room temperature and then, 300 μL phosphate buffer was added. A 10-μL aliquot was used for the determination of total cholesterol.

For correlation studies, cholesterol was determined in serum samples using the American Monitor Corporation method (a modified Trinder) applied on a Perspective Analyzer (M12) at SAGH.
II.4.4.2. Determination of cholesterol using dry blood spot samples

Blood spots were obtained by placing about 10 µL of blood from freshly mixed lavender top tubes (same type as described in part II.3.5.) and from serum control on Whatman no.1 filter paper. After they were left to dry at room temperature, a disc, about 6.5 mm in diameter was punched from as close as possible to the edge of the spot and placed directly into a 10 x 75 mm borosilicate glass test tube. After extraction with isopropanol (2 x 0.5 mL), the isopropanol was evaporated, and the residue resuspended in 90 µL phosphate buffer, pH 6.4, containing 10% Triton X-100, to which 10 µL CE was added. After 10 min incubation, 10-µL samples were aliquoted for cholesterol determination. Controls were treated the same way and used for calculation of the unknown samples. The plasma from the lavender tubes was separated by centrifugation and analyzed for cholesterol as described in part II.4.1.

II.4.5. Interference and standard addition studies

Pools of clear, non-hemolyzed sera were used for interference and standard addition studies (II3). Aliquots of 500 µL were placed in test tubes and the interference (bilirubin, hemoglobin, or triglycerides solutions) or standard (glucose or cholesterol solutions) were added to them according to the scheme
presented in Table I.

The interferant or standard in each test tube is the sum of that added plus its base level in the pooled sera. The six tubes are homogeneous in all other constituents (113). Test tube no. 1 was used to measure the amount of analyte and/or interference in the initial serum, and test tubes 2-6 were used to assess the recoveries of the added analyte or the effects of interference on the analyte measurement. Each experiment was performed in triplicate.

Error bars in all figures represent standard error. Their absence in some figures indicates that their size is smaller than that of the symbol.
TABLE I
PREPARATION OF SAMPLES FOR
STANDARD ADDITION AND INTERFERENCE STUDIES

<table>
<thead>
<tr>
<th>Test tube (nr)</th>
<th>Serum (µL)</th>
<th>Stock standard or interference (µL)</th>
<th>Solvent a (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>500</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>500</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

aThe added solvent was the one used to prepare the stock standard or interference.
CHAPTER III
RESULTS AND DISCUSSION


The variation of fluorescence intensity with pH is known for many fluorescent substances (59). In the case of scopoletin (47, 48) and PHPAA (9), the reaction between the hydrogen peroxide and the peroxidase substrate is performed at pH 4.5 and then, before fluorescence measurement, the pH is changed to 10 by appropriate buffer addition. This methodology implies an additional step and complicates the blanking and the fluorescence measurement itself. Since our intent was to develop a simple method for the determination of hydrogen peroxide that can be easily adapted to enzyme-coupled assays for analytes in biological fluids, with the possibility of determining more than one analyte in the same cuvette, a study of the variation of resorufin fluorescence intensity with pH was performed. As can be seen in Fig. 6, there is a strong decrease in the intensity of fluorescence as the pH decreases, with a plateau between pH 6.6 and 7.0, and a change in slope as the pH changes from basic to acidic. A shift in the absorption maxima and disappearance of fluorescence was observed at pH below 5.5. (Fig. 7).

Although the intensity of resorufin fluorescence decreases as the pH decreases, an increase in the number of moles of resorufin which react with hydrogen peroxide
FIGURE 6

VARIATION OF FLUORESCENCE INTENSITY OF RESORUFIN WITH pH

Legend

The fluorescence intensity is calculated as percentage of the fluorescence intensity at pH 9.5. The buffers used were: pH 4.5, citrate; pH 5.8-8.1, phosphate; pH 8.1-9.5, borate. λex and λem were adjusted between 560 to 572, and 582 to 585, respectively, in order to obtain maximum fluorescence intensity at each pH.

The measurements were performed in triplicate, and the averages were used for plotting.
FIGURE 7

ABSORPTION PEAK OF RESORUFIN AT ACID AND BASIC pH

**Legend**

A: pH = 4.5
B: pH = 8.5

Spectra were taken using resorufin solutions in phosphate buffers.
Distilled water was used for blank.
was observed when the pH was decreased (Fig. 8). Figure 8 does not show the real increase in the yield because of the lowering of the fluorescence intensity of resorufin due to the decrease of the pH (see Fig. 6).

Using small amounts of enzyme, so that the rate of reaction measurements could be performed, it was found that the rate of reaction continues to increase as the pH decreases, even below pH 5.8 (Fig. 9). Since the pKa for resorufin is given as 5-6 (59), it can be concluded that protonation of the heterocyclic nitrogen may increase the rate and the yield of the reaction.

Because the curve of decrease in fluorescence for the same amount of hydrogen peroxide starts to flatten at about pH 6.4, this pH was chosen as one of the assay conditions. A lower pH will not increase the signal per mole of hydrogen peroxide, and the buffering capacity of the phosphate buffer will be diminished.

Figure 10 shows that there is a linear response of the fluorescence intensity versus concentration of resorufin at pH 6.4. The increment of the signal for an increase in concentration of 42.5 pmoles/cuvette for resorufin concentrations between 42.5 pmoles/cuvette (85 nmol/L) and 722 pmoles/cuvette (1435 nmol/L) was found to be 51.6 units with a coefficient of variation of 6.5% for the whole range.

In similar conditions, 4-hydroxycoumarin (which has
FIGURE 8

APPARENT VARIATION OF THE EXTENT OF REACTION
(NUMBER OF MOLES) OF RESORUFIN WITH HYDROGEN PEROXIDE
AS A FUNCTION OF pH

Legend

The decrease in fluorescence for each pH value is expressed relative to the value obtained at pH 5.6. The concentrations in cuvette were: 765 nmol/L resorufin; 400 nmol/L hydrogen peroxide; and 1500 U/L HRP.

The experiment was performed in triplicate, and the averages were used for plotting.
FIGURE 9
VARIATION IN THE RATE OF REACTION OF RESORUFIN AND HYDROGEN PEROXIDE WITH pH

Legend
Cuvette concentrations were: 755 nmol/L resorufin; 400 nmol/L hydrogen peroxide; and 700 U/L HRP.
Each experiment was repeated three times, and the obtained values were averaged for plotting.
FIGURE 10

FLUORESCENCE INTENSITY AS A FUNCTION OF RESORUFIN CONCENTRATION

Legend

The determinations at pH 5.6 (Δ) and pH 9.2 (□) were performed by setting the fluorescence intensity at an arbitrary zero for the concentration of 42.1 nmol/L resorufin.

The results are averages from experiments performed in triplicate.
the same spectral properties as scopoletin) produces a signal of only 21 units (2.4 times less than resorufin).

Since we were interested in determining very low amounts of hydrogen peroxide, concentrations of about 850 nmol/L resorufin in the reaction mixture were used in most experiments. This amount of resorufin produces a fluorescence intensity close to the spectrofluorometer’s upper limit of measurement when the setting for the sensitivity control is 4 and the selector control is at x10. Higher sensitivity settings increase the noise and CV of the determinations, and due to the lower amounts of resorufin that have to be used, decrease the rate of reaction and the range of the hydrogen peroxide concentrations that can be measured.

From the slope of the standard curve for hydrogen peroxide (Fig. 11), a 53% yield can be calculated, that is, for each mole of resorufin transformed, about two moles of hydrogen peroxide are required. The within-run coefficient of variation was found to be 1.7% for a solution of hydrogen peroxide containing 200 nmol/L and 7.6% for a solution of hydrogen peroxide containing 40 nmol/L (Table II). The limit of quantitation, calculated as three times the value of SD, is 9 nmol/L (4.5 pmoles of hydrogen peroxide/cuvette).

By using a two-step procedure (Figs. 10 and 11, and Table II), a yield of about 100% was obtained (one mole of
FIGURE 11

STANDARD CURVES FOR HYDROGEN PEROXIDE

Legend

One-step procedure (Δ), \( Y = 0.53X + 0.8 \);
Two-step procedure (□), \( Y = 0.98X + 2.3 \).
TABLE II

METHOD PRECISION FOR DETERMINATION OF HYDROGEN PEROXIDE IN AQUEOUS SOLUTIONS

<table>
<thead>
<tr>
<th>Sample (nmol/L)</th>
<th>Mean (nmol/L)</th>
<th>SD (nmol/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>200</td>
<td>3.4</td>
<td>1.7</td>
</tr>
<tr>
<td>40</td>
<td>39.5</td>
<td>3.0</td>
<td>7.6</td>
</tr>
<tr>
<td>20</td>
<td>20.2</td>
<td>1.3</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*a Obtained by dilutions from standardized hydrogen peroxide solutions. The values for 200 nmol/L and 40 nmol/L samples were obtained using the one-step procedure; the values for 20 nmol/L samples were obtained using the two-step procedure. Six determinations were performed for each concentration.
hydrogen peroxide is required for the transformation of one mole of resorufin), similar to that reported for scopoletin (46), and the within-run CV for a solution containing 20 nmol/L hydrogen peroxide was found to be 6.7%. The limit of quantitation (allowing a 50% dilution due to buffer additions for pH adjustments) was calculated to be 4.4 nmol/L (2.2 pmoles of hydrogen peroxide/cuvette).

The sensitivity of the two-step procedure is about 3.3 times higher than that of the one-step procedure, and both are at the lowest end of the range reported for determination of hydrogen peroxide using fluorometric methods (11,17,46,48).

The amount of HRP used is important for the completion of the reaction (Fig. 12). It is possible that certain impurities present in reagents are inhibiting the enzyme, since not only the rate of the reaction decreases, but also the number of moles of resorufin transformed is lower if less than 0.6 units/cuvette (1,200 U/L) of HRP are used. If more enzyme is added to the reaction mixture, the reaction will continue.

The reaction was performed at room temperature. The magnitude of the signal was identical at either 25°C or at 37°C. The slight increase in the number of moles of resorufin transformed might be levelled off by the decrease in fluorescent signal to the temperature increase.
FIGURE 12
VARIATION OF APPARENT YIELD OF REACTION OF RESORUFIN AND HYDROGEN PEROXIDE WITH HRP CONCENTRATION

Legend

The measurements were performed, in triplicate, at pH 6.4. Cuvette concentrations were: 850 nmol/L resorufin; and 400 nmol/L hydrogen peroxide.
Small amounts of hydrogen peroxide in the reagent water are reported as causing a problem in the determination of hydrogen peroxide in trace amounts (12), and a careful treatment of the reagent water has been described (12). Purification of reagent water is also required (46) because the presence of impurities might catalyze the decomposition of hydrogen peroxide in samples during dilutions.

The addition of HRP to a solution of resorufin in buffer did not cause a decrease in the signal larger than that obtained when distilled deionized water is added to the resorufin solution. This suggests that the water used during this work does not contain hydrogen peroxide. However, due to the fact that prior to hydrogen peroxide addition, the fluorescence intensity of the solution of resorufin and HRP is measured as a baseline, the presence of trace amounts of hydrogen peroxide in reagent water will not interfere significantly.

A great deal of effort was spent in trying to isolate and characterize the product of the reaction of resorufin with hydrogen peroxide, or at least to get some information in order to propose a structure for the reaction product. By performing the reaction in distilled water followed by separation of the enzyme by filtration through an Amicon filter (cut off 10,000) we isolated a
dilute solution of the reaction product, and succeeded in isolating some traces of organic material from this solution using organic solvents for extraction. However, the IR, NMR, and mass spectra performed on this product were not meaningful, suggesting a possible degradation either under the action of HRP or due to the manipulation.

The product of the reaction of scopolletin is not described in the literature. However, it is reported that esculetin is also a substrate for HRP, whereas umbelliferone (7-hydroxycoumarin) is described as resistant to the oxidative action of hydrogen peroxide in the presence of HRP (47). We found that 4-hydroxycoumarin is also a substrate for HRP, but a correlation of the structure of these compounds with their ability to serve as substrates for HRP is difficult.

There are at least three ways to obstruct the conjugation of the \( \pi \) electrons in the case of the resorufin molecule under the experimental conditions used in this study (Fig. 13): the transformation of the heterocyclic nitrogen into an N-oxide; the destruction of a double bond; and the substitution of the phenolic hydrogen. We found that the product of the reaction is not resorufin (a blue colored compound). The formation of a peroxo dimer conflicts with our finding (and others, for scopolletin (47)) that the stoichiometry of the reaction of hydrogen peroxide with resorufin is 1:1.
FIGURE 13
MODIFICATIONS OF RESORUFIN STRUCTURE
WHICH RESULT IN LOSS OF FLUORESCENCE

Legend
A: Disruption of $\pi$ electron resonance by forming an epoxy derivative;
B: Removing of $n$ electrons of nitrogen from the resonance with the $\pi$ electrons by forming a N-oxide;
C: Formation of a dimer involving the phenolic hydroxyl group.
The main mechanism for the action of HRP is through radicals, and this explains why there is a wide range of dimers, trimers, and other adducts described as compounds resulting from the action of HRP on its substrates (75). However, the formation of peroxides under the action of HRP is not reported in the literature.

The most possible alternative seems to be the formation of epoxyresorufin, but the fact that the reaction is stoichiometric excludes a mechanism in which molecular oxygen from the solvent is involved as is the case with cooxidation of styrene to epoxy styrene (84).

The fact that the reaction will not occur without the presence of HRP at any pH was proven by an experiment in which resorufin and hydrogen peroxide (100 times in excess) were incubated at room temperature at pH between 4.5 and 9.5. Blanks containing only resorufin were used to assess the possible degradation of resorufin. The absorption spectra of the samples and blanks were monitored every hour. In these experiments, there was no decrease or increase in the resorufin absorbance due to the presence of hydrogen peroxide at any pH over a period of six hours. At pH under 5.5, there is a shift in the absorption maximum of resorufin from 572 nm to 468 nm, and the fluorescence of resorufin is very weak. When after six hours of incubation, HRP was added to these solutions of resorufin and hydrogen peroxide, the absorption peak
disappeared without the appearance of other absorption peaks at any wavelengths between 280 and 700 nm. This proves that there is a structural change of resorufin, that the change requires hydrogen peroxide and HRP in order to occur, and that the reaction product is colorless.

III.2. **Determination of Glucose**

Almost every time a new substrate is described for peroxidase, the first application to the determination of analytes in body fluids is the measurement of glucose using GOX (25,29,30,37). This is not necessarily due to a need for more sensitive methods for the determination of glucose, but rather due to the fact that glucose is the most frequently ordered test in a clinical chemistry laboratory. Glucose oxidase is not expensive and has a broad range of pH optima, and its properties are well studied.

The determination of glucose using GOX and HRP is affected by the presence of bilirubin, hemoglobin, and triglycerides, common constituents of serum and plasma (66). A more sensitive method, which could allow for the dilution of the original sample several times may reduce the magnitude of the interferences.

III.2.1 **Method optimization**

The first approach towards establishing a method for the determination of glucose using the following reaction
sequence:

\[ \text{D-GLUCOSE} + O_2 + H_2O \xrightarrow{\text{GOX}} \text{D-GLUCONIC ACID} + H_2O_2 \]

\[ H_2O_2 + \text{RESORUFIN} \xrightarrow{\text{HRP}} \text{REACTION PRODUCT (NONFLUORESCENT)} \]

was to use an end-point procedure. Such a method requires micromolar concentrations of glucose since solutions containing more than 1 mmol/L resorufin are not useful in fluorometric determinations due to the inner filter effect.

pH 6.4 is well within the range of maximum activity for glucose oxidase (4.5-7.0), so it was convenient to work at this pH at which the determination of hydrogen peroxide was optimized. However, using glucose concentrations between 2 and 20 μmol/L (about 1000 times smaller than that in serum) we did obtain a non-linear calibration curve, with A F/mol of glucose increasing as the concentration of glucose was increased. Moreover, due to the high Michaelis constant of GOX, large amounts of enzyme had to be used, so we had to consider a kinetic method.

By using a kinetic method, the concentration of resorufin could be lower than that of glucose, as long as the overall reaction rate is not limited by the reaction of hydrogen peroxide with resorufin. The amounts of glucose and GOX were varied until a linear response of the rate of the reaction versus the glucose concentrations was
obtained. At the optimum conditions, solutions containing 1.35-25 mmol/L glucose could be measured by using 2000 U/L glucose and allowing a 1:49 dilution of sample in the final solution (10 µL sample in 490 µL buffer containing the reagents). We found that when serum is added to the resorufin a certain degree of quenching results, therefore it was necessary to trigger the reaction with GOX rather than with the serum sample. Using the optimum conditions, a standard curve for glucose in aqueous solutions was generated (Fig. 14). The regression line equation for this curve was \( Y = 10.77X - 0.22 \) (\( r = 0.998 \)).

For glucose measurement in serum, we used two serum calibrators, rather than aqueous calibrators. The two calibrators, Omni Cal I and II, containing 2.7 and 16.0 mmol/L glucose, were analyzed three times each and an average value expressed as ΔF/min/mmol glucose was determined. The concentration of glucose in serum was calculated by dividing the ΔF/min value obtained for unknown samples by the ΔF/min/mmol value obtained for calibrators. Control sera (Serachem, Level I and II) were analyzed with every ten serum samples and the results were used to assess the stability of the calibration.

III.2.2. Analytical recovery

Percent recoveries for five different levels of glucose varied between 97 and 102.8%, and indicate that
FIGURE 14

STANDARD CURVE FOR THE DETERMINATION OF
GLUCOSE.

Legend

Concentrations in cuvette were:

GOX, 2000 U/L;

HRP, 7500 U/L;

Resorufin, 8.5 μmol/L.

ΔFluorescence/min is the decrease of fluorescence/min.

The equation of the regression line is:

\[ Y = 10.77X - 0.22, \quad (r = 0.998) \]

The measurements were performed in triplicate.
there is no matrix effect in the determination of glucose using this method (Table III).

III.2.3. Correlation studies

Serum samples (N = 60) were analyzed using both a modified Trinder method (66) and the proposed method. A correlation coefficient of $r = 0.993$ and a regression equation of $Y = 1.03X - 0.06$, were obtained (Fig. 15). The values above 15 mmol/L were obtained by increasing the sample size, since serum samples containing such high levels of glucose were not available during these studies.

III.2.4. Precision studies

Two control sera were used to determine the within-run and between-run variations (Table IV). The within-run CVs for the two sera, containing (mean ± SD) 4.60 ± 0.07, and 14.26 ± 0.2 mmol/L glucose were 0.5% and 1.4%, respectively, and the between-run CVs for the same sera were 1.7% and 2.64%, respectively.

III.2.5. Interference studies

Since the reaction of resorufin with hydrogen peroxide is almost instantaneous, it was expected that less interference would be observed for this method when compared with the colorimetric methods.

The main interfering substances in serum which affect the oxidase-peroxidase coupled assays are bilirubin, hemoglobin, and triglycerides (lipemia). It was found that the resorufin method is free of interference from lipemia.
TABLE III

RESULTS OF ANALYTICAL RECOVERY

EXPERIMENTS FOR GLUCOSE

<table>
<thead>
<tr>
<th>Initial concn (mmol/L)</th>
<th>Added (mmol/L)</th>
<th>Found (mmol/L)</th>
<th>Recovered (mmol/L)</th>
<th>Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.90</td>
<td>1.82</td>
<td>8.67</td>
<td>1.77</td>
<td>97</td>
</tr>
<tr>
<td>6.90</td>
<td>3.63</td>
<td>10.63</td>
<td>3.73</td>
<td>102.8</td>
</tr>
<tr>
<td>6.90</td>
<td>5.45</td>
<td>12.24</td>
<td>5.34</td>
<td>98</td>
</tr>
<tr>
<td>6.90</td>
<td>7.27</td>
<td>14.20</td>
<td>7.29</td>
<td>100.3</td>
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<tr>
<td>6.90</td>
<td>9.09</td>
<td>19.95</td>
<td>9.05</td>
<td>99.5</td>
</tr>
</tbody>
</table>

These results are the average of triplicate determinations.

The recovery studies were carried out by adding 50 μL aqueous standard solution of glucose to 500 μL pooled sera. The initial concentration was determined in an aliquot from the same pool of sera to which 50 μL of saline was added.
FIGURE 15
COMPARISON OF THE PROPOSED METHOD WITH A TRINDER METHOD FOR GLUCOSE

Legend

The equation of the regression line is:

\[ Y = 1.03X - 0.06, \quad (r = 0.993, \quad N = 60) \]

Experimental details are in part II.4.2.
### TABLE IV

**METHOD PRECISION FOR DETERMINATION OF GLUOSE**

<table>
<thead>
<tr>
<th>Within-run</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Level I</strong></td>
<td><strong>Level II</strong></td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
</tr>
<tr>
<td>4.57</td>
<td>14.05</td>
</tr>
<tr>
<td>4.65</td>
<td>14.25</td>
</tr>
<tr>
<td>4.72</td>
<td>14.00</td>
</tr>
<tr>
<td>4.59</td>
<td>14.53</td>
</tr>
<tr>
<td>4.51</td>
<td>14.38</td>
</tr>
<tr>
<td>4.56</td>
<td>14.38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>6</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.60</td>
<td>14.26</td>
</tr>
<tr>
<td>SD</td>
<td>0.07</td>
<td>0.20</td>
</tr>
<tr>
<td>CV (%)</td>
<td>0.50</td>
<td>1.40</td>
</tr>
</tbody>
</table>


(Fig. 16), but hemoglobin in concentrations larger than 1.2 g/L (Fig. 17), and bilirubin in concentrations larger than 100 μmol/L (Fig. 18) interfered significantly with the glucose measurements. However, when compared with the Trinder method (66-69), the resorufin method seems to be advantageous because of its lack of interference from lipemia and from low amounts of bilirubin.

The bilirubin interference in oxidase-peroxidase coupled assays is considered to be a combination of spectral effects and chemical interaction (alternative peroxidase substrate, destruction of a primary oxidase reaction intermediate, or destruction of a peroxidase reaction intermediate) (72), and it is suggested that the spectral problems can be lessened by measuring at a wavelength greater than 500 nm. Since fluorescence measurements for glucose determination were performed at λex 568, and λem 582 nm, this could explain why moderate levels of bilirubin do not interfere in the resorufin method, due to the fact that for a chemical interference a higher concentration of bilirubin may be required.

III.3. Determination of Uric Acid

The determination of uric acid involved the following reaction sequence:

\[ \text{URIC ACID} + O_2 + H_2O \xrightarrow{\text{URICASE}} \text{ALLANTOIN} + H_2O_2 \]
\[ H_2O_2 + \text{RESORUFIN} \xrightarrow{\text{HRP}} \text{REACTION PRODUCT (NONFLUORESCENT)} \]
FIGURE 16
INTERFERENCE OF TRIGLYCERIDES ON GLUCOSE DETERMINATION

Legend

A clear serum pool was used to prepare the samples as described in part II.4.5. Test tube nr. 1 contained 6.97 mmol/L glucose. Each sample was analyzed three times and the average was used for plotting.
FIGURE 17
INTERFERENCE OF HEMOGLOBIN ON
GLUCOSE DETERMINATION

Legend

A clear, nonhemolyzed, serum pool was used to prepare the samples as described in part II.4.5. Test tube nr. 1 contained 6.50 mmol/L glucose. Each sample was analyzed three times and the average was used for plotting.
FIGURE 15
INTERFERENCE OF BILIRUBIN ON
GLUCOSE DETERMINATION

Legend
A clear, nonhemolyzed, serum pool, containing less than 0.9 μmol/L bilirubin, was used to prepare the samples as described in part II.4.5. Test tube nr. 1 contained 6.56 mmol/L glucose. Each sample was analyzed three times and the average was used for plotting.
At first, attempts were made to establish an end-point procedure. Uricase was added to a solution containing resorufin, HRP and uric acid in phosphate buffer in order to trigger the reaction, but no decrease in fluorescence could be observed. The uricase used was from pig liver and contained one copper atom per molecule (98). It is known (114) that substances which react with copper inhibit the copper-containing uricase.

A reaction between resorufin and copper is not reported in the literature, but the structure of resorufin might allow for the formation of a chelate. Indeed, when copper solutions are added to the resorufin solution, a quenching of the fluorescence is observed. However, the concentration of the copper in the final solution has to be quite high (in the mmol/L range) for the quenching to be observed. As a result of the above facts, the use of uricase from microbial sources was considered, but again, no generation of hydrogen peroxide could be observed at any pH between 6.4 and 8.2.

During the optimization studies for glucose determination, it was observed that serum quenches some of the resorufin fluorescence, and this suggested that there may be some interaction between uric acid and resorufin. If uric acid is added to a solution of resorufin, no quenching occurs. However, when HRP is present a very fast quenching occurs, suggesting a reaction between uric acid
and resorufin. It is known that uric acid acts as a reducing agent and that resorufin can be reduced to hydroxyresorufin (Fig. 19), therefore, it is possible that such a redox reaction may take place. The requirement for HRP for such a reaction to occur is difficult to explain. Certainly, the uric acid is transformed into a substance which is no longer a substrate for uricase.

When aqueous solutions of uric acid were used, a standard curve with the equation $Y = 19.7X + 0.2$ ($r = 0.999$) was obtained (Fig. 20). Better sensitivities could be obtained when working at a higher pH.

This method could not be applied to serum. When serum is added to resorufin either in the presence or absence of HRP some quenching occurs, but the decrease in fluorescence could not be correlated with the concentration of uric acid. The easiest way to avoid the uric acid interference in the measurement of other analytes in serum is by measuring the fluorescence of the reagent mixture containing the serum sample, HRP, and resorufin, and triggering the reaction using the appropriate oxidase. The decrease in fluorescence obtained will be due only to the reaction of hydrogen peroxide produced with resorufin.

III.4. Determination of Cholesterol

The use of CE in combination with COX to measure total cholesterol in serum has been previously described
Figure 19

Proposed Reaction for Uric Acid with Resorufin

Legend

In this reaction, uric acid supposedly acts as a reducing agent for resorufin. Hydroxyresorufin is reported to be a colorless, nonfluorescent substance (60).
FIGURE 19.

HYDROXYRESORUFIN

RESORUFIN

URIC ACID

HRP
FIGURE 20
STANDARD CURVE FOR DETERMINATION
OF URIC ACID

Legend
Concentrations in cuvette were:

HRP, 7500 U/L;
Resorufin, 8.5 μmol/L.

Each standard solution of uric acid was analyzed three times, and the average was used for plotting. The equation of the regression line is \( Y = 19.7X + 0.2 \) (\( r = 0.999 \)). ΔFluorescence is the difference in the fluorescence intensity between the initial measurement and the end point.
(21,27), and a more sensitive indicator reaction is considered desirable (21). An increase in the sample size was reported necessary in order to measure cholesterol in various serum fractions, such as high density lipoproteins, due to the low concentrations in these fractions (21). The coupling of a more sensitive method for determination of hydrogen peroxide to the CE-COX system was considered in order to reduce both the sample size and interferences.

III.4.1. Method optimization

The following reaction sequence was used for cholesterol determination in serum:

\[
\text{CHOLESTEROL ESTERS} + \text{H}_2\text{O} \xrightarrow{\text{CE}} \text{CHOLESTEROL} + \text{FATTY ACIDS}
\]

\[
\text{CHOLESTEROL} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{COX}} \text{4-CHOLESTEN-3-ONE} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{RESORUFIN} \xrightarrow{\text{HRP}} \text{REACTION PRODUCT (NONFLUORESCENT)}
\]

When aqueous solutions of cholesterol were used, the first reaction was omitted.

Because the use of CE to hydrolyze cholesterol esters is well documented in the literature (21,99), we did not attempt to improve this part of the reaction sequence, but rather used the conditions described elsewhere (99). However, in order to make sure that the hydrolysis of the cholesterol esters is complete, serum samples with high (10 mmol/L) and low (1.4 mmol/L) concentrations of
cholesterol were analyzed after longer incubation times with CE, and/or with larger amounts of CE. The concentrations determined for total cholesterol in these conditions were not significantly different from those obtained when the 1000 U/L CE and 10-min incubation times were used (99).

The hydrolysis of cholesterol esters was performed before the final dilution, so that low amounts of CE per test were necessary.

Cholesterol oxidase from a variety of sources is commercially available, but the one obtained from *Streptomyces species*, which has an optimum activity at pH 6.5 - 7.0 (Sigma Chemical Company, personal communication, December, 1987) was considered suitable for this oxidase-peroxidase coupled assay. In this way, the determination of cholesterol could be performed at the optimum conditions determined for hydrogen peroxide. Using aqueous solutions of standards and an end-point procedure, a standard curve for cholesterol was obtained (Fig. 21). The regression line equation for this curve was

\[ Y = 14.7X - 0.1 \]

and the correlation coefficient, \( r = 0.998 \).

The concentration of COX was adjusted in such a way that the reaction rate was high enough for the end point to be reached in 1-2 min.

When the method was applied to serum samples, serum calibrators, treated in the same way as the samples, were
FIGURE 21
STANDARD CURVE FOR DETERMINATION OF
CHOLESTEROL

Legend

Concentrations in cuvette were:

HRP, 1500 U/L;
COX, 2000 U/L;
Resorufin, 8.5 µmol/L.

Each standard solution of cholesterol was analyzed
twice times, and the average was used for plotting. The
equation of the regression line is \( Y = 14.7X - 0.1 \)
\( (r = 0.998) \). \( \Delta \) Fluorescence is the difference in the
fluorescence intensity between the initial measurement and
the end point.
used for cholesterol quantitation, rather than the standard curve obtained with cholesterol in aqueous solutions. The two calibrators, Omni Cal I and II, containing 1.4 and 6.37 mmol/L cholesterol, were analyzed three times each, and an average ΔF/mmol cholesterol was calculated. The concentration of cholesterol in serum was calculated by dividing the ΔF value obtained for unknown samples by the ΔF/mmol value obtained for calibrators. Although in the final solution the original 10 μL serum is diluted 2000 times, we triggered the reaction sequence with COX in order to avoid any interference from the quenching effect of some serum components on resorufin fluorescence.

III.4.2: Analytical recovery

Table V shows the results of standard addition studies performed on a pool of sera, containing 5.87 mmol/L cholesterol, to which three different amounts of cholesterol were added. Percent recoveries of 94.5 to 106% were considered acceptable (because all volume measurements were performed manually) and indicated that there is no matrix effect in cholesterol determination using the proposed method.

III.4.3: Correlation studies

A modified Trinder reaction (112) was used for correlation studies with the resorufin method.

A correlation coefficient of $r = 0.98$ (N = 56), and a
### TABLE V

RESULTS OF ANALYTICAL RECOVERY

EXPERIMENTS FOR CHOLESTEROL

<table>
<thead>
<tr>
<th>Initial concn (mmol/L)</th>
<th>Added (mmol/L)</th>
<th>Found (mmol/L)</th>
<th>Recovered (mmol/L)</th>
<th>Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.87</td>
<td>2.0</td>
<td>7.86</td>
<td>2.01</td>
<td>100.5</td>
</tr>
<tr>
<td>5.87</td>
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<tr>
<td>5.87</td>
<td>2.0</td>
<td>7.93</td>
<td>2.06</td>
<td>103.0</td>
</tr>
<tr>
<td>5.87</td>
<td>4.0</td>
<td>10.03</td>
<td>4.16</td>
<td>104.0</td>
</tr>
<tr>
<td>5.87</td>
<td>4.0</td>
<td>10.11</td>
<td>4.24</td>
<td>106.0</td>
</tr>
<tr>
<td>5.87</td>
<td>4.0</td>
<td>10.04</td>
<td>4.17</td>
<td>103.0</td>
</tr>
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<td>5.87</td>
<td>6.0</td>
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<td>5.87</td>
<td>6.0</td>
<td>12.20</td>
<td>6.33</td>
<td>105.5</td>
</tr>
<tr>
<td>5.87</td>
<td>6.0</td>
<td>12.01</td>
<td>6.14</td>
<td>102.3</td>
</tr>
</tbody>
</table>

The recovery studies were carried out by adding 50 µL aqueous standard solution of cholesterol to 500 µL pooled sera. The initial concentration was determined in an aliquot from the same pool of sera to which 50 µL of saline was added.
regression equation \( Y = 1.02X - 0.13 \) were obtained (Fig. 22). One sample which was significantly off the regression line was analyzed and discarded as an outlier (115).

III.4.4. Precision studies

Two control sera were used to determine within-run and between-run variations (Table VI). The within-run CVs for the two sera containing (mean ± SD) 3.19 ± 0.06, and 5.66 ± 0.05 mmol/L cholesterol were 1.66% and 0.86%, respectively, and the values for the between-run CVs were 2.60% and 1.22%, respectively.

III.4.5. Interference studies

Since the optimized method for cholesterol determination in serum allows for a 2000-times dilution of a 10-μL sample, it was expected that interferences from bilirubin, hemoglobin, and lipemia would be minimal.

It was found (Fig. 23) that the interference from bilirubin is linear for bilirubin concentrations up to 450 μmol/L, with a 0.002 mmol/L decrease in the measured cholesterol for each μmol/L of bilirubin. This interference is significant for bilirubin concentrations larger than 112 μmol/L. Above 450 μmol/L bilirubin, there is a sharp increase in interference.

The interference from triglyceride concentrations up to 10.25 mmol/L is insignificant (Fig. 24).

Hemoglobin in concentrations of up to 5.6 g/L does
FIGURE 22

COMPARISON OF THE PROPOSED METHOD WITH A TRINDER
METHOD FOR CHOLESTEROL

Legend
The equation of the regression line is:
\[ Y = 1.02X - 0.13 \quad (r = 0.98; \quad N = 96) \]

T: Trinder reaction.
### TABLE VI

**METHOD PRECISION FOR DETERMINATION OF CHOLESTEROL**

<table>
<thead>
<tr>
<th></th>
<th>Within-run</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level I</td>
<td>Level II</td>
</tr>
<tr>
<td></td>
<td>(mmol/L)</td>
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<tr>
<td>3.25</td>
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<td>3.11</td>
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<tr>
<td>SD</td>
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</tr>
<tr>
<td>CV (%)</td>
<td>1.88</td>
<td>2.60</td>
</tr>
</tbody>
</table>
Figure 23
INTERFERENCE OF BILIRUBIN ON CHOLESTEROL DETERMINATION

Legend

A clear, nonhemolyzed pool of sera, containing less than 0.9 µmol/L bilirubin, was used to prepare samples as described in part II.4.5.

Test tube nr. 1 contained 5.59 mmol/L cholesterol. Each sample was analyzed three times and the average was used for plotting.
Figure 24
INTERFERENCE OF TRIGLYCERIDES ON CHOLESTEROL DETERMINATION

Legend

A clear, nonhemolyzed, pool of serum was used to prepare samples as described in part II.4.5.

Test tube nr. 1 contained 5.75 mmol/L cholesterol. Each sample was analyzed three times and the average was used for plotting.
not significantly interfere with the measurement of cholesterol (Fig. 25) using the resorufin method.

The bilirubin interference in cholesterol determination, as in the case of glucose determination, seems to be eliminated only due to the dilution and not due to a different reaction mechanism of production or use of hydrogen peroxide. The lack of interference from hemoglobin and lipemia could be attributed to the large dilution of samples. The short pathway (0.2 cm) of the emitted light through the solution, and its long wavelength may also contribute to the reduction of interferences.

III.4.6. Determination of cholesterol using dry blood spot samples

The necessity for mass screening of the North American population for blood cholesterol levels was recently stressed (100), but the standardization of screening procedures for cholesterol is considered a difficult task (100). One way to standardize the population screening methods is the collection of samples as dry blood spots (116-118), followed by the determination of analyte levels in a reference laboratory which can handle a large number of samples.

Since the determination of cholesterol using the resorufin method proved to be sensitive enough for determining micromolar concentrations of cholesterol, a
FIGURE 25
INTERFERENCE OF HEMOGLOBIN ON
CHOLESTEROL DETERMINATION

Legend
A clear, nonhemolyzed, pool of serum was used to prepare samples as described in part II.4.5.
Test tube nr. 1 contained 5.59 mmol/L cholesterol. Each sample was analyzed three times and the average was used for plotting.
preliminary attempt was made to test if this method could be applied for a screening procedure. We should stress that the adaptation of the cholesterol method to the determination of this analyte in dry blood spot samples was not optimized, and that the specimen handling was adapted from methods used elsewhere (116).

The correlation of the cholesterol results for dry blood spot samples with the results obtained for plasma for 28 samples (Fig. 26) produced a regression equation $Y = 0.92X + 0.43$, and the within-run CVs for two control sera found to contain $2.98 \pm 0.40$ mmol/L (target value, 3.14-3.42 mmol/L) and $5.40 \pm 0.78$ mmol/L (target value, 5.39-5.79 mmol/L) were 13.4% and 14.4%, respectively.

The poor correlation and the large CVs could be improved by studying the proper solvent for extraction of cholesterol from the filter paper and by finding an efficient way to dissolve the extracted cholesterol after the solvent is evaporated. In our case, only 10-15% of the original cholesterol in plasma was recovered.

These preliminary results indicate that a study for the optimization of a method for cholesterol determination in dry blood spot samples may be successful and suitable for population mass screening.
FIGURE 26
COMPARISON OF THE CHOLESTEROL RESULTS FOR
DRY BLOOD SPOT SAMPLES AND PLASMA

Legend

The equation of the regression line is:
\[ Y = 0.93X + 0.92 \ (r = 0.92, \ N = 28) \]

R: The result of cholesterol determination in plasma using the resorufin method.
CHAPTER IV
CONCLUSIONS

A sensitive fluorometric method has been developed for the determination of hydrogen peroxide. The measurement of some important analytes in body fluids is often performed using enzyme-coupled assays in which the determination of hydrogen peroxide is part of the indicator reaction. The measurement of hydrogen peroxide at very low concentrations is also important in the analysis of some environmental samples (clouds, rain water, ground water, etc.). In pursuing our interest in the possible application of the resazurin-resorufin system to clinical chemistry, we have found that the highly fluorescent compound resorufin is a substrate for horseradish peroxidase (HRP). In the presence of HRP, resorufin reacts with hydrogen peroxide to form a nonfluorescent product.

This method can be applied either using a one-step procedure at pH 6.4, or employing a two-step procedure in which a change in pH is required.

For the one-step procedure, the within-run CVs for final concentrations of hydrogen peroxide of 200 nmol/L and 40 nmol/L were 1.65% and 7.6%, respectively, and the limit of quantitation was 9 nmol/L.

The two-step procedure is even more sensitive and has a limit of quantitation of 4.4 nmol/L with a within-run CV.
of 6.5% for a final hydrogen peroxide concentration of 20
nmol/L. The method is linear at least up to 1 µmol/L.

The determination of hydrogen peroxide using the
resorufin-peroxidase system is a simple, precise, and very
sensitive method. It requires small amounts of HRP,
practically no incubation time, and can be easily
automated. It offers an alternative to the existing
fluorometric methods for the determination of hydrogen
peroxide, being at least as sensitive and allowing
fluorescence measurements at longer wavelengths, thereby
minimizing the interference from the natural fluorescence
of serum.

The reaction of resorufin with hydrogen peroxide was
further applied to the determination of glucose,
cholesterol, and uric acid in serum.

A glucose oxidase-peroxidase coupled kinetic method
was developed for the determination of glucose. This
method, in which the ratio of serum to reagent in the
final solution was 1:49, permitted the determination of
glucose for serum levels between 1.35-24 mmol/L.

The within-run and between-run CVs for a control
serum containing 4.6 mmol/L glucose were 0.5 and 1.7%,
respectively, and for a control serum containing
14.3 mmol/L glucose were 1.43, and 2.64%, respectively.

Interferences from triglycerides up to 10.25 mmol/L,
bilirubin up to 150 µmol/L and hemoglobin up to 1.5
g/L were insignificant, but higher levels of bilirubin and hemoglobin decreased the levels of glucose determined.

Recoveries for standard additions between 1.8 and 9.1 mmol/L were between 97 and 102.8%.

Serum samples were analyzed using both a modified Trinder method and the proposed method. A correlation coefficient of $r = 0.993$ and a regression equation

$$y = 1.03x - 0.06,$$

were obtained ($N = 60$).

A very sensitive end-point method for the determination of cholesterol using COX and the resorufin-HRP system was developed. The ratio of serum to reagent was 1:1999 which permitted the determination of cholesterol concentrations between 0.6 and 10 μmol/L in the final solution.

The within-run and between-run CVs for two control sera containing 3.19 and 5.62 mmol/L cholesterol were 1.9 and 2.5%, and 0.88 and 1.7%, respectively. Interferences from triglycerides up to 10.25 mmol/L, and hemoglobin up to 5.6 g/L were insignificant, whereas the bilirubin interference is significant starting from levels as low as 115 μmol/L.

Recoveries were 94.5 to 104.3 for 2 to 6 mmol/L standard additions to a pooled serum containing 5.67 mmol/L cholesterol.

Serum samples were analyzed using a modified Trinder method and the proposed method. A correlation coefficient
of \( r = 0.98 \) (\( N = 56 \)) and a regression line equation
\[ Y = 1.02X - 0.13 \] were found between the two methods.

The methods for the determination of glucose and cholesterol are simple, reliable, and can be easily automated.

The cholesterol method was tentatively applied for the determination of this analyte in dry blood spot samples. Preliminary results suggest that it will be possible for such a method to be considered suitable for a mass screening program.

The determination of uric acid in serum was not successful due to the fact that the uric acid reacts with resorufin, producing a decrease in fluorescence. The decrease is linear for aqueous uric acid solutions, but does not correlate with the uric acid concentrations when sera are used.

The peroxidase substrate resorufin can be used for the sensitive fluorometric determination of biological analytes in body fluids. This investigation details the successful application of our resorufin method to the measurement of glucose and cholesterol. In addition, we can suggest that in the enzyme-coupled assays which transform nonfluorescent resorufin derivatives into fluorescent resorufin, hydrogen peroxide and peroxidase-like substances, and/or uric acid may constitute major interferences.
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VITA AUCTORIS

GEORGE BROTEA

DATE OF BIRTH: January 7, 1950.

CITIZENSHIP: Canadian

EDUCATION:

DOCTOR OF PHILOSOPHY
(Pending: July, 1988)
Clinical Chemistry (1984-1988), University of Windsor, Windsor, Ontario

MASTER OF SCIENCE
Organic Chemistry (1969-1973), Faculty of Chemistry, University of Bucharest, Bucharest, Romania.

AWARDS:


University of Windsor Tuition Scholarship (1985-1986).


PROFESSIONAL SOCIETIES MEMBERSHIP:

Canadian Society of Clinical Chemists
American Association for Clinical Chemistry

EMPLOYMENT:

TEACHING ASSISTANT, Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario (1984-1988)


RESEARCH SCIENTIST, Department of Phytochemistry, Chemical and Pharmaceutical Research Institute, Bucharest, Romania (1977-1982).

PUBLICATIONS AND PRESENTATIONS:


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PATENTS:
