The immobilization and clinical evaluation of enzymes on nylon tubing modified with long spacer arms peroxidase, glucose oxidase and oxalate oxidase.

Sheila Carol Boss

University of Windsor

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THE IMMOBILIZATION AND CLINICAL EVALUATION OF ENZYMES
ON NYLON TUBING MODIFIED WITH LONG SPACER ARMS:
PEROXIDASE, GLUCOSE OXIDASE AND OXALATE OXIDASE

by

SHEILA CAROL BOSS

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

Windsor, Ontario, Canada
1986
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ABSTRACT

IMMOBILIZATION AND CLINICAL EVALUATION OF ENZYMES ON NYLON TUBING MODIFIED WITH LONG SPACER ARMS:
PEROXIDASE, GLUCOSE OXIDASE AND OXALATE OXIDASE

by

SHEILA CAROL BOSS

The immobilization of horseradish peroxidase (POD) and glucose oxidase (GO) from *Asperigillus niger* and their coimmobilization to nylon modified with long diamino arms is described.

Limited densities of ε,ω-poly(oxyethylene)diamine arms on the support were prepared by minimizing the extent of activation of the nylon. Activation of the nylon tubing was controlled by initial O-alkylation with a solution containing 200 nmoles of triethylxonium tetrafluoroborate (TEOTFB) per meter of tubing.

Immobilization of glycoenzymes was carried out by Schiff base formation between the amino groups on the support and aldehydic functions derived from oxidized preparations of peroxidase or glucose oxidase.

Quantitation of enzyme activities and serum glucose levels was completed by the oxidative coupling of 2-hydroxy-3,5-dichlorobenzenesulfonyl acid (HDCBS) and 4-aminoantipyrine.
(4-AAP). Peroxidase activities were measured in the presence of hydrogen peroxide as substrate, while glucose oxidase activities were determined with glucose as substrate and in the presence of soluble peroxidase. The "total" coupled activity on coimmobilized tubes was measured using glucose as substrate, however, no soluble peroxidase was added to the system.

Evaluation of the effect of arm length on the activity of immobilized peroxidase showed that at greater distances from the carrier surface the enzyme's behavior approximates that expressed by soluble POD as the apparent $K_m$ approaches that of the native enzyme. The stability of the bound enzymes to otherwise denaturing conditions such as elevated temperature and variation in pH was superior to that observed for the soluble analogues of POD and GO.

Coimmobilization of POD and GO was completed by three different methods based on:

(a) Mixing aliquots of the oxidized solutions and coupling in a random fashion.

(b) Initial formation of a GO-POD conjugate and then coupling.

(c) Preparation of a GO-Dihydrazide-POD derivative followed by coupling to the support.

Evaluation of the proposed methods for use as analytical methods to determine serum glucose concentrations was completed on peroxidase and glucose oxidase tubing and on a coimmobilized derivative prepared by mixing aliquots of the two glycoenzymes.
The immobilized enzyme preparations were found to be reliable methods for quantitation of this analyte in a biomatrix.

For immobilization of enzymes not carrying carbohydrate, nylon was modified with amino-terminated arms to a limited extent as above and these were capped by glutaraldehyde treatment. The resulting aldehydo arms were allowed to undergo Schiff base formation with enzyme amino groups. Oxalate oxidase (DOx) was chosen for demonstration of this approach.

Quantitation of oxalate oxidase and urinary oxalate concentrations was completed by the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone with its formaldehyde azine to form a tetraazapentamethine dye.
ACKNOWLEDGEMENTS

I would like to express a depth of gratitude to Dr. K.E. Taylor for his patient direction and encouragement for the duration of my program.

I would also like to thank Dr. R.J. Thibert for his guidance and positive reinforcement over my stay at the University of Windsor.

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I would like to thank Dr. D. Stephan and the members of his research group for their generosity in providing us with the dry methylene chloride necessary in the completion of this work.

Finally, I would like to thank my friends and family for their moral support which ultimately enabled the successful completion of this manuscript and made my stay in Windsor an enjoyable one.
DEDICATION

To
My Parents

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<tr>
<td>4-AAP</td>
<td>4-aminoantipyrine</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DMS</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMA</td>
<td>Dimethylaniline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GO</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HALPS</td>
<td>Sodium N-sulfopropylamine</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HDCBS</td>
<td>2-Hydroxy-3,5-dichlorobenzenesulfonate</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>MBTH</td>
<td>3-Methyl-2-benzothiazolinone hydrazone</td>
</tr>
<tr>
<td>n</td>
<td>Number of samples</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>OOX</td>
<td>Oxalate oxidase</td>
</tr>
<tr>
<td>p</td>
<td>Level of significance</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TEOTFB</td>
<td>Triethylxoxonium tetrafluoroborate</td>
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<td>x</td>
<td>Mean</td>
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CHAPTER I

INTRODUCTION

Enzymes have been used as analytical tools for over a century because of their unique ability to catalyze reactions specifically and efficiently under mild conditions. Still problems associated with their preparation, instability in solution and expense limit their usefulness. Some of these disadvantages may be avoided by immobilization to a polymeric support.

"Enzyme immobilization refers to the attachment of enzymes to a soluble or insoluble macromolecular support. Generally, immobilization may be achieved by chemical or physical attachment to a support or by confining the enzyme by means of a semi-permeable matrix."(1) A major breakthrough in this technology occurred in 1916 when Nelson observed that invertase retained most of its activity when adsorbed to activated charcoal (2). About fifty years later scientists began to direct their attention to the analytical implications of this finding when Reisel reported the use of immobilized urease in the quantitation of serum urea (2). Since then immobilized enzyme technology has taken off and hundreds of publications describing novel techniques and applications have appeared in the literature (3-8).

The advantages in using this technology are numerous:
1. The enzyme can be readily separated from the reaction mixture and reused.
2. Operational and storage stabilities minimize costs and problems associated with the preparation of enzymes in solution.
3. Products are enzyme free and characterization is therefore easier.
4. Attachment quite often renders the enzyme more resistant to unfolding in heat or pH extremes and less sensitive to activators or inhibitors that affect its soluble analogue.
5. Continuous analysis at the industrial scale becomes practical.
6. Any detection system may be used.
7. Sensitive and specific reactors are produced.

The chemical and physical methods of immobilization include adsorption to a rigid matrix, entrapment within the pores of a support, copolymerization of the enzyme to itself and to other proteins, encapsulation and covalent attachment to a matrix (5). The choice of method depends to a large extent on the expected use of the preparation and availability of suitable polymeric supports. Covalent coupling is still, however,
the most popular of these techniques. The biotechnologist has access to many suitable support materials allowing a great deal of flexibility in design of a reactor. The capacity of the matrix or technique to bind enzyme, its mechanical and chemical stabilities and ease of derivatization are all important considerations when choosing an enzyme support. The availability and expense of the matrix, flow characteristics, hydrophilicity and how easily it may be adapted into an enzyme analysis system must also be evaluated (5). Of the materials obtainable - beads, membranes, filters and tubes - none fulfill all of these requirements and compromises must be made.

A. APPLICATIONS OF IMMobilIZATION TECHNOLOGY

There has been a great deal of excitement regarding the practical applications of enzyme immobilization in the treatment of industrial wastes, the production of food and drugs, in clinical analysis and as therapeutic agents. Immobilized enzymes have been employed both as enzyme sensors and as an integral part of continuous flow reactors (2-8). They may be designed as packed bed sensors, single bead STRING reactors or open tubular gas segmented reactors (8-11).

1. Industrial Applications

Immobilized biocatalysts hold particular promise for industry. Lactase immobilized on ceramic columns permits
hydrolysis of lactose in whey to produce sweeteners and would also allow removal of lactose from milk for individuals suffering from lactose intolerances (1). Glucose isomerase reactors have been successfully employed in the industrial scale production of high fructose syrups (5,12). Immobilized enzyme reactors have also been introduced for antibiotic production with the immobilization of penicillin amidase (5,13). Chibata and co-workers developed an enzymatic method for the conversion of racemic mixtures to pure L-amino acids such as, L-lysine, L-aspartate and L-citrulline (5,14). Another interesting application of immobilized enzymes has been the development of a sensor capable of monitoring air for the presence of pesticides such as organophosphates and carbamates. In the presence of these compounds a sensor pad containing immobilized acetylcholinesterase is irreversibly inhibited and the decrease in activity quantitated (8). The advantages of enzyme immobilization over solvent extraction in the industrial scale removal of phenol derivatives from waste waters can be easily imagined (13).

2. Therapeutic Applications

Most studies of the medical uses of artificial cells and immobilized enzyme transducers have focused on removing undesirable products from the blood. This can be accomplished with the administration of immobilized enzymes into the body or through an extracorporeal device (15). Clearly, the constraints
of these types of electrodes are unique: they must be reliable, biocompatible and in some cases totally dependent on the physiological milieu for supply of all substrate requirements for analysis (15).

In diseases involving hereditary enzyme deficiencies the patient may be treated by injecting soluble enzyme, an approach which often results in hypersensitivity, production of antibodies and the rapid removal of the enzyme. Encapsulation of the enzyme may circumvent some of these problems and protect and stabilize the enzyme upon administration (14). This approach to enzyme replacement therapy was first demonstrated in the treatment of acatasemic mice with microencapsulated catalase (14). Later, it was employed in the treatment of asparaginase dependent lymphomas by intraperitoneally injecting encapsulated asparaginase (13).

Artificial cells have been used to clear metabolites such as urea or heparin from the bloodstream through an extracorporeal device (14). More recently Lavin et al. introduced an effective and efficient means of clearing bilirubin from the blood by circulation through a bilirubin oxidase column (15). Another group illustrated the use of a nylon net chemically bound with L-lactate oxidase to continuously monitor the concentration of lactate in diabetics (16).

3. Analytical Applications

Immobilized enzymes offer several advantages that warrant
their investigation as analytical tools. Three of the most important assets lie in their reusability, stability and flexibility in detection systems which offset the effort and expense of preparation by offering extended use and greater economy. In addition, covalent attachment is ideally suited for clinical analysis as it avoids the disadvantage of leaching, frequently seen with adsorption or entrapment methodologies.

In an attempt to evaluate the clinical applicability of the immobilized enzymes developed here an AutoAnalyzer™ was adapted. Nylon tubing is perhaps the most suitable polymeric support for incorporation into a continuous flow analyzer (18). It maintains good flow characteristics and wetting properties, it is compact, easy to derivatize and inexpensive (17). Immobilization of enzymes on this type of support has been utilized as both a reactor and enzyme sensor in the quantitation of a variety of serum analytes including: creatinine (17), creatine (17), cholesterol (19), glucose (20-26), 3α-hydroxysteroids (27,28), lactate (17), pyruvate (17), oxalate (29-31), urea (32,33) and uric acids (21,34-36).

While continuous flow systems may be passé with respect to the discrete analyzers on the market today, the AutoAnalyzer is a robust instrument and allows a starting point for further development. If immobilized enzyme technology is to achieve commercial success in the 1980’s reactors must be designed with the features of the discrete analyzers in mind. These systems offer the clinical chemist greater economy, accuracy and speed
along with the desired flexibility in assay procedures. Some maintain random access and patient oriented testing while others offer batch analysis or a combination of all three modes.

Systems with reusable cuvets and a batch analysis option may be a worthwhile starting point for enzyme immobilization. If the instrument automatically washes and scans the cuvet for cleanliness and acceptability, the activity of an enzyme coupled to the cuvet walls may be monitored continuously. The BMC Hitachi\textsuperscript{TM} or Coulter Dacos\textsuperscript{TM} are examples of instruments employing reusable cuvet blocks. The Paramax\textsuperscript{TM} (American Scientific Products) manufactures an instrument with separate reaction and reading compartments and would be ideally suited for this technology.

The geometry of the system is, of course, an important consideration. Centrifugal analyzers such as Electro-Nucleonics FLEXIGEM\textsuperscript{TM} or GEMINI\textsuperscript{TM} may seem ideal for use as an immobilized enzyme batch analyzer. The reagent well of the disk could be derivatized with an enzyme which could permit some versatility in analysis to offset the potential expense of "designating" the disk to one enzyme. For example, high volume tests employing Trinder type coupled reactions may be simplified using this technology. Analysis of cholesterol, glucose, triglycerides, phospholipids, creatinine and uric acid as well as galactose, creatine, 5'-nucleotidase, phosphatidyl glycerol and sphingomyelin measurements could all be completed on the same disk. The programming flexibility of these instruments
would also allow variation in the incubation times for equilibrium analysis, temperatures and spectral functions.

Unfortunately, however, the cuvets were not designed for reuse and a washing mode was not incorporated into the instrumentation. Other centrifugal analyzers, such as the CentrifichiChem™ (Union Carbide Corporation) or the ROTOCHEM II™ (American Instrument Corporation) may avoid some of these difficulties. The latter, for example, has a central removal transfer disk of Teflon for reagents and samples and an extra rotor containing optically clear material. Upon measurement, the rotor is purged, washed and dried automatically.

One interesting use for immobilized enzymes has been their incorporation into HPLC systems as either a pre- or postcolumn detection system for the presence of serum drugs (12).

This technology has also been used in the quantitation of drugs in Dade’s STRATUS™. The operation of this instrument is based on a competitive assay between enzyme labelled drugs and the sample for antibody immobilized on glass fiber paper. Patient drug levels are quantified by the fluorescence produced when the "tab" is washed with a fluorophore. The instrument does grant some multiplicity in programming to permit the introduction of new chemistries. The immobilization of immunoglobulins and monoclonal antibodies is under current investigation in this laboratory although the matrix design is not one which would permit incorporation into this instrument.
B. THE EFFECTS OF IMMOBILIZATION ON THE ENZYME

Physically confining an enzyme to a support may have a number of effects on the characteristics of enzyme catalysis and its behavior in the presence of denaturing agents. These effects may result from interaction between the polymer matrix and the enzyme such that the mechanism of catalysis is altered by conformational changes or steric effects that limit accessibility to catalytic or allosteric sites of action. Fixed electrostatic charges on the support may further alter the microenvironment surrounding the enzyme by partitioning substrates, products or inhibitors toward (or repelling them away from) the matrix surface thus concentrating (or depleting) these species in the immediate vicinity of the enzyme (6).

The support may present itself as a barrier to free diffusion of molecules to and from the enzyme, generating concentration gradients of substrate and product that do not exist in a soluble homogeneous solution. Such effects are caused by external and/or internal diffusion barriers. The former is a result of a thin, unstirred layer around the support particle while internal resistance is caused by limitations in free diffusion imposed by the matrix itself. Immobilization of enzymes on inorganic supports such as TiO\textsubscript{2} or SiO\textsubscript{2} ceramic and glass beads must be optimized for diffusional limitations by varying pore diameter and particle size (37).

Partitioning and diffusion phenomena exist in all forms of
immobilized enzymes acting alone or together in either a synergistic or antagonistic fashion. The intrinsic activities measured in the microenvironment may be expected to differ from those observed for the free enzyme in solution due to conformational or steric effects that the enzyme may incur upon immobilization to the support. In practice measurements are taken from the bulk phase to provide the researcher with inherent activities (4).

1. Kinetics

In open tubular enzyme reactors, such as those to be developed in this study, the enzyme is present in an unimolecular layer at the surface so that diffusional effects within the support are not involved (4). However, diffusion in the substrate solution flowing through the tube will have an important effect on the enzyme reactor rates and kinetic constants (17).

If diffusional effects are limited immobilized enzyme systems will obey Michaelis-Menten kinetics. For accurate determination of kinetic constants the following conditions must be maintained: (3,10,11,34,38-41).

1. Enzyme concentration is limited such that catalysis is sufficiently slow and diffusion of the substrate can keep up.
   Short reactor lengths are employed.
2. Substrate concentrations for activity
determinations are maintained much greater than the $K_m$. Radial mass transfer is promoted with the coiling of the tube measured.

3. Small diameter tubing is employed.

4. High flow rates are maintained increasing the rate of diffusion of substrate to the surface and reducing the thickness of the unstirred layer.

In biochemical engineering the immobilization factor (IF) has been adopted as a numerical expression of the influence of diffusion upon expressed activities and the deviations in intrinsic activities due to immobilization (42). It is defined as:

$$IF = \frac{\text{Specific Activity Of The Immobilized Enzyme}}{\text{Specific Activity Of The Enzyme In Solution}}$$

2. Stability

Coupling of an enzyme to any charged support generally causes a shift in the apparent pH optimum. Briefly, charges on the carrier or the enzyme itself may be sufficient to result in a more acidic or basic microenvironment for the enzyme. Measurements taken from the bulk solution would reflect this difference as an apparent swing in the pH profile (4,5).

In discussing the effects of immobilization on stability, it should be recognized that there are several types of "stability" that can be measured. In an analytical sense enzyme storage and operational stability are of primary interest. The
latter is a function of not only the enzyme, but also of the carrier durability, pH and inhibitor concentrations in the reagent stream and the analyte solutions.

Quite often immobilization of the enzyme enhances its thermal characteristics, perhaps by increasing the rigidity of the molecule to prevent alterations in the tertiary structure upon heating. It may be envisioned that multiple bond formations may stress the conformation of the protein which may or may not affect overall stability and catalytic properties.

The physical properties of the immobilized enzyme will differ from those of the native enzyme primarily because of the influences of the restraining matrix and steric effects resulting from the close proximity of the carrier and other molecules. Conformational changes may also be incurred upon immobilization due to the heterogeneity in binding to the support. Random covalent bonding of the enzyme to the support surface can result in multiple orientations of the enzyme on the surface. The active site may be correctly oriented to face the bulk solution, or may be partially or totally obscured by cross-linkages to the matrix. Heterogeneity can also arise from the distribution of functionalities that bind each enzyme molecule to the support. The majority of studies related to conformational changes and flexibility upon immobilization deal with investigation of stability to otherwise denaturing conditions such as, pH or thermal extremes and comparison to the native enzyme in aqueous solution (3-7).
C. ENZYME IMMOBILIZATION

1. Activation And Derivatization Of The Support

The nylon is a family of linear polymers consisting of repeating methylene groups connected by secondary amide linkages. Native nylon has few free end groups and must be pretreated to generate potentially reactive centers capable of covalently binding with enzyme molecules. Activation of this matrix may be effected by initial acid hydrolysis or direct activation of the native nylon.

The first approach consists of a controlled partial hydrolysis of nylon by acid to release a compliment of amine and carboxy groups. Activation of the carboxy groups with benzidine or hydrazine using carbodiimide, or the amine functionalities with glutaraldehyde or phosgene and cyanuric chloride can be used to prepare nylon-enzyme conjugates (34-36, 43) . A second approach has involved cleavage of the nylon with N,N'-dimethyl-1,3-diaminopropane to obtain free amino and amide groups (43). A positively charged support is produced which may exhibit electrostatic interactions at neutral pHs. The surface area available for immobilization is, however, quite small and to alleviate this problem the methods of activation were improved by Hornby et al. (44) with the incorporation of an initial pitting procedure using a CaCl2-methanol mixture to remove amorphous nylon. Still, the hydrolysis procedures result in
partial depolymerization of the backbone and therefore compromises the integrity of the support (3,13).

Alternatively, the support may be activated directly with alkylating reagents to produce imidate salts (45). In this way it is possible to introduce reactive centers without breaking any backbone secondary amide bonds in the process. Hornby and coworkers introduced this approach, employing dimethyl sulphate in the immobilization of glucose oxidase (3,20). Morris et al. described another procedure using triethylxonium tetrafluoroborate (TEOTFB) as the alkylating reagent (24). This method allows an efficient and controlled reaction without the high temperatures and toxicity which accompany activations with dimethyl sulphate.

The first product of O-alkylation with TEOTFB, the imidate salt of nylon, represents a versatile intermediate allowing several routes for immobilization (Figure 1). The enzyme may be coupled directly but it has been observed that this approach does not yield the most active derivatives (46a).

Alternatively, the support may be modified in such a way that the enzyme is coupled through a flexible alkyl arm. Campbell et al. (20) noted that in using lysine or its diamine derivatives as spacer arms the enzyme was removed from the surface and the intrinsic activity expressed increased. Use of long "arms" would effectively remove the enzyme from a fairly hydrophobic nylon surface and provide a more acceptable microenvironment for substrate binding. Previous work in this
FIGURE 1

THE ACTIVATION OF NYLON TUBING
BY O-ALKYLATION WITH
TRIETHYloxonium TETRAFLUOROBORATE

Legend

The activation of nylon-6 support by O-alkylation with triethylxonium salts is illustrated. The imidate salt of nylon can then be reacted directly with the enzyme or may be initially derivatized with alkyl amines to yield amino-substituted nylon or acid hydrazides to produce a hydrazide substituted support.
laboratory has dealt with an evaluation of the structural features of long hydrophilic spacer arms and subsequent immobilization of peroxidase through derivatization of beads with these arms (46b-48). The spacer arms employed in the structural studies and the present work were of the poly-(oxyethylene) type illustrated in Figure 2A.

2. Enzyme Coupling

Enzyme coupling has generally been through amino acid residues in the protein component (3,49). Direct immobilization to the support has the advantage that no prior manipulation of the enzyme is required, however, in some cases the amino acids involved in immobilization are also important to substrate binding and catalysis. This problem may be avoided by making use of other functionalities available on the enzyme. Researchers have shown that the carbohydrate content of some glycoproteins is not essential to the catalytic properties of the enzyme and immobilization through these residues is a viable alternative to direct coupling (50-52). This approach is demonstrated in Figure 2B where the immobilization of glycoenzymes is carried out by Schiff base formation between an amino derivatized support and the periodate treated enzyme. The coupled enzyme, indicated as "C" in Figure 2, may be irreversibly bound by reductive amination to form a secondary amine in the presence of sodium cyanoborohydride (NaBH₃CN) or borohydride (NaBH₄) (Scheme 1). Reduction of the imine with
FIGURE 2

THE IMMobilization OF PERIODate OXIDIZED GLYCOEnzymES TO AMINO-SUBSTITUTed NYLON TUBING

Legend

A. Reaction A represents nylon tube supported α,ω-poly(oxyethylene)diamine spacer arms used throughout this study. Variation in the values of the subscripts a, b and c indicate spacer arms of specific lengths. The approximate arm lengths are indicated below:

<table>
<thead>
<tr>
<th>Spacer Arm</th>
<th>Approximate Value</th>
<th>Arm Length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b</td>
<td>a+c</td>
</tr>
<tr>
<td>ED-600</td>
<td>8.5</td>
<td>2.5</td>
</tr>
<tr>
<td>ED-2000</td>
<td>40.5</td>
<td>2.5</td>
</tr>
<tr>
<td>ED-6000</td>
<td>135.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

B. Oxidation of the carbohydrate moiety of the glycoenzyme with sodium metaperiodate yields aldehydic groups(B).

C. Immobilization of the enzyme (C) is then possible through Schiff base formation between the oxidized enzyme and the amino-substituted support (A).
FIGURE 2

A.

B. Native Enzyme \[ \rightarrow \text{Oxidized Enzyme} \]

C. Immobilized Glycoenzyme
the former is generally achieved at neutral pH while sodium borohydride reactions proceed at more alkaline pHs (53,54).

![Chemical Reaction]

Scheme 1.

In this study glycoenzymes were oxidized with periodate and coupled to amino-substituted nylon as demonstrated in Figure 2.

D. ENZYMES IMMobilIZED IN THIS STUDY

The immobilization of three enzymes was investigated in the study: two, glucose oxidase and peroxidase, were glycoproteins of known carbohydrate content, the third was a nonglycosylated protein, oxalate oxidase.

1. Glycoenzymes

A large number of enzymes contain covalently linked carbohydrate residues as part of their molecular structure. This group of glycoenzymes occur in a variety of biological materials including plant tissue, animal organs or microbial sources and may be soluble or membrane bound, extracellular or intracellular (55). The carbohydrate content of, some
glycoenzymes is indicated in Table I (55).

The carbohydrate moiety is asymmetrically distributed across the membrane bilayer and is attached to the protein by an N-glycosyl link to the amide group of asparagine or an O-glycosyl bond between the carbohydrate and the hydroxy function of serine, threonine or hydroxyllysine (55,56). Structurally, it has been found that glycoproteins contain only a few different monosaccharides and vary little in structural patterns but differ greatly in their total carbohydrate content, the number and distribution of these residues and in the degree of branching (56). As a rule, the oligosaccharide units of proteins containing N-glycosyl linkages possess a common core consisting of a pentasaccharide, trimannosyl-di-N-\(-acetylglicosamine [Man3(Glc-NAc)2] and further substitution of the peripheral mannose residues is either by mannose rich oligosaccharides or more complex N-acetylactosamine sialic acid derivatives (57). Few generalizations can be made regarding the structure of the O-glycosyl units (57).

The function of the carbohydrate residues in glycoproteins is of particular interest to researchers. It is apparent from accumulated evidence that the catalytic activity of glycoenzymes is not in any way associated with their carbohydrate content (58). Glycoproteins are generally more resistant to proteolysis and denaturation than other proteins leading to the suggestion that the carbohydrate moiety is present in a protective capacity. Carbohydrate units attached to amino acids close to
### TABLE I
CARBOHYDRATE CONTENT OF SOME GLYCOENZYMES (55)

<table>
<thead>
<tr>
<th>Source</th>
<th>Carbohydrate %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrolases</strong></td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td><em>Candida albicans</em> 87.0</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>human placenta membrane 4.1</td>
</tr>
<tr>
<td>α-Amylase</td>
<td><em>A. niger</em> 3.5</td>
</tr>
<tr>
<td>Bromelain</td>
<td><em>Pineapple stem</em> 3.0</td>
</tr>
<tr>
<td>Cholinesterase</td>
<td>-</td>
</tr>
<tr>
<td>Deoxyribonuclease A</td>
<td><em>Bovine pancreas</em> 4.3</td>
</tr>
<tr>
<td>Dipeptidase</td>
<td>Pig intestine 0.3</td>
</tr>
<tr>
<td>Enterokinase</td>
<td>Pig duodenal mucosa 37.0</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td><em>A. fumigalus</em> 8.0</td>
</tr>
<tr>
<td>Glucoamylase A</td>
<td><em>A. niger</em> 10.0</td>
</tr>
<tr>
<td>Invertase</td>
<td><em>Saccharomyces</em> 53.0</td>
</tr>
<tr>
<td>Lipase A</td>
<td><em>Porcine Pancreas</em> -</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>-</td>
</tr>
<tr>
<td>Phospholipase</td>
<td>-</td>
</tr>
<tr>
<td>Protease</td>
<td><em>Mucor nichei</em> 4.9</td>
</tr>
<tr>
<td>Sulfatase</td>
<td><em>ox liver</em> 9.4</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>-</td>
</tr>
<tr>
<td><strong>Oxidoreductases</strong></td>
<td></td>
</tr>
<tr>
<td>Alcohol Dehydrogenase</td>
<td><em>Pseudomonas</em> -</td>
</tr>
<tr>
<td>Amine oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Glucose dehydrogenase</td>
<td><em>A. oryzae</em> -</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td><em>A. niger</em> 16.0</td>
</tr>
<tr>
<td>Luciferase</td>
<td><em>Phoeas diactylus</em> 11.0</td>
</tr>
<tr>
<td>Peroxidase</td>
<td><em>horseradish</em> 18.0</td>
</tr>
<tr>
<td><strong>Transferases</strong></td>
<td></td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td><em>Bovine colostrum</em> -</td>
</tr>
<tr>
<td>Ribonuclease B</td>
<td><em>Porcine pancreas</em> 31.0</td>
</tr>
</tbody>
</table>
the surface may protect the three dimensional structure of the protein by sterically hindering interaction with and hydrolysis of the enzyme by proteolytic agents. Resistance to heat extremes may also be attributed to steric effects that prevent molecular transformations necessary for denaturation (56,57). That the carbohydrate may contribute to the antigenicity of the enzyme and could be involved in transport mechanisms have also been postulated (58,59).

**Glucose Oxidase**

Glucose oxidase from *Aspergillus niger* is a flavoprotein consisting of two identical polypeptide subunits (mol wt 95,000) covalently linked by disulfide bonds. Each subunit of this globular protein contains one mole of iron and one mole of FAD (60-65). Although amino acid sequencing and crystallographic data are incomplete, the enzyme is known to contain 78% protein, 18% neutral sugar and 4% amino sugars (60,66).

Glucose oxidase is specific for β-D-glucose, the only notable exception being its action on 2-deoxy-D-glucose which is catalyzed at only 12% of the rate of glucose (60). The overall reaction catalyzed by glucose oxidase is as follows:

\[
\text{Glucose} + O_2 \rightarrow \text{Gluconic acid} + H_2O_2
\]

The mechanism illustrated in Scheme 2, involves a four step reduction of the FAD moiety in glucose oxidase (64,67-69).
where, E = Oxidized Enzyme  
G = β-D-Glucose  
L = Gluconolactone  
R = Reduced Enzyme

Scheme 2

The enzymatic methods of glucose determination using glucose oxidase are numerous. Free wrote an excellent review of the history and properties of glucose oxidase and its application to the measurement of glucose in biologic matrices (70). Fluorometric methods utilizing POD and homovanillic acid (71) and polarographic electrodes permit direct determination of glucose (72). Photometric measurements of glucose are possible by peroxidase coupled reaction to a chromogenic oxygen acceptor such as o-tolidine, o-danisidine and indophenol (73). Another approach introduced by Trinder, involved the oxidative coupling of 4-aminoantipyrine (4-AAP), an auxochrome such as
phenol or a phenol derivative and peroxidase to yield a red chromogen (74-76).

Glucose oxidase has been immobilized by adsorption on a solid carrier, entrapment in gel particles and by hybrid covalent-crosslinking methods employing bifunctional reagents (20,22,43,77-79). The more popular methods entail covalent coupling to carriers such as Sepharose, cellulose, polystyrene, polyacrylamide, nylon or amino-silanized porous glass (23,42,43,80-85). These methods all involve coupling through the amino acid residues of glucose oxidase and in some cases a low intrinsic activity of the immobilized enzyme was reported suggesting partial inactivation (42,84,85).

The work of Nakamura and Pazur has revealed that the sugar moieties of glucose oxidase are not involved in its catalytic properties but act in a protective capacity in the presence of temperature extremes or denaturing agents (57,61). Because of these observations Zaborsky and Ogletree introduced the immobilization of the enzyme to an amino containing support, p-aminostyrene (45). Valentova also applied this approach in the immobilization of the same enzyme on chemically modified beaded cellulose and glycidylmethacrylate copolymers (86).

**Peroxidase**

Horseradish peroxidase is a globular glycoprotein (diameter 50 Å) of molecular weight 40,000. The enzyme consists of 308 amino acid residues, a hemin group and eight neutral
carbohydrate side chains attached through N-glycosyl linkages with asparagine (87,88). The carbohydrate moiety of this enzyme constitutes about 18% of the molecular weight.

The crude extract of the plant enzyme contains 7 major and 13 minor isoenzymes, the most abundant of which is isoenzyme C (87). Peroxidase has been extensively studied with respect to structure, physical and catalytic properties and the complete amino acid sequence is available (87-91). Attempts at X-ray crystallography have proven unsuccessful for the plant peroxidases however, recent work has led to the availability of this data for yeast cytochrome C peroxidase, a possible structural homologue of the plant enzyme (92,93). Poulos and Kraut studied the stereochemical basis for catalysis and presented a convincing argument for generalization of this cytochrome C POD model to other peroxidases (92). The model is based on the complementarity of the carboxylate residues in peroxidase and the lysine groups in cytochrome C and an acid-base catalysis by the invariant distal histidine (His-52).

This work supports the essential role for arginine in catalysis, as proposed by others in the field and indicates the function of histidine and a glutamate-glutamine pair (Glu-187,Gln-239) in stabilization of the higher oxidation states of the heme iron (88,92,94,95). It should be noted that while the structural features common to both cytochrome C and horseradish peroxidase are remarkable some differences do arise. For example, unlike the yeast source, the plant peroxidases contain disulfide bonds
and bind two moles of calcium each per molecule. In addition, the plant peroxidases are glycoproteins and react with small aromatic hydrogen donors but not with cytochrome C (92,93). Still, from this work and that of Welinder, prediction of the structural form and active site geometry of the plant enzyme has been introduced. A general model for peroxidase focusing on helices, disulfide linkages and carbohydrate attachments as proposed by Welinder is illustrated in Figure 3A (93).

Although the carbohydrate chains have not been sequenced, they are known to be mannose rich derivatives and because of their compositional similarity are believed to resemble those of another plant glycoprotein, pineapple stem bromelain (93,96,97). Figure 3B shows the 2 N-acetyl-glucosamine, 3 mannose glycan core found in bromelain.

Peroxidase is highly specific for the hydrogen acceptor, only H₂O₂, CH₃OOH and C₂H₅OOH of a number of compounds tested are active. Peroxidase may act as an oxidase with indirect electron transport to the heme by aromatic amines or phenols. Alternatively, inorganic ions such as nitrite interact directly with the heme to transfer an electron from the donor. These mechanistic differences are manifested in varying pH dependencies and sensitivity to activators or inhibitors. This broad specificity may also be associated with the basicity of the substrate, solubility and steric factors (93).

The overall reaction catalyzed by peroxidase is:
FIGURE 3

A STRUCTURAL MODEL OF HORSERADISH PEROXIDASE

Legend

A. The structural model of peroxidase presented by Welinder (93) is illustrated. Cylindrical projections A–J represent helices of the enzyme. Extended structure $\beta_1$, $\beta_2$ and $\beta_3$ are indicated by arrows while the reverse turns and coils are shown as connecting lines. The amino and carboxy terminals are labelled as N and C, respectively. The three disulfide bridges of plant peroxidases are indicated by $-\text{SS}-$ and the corresponding residue numbers. The hæmin prosthetic group is indicated by the central rectangle, while the points of carbohydrate attachment are illustrated by a fork shaped symbol and numbers (93).

B. A schematic representation of the carbohydrate moieties of pineapple stem bromelain is indicated. As with the plant peroxidases this unit of bromelain is linked to the protein through asparagine residues (97).
Donor + \( \text{H}_2\text{O}_2 \) \( \xrightarrow{\text{POD}} \) Oxidized Donor + 2\( \text{H}_2\text{O} \)

A simplified scheme for the reaction mechanism of horseradish peroxidase may be described by the following equations: \( (81,85,90,91) \)

\[
E + S_1 \xrightarrow{k_1} EI + \text{H}_2\text{O}
\]

\[
EI + S_2 \xrightarrow{k_2} EI-S_2 \xrightarrow{k_3} EII-P' \xrightarrow{k_4} EII + P'
\]

\[
EII + S_2 \rightarrow E + P'
\]

Where, \( E \) = Peroxidase

\( EI \) = Enzyme Compound I

\( EII \) = Enzyme Compound II

\( P' \) = Product

\( S_1 \) = Substrate, eg. \( \text{H}_2\text{O}_2 \)

\( S_2 \) = Hydrogen Donor

Scheme 3.

Peroxidase has been immobilized on a variety of organic and inorganic materials. Coupling to porous glass has been accomplished with glutaraldehyde, isothiocyanate, fluorodinitro-
benzene, acrylic copolymers and diazonium linkages (100). Others have employed covalent cross-linking with poly(methylacryl) benzoic acid carriers, adsorption to carboxymethyl cellulose membranes and embedding the enzyme in polyacrylamide gels (101-104). Ugarova effected coupling of peroxidase to gels through glutaraldehyde and observed a concomitant decrease in activity and thermal stability upon immobilization (104,105).

Oxidation of the native enzyme causes a shift of the Soret band from 404 nm to $\lambda_{max}=420$ nm but no decrease in activity was evident (106-108). This observation led researchers to attempt immobilization of this enzyme through the carbohydrate moieties (108,109). One interesting development from this approach has been the preparation of peroxidase-antibody conjugates for enzyme immunoassays. Nakane et al. (100) and later Tijssen et al. (109) described methods of conjugation based on initial periodate oxidation of peroxidase followed by antibody coupling.

2. Non-glycosylated Proteins

Not all enzymes contain a carbohydrate component and therefore immobilization must be directed more conventionally through the protein moiety (3,110). One enzyme studied in this regard was oxalate oxidase.

**Oxalate Oxidase**

Oxalate oxidase found in the roots of barley seedlings is a
protein consisting of two identical subunits of molecular weight 75,000 (111). Data concerned with the structural characteristics and mechanistic information for the soluble enzyme is limited and although amino acid sequencing is incomplete, the amino acid composition is available (112). Oxalate oxidase is activated by flavins, FMN and FAD, however, an apoprotein cannot be separated from the pure enzyme (113,114). The mechanism of the effect of the added flavins is unclear and no evidence for an electron transfer pathway is apparent. No studies identifying a carbohydrate moiety or other structural residues have been published to date.

The enzyme, specific for oxalic acid, produces carbon dioxide and hydrogen peroxide according to the general equation: (111-115).

\[
\text{C}_2\text{H}_2\text{O}_4 + \text{O}_2 \xrightarrow{\text{Ox}} 2\text{CO}_2 + \text{H}_2\text{O}_2
\]

Determination of oxalate in urine or plasma is important in the diagnosis and treatment of primary hyperoxaluria and enteric hyperoxaluria (116-118). Enzymatic assays employ initial decarboxylation with oxalate decarboxylase or oxidation by oxalate oxidase (119). The former reaction has been used by various authors but has proved unsuitable for routine purposes (120-124).

Methods using oxalate oxidase have involved quantitation of both oxidation products. If CO\textsubscript{2} is measured, the reaction is
twice as sensitive as that based on decarboxylation, however, there are difficulties in the determination of oxalate in serum due to endogenous CO₂ concentrations (124–126). Alternatively, peroxide may be measured by coupling catalase and aldehyde dehydrogenase to produce NADH (127). Other substrates for oxalate quantitation include the coupling of 3-methyl-2-benzo-thiazolinone hydrazone (MBTH) with N,N-dimethylaniline (128,129), 3-(dimethylamino)benzoic acid (130) or sodium N-sulfopropylaniline (131) to determine hydrogen peroxide.

Recently, Capaldi and Taylor described procedures to determine hydrogen peroxide and cell surface sialic acid involving the oxidative coupling of MBTH with its formaldehyde azine to form a tetraazapatamethine dye (132,133). This reaction offers the sensitivity (ε = 52 mM⁻¹cm⁻¹) desired for oxalate quantitation and is particularly amenable to automation since it proceeds close to the pH optimum of oxalate oxidase, 3.5. This reaction scheme was therefore employed in the assay of oxalate oxidase and urinary oxalate. (Figure 4)

Immobilization of oxalate oxidase has been completed on diaminomethane modified nylon tubing initially O-alkylated by TEOFB and then reactivated with glutaraldehyde (134,135). A similar approach was taken by Kasidas and Rose for immobilization through polyethyleneimine derivatized tubing (136,137). Rahni et al. introduced chemical cross-linking of oxalate oxidase to a membrane mounted on the tip of an oxygen electrode for the amperometric determination of oxalate in urine.
FIGURE 4

ASSAY SEQUENCE FOR THE DETERMINATION OF
OXALATE OXIDASE

Legend

Standard solutions of oxalate were oxidized to hydrogen peroxide and carbon dioxide in the presence of oxalate oxidase. The azine is formed in a separate reaction by condensation of 3-methly-2-benzothiazolinone hydrazone (MBTH) with formaldehyde. Further coupling of the azine with MBTH in the presence of peroxidase and hydrogen peroxide yields a blue cationic dye that is measured by spectrophotometric analysis (132,133).
\[
\text{(COOH)}_2 + O_2 \xrightarrow{O\&x} 2\text{CO}_2 + \text{H}_2\text{O}_2
\]

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{MBTH} & + \quad \text{AZINE} \\
\text{POD} & \quad \text{H}_2\text{O}_2 \\
\text{TETRAAZAPENTAMETHINE}
\end{align*}
\]
Immobilization of oxalate oxidase in this study was
effected using the amino arms modified as illustrated in Figure
5.

E. APPROACHES TAKEN IN THIS STUDY

The primary objective of this study was to further
characterize and optimize covalent methods of immobilization.

Quite often enzyme immobilization leads to a compromise in
the activity when compared to its soluble analogue. Multiple
bond formation and uneven distribution of cross-linking groups
are two explanations of this phenomena, however, the degree of
support derivatization may also be important to the recovery of
enzyme activity. This work investigated the possibility of
minimizing heterogeneity and activity losses at the activation
step to ultimately control the functional group density available
for enzyme coupling. In this approach limited concentrations of
alkylating reagent, TEDTFB, were provided to the nylon support
yielding a restricted number of reactive sites for spacer arm
attachment. Subsequent treatment with excess diamine arms
produced amino-substituted polymers with varying group densities
capable of enzyme coupling.

The effect of arm length was also of interest as other
authors have found that enhanced activities may be expected with
the use of spacer arms (45). In this study the effects of arm
FIGURE 5

THE IMMOBILIZATION OF NON-GLYCOSYLATED PROTEINS THROUGH AMINO-SUBSTITUTED NYLON TUBING

Legend

Nylon tubing modified with ED-600, \( \omega, \omega \)-poly-(oxyethylene)diame, spacer arms (A) was reactivated through the distal amine (*) with a 5% glutaraldehyde solution (B) to yield free aldehyde groups. In reaction C, free amine functionalities on the native enzyme were coupled with the aldehydic groups to produce an imine bond with the support.
Figure 5

A. Nylon Tube Supported ED-600 Arms

B. OHC(CH$_2$)$_3$CHO

C. Enzyme = NH$_2$
length on chemical kinetics and intrinsic activities were investigated. The spacer arms employed were of the \( \epsilon, \omega \)-poly-(oxyethylene)diamine type and ranged in length from 60 to 630 Å.

Limited substitution with these diamine arms was applied in the immobilization of peroxidase, glucose oxidase and their coimmobilization. The approach taken involved the conventional sodium metaperiodate oxidation of enzyme carbohydrate moiety followed by support coupling through the generated aldehydic functions to form a Schiff base with the carrier (139).

Evaluation of POD and GO characteristics before and after immobilization, as well as serum glucose measurements was completed using the approach of Artiss et al. illustrated in Figure 6 (75,76). Clinical applicability of the immobilized systems studied was evaluated using a Technicon AutoAnalyzer I, by determination of serum glucose in a "normal" population.

To demonstrate the diversity of this approach, using long spacer arms of limited group density, the immobilization of a protein, oxalate oxidase, was effected. In this case, the amino-substituted nylon was further derivatized with glutaraldehyde and enzyme bonding accomplished through the amine groups on the native enzyme (Figure 5).

Quantitation of oxalate oxidase activity was completed using a modification of the reaction sequence developed in this laboratory, by Calpaldi and Taylor (132,133) (Figure 7).
FIGURE 6

A SCHEMATIC REPRESENTATION OF THE APPROACH TAKEN IN ACTIVITY ASSAYS OF PEROXIDASE AND GLUCOSE OXIDASE

Legend

A. Immobilized glucose oxidase (GO) was assayed by the peroxidase catalyzed coupling of 4-aminoantipyrine (4-AAP) and 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) with glucose as substrate to form a red product (75,76).

B. Immobilized peroxidase (POD) was assayed employing the same chromogenic system, 4-AAP and HDCBS with hydrogen peroxide as substrate.

C. Coimmobilized coupled activity (POD+GO) was measured with glucose substrate using the modified Trinder reaction described above for peroxidase and glucose oxidase. In this case no soluble enzyme was present.
A SCHEMATIC REPRESENTATION OF THE APPROACH TAKEN IN ACTIVITY ASSAYS OF OXALATE OXIDASE

\textbf{Legend}

Interaction of immobilized enzyme and oxalate produces H$_2$O$_2$ which is captured by soluble peroxidase and used in the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and its formaldehyde azine to yield a blue tetraazepentamethine dye.
FIGURE 7

- **OOx**
- **Pod**
- **Blue Chromogen**
- **MBTH HCHO**
- **Oxalate**
CHAPTER II

EXPERIMENTAL

A. ANALYTICAL INVESTIGATION

1. Materials

Glucose oxidase (Aspergillus niger) [β-D-Glucose:Oxygen 1-oxidoreductase; EC 1.1.3.4] Type X was purchased from Sigma Chemical Company, St. Louis MO, 63178.

Peroxidase (Horseradish) [Donor: Hydrogen peroxide oxidoreductase; EC 1.11.1.7] Grades I, II, and immunochemical quality and Oxalate oxidase (Barley seedlings) [Oxalate: Oxygen oxidoreductase; EC 1.2.3.4] were obtained from Boehringer Mannheim Canada, Dorval PQ, H9P 1A9.

The enzyme activities quoted are those of the supplier. Unit definitions are as follows: GO, one unit is the amount of enzyme that will oxidize one μmole of β-D-glucose per min at pH 5.1 and 35°C; POD, one unit is the amount that will catalyze the oxidation of 1 μmole of guaicol by H₂O₂ per min at 25°C pH 7.0; OD₅₅₀, one unit is the amount of enzyme which will oxidize 1 μmole of oxalate to carbon dioxide and H₂O₂ per minute at 37°C, pH 5.0.

Nylon tubing was obtained from Portex Ltd., Hythe, Kent, England.

Triethylxonium tetrafluoroborate was purchased from Fluka
Chemical Corp., Hauppauge NY, 11788. Methylene chloride required for activation of the support was obtained from Fisher Scientific (ACS grade), Fair Lawn NJ, 07410.

Poly(oxyalkylene)diamine arms Jeffamine ED-series 600, 2000 and 6000 were provided by Texaco Chemical Inc., Belaire TX, 77401. Ethylenediamine was purchased from Fisher Scientific.

Amicon Corporation Scientific Systems Division, Danvers MA, 01923 was the source of the ultrafiltration membranes YM-30, XM-50, YM-100 and XM-300 used throughout the study.

The analytical grade reagents purchased from Aldrich Chemical Co., Milwaukee WI, 53233 were: adipic dihydrazide, 4-aminoantipyrine, sodium 2-hydroxy-3,5-dichlorobenzene-sulfonate and 3-methylbenzothiazolinone hydrazone hydrochloride monohydrate.

Brij 35 wetting agent, dimethylformamide, hydrazine hydrate, phenol reagent (Folin and Ciocalteu) and sodium periodate were obtained from BDH Chemical, Toronto ON, M8Z 1K5.

The following reagents were purchased from Fisher Scientific Co.: L-ascorbic acid, standard buffer solutions, calcium chloride, ethylene glycol, formaldehyde (37% w/w), D-glucose, hydrogen peroxide (30% w/v), methanol and sodium azide.

Glutaraldehyde (Grade II), oxalic acid and sodium cyanoborohydride were obtained from Sigma Chemical Co.

2. Instrumentation
For the purification of the oxidized enzyme preparations an Amicon ultrafiltration cell (10 mL volume) was employed. An operating pressure of 4 atm nitrogen and temperature of 4°C were maintained throughout the procedure. Membranes were soaked in distilled water for one hour and preconditioned with 1 mg/mL BSA before use. For reuse, membranes were stored in 10% ethanol at 4°C.

All spectrophotometric measurements and recordings were made on the Beckman 35 UV-Visible Spectrophotometer available from Beckman Instruments Inc., Irvine CA, 92713 or the 8451A Diode Array Spectrophotometer with 7470 Plotter from Hewlett Packard, Palo Alto, CA 94304.

Activity assays and kinetic measurements were completed on the Beckman 35 equipped with a 1 cm flow cell using an LKB 2115 Multiperpex Pump, Bromma, Sweden.

The micropipettors used in this study were Gilson Models P-200 D and P-1000 D with disposable pipet tips available from Mandel Scientific Company Ltd., Rockwood ON.

For weights above one gram, a Mettler PC 4400 Delta Range electronic balance was used. For weights below one gram, a Mettler H1b semi-automatic balance, Fisher Scientific Co., was employed.

Verification of pH measurements were completed on a Fisher Accumet pH meter, Model 800.

3. Reagents
Reagents For Immobilization

Alkylation Reagent: A solution containing 200 nmoles triethylxonium tetrafluoroborate in dry CH₂Cl₂ was prepared immediately before use. This compound is extremely hygroscopic and meticulous care must be taken to ensure that all glassware and solvents are absolutely dry to avoid degradation of the material to ethanol and diethyl ether. To this end, all reaction vessels were dried at 100°C and cooled to room temperature in a dessicator. In addition, methylene chloride was redistilled over calcium hydride and nylon tubing dried for at least a week in a dessicator over phosphorous pentoxide before use. Although dry weights of alkylating material were determined in a dry box, no attempt was made to prepare exact solutions. An aliquot approximating the desired weight was taken, the weight recorded and dilutions prepared in dry solvent as quickly as possible.

Glucose Assay Reagents

HDCBS: A solution with a concentration of 18 mM HDCBS and 1mL/L Brij 35 in 0.1 M phosphate buffer, pH 7.5 was prepared. This solution is stable for one week when stored in the dark at 4°C.

4-AAP: This solution was prepared to contain 8 mM 4-aminoantipyrine, 1mL/L Brij 35 and 0.30 mM sodium azide in either 0.1 M phosphate buffer at pH 7.5 (PDD assay) or 0.05 M
acetate buffer, pH 5.5 (GO assay). This solution is stable for several weeks when stored in the dark at 4°C.

**Hydrogen Peroxide Standards:** A 60 mM stock solution was prepared in distilled water. The stock and appropriate dilutions were standardized with potassium permanganate before use (140).

**Glucose Standards:** A stock solution containing 1 M D-glucose in 1 g/L benzoic acid was permitted to mutarotate 24 h prior to use. Dilutions of the stock were prepared in the same solvent.

**Enzyme Solutions:** In determinations of glucose oxidase activity, a solution containing a final cuvet concentration of $3 \times 10^3$ U.L$^{-1}$ PDD in 0.10 M phosphate buffer at pH 7.5 was prepared and immobilized glucose oxidase was used in lieu of its soluble analogue.

Immobilized peroxidase activities were measured in the presence of hydrogen peroxide and an appropriate segment of nylon coupled peroxidase.

Coimmobilized activities were assayed in the absence of soluble enzyme. Measurements of GO bound in these systems were carried out as described above, that is, in the presence of excess soluble peroxidase with glucose as substrate. Quantitation of peroxidase activity coupled to coimmobilized
tubing was effected using H₂O₂ substrate and no soluble enzyme.

**Oxalate Oxidase Reagents**

**Chromogen System:** This reagent was prepared to contain 0.85 mM formaldehyde and 0.88 mM MBTH in distilled water and allowed to stand for at least one hour before use. This solution was observed to be stable for several days at room temperature.

**Peroxidase Solution:** A solution containing 10.3 x 10³ U.L⁻¹ PDD and 6.2 x 10⁻⁴ M 8-hydroxyquinoline in 0.05 M succinate buffer, pH 3.6 was prepared and stored at 4°C when not in use.

**Oxalate Standards:** A stock solution of 20 mM oxalic acid, disodium salt was prepared in 0.1 M HCl. Working standards were prepared by diluting the above stock in 0.1 M HCl.

**Oxalate Oxidase Reagent:** To measure urinary oxalate concentrations, 700 U.L⁻¹ soluble oxalate oxidase was dissolved in 0.05 M succinate buffer, pH 3.6. Alternatively, a nylon coil of immobilized oxalate oxidase was employed in absence of the soluble enzyme.

4. **Methods**

**Support Activation And Amination**

**O-Alkylation of Nylon Tubing:** A 1-m length of nylon-6 tubing, 1 or 1.5 mm bore was perfused with a mixture containing
18.6% w/w calcium chloride and 18.6% w/w water in methanol and heated at 60°C for 20 min. This process etches the inside of the nylon tubing, removing amorphous nylon and increasing the surface area available for coupling. The tubing was then dried for one week in a dessicator over phosphorous pentoxide.

The dried tubing was filled with triethylxoxonium tetrafluoroborate solution in methylene chloride and incubated at room temperature for 50 min. Unreacted alkylating reagent was removed and the tubing washed with dry methylene chloride.

**Amination:** The O-alkylated tubing was immediately filled with a 10% v/v solution of ε,ω-poly(oxyethylene)diamine arms in dimethylformamide and incubated for 4 h at 25°C. The amine substituted nylon was then washed with 75 mL of each of the following solutions: dimethylformamide, water, 0.1 M HCl, 0.1 M NaHCO₃, 3.0 M NaCl, 0.2 M KCl and excess water. Thereafter the tubing was washed exhaustively with 0.1 M phosphate buffer, pH 7.5. The modified tubing was filled with buffer, pH 7.5, and stored at 4°C until use.

**Glutaraldehyde Activation:** To effect the immobilization of non-glycosylated proteins the aminated tubing was perfused (2 mL/min) with 5% w/v glutaraldehyde in 0.2 M borate buffer, pH 8.5 for 15 min at room temperature. The tube was then washed with 0.15 M NaCl in borate buffer for 15 min.
Enzyme Immobilization

Glycoenzymes: A fresh solution containing 1-2 mg/mL POD or GO was made in 0.1 M phosphate buffer pH 7.5. The initial concentration of enzyme was calculated spectrophotometrically using an extinction coefficient of $9.1 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ at 404 nm for peroxidase and $2.82 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ at 450 nm for glucose oxidase (92,63). In coimmobilization procedures initial and final concentrations of POD and GO were determined by a multicomponent analysis available as software on the Hewlett Packard 8451 spectrophotometer. Analysis is based on the spectral differences of the two enzymes.

Oxidation of the enzyme was initiated by mixing 1 mL each of 12.0 mM sodium metaperiodate and enzyme. The reaction was stirred for 90 min at 25°C in the dark. Excess periodate was destroyed with the addition of 1 mL of 0.32 M ethylene glycol. The reaction was incubated for 60 min at 25°C with constant stirring.

The oxidized enzyme was isolated by ultrafiltration at 4°C using a YM-30 Amicon membrane for peroxidase and a XM-50 membrane with glucose oxidase. The enzyme was washed twice with two 10 mL volumes of coupling buffer before removing it from the Amicon cell. Concentration of the oxidized enzymes were checked again by spectrophotometric analysis before coupling to the amino substituted nylon.

Nylon tubing was then filled with the oxidized enzyme and incubated for 6 or 18 h at 4°C for POD and GO, respectively.
Excess enzyme and noncovalently bound enzyme were removed by perfusion with the coupling buffer, 0.1 M phosphate buffer, pH 7.5. The immobilized enzyme coil was filled with buffer and stored at 4°C when not in use.

**Coimmobilization Of Glycoenzymes:** Coimmobilization of oxidized GO and POD to amine-substituted nylon was completed using three different approaches:

1. Mix aliquots of periodate treated enzymes and couple directly.
2. Prepare a GO-POD conjugate, reoxidize and couple.
3. Prepare a GO-Dihydrazide-POD conjugate, reoxidize and couple.

Quantitation of coupling yields was performed by multi-component analysis on the post immobilization eluates in all coimmobilization experiments.

**Mix Aliquots And Couple:** Samples were individually oxidized, mixed in known ratios and incubated with the amino derivatized nylon support for 18 h at 4°C. Post immobilization eluates were collected and subjected to multi-component analysis. The tubing was then washed and stored as described previously.
GO-POD Conjugate: Oxidized GO was coupled with native POD for 18 h at 4°C in 0.1 M phosphate buffer, pH 7.5. The "enzyme conjugate" was reoxidized with sodium periodate as described previously. Ultrafiltration with XM-300 and XM-50 membranes permitted purification of the enzyme derivative. The pure GO-POD conjugate was reconstituted in 0.1 M phosphate buffer, pH 7.5 and coupled to amino-substituted nylon for 18 h at 4°C. Post immobilization eluates were collected and subjected to multi-component analysis. The tubing was washed and stored as previously indicated.

GO-Dihydrazide-POD Conjugate: Oxidized GO was incubated with a solution containing a final concentration of 10 mM adipic dihydrazide and 10 mM NaBH₃CN at pH 7.5 in coupling buffer for 2 h at room temperature. Excess reagent was removed by ultrafiltration using XM-300 and XM-50 membranes. The dihydrazide modified GO was permitted to couple with "mildly oxidized" POD overnight at 4°C, pH 7.5. Mild oxidation of POD was effected employing a final concentration of 1 mM sodium periodate for 90 min at 25°C. All other conditions for oxidation were as described in the text. Free POD was removed by ultrafiltration with a XM-50 membrane. Upon reoxidation under normal conditions, the GO-Dihydrazide-POD conjugate was filtered again with a YM-30 membrane and coupled to amino-substituted nylon for 18 h at 4°C. Washing and storage procedures were as described earlier.
Non-glycosylated Proteins: Glutaraldehyde activated tubing (2m) was filled with a solution containing 20 U of oxalate oxidase in 0.25 M potassium phosphate buffer, pH 7.0 with 0.02 M KCl and incubated 18 h at 4°C. Excess protein and noncovalently bound enzyme were removed by washing with 0.05 M succinate buffer, pH 3.5. For storage the bound enzyme tubing was filled with the same buffer and maintained at 4°C.

Biochemical Investigation

Evaluation of bound enzyme was completed in three fashions:

1. Differential calculations from spectral data for concentrations of enzyme bound.
2. Elution methods for the determination of enzyme bound.
3. Activity assays for the units of active enzyme per meter.

Differential Calculations From Spectral Data For Concentration Of Enzyme Bound: Bound enzyme was calculated by difference from the spectra of the enzyme before and after incubation with derivatized tubing. The same approach was taken for coimmobilized enzymes employing a multi-component analysis based on spectral differences. Peroxidase contains a heme molecule which gives rise to a characteristic \( \lambda_{\text{max}} \) at 404 nm, \( \epsilon = 9.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \) (92). Glucose oxidase contains two FAD
molecules per mole of enzyme giving rise to absorbance peaks at 350 and 450 nm, at $\lambda_{\text{max}} = 450$ nm, $\epsilon = 2.82 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ (63). Enzyme eluted in washing solutions was added to the concentration of unbound enzyme.

Protein concentrations before and after coupling were determined by the method of Lowry et al. using BSA standards. (141)

**Elution Method For Determination Of Enzyme Bound:** Bound enzyme was estimated by the concentration of enzyme released when a length of unreduced tubing was incubated with 10 mM hydrazine in 0.1 M phosphate buffer, pH 7.5 for 2 h at room temperature. The concentration of enzyme eluted was measured at $\lambda_{\text{max}} = 404$ and 450 nm for POD and GO, respectively. Activities remaining on the tube were also evaluated after removal of the hydrazine containing solution.

**Activity Assay For Units Of Active Enzyme Per Meter:** Immobilized enzyme activities and kinetic data were studied using the modified gradientless recirculation reactor illustrated in Figure 8 (38-40).

Table II indicates the volumes and final concentrations used in the activity assays for both immobilized and soluble peroxidase, glucose oxidase and coimmobilized enzymes. Reagents were mixed and the reaction initiated with the addition of the enzyme assayed. The strategies involved in the immobilized
A gradientless reactor was used in the calculation of activities and in kinetic studies. To ensure steady diffusion of substrates and products, constant stirring using a magnetic stir bar and a flow rate of 30 mL.min\(^{-1}\) was maintained throughout the assay. Calibration of the pump scale avoided the necessity of a flow meter. The initial rates were recorded at room temperature on the Beckman JS, equipped with a 1 cm flow cell. Immobilized enzyme tubes assayed for activity (U/m) were typically 5-10 cm in length and possessed an inner diameter of 1 or 1.5 mm.

To estimate leaching of the enzyme from the coupled tubing, reagents were recirculated to the reaction vessel, the pump stopped and the change in absorbance was monitored over time.
# TABLE II

**ACTIVITY ASSAYS**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Volume (mL)&lt;sup&gt;a,b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>HDCBS</td>
<td>9.0</td>
<td>10</td>
</tr>
<tr>
<td>4AAP</td>
<td>2.4</td>
<td>4</td>
</tr>
<tr>
<td>NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.3</td>
<td>6</td>
</tr>
<tr>
<td>Glucose</td>
<td>30.0</td>
<td>-</td>
</tr>
<tr>
<td>POD</td>
<td>Soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Immob</td>
<td>-</td>
</tr>
<tr>
<td>GO</td>
<td>Soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Immob</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Activity of the soluble enzymes was initiated with the addition of 20μL of POD or GO.

<sup>b</sup>Activity of the immobilized enzymes was initiated by passage through 5-10 cm of coiled nylon tubing.
enzyme assays are pictorially illustrated in Figure 4. Absorbance was monitored continuously against a reagent blank, containing water in lieu of substrate.

Oxalate oxidase activity was measured by the following method: A 10 mL solution containing 0.85 and 0.83 mM MBTH and formaldehyde was mixed with 2.5 mL of 30 mM oxalate standard and 2.5 mL of peroxidase solution. The reaction was initiated with the addition of 0.5 mL soluble oxalate oxidase or by pumping the mixture through a 10 cm length of immobilized oxalate oxidase. Initial rates were recorded at 630 nm on the Beckman 35.

In all assays the length of nylon tube supported enzyme incorporated into the recirculation system was chosen such that the amount of substrate converted was less than 2 % of the total amount initially present. In practice lengths of 5 or 10 cm were routinely employed.

8. CLINICAL INVESTIGATION

1. Materials

All materials were those used for the ANALYTICAL INVESTIGATION described in CHAPTER IIA, p.44.

2. Instrumentation

Clinical evaluations were completed using a Technicon AutoAnalyzer I equipped with a Fisher series 5000 recorder. Manifolds for determination of serum glucose by immobilized POD,
60

60 and coimmobilized POD and GO are indicated in Figures 9-11.

Serum separation tubes were vacutainers without
anti-coagulant and are available from Becton-Dickinson Ltd.,
Rutherford, NJ 07070. Serum isolation was effected using a
Beckman TJ-6 tabletop centrifuge from Beckman Instruments Inc.,
Falo Alto, CA 94304.

All other apparatus were as described for the ANALYTICAL
INVESTIGATION in CHAPTER IIA, p.45.

3. Reagents

Standard solutions and chromogen reagents, 4-AAP and HDCBS
were prepared as described in the ANALYTICAL INVESTIGATION,
CHAPTER IIA, p.46, with the following modifications:

**Immobilized Peroxidase Manifold**

A final concentration of 60 x 10^3 U.L^-1 soluble glucose
oxidase was added to the 4-AAP reagent, pH 5.5, 0.05 M acetate
buffer.

No soluble peroxidase was added to the system.

**Immobilized Glucose Oxidase Manifold**

A final concentration of 3 x 10^3 U.L^-1 soluble peroxidase
was added to the HDCBS solution in 0.1 M phosphate buffer, pH
7.5.

Soluble glucose oxidase is absent from this system.
The manifold employed in the determination of serum glucose using immobilized peroxidase is illustrated. Samples were initially diluted and then coupled with 4-aminoantipyrine (4-AAP) and glucose oxidase solution. The reagents were mixed and reacted with peroxidase coupled to nylon upon passage through a 1 m length enzyme coil maintained at 35°C. Addition of HDCBS caused the development of a red product. The intensity of this product was recorded at 505 nm using a 15 mm flow through cell. A sampling rate of 60 per hour at a sample to wash ratio of 1:1 was maintained.
FIGURE 10

MANIFOLD FOR SERUM GLUCOSE DETERMINATION USING
IMMOBILIZED GLUCOSE OXIDASE

Legend

The manifold utilized in the quantitation of serum glucose using nylon coupled glucose oxidase is shown. Sera were diluted in an air segmented stream before an aliquot was redrawn and coupled with 4-AAP and sodium azide (NaN₃). Peroxide was produced with passage of the stream through the immobilized GO coil (1 m), maintained at 35°C. Soluble peroxidase and HDCBS reagent were added immediately after the GO coil and mixed to permit development of a red quinoneimine dye. Product transmittance was measured at 505 nm using a 15 mm flow cell. Samples were introduced at a rate of 50 per hour and a 1:1 sample to wash ratio.
FIGURE 10

Flow Rate
50
1/1

0.32 mL/min

1.00
H₂O

0.32
Air

W

A

0.16
AAP: NaN₃

1.00

0.32
Air

GO COIL

W

1.20
HDCBS; POD

2.00
H₂O

505 nm

Recorder
FIGURE 11

MANIFOLD FOR SERUM GLUCOSE DETERMINATION
USING COIMMOBILIZED ENZYMES

Legend

The manifold employed in the determination of serum glucose with coimmobilized glucose oxidase and peroxidase is illustrated. Samples were introduced to an air segmented stream of water at a rate of 50 per hour (1:1 Sample:Wash). An aliquot of the diluted sample was then mixed with 4-AAP and NaN₃ reagent followed by an HDCBS line. The stream was then passed through a coimmobilized coiled tube 1 m in length. A second mixing coil was added (7 turns) before debubbling and the transmittance read at 505 nm. A 15 mm flow cell was utilized in all measurements.
FIGURE 11

Flow Rate

50
1/1

0.32 ml/min

0.32

Air

0.80

H₂O

0.16

AAP:NaN₃

1.00

0.32

Air

1.20

HDCBS

2.00

H₂O

COIMMOBILIZED COIL

505 nm

Recorder
Coimmobilized Peroxidase And Glucose Oxidase Manifold

No soluble enzymes were added, reagents were prepared as described for activity assays.

4. Methods

Glucose analysis on serum samples was completed on the AutoAnalyzer using the appropriate manifold. (Figures 9-11)

Oxalate levels were quantitated by a soluble oxalate oxidase method as follows: Mix 200 µL MBTH reagent, 50 µL oxalate standard (or water for blank), 50 µL POD and 50 µL oxalate oxidase containing 8-hydroxyquinoline. Incubate the solution for 7 min at 35°C and read absorbances at 630 nm.

5. Specimen Collection

Blood samples utilized in the collection of "normal" patient data were split specimens drawn for routine analytical purposes making patient authorization unnecessary. An identification scheme different from that employed by the hospital laboratory was used to insure patient confidentiality.

For study of glucose levels on "normal" individuals, 125 fasting serum samples were collected in vacutainer tubes without anti-coagulant. Samples were allowed to clot at room temperature and centrifuged at 3000 x g for 5 min at 4°C. Samples were maintained at 4°C for no longer than 24 h prior to assay. All patient data were samples obtained from the Chemistry Laboratory at Salvation Army Grace Hospital, Windsor,
Ontario. The "normal" individuals were ambulatory patients believed to be free of any disease state that might alter serum glucose levels.

Urinary oxalate levels were quantitated on 24 h samples acidified with 0.1 M HCl to pH < 3.0. Patient samples were obtained from the Chemistry Laboratory at Salvation Army Grace Hospital, Windsor, Ontario. Upon collection the samples were mixed and oxalate precipitated with CaCl₂ exactly as described by Mazzachi et al. (142).
CHAPTER III

RESULTS AND DISCUSSION

A. IMMobilization of Peroxidase AND Glucose Oxidase

1. Analytical Investigation

The assay sequence employed in the calculation of peroxidase and glucose oxidase activities involved the oxidative coupling of HDCBS and 4-AAP (75,76). This scheme has been extensively applied in the quantitation of peroxide in peroxide generating reactions. B.Dulay, working in this laboratory, further extended the application of this indicator reaction to the assay of peroxidase activities (48). A schematic of the concept involved in the determination of nylon coupled peroxidase and glucose oxidase has been given earlier in Figure 4A and B.

Reactor Design

Immobilized enzyme activities were measured using the gradientless reactor illustrated earlier in Figure 8. The essential features of this reactor include the peristaltic pump, a spectrophotometer equipped with a 1 cm flow cell and a segment of immobilized enzyme derivative which is coiled to enhance
radial diffusion.

Serious deviations in the kinetic behavior of the immobilized enzyme may be expected if the reaction is carried out under conditions permitting diffusion control. Laidler (in Ref 3) suggested that kinetic experiments be conducted utilizing minimal radius and length of tubing. Under these conditions, mass transfer of substrate to the surface and diffusion of the substrate within the diffusion layer is maximized. Enzyme coupling was, therefore, completed on lengths of tubing possessing an inner diameter of 1-1.5 mm. In addition, the length of enzyme coupled nylon necessary for activity measurements was considered. At a fixed flow rate it is possible to mathematically estimate the length of support required to achieve a given degree of substrate conversion (34). In measuring intrinsic activities a conversion of 2 % was considered ideal and lengths of 3-10 cm were calculated from theorems developed in Horvath's laboratory (34). Assay of support bound POD demonstrated an apparent decrease in initial rates when the length of carrier assayed varied in length from 3 to 60 cm (Table III). Optimal results were obtained using segments less than 10 cm in length, henceforth all activity and kinetic assays were conducted utilizing 5-10 cm of support.

Koyayashi and Laidler (38) have also demonstrated that at high flow rates the effective thickness of the unstirred Nernstian layer is decreased as the rate of diffusion of substrate to the surface is enhanced. Under these
### TABLE III

THE EFFECT OF REACTOR LENGTH ON APPARENT ACTIVITIES

<table>
<thead>
<tr>
<th>Reactor Length (cm)</th>
<th>Apparent Activity (U/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.51</td>
</tr>
<tr>
<td>5</td>
<td>3.11</td>
</tr>
<tr>
<td>10</td>
<td>3.37</td>
</tr>
<tr>
<td>20</td>
<td>2.18</td>
</tr>
<tr>
<td>40</td>
<td>1.51</td>
</tr>
<tr>
<td>60</td>
<td>1.37</td>
</tr>
</tbody>
</table>

*a*Nylon tubing modified with amine groups was derivatized with a solution of oxidized peroxidase. After a 4 h incubation, unbound peroxidase was removed and the support washed with 0.1M phosphate buffer, pH 7.5. Lengths of tube from 3-60 cm were cut and the activity measured utilizing 0.30 mM \( \text{H}_2\text{O}_2 \), 9.0 mM HDCBS and 2.4 mM 4-AAP. Measurements were completed on a gradientless reactor at a pump setting of 8.5.*
circumstances the rate of product formation depends only on the chemical interaction between enzyme and the substrate at the support surface. A study of the effect of flow rate on the rate of reaction was therefore undertaken. The results of Figure 12 show that while maintaining all other conditions for immobilization a linear increase in velocity can be observed up to a flow rate of 25 mL.min⁻¹ (Pump scale= 8). A flow rate of 30 mL.min⁻¹ (Pump scale= 8.5) was chosen for all activity and kinetic assays.

Initial rates were calculated from tangents taken to growth curves for each standard.

**Peroxidase Assay**

Experiments conducted by B. Dulay showed that \( \text{H}_2\text{O}_2 \) concentrations greater than 0.3 mM demonstrate an inhibitory effect on peroxidase activity (48). These observations were corroborated by this work and a final concentration of 0.3 mM hydrogen peroxide was considered an optimal excess of substrate for kinetic assay. The remaining conditions for use of this indicator reaction remained unchanged from that described by Artiss et al. (75,76). Using 0.3 mM \( \text{H}_2\text{O}_2 \), 9.0 mM HDCBS and 2.4 mM 4-AAP a linear relationship between the rate of color formation and POD concentration can be realized (Figure 13). Peroxidase activities (U/m) were routinely calculated using the extinction coefficient derived from the standard curve for this chromogenic system.
The effect of flow rate upon initial rates of reaction is illustrated. Initial rates were measured using the gradientless reactor indicated in Figure 8. A 5 cm segment of nylon tube coupled peroxidase (1 mm ID) was employed in this study. Flow rates were calculated by calibration of the pump scale.

The experiment was repeated twice utilizing final concentrations of 0.3 mM hydrogen peroxide, 9.0 mM HDCBS and 2.4 mM 4-AAP.
Assay of peroxidase activity was carried out in duplicate using 0.30 mM hydrogen peroxide with 9.0 mM HDCBS and 2.4 mM 4-AAP. Initial rates were recorded at 510 nm using a Beckman 35 spectrophotometer and 1 cm flow through cell.

Linear regression analysis of this relationship indicated a slope of $0.1007 \pm 0.0046$ nM$^{-1}$·min$^{-1}$, y-intercept of $-0.0107 \pm 0.0005$ and a correlation coefficient of 0.9922 when cuvette concentrations were used of peroxidase were used.

Concentrations of POD were verified by spectrophotometric analysis at 404 nm, $\varepsilon = 9.1 \times 10^4$ M$^{-1}$ cm$^{-1}$. 
FIGURE 13

ΔA 510/min vs. POD (nM)
Glucose Oxidase Assay

In the reconstruction of the peroxidase reaction for glucose oxidase activity measurements 9.0 mM HDCBS, 2.4 mM 4-AAP and 3 U.mL⁻¹ soluble POD were mixed as described by Artiss et al. (75,76).

Catalase, present as a trace level contaminant in commercial glucose oxidase preparations will diminish the concentration of peroxide available for reaction by causing the degradation of peroxide to oxygen and water. In a homogeneous reaction catalase may be expected to maintain a limited effect on the reaction as the coupling of POD with H₂O₂ and the chromogenic system would be rapid compared to the degradation of H₂O₂ by catalase. However, this reaction may become an acute problem in the stepwise reactions employed in the determination of serum glucose with immobilized enzymes. Thompson et al. have shown that the addition of millimolar concentrations of sodium azide demonstrates a mixed type noncompetitive inhibition of catalase (144,145). These authors have found that peroxidase is unaffected by this reagent and the catalase inhibition appears to be only weakly reversible.

An investigation of the effect of azide on the amount of peroxide converted to product indicated that levels of 0.30 mM were sufficient to permit a mean recovery of 90.0 % of substrate (Figure 14) and this concentration of inhibitor was routinely added to all assays. This result is in the range found by
FIGURE 14

THE EFFECT OF SODIUM AZIDE CONCENTRATION ON ABSORBANCE

Legend

The effect of varying concentrations of azide on glucose assay results are shown. Glucose standards were mixed with 9.0 mM HDCBS and 4-AAP using the immobilized peroxidase manifold. In effect this experiment represents a stepwise reaction although the lag time between passage through the immobilized coil and encounter with HDCBS reagent was limited. Concentrations of sodium azide ranging from 0.1 to 0.5 mM were added to the 4-AAP reagent. Final cuvette concentrations of substrate and sodium azide are indicated.
FIGURE 14

[Graph showing the relationship between absorbance and NaN₃ concentration for different glucose concentrations.]
Thompson et al. (144) who suggested that an optimal concentration of 1 mM be employed. In addition, to avoid color loss due to progressive catalase activity in serum glucose determinations, reagent lines for HDCBS were placed immediately after the immobilized enzyme coil.

Under these conditions, variation of GO concentrations from 0.7 to 60 nM indicated a linear correlation between absorbance and concentration (Figure 15). As with POD bound and free GO activities were calculated from the extinction coefficient for the HDCBS:4-AAP coupling system.

**Enzyme Immobilization**

**O-Alkylation Of The Support:** Triethylxonium tetrafluoroborate (TEOTFB) offers several advantages to other alkylating reagents. O-alkylation can be carried out at room temperature in contrast to the harsh conditions necessary for use of dimethyl sulfate. In addition, use of TEOTFB avoids risk of exposure to poisonous vapours and burns which accompany use of the diester (24). Finally, the extent of reaction can be controlled to give a more reproducible product.

The literature indicates that a significant degree of denaturation may be expected upon immobilization of the enzyme to a support (3-6,27,28,42). In attempt to minimize multiple bond formation we have limited the number of spacer arms available for enzyme coupling. This was accomplished at the activation step where restricted concentrations of the
FIGURE 15

STANDARD CURVE FOR GLUCOSE OXIDASE

Legend

The assay was conducted on duplicate aliquots of GO employing the enzyme assay conditions described in the Methods section. Initial rates were calculated from growth curves using 30 mM glucose at anomeric equilibrium. Recordings of growth curves were made using the Beckman 35. Concentrations of glucose oxidase were verified spectrophotometrically, \( \varepsilon = 2.84 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \), \( \lambda_{\text{max}} = 450 \text{ nm} \).

Linear regression analysis gave the following characteristics: slope \( =0.050 \pm 0.0040 \text{ nM}^{-1}\text{min}^{-1} \), y-intercept \( = 0.057 \pm 0.0020 \) and correlation coefficient of 0.9967 when cuvette concentrations of GO were used.
alkylating reagent, TEDITFB, were exposed to the CaCl₂ etched nylon tubing. The support was subjected to varying levels of activation and the effect on peroxidase immobilization monitored. The concentrations of alkylating reagent examined spanned a range up to the lower limit of that employed in the literature (5300 nmoles) (24,27,29,30,134-137).

Because of the hygroscopic nature of TEDITFB extreme caution must be exercised in the preparation of solutions of this reagent. Serial dilutions in different glassware does possess a limitation in that hydrolysis of the chemical may be progressively compounded. Attempts in limiting this factor were made by transferring an aliquot approximating the desired concentration and preparing an appropriate dilution in a dry box. Solvents and vessels were carefully dried and all weighings, transfers and dilutions completed in a dry box.

It was observed that greater concentrations of peroxidase were apparently coupled at elevated levels of alkylating reagent (Table IV). The differences in enzyme coupled between the highest (5300 nmoles) and the lowest (15 nmoles) levels studied, however, represented only a 2.5-fold enhancement and the expressed activity did not increase proportionally. Furthermore, the lower limits tested here gave no worse an intrinsic activity than the lower limits, 5300 nmoles/m, employed in the literature (24,27,29,30,134-137). As a result of these findings, a level of 200 nmoles/m (200 μM) TEDITFB was utilized throughout the study.
TABLE IV

VARIATION OF THE LEVEL OF TRIETHYLOXONIUM TETRAFLUOROBORATE: THE EFFECT ON PEROXIDASE ACTIVITY

<table>
<thead>
<tr>
<th>Activating Reagent</th>
<th>Area per Site</th>
<th>Coupled Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmoles</td>
<td>Å x Å</td>
<td>µM</td>
<td>U/mL</td>
</tr>
<tr>
<td>5300</td>
<td>1 x 1</td>
<td>4.11</td>
<td>1.76</td>
</tr>
<tr>
<td>300</td>
<td>3 x 3</td>
<td>2.95</td>
<td>2.08</td>
</tr>
<tr>
<td>310</td>
<td>4 x 4</td>
<td>1.13</td>
<td>1.50</td>
</tr>
<tr>
<td>53</td>
<td>10 x 10</td>
<td>2.10</td>
<td>2.06</td>
</tr>
<tr>
<td>15</td>
<td>20 x 20</td>
<td>1.67</td>
<td>2.52</td>
</tr>
</tbody>
</table>

aStock solutions of TEOFB were prepared in dry CH<sub>2</sub>Cl<sub>2</sub> and dilutions prepared. All reagents and glassware were dried and utilized as described in the EXPERIMENTAL. Concentrations indicate the level (nmoles) exposed to 1 m tubing.

bFunctional group densities were calculated from surface area available for immobilization. (Appendix A).

cCoupled enzyme levels were derived from the material balance of POD before and after immobilization as indicated by spectrophotometric investigation.

dEnzyme activities were determined in duplicate according to the POD activity procedure described in Methods (Table II). The experiment was repeated twice. Enzyme activity available for immobilization was 90 U/mL (18.7 µM) for all experiments. Total volume to fill a 1 m length of tubing was taken as 1 mL.
These observations support the premise that greater cross sectional areas per spacer arm can be effectively used to sustain more active enzyme derivatives. The disproportionality between enzyme activity (U/m) and the concentrations apparently bound (µm) at higher levels of TEOTFB can be explained by steric interactions or partial occlusion of the active site by multiple bond formation. A direct measure of this type of heterogeneity is not possible, but limited information may be gained from reproducibility studies. To this end, two 1 m lengths of limited amine-substituted nylon tubing were prepared. Peroxidase was coupled to one tube and glucose oxidase to the other. They were then cut into 10 cm segments and assayed for their respective activities. The POD derivatized carrier was found to have an activity of 2.0 ± 0.08 U/m and the GO coupled support an activity of 5.41 ± 0.58 U/m. These results show that the techniques used for preparation of nylon coupled enzymes yield products in which the enzyme is consistently distributed over the entire length of nylon. Activity assays were routinely determined on a 5-10 cm segment cut from the center of the tube.

Variation Of The Time Of Alkylation: In this experiment the period of alkylation was varied from 15 to 120 min while maintaining the degree of activation. The results in Table V show the post immobilization activities obtained from the eluates and the support for peroxidase labelled nylon. It is
<table>
<thead>
<tr>
<th>Alkylation Time&lt;sup&gt;a&lt;/sup&gt; (min)</th>
<th>Peroxidase Immobilized&lt;sup&gt;b&lt;/sup&gt; (μM)</th>
<th>Tube Activity&lt;sup&gt;c&lt;/sup&gt; (U/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>9.59</td>
<td>0.24</td>
</tr>
<tr>
<td>30</td>
<td>10.80</td>
<td>0.63</td>
</tr>
<tr>
<td>45</td>
<td>10.58</td>
<td>0.53</td>
</tr>
<tr>
<td>60</td>
<td>10.85</td>
<td>0.83</td>
</tr>
<tr>
<td>120</td>
<td>10.63</td>
<td>0.55</td>
</tr>
</tbody>
</table>

<sup>a</sup>A 4 m length of tubing was filled with a 200 μM solution of activating reagent. At various times 50 cm lengths were cut off and washed with methylene chloride before derivatization with spacer arms and finally, oxidized peroxidase solution.

<sup>b</sup>Concentration of POD coupled was calculated by difference spectrophotometry using ε = 9.1 x 10<sup>4</sup> M<sup>-1</sup>cm<sup>-1</sup>. Enzyme available for immobilization was 18.69 μM.

<sup>c</sup>Intrinsic peroxidase activities were determined by colorimetric assay of a 10 cm segment of the enzyme labelled tube utilizing 0.30 mM H<sub>2</sub>O<sub>2</sub>, 9.0 mM HDCBS and 2.4 mM 4-AAP. Measurements were completed using the gradientless reactor (Fig. 8). Initial activities available for immobilization was 90 U.mL<sup>-1</sup>. Total volume of 1 m length of tubing (1 mm ID) was taken as 1 mL.
apparent that after 45 min no additional alkylation occurred and the amount of peroxidase coupled stabilized. From this data it was felt that a 60 min incubation would permit maximal enzyme immobilization with limited arm density. Morris et al. (24) have shown that a similar observation could be made after a 10 min incubation. These authors were, however, employing saturating concentrations of TEOFB corresponding to a 1 M solution (24).

**Effect Of Arm Length On Peroxidase Activity:** Researchers have shown that removing the enzyme from the matrix will expose the enzyme to a better microenvironment for expression of its activity (3,20,43). As a result of studies completed in this laboratory, some of the structural features necessary for optimal activity have been identified (46-48). These workers found that a degree of hydrophilicity was a desirable characteristic while others observed greater immobilization capabilities using spacer arms possessing diamine or polyamine groups (20). We have chosen to investigate the effects of spacer arm substitution employing o,ω-poly(oxyethylene)diamine arms available as surfactants from Texaco Inc. The structure of these spacer arms, which range in length from 60 to 630 Å (ED-600 to ED-6000), are those illustrated previously in Figure 2.

In our hands, comparable levels of enzyme were immobilized as arm length increased from ethylenediamine (≈6 Å) to ED-6000
(≈630 Å) \(\varepsilon,\omega\)-poly(oxyethylene)diamine arms. A decrease in \(K_m\) apparent to approach the Michaelis constant of the native soluble enzyme is also indicated from Table VI (Appendix B). Horvath (34) has indicated that deviations from Michaelis-Menten kinetics due to diffusional effects can be more readily discerned by Eadie-Hofstee replots. Hofstee plots of the data yielded straight lines with values of \(K_m\) and \(V_{max}\) close to those obtained from the Lineweaver-Burk plots. Therefore, it was concluded that these experiments represent kinetically controlled reactions and the \(K_m\) data derived from this study represent the true kinetic parameters within experimental error. It would appear that use of arms effectively removes the enzyme from the support surface to avoid steric interactions which limit accessibility to the active site. Still, some degree of chemical modification of POD is apparent by the high \(K_m\) for immobilized peroxidase using ED-600 arms. This phenomenon could be particularly important if the substrate were large and binding of the enzyme altered the conformation of the enzyme. These data are shown in Table VI and Figure 16. The rates illustrated in Figure 16 and subsequent Lineweaver-Burk plots are not normalized with respect to protein concentrations and are included to demonstrate the behavior of the data obtained.

In the remaining studies ED-600 spacer arms, approximately 60 Å in length, were used.

Michaelis constants derived in the literature for the
TABLE VI
THE EFFECT OF ARM LENGTH ON IMMOBILIZED PEROXIDASE

<table>
<thead>
<tr>
<th>Immobilized Enzyme Activity</th>
<th>Immobilized Enzyme Activity</th>
<th>Immobilized Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/m</td>
<td>Kₘ (mM ± SD)</td>
<td>Vₘₐₓ (mM.min⁻¹.mg⁻¹ ± SD)</td>
</tr>
<tr>
<td>Native</td>
<td>0.059 ± 0.005</td>
<td>0.399 ± 0.006</td>
</tr>
<tr>
<td>Oxidized</td>
<td>0.086 ± 0.027</td>
<td>0.271 ± 0.011</td>
</tr>
<tr>
<td>Ethylenediamine</td>
<td>0.533</td>
<td>1.060 ± 0.226</td>
</tr>
<tr>
<td>ED-600</td>
<td>0.355</td>
<td>0.282 ± 0.063</td>
</tr>
<tr>
<td>ED-2000</td>
<td>0.423</td>
<td>0.231 ± 0.010</td>
</tr>
<tr>
<td>ED-6000</td>
<td>0.325</td>
<td>0.125 ± 0.030</td>
</tr>
</tbody>
</table>

Results are the mean of duplicate experiments. Substrate concentrations ranged from 0.016-0.640 mM hydrogen peroxide utilizing a 20 µL aliquot of soluble enzyme or 10 cm of peroxidase coupled nylon.

Nylon tubing was derivatized with arms ranging in length from 6Å to approximately 630 Å. The amine-substituted nylon was then coupled with oxidized peroxidase for 6 h at 4°C. Immobilized activity was calculated using a substrate concentration of 0.3 mM H₂O₂.

Vₘₐₓ data was normalized with respect to mg protein in the stock solution of native and oxidized preparations. For immobilized enzymes, the data was normalized with respect to the concentration (mg protein) that disappeared from the starting solution.
FIGURE 16

LINEWEAVER-BURK PLOTS OF KINETIC DATA OF THE VARIATION OF ARM LENGTH UTILIZING IMMOBILIZED PEROXIDASE

Legend

Five 1-m lengths of tubing were alkylated and treated with 10 % v/v DMF solutions of spacer arms: ethylenediamine, ED-600 (60 Å), ED-2000 (210 Å) or ED-6000 (630 Å). Long arm incorporation was terminated after 6 h with the washing procedure described in Methods.

An oxidized peroxidase solution was prepared and incubated with the amine-substituted tubing. Post immobilization eluates were collected and assayed for enzyme activity. Kinetic activity of the coupled tubing was evaluated utilizing H2O2 concentrations of 0.016 – 0.32 mM. Measurements were made using the gradientless reactor assay described in Methods.

Lineweaver-Burk plots are shown for each of the four spacer arm lengths tested, data are not normalized with respect to protein concentrations. In this representation, Panel A includes plots for ethylenediamine (closed circles) (r = 0.9994) and ED-600 (open circles) (r = 0.9986). Panel B: ED-2000 (open circles) (r = 0.9993) and ED-6000 (closed circles) (r = 0.9974).
native soluble enzyme have been as low as 0.040 mM utilizing
\( \alpha \)-dianisidine (143). Data for the oxidized analogue of
peroxidase show that a substrate concentration of 0.039 mM was
necessary to attain velocities of one-half \( V_{\text{max}} \) (99, 104). Our
investigation demonstrated \( K_m \)'s of 0.059 ± 0.005 mM and 0.086 ±
0.014 mM for native and oxidized peroxidase, respectively.
These data are illustrated in Figure 17.

Kinetic investigations for glucose oxidase (Figure 18) were
obtained using ED-600 diamine arms. In solution, literature
values of 33 mM have been reported using an oxygen electrode for
detection of enzyme (66, 67). Nakamura et al. (61) have reported
a \( K_m \) of 28 mM for the native enzyme and 26 mM for the oxidized
analogue. Our study demonstrated \( K_m \) values for glucose oxidase
preparations which were much lower than these calculations. The
\( K_m \) for the soluble enzyme was calculated to be 13.3 ± 2.5 mM and
for the oxidized preparation, a \( K_m \) of 13.0 ± 2.18 mM was
obtained utilizing glucose standards at anomeric equilibrium.
The dissimilarities in the methods of quantitation may account
for the differences from the literature values.

The nylon coupled enzyme showed a Michaelis constant of
7.25 ± 0.74 mM (Figure 18). Eadie-Hofstee replots suggested
that diffusional effects were not limiting in this
determination. The observed kinetic behavior of an immobilized
enzyme may differ from that of the same enzyme in solution even
in the absence of diffusional effects. Modified behavior may be
attributed to the presence of charged species, substrate,
FIGURE 17

LINEWEAVER-BURK PLOTS OF KINETIC DATA FOR SOLUBLE PEROXIDASE

Legend

Double reciprocal plots of data collected for the native (closed circles) and oxidized (open circles) forms of soluble peroxidase are shown. The data represented are not normalized with respect to protein concentrations. Duplicate assays were completed as described in Methods for peroxidase activity using peroxide concentrations between 0.03 and 0.30 mM.

The \( K^\text{app} \) for native peroxidase was calculated by linear regression to be 0.059 ± 0.005 mM and \( V_{\text{max}} \), 0.399 ± 0.006 mM.min\(^{-1}\).mg\(^{-1} \) (\( r = 0.9983 \)).

Kinetic constants obtained for the oxidized derivative of peroxidase were: \( K_m = 0.086 ± 0.014 \) mM and \( V_{\text{max}} = 0.271 ± 0.011 \) mM. min\(^{-1}\).mg\(^{-1} \) (\( r = 0.9933 \)).
LINEWEAVER-BURK PLOTS OF KINETIC DATA
FOR SOLUBLE AND NYLON COUPLED GLUCOSE OXIDASE

Legend

Double reciprocal plots of data collected for the
native (closed circles) and oxidized (open circles)
derivatives of glucose oxidase are shown. The data
illustrated here are not normalized with respect to
concentration of protein. Initial rates were collected by
colorimetric assay for GO activity using 9.0 mM HDCBS, 2.4
mM 4-AAP and 3 U.mL⁻¹ POD. Substrate concentrations varied
from 2 to 30 mM glucose. Similarly, assay of rates using
limited diaminetetra substituted nylon tubing was carried
out employing a reactor length of 5 cm (C) (closed
rectangles).

$K_{m}^{app}$ for native GO was calculated to be $13.3 \pm 2.55$
mM and $V_{max} = 0.323 \pm 0.028$ mM. min⁻¹.mg⁻¹ ($r = 0.9958$).
The Michaelis constants obtained for the oxidized
derivative of glucose oxidase were: $K_{m} = 13.0 \pm 2.18$ mM
and $V_{max} = 0.673 \pm 0.051$ mM. min⁻¹.mg⁻¹ ($r = 0.9902$).

Immobilized glucose oxidase (closed rectangles) gave
the following kinetic data were derived: $K_{m} = 7.25 \pm 0.74$
mM, $V_{max} = 3.298 \pm 0.532$ mM.min⁻¹.mg⁻¹ ($r = 0.9941$).
products or effectors in the domain of the bound enzyme. An electrostatic environment different from that in free solution could, therefore, be created by interaction with residual charges on the support.

Variation Of The Conditions Of Enzyme Immobilization: As limiting arms were employed in the immobilization of peroxidase and glucose oxidase, no attempt was made to limit the concentration of enzyme available for coupling. Generally, a solution of approximately 2 mg.mL\(^{-1}\) of enzyme was prepared and its concentration verified spectrophotometrically. Similar levels of enzyme were utilized by Zaborsky (51) in the preparation of GO derivatives and by Nakane and Kawooci (108) for POD-antibody conjugates. These researchers completed enzyme immobilization through the carbohydrate component of GO and POD, respectively, and it was with this approach that our investigation began.

The oxidation of the sugar residues of a glycoenzyme may be initiated with sodium periodate treatment. This reagent permits a high yield of aldehydic functions under mild conditions and neutral pH (108,109). A stronger oxidizing agent may result in conformational changes and inactivation of the enzymes by interaction with the amino acid residues of the backbone. In addition, carboxyl groups may be produced instead of the desired aldehydic functionalities. The conditions necessary for optimal periodate oxidation were investigated by B. Dulay in
this laboratory (48) and by Tijssen et al. (109). In an attempt
to evaluate the effect of periodate oxidation on the enzymes in
solution, a number of control experiments were conducted.

A solution containing 0.4 mg.mL\(^{-1}\) pure peroxidase was
subjected to oxidation with 6 mM sodium periodate as described
by B. Dulay (48). The data, presented in Table VII, show minute
losses in specific activity even after two successive treatments
with this reagent. When the oxidized sample was coupled in
solution with ethanolamine to yield a Schiff base 91\% of the
activity was retained even after the second oxidation.

A similar investigation was undertaken to evaluate the
relationship of periodate oxidation upon the specific activity
of glucose oxidase. The results of Table VIII show a
quantitative recovery of enzyme activity after two successive
oxidations.

These conclusions support the findings of other workers,
who suggested that the carbohydrate moiety maintains minimal, if
any, effect on the catalytic activity of the glycoenzymes
studied (51,33-59,61,62). Nakamura et al. (61) demonstrated the
similarities in physical and chemical characteristics of the
native and oxidized derivatives of glucose oxidase. These
researchers did observe a significant difference in the
stability of the two forms to denaturing agents such as SDS and
urea, suggesting a protective role for the carbohydrate moiety
(61,62). Similar findings were reported with the oxidation of
peroxidase in solution (107-109).
TABLE VII

THE EFFECT OF PERIODATE OXIDATION ON
PEROXIDASE ACTIVITY

<table>
<thead>
<tr>
<th>Enzyme Solution&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein&lt;sup&gt;b&lt;/sup&gt; mg/mL</th>
<th>Spectra&lt;sup&gt;c&lt;/sup&gt; µM</th>
<th>Specific Activity U/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure POD</td>
<td>0.41</td>
<td>15.3</td>
<td>170</td>
</tr>
<tr>
<td>A. Oxidation #1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POD-CHO</td>
<td>0.31</td>
<td>10.9</td>
<td>180</td>
</tr>
<tr>
<td>No Reduction/E'amine</td>
<td>0.29</td>
<td>10.4</td>
<td>175</td>
</tr>
<tr>
<td>With Reduction/E'amine</td>
<td>0.34</td>
<td>10.8</td>
<td>114</td>
</tr>
<tr>
<td>B. Oxidation #2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POD-CHO</td>
<td>0.19</td>
<td>6.8</td>
<td>170</td>
</tr>
<tr>
<td>No Reduction/E'amine</td>
<td>0.20</td>
<td>6.3</td>
<td>155</td>
</tr>
<tr>
<td>With Reduction/E'amine</td>
<td>0.22</td>
<td>6.0</td>
<td>106</td>
</tr>
</tbody>
</table>

<sup>a</sup>Duplicate peroxidase solutions were exposed to 6 mM sodium periodate for 2 hours at room temperature in the dark. Excess oxidizing reagent was destroyed by reacting with 0.16M ethylene glycol for 1 hour at room temperature. Purification of the oxidized enzyme was effected by ultrafiltration with a YM-30 membrane. The oxidized enzyme was subsequently incubated with 10 mM ethanolamine (E'amine) in 100 mM phosphate buffer at pH 7.5 to form Schiff bases. Reduction of the conjugate was performed in the presence of reducing agent, 10 mM NaCNBH<sub>3</sub>, in 100 mM phosphate buffer at pH 7.5. Reducing agent and unbound ethanolamine were removed by YM-30 ultrafiltration.

<sup>b</sup>Protein concentrations were determined using the method of Lowry et al. (141).

<sup>c</sup>Concentrations of enzyme were calculated from the extinction coefficient at 404 nm, ε = 9.1 x 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup>.

<sup>d</sup>Soluble peroxidase activities were measured using 0.30 mM H<sub>2</sub>O<sub>2</sub>, 9.0 mM 4H CBS and 2.4 mM 4-AAP in the presence of a 20 µL aliquot of soluble POD.
# TABLE VIII

THE EFFECT OF PERIODATE OXIDATION ON GLUCOSE OXIDASE ACTIVITY

<table>
<thead>
<tr>
<th>Enzyme Solution</th>
<th>Protein mg/mL</th>
<th>Spectra μM</th>
<th>Specific Activity U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure GO</td>
<td>2.23</td>
<td>19.0</td>
<td>194</td>
</tr>
<tr>
<td>A. Oxidation #1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO-CHO</td>
<td>2.25</td>
<td>18.2</td>
<td>204</td>
</tr>
<tr>
<td>No Reduction/</td>
<td>1.84</td>
<td>17.7</td>
<td>176</td>
</tr>
<tr>
<td>E'amine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With Reduction/</td>
<td>1.82</td>
<td>15.4</td>
<td>143</td>
</tr>
<tr>
<td>E'amine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>B. Oxidation #2</td>
<td>1.38</td>
<td>11.4</td>
<td>184</td>
</tr>
<tr>
<td>GO-CHO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Reduction/</td>
<td>1.30</td>
<td>10.3</td>
<td>180</td>
</tr>
<tr>
<td>E'amine</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>With Reduction/</td>
<td>1.14</td>
<td>9.3</td>
<td>155</td>
</tr>
<tr>
<td>E'amine</td>
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</tbody>
</table>

aDuplicate solutions of glucose oxidase were incubated with 6 mM sodium periodate for 2 hours at room temperature in the dark. Excess oxidizing reagent was destroyed by reacting with 0.16 M ethylene glycol for 1 hour at room temperature. Purification of the oxidized enzyme was effected using a XM-50 membrane. Schiff bases were formed in solution by incubating the oxidized enzyme with 10 mM ethanolamine (E'amine) in 100 mM phosphate buffer at pH 7.50. Reduction of the Schiff base was performed in the presence of 10 mM NaCNBH₃ in 100 mM phosphate buffer at pH 7.5. Reducing agent and unbound ethanolamine were removed by XM-50 ultrafiltration.

bProtein concentrations were determined using the method of Lowry et al. (141).

cConcentrations of enzyme were calculated from the extinction coefficient at 450 nm, ε = 2.82 x 10⁴ M⁻¹ cm⁻¹.

dSoluble glucose oxidase activities were ascertained in the manner described for the immobilized enzyme in the Experimental. A 20 µL aliquot of soluble GO was assayed.
When unoxidized samples of each glycoenzyme were incubated with long arm amino-substituted nylon, limited enzyme binding was observed (3.5 %, 0.15 U/m POD; 2.0 %, 0.25 U/m GO). After removing the unbound enzyme, the tubes were washed with 50 mL of 0.1 M phosphate buffer and an experiment was conducted to evaluate the degree of enzyme leaching from the support. Ten successive washes of the support after immobilization demonstrated POD and GO activity in the first seven. These results suggest that weak binding forces such as hydrophobic and/or electrostatic interactions may be involved in the coupling process. When replaced with their oxidized analogues, 35 % of the concentration of peroxidase available for immobilization disappeared and 2.0 U/m of bound POD activity was expressed. Likewise, a coupling yield of 15.5 % and 1.7 U/m was found for tubes labelled with glucose oxidase. Using oxidized enzyme preparations a calculation of enzyme activities released in ten successive washings of the coupled support revealed activity in only the first three eluates for peroxidase, while residual concentrations could be detected after five washes for the GO coupled support (Table IX).

Oxidized enzyme preparations of GO and POD were then incubated with either a segment of nylon support possessing diamine arms or carrier which had been alkylated but not derivatized with spacer arms. It was clear from this control that the presence of amine functionalities on the matrix were imperative for enzyme coupling. In the absence of amine groups,
### TABLE IX

**ENZYME LEACHING AFTER IMMOBILIZATION:**
**WASHING CHARACTERISTICS**

<table>
<thead>
<tr>
<th>Number of Washes</th>
<th>Enzyme Concentration in Eluate (μM)</th>
<th>Enzyme Activity Remaining on Tube (U/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POD</td>
<td>GO</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.125</td>
<td>0.620</td>
</tr>
<tr>
<td>.5</td>
<td>0.003</td>
<td>0.029</td>
</tr>
<tr>
<td>10</td>
<td>0.005</td>
<td>0.009</td>
</tr>
</tbody>
</table>

*Enzyme coupling was effected using oxidized preparation of peroxidase and glucose oxidase. Reduction of the resultant imine linkages was not undertaken in this study. Upon completion of the immobilization period, the tubes were washed with 50 mL of 0.1 M phosphate buffer, pH 7.5 and activities measured as the initial "0" reference point.*

*Two 1 m segments of immobilized peroxidase and glucose oxidase were washed using 10 mL aliquots of 0.1 M phosphate buffer, pH 7.0. One mL samples were then assayed for enzyme activity by spectrophotometric analysis of the enzyme spectra and by quantitative evaluation of enzyme coupled to the support as described in the Methods section.*
the support was unable to bind enzyme and near quantitative recoveries were found in the eluates following the immobilization period.

Exposure to a concentration of sodium chloride capable of acting as an ion-exchanger to the bound enzyme did not have a significant effect on the immobilized enzymes. In this experiment an 8% loss in tube activity was observed when immobilized peroxidase was treated with 1.0 M NaCl. A decrease of 8.4% was experienced for the GD derivatized tube. This leads to the suggestion that electrostatic forces are not the primary mode of coupling. In addition, when these tubes were further exposed to a strong nucleophile, 10 mM hydrazine, for 2 h the enzyme was exchanged. Enzyme activity remaining on the matrix was only 18.3% of its original level for peroxidase and 13.0% for glucose oxidase. Concentrations appearing in the eluate recovered 70% of the bound enzyme as calculated by spectrophotometric material balance (Table X).

Enzyme immobilization proceeds by Schiff base formation between the aldehydic group of the modified enzyme and the amine functionalities of the long spacer arms. Stabilization of the Schiff bases can be achieved by reduction with reagents such as borohydride or cyanoborohydride (53,54). The latter is more stable to acidic media and will proceed at pH values as low as 6.0 (54) and, furthermore, is selective for imines (or imine salts) in the presence of aldehydes. Some researchers have
### TABLE X

**SODIUM CHLORIDE AND HYDRAZINE ELUTION DATA FOR PEROXIDASE AND GLUCOSE OXIDASE**

<table>
<thead>
<tr>
<th></th>
<th>Enzyme Coupled&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Enzyme Released in Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Active Bound Enzyme Remaining on Tube&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Activity Remaining %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Pre Treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POD</td>
<td>8.65</td>
<td>3.00 ± 0.28</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>7.95</td>
<td>2.28 ± 0.45</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>B. Post Treatment with 1.0 m NaCl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POD</td>
<td>-</td>
<td>0.11</td>
<td>2.76 ± 0.05</td>
<td>92</td>
</tr>
<tr>
<td>GO</td>
<td>-</td>
<td>ND</td>
<td>2.09 ± 0.01</td>
<td>92</td>
</tr>
<tr>
<td><strong>C. Post Treatment with 10 mM Hydrazine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POD</td>
<td>-</td>
<td>6.02</td>
<td>0.55 ± 0.02</td>
<td>20</td>
</tr>
<tr>
<td>GO</td>
<td>-</td>
<td>5.69</td>
<td>0.29 ± 0.05</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enzyme coupling was effected using oxidized preparations of POD and GO. Reduction of the resultant imine linkages was not undertaken in this study. Upon completion of the immobilization period, the tubes were washed with 50 mL of 0.1 M phosphate buffer, pH 7.5 and activities measured as the "pre treatment" reference point. Concentrations of enzyme coupled and released by treatment with 1.0 m NaCl or 10 mM hydrazine in 0.1 M phosphate buffer, pH 7.5 were calculated from their spectral characteristics. ND = none detectable.

<sup>b</sup> Active enzyme remaining bound to the support after treatment was measured by routine activity assays using 10 cm of carrier. Uncertainties are due to duplicate assays.
found that reduction to a secondary amine was imperative to the longevity of the immobilized enzyme preparation (48,108,109). Segments of nylon coupled POD and GO were prepared in the presence and absence of NaCNBH₃ at 4°C for 2 h as described by Means and Feeney (53). Reduction of the imine was indicated by the inability to undergo an exchange reaction with hydrazine, however, no apparent advantage to this procedure could be realized (Tables IX-XI). It was the finding of this laboratory, that enzyme leaching was insignificant in the absence of reducing agent (Table IX). Leakage of enzyme from the support was checked by the following modification to the routine activity assay protocol: upon measurement of initial rates the assay solution was recirculated for 5 min whereupon the pump was turned off and change in absorbance monitored over approximately 5 min. The units of enzyme released from the support can therefore be determined. Theoretically, the slope of this experiment should approach zero if the enzyme-support linkage is stable and no free enzyme is present in the flowcell. In addition, enzyme activities eluted in the post immobilization washing procedures were quantitated.

Table XI shows only a 1.5-to 2-fold difference in enzyme leaching expressed as the units of enzyme released per meter in the presence or absence of reducing agent. The amount of material lost using either treatment was found to be negligible. In addition, the bound activity (U/m) of peroxidase or glucose oxidase for the non-reduced carriers was no worse than that
TABLE XI

THE EFFECT OF REDUCTION ON THE IMMOBILIZATION OF
PEROXIDASE AND GLUCOSE OXIDASE

<table>
<thead>
<tr>
<th></th>
<th>Soluble Enzyme^b</th>
<th>Coupled Enzyme^c</th>
<th>Immobilization Factor^d</th>
<th>Leaching^e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>U/M</td>
<td>µM</td>
<td>U/m</td>
</tr>
<tr>
<td>Peroxidase^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Reduction</td>
<td>12.86</td>
<td>5.46</td>
<td>10.08</td>
<td>2.0 ± 0.08</td>
</tr>
<tr>
<td>Reduction</td>
<td>12.86</td>
<td>5.46</td>
<td>10.16</td>
<td>1.3 ± 0.10</td>
</tr>
<tr>
<td>Glucose Oxidase^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Reduction</td>
<td>28.83</td>
<td>11.22</td>
<td>7.20</td>
<td>5.41 ± 0.58</td>
</tr>
<tr>
<td>Reduction</td>
<td>28.83</td>
<td>11.22</td>
<td>11.30</td>
<td>6.04 ± 0.67</td>
</tr>
</tbody>
</table>

^a Enzyme coupling to nylon tube derivatized with amine spacer arms was accomplished in the presence or absence of 10 mM NaCNBH₃ in 0.1 M phosphate buffer, pH 7.5.

^b The concentration of enzyme available for coupling was calculated by spectrophotometric analysis. Units per meter (µmoles per min per meter) tubing was calculated assuming a total volume of 1.0 mL/m. Activity assays were conducted using the standard protocol.

^c Bound enzyme levels were determined by assay of a 5 cm segment of tubing (U/m). Enzyme bound (µM) was derived from the material balance of enzyme present before and after immobilization. Uncertainties are the result of duplicate assays.

^d The immobilization factor was taken as the ratio of specific activities of the coupled enzyme to that calculated for its soluble counterpart.

^e Enzyme leaching from the enzyme derivatized carrier was calculated as the units released per m tubing.
experienced with their reduced counterparts.

In both the reduced and non-reduced immobilizations, comparable enzyme activity was found in the first two washings (10 mL each) after coupling. Subsequent washing of the reduced segments showed no further loss of enzyme while measurable activity losses were found in the following three eluates of the non-reduced tubes. Comparable degrees of enzyme inactivation were observed with both treatments. The immobilization factor, IF, suggests that a significant amount of inactivation has occurred with the coupling of enzyme to a support. Others have experienced this problem (3, 28, 42, 81), while some have indicated no activity losses whatsoever (51). In this laboratory, we have never been able to reproduce the latter result and at best an IF value of 0.62 for peroxidase and 0.88 for glucose oxidase were observed. Still, derivatives which maintained their activity over several months were prepared in the absence of reduction.

The effect of NaCNBH₃ on the glycoenzymes in solution was examined. In Tables VII and VIII, oxidized solutions of POD and GO were coupled with ethanolamine to yield a Schiff base linkage. A partial reduction in the activity (≤10 %) of both enzymes was noted upon coupling to the amine. More significant, however, is the loss observed when this conjugate was reduced with 10 mM NaCNBH₃. An activity loss corresponding to 30 % of the initial activity of oxidized POD and GO was found. As a result of these studies and the observations made using the coupled enzymes, reduction with NaCNBH₃ was not incorporated as
a routine step in the immobilization of either POD or GO.

In an effort to obtain more active derivatives an investigation of pH and time variables was undertaken. Coupling buffers ranging in pH from 3.6 to 8 were studied. For both POD and GO optimal degrees of coupling were observed at pHs between 6 and 7.5 and as a result enzyme immobilization was carried out at pH 7.5 (Tables XII and XIII).

To monitor the effect of immobilization period on coupling yields, 5-50 cm lengths of nylon tubing derivatized with amine functionalities were prepared. One was exposed to a solution of oxidized peroxidase, the other to an oxidized glucose oxidase preparation. At the time intervals indicated in Tables XIV and XV the tubing was washed and the eluates collected for further analysis. For peroxidase 6 hours was chosen as the interval necessary to permit maximum immobilization yields, while an 18 hour incubation period was routinely utilized in GO coupling experiments.

**Thermal Stability Of The Immobilized Conjugates**

Enzyme immobilization has been found to enhance the enzymes stability by restricting intermolecular contact and structural unfolding in the presence of heat or pH extremes.

When immobilized and soluble solutions of POD and GO were assayed for activity at temperatures ranging from 25-60°C, no significant differences between the native and supported enzyme was noted. A more dramatic representation of the enhanced
<table>
<thead>
<tr>
<th>pH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Enzyme in Starting&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>Enzyme&lt;sup&gt;b&lt;/sup&gt; Coupled</th>
<th>Immobilized&lt;sup&gt;c&lt;/sup&gt; Activity</th>
<th>μM</th>
<th>U/m</th>
<th>μM</th>
<th>U/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>13.90</td>
<td>30.69</td>
<td>5.42</td>
<td>2.98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>15.11</td>
<td>23.07</td>
<td>6.04</td>
<td>4.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>16.09</td>
<td>44.91</td>
<td>6.13</td>
<td>4.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>16.46</td>
<td>20.01</td>
<td>6.96</td>
<td>5.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>17.05</td>
<td>30.69</td>
<td>7.68</td>
<td>5.12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Enzyme immobilization was permitted to proceed for 6 h using coupling buffers ranging from 3.6 - 8.0.

<sup>b</sup>The concentration of enzyme in solution and in the enzyme eluate (μM) was calculated spectrophotometrically. Coupled enzyme was determined by difference before and after immobilization.

<sup>c</sup>Activities on nylon were measured by the standard protocol for peroxidase activity using a 0.30 mM H<sub>2</sub>O<sub>2</sub> standard. A 10 μL aliquot of soluble POD was assayed or a 10 cm length of POD bound nylon was assayed.
TABLE XIII

THE EFFECT OF pH VARIATION ON GLUCOSE OXIDASE IMMobilIZATION

<table>
<thead>
<tr>
<th>pH^a</th>
<th>Enzyme in Starting Solution</th>
<th>Enzyme Coupled</th>
<th>Immobilized Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>U/m</td>
<td>µM</td>
</tr>
<tr>
<td>3.6</td>
<td>8.05</td>
<td>16.47</td>
<td>4.77</td>
</tr>
<tr>
<td>4.6</td>
<td>8.78</td>
<td>19.98</td>
<td>5.74</td>
</tr>
<tr>
<td>6.0</td>
<td>8.97</td>
<td>19.19</td>
<td>6.42</td>
</tr>
<tr>
<td>7.0</td>
<td>10.19</td>
<td>21.07</td>
<td>6.44</td>
</tr>
<tr>
<td>8.0</td>
<td>8.21</td>
<td>15.97</td>
<td>4.01</td>
</tr>
</tbody>
</table>

^a Enzyme immobilization was permitted to proceed for 18 h using coupling buffers ranging from 3.6 - 8.0.

^b Enzyme concentrations (µM) in the starting solution and post immobilization eluates were calculated spectrophotometrically. The concentration of enzyme coupled was calculated by difference using data from this method of analysis.

^c GO activity was measured using a 30 mM glucose standard with the standard protocol for GO activity determination. A 10 µl aliquot was utilized for the soluble enzyme while a 10 cm length was assayed for bound activities.
### TABLE XIV

**THE EFFECT OF TIME VARIATION ON PEROXIDASE IMMOBILIZATION**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Enzyme Coupled&lt;sup&gt;a,b&lt;/sup&gt; (μM)</th>
<th>Protein Coupled&lt;sup&gt;b&lt;/sup&gt; (mg/mL)</th>
<th>Immobilized Activity&lt;sup&gt;c&lt;/sup&gt; (U/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.51</td>
<td>0.170</td>
<td>2.03 ± 0.25</td>
</tr>
<tr>
<td>4</td>
<td>3.23</td>
<td>0.269</td>
<td>3.17 ± 0.13</td>
</tr>
<tr>
<td>6</td>
<td>8.86</td>
<td>0.353</td>
<td>3.94 ± 0.20</td>
</tr>
<tr>
<td>8</td>
<td>9.58</td>
<td>0.484</td>
<td>3.09 ± 0.10</td>
</tr>
<tr>
<td>18</td>
<td>7.20</td>
<td>0.421</td>
<td>2.44 ± 0.08</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enzyme coupling to diamine substituted nylon was permitted to proceed for the length of time indicated at 4°C and pH 7.0.

<sup>b</sup> Enzyme coupled (μM) and protein coupled was calculated by difference between concentrations found in the eluate after immobilization and concentrations presented in solution before immobilization.

<sup>c</sup> Enzyme activity available for immobilization was 20.0 U/m (16.5 μM) for all experiments as determined by POD activity assay of the pre immobilization solution. Errors indicate uncertainties in duplicate assays.
### Table XV

**The Effect of Time Variation on Glucose Oxidase Immobilization**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Enzyme Coupled&lt;sup&gt;a,b&lt;/sup&gt; (μM)</th>
<th>Protein Coupled&lt;sup&gt;b&lt;/sup&gt; (mg/mL)</th>
<th>Immobilized Activity&lt;sup&gt;c&lt;/sup&gt; (U/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.25</td>
<td>0.665</td>
<td>1.52 ± 0.20</td>
</tr>
<tr>
<td>4</td>
<td>5.24</td>
<td>0.640</td>
<td>2.21 ± 0.15</td>
</tr>
<tr>
<td>8</td>
<td>8.70</td>
<td>0.724</td>
<td>4.04 ± 0.20</td>
</tr>
<tr>
<td>18</td>
<td>14.45</td>
<td>0.960</td>
<td>4.60 ± 0.21</td>
</tr>
<tr>
<td>24</td>
<td>10.24</td>
<td>0.656</td>
<td>2.33 ± 0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Enzyme coupling to diamine substituted nylon was permitted to proceed for the length of time indicated at 4°C pH 7.0.

<sup>b</sup>Enzyme coupled (μM) and protein coupled was calculated by difference between concentrations found in the eluate after immobilization and concentrations presented in solution before immobilization.

<sup>c</sup>Enzyme activity available for immobilization was 21.1 U/m (10.2 μM) for all experiments as determined by GO activity assays of the starting solution. Errors indicate uncertainties in duplicate assays.
stability of immobilized enzymes was, however, indicated by a thermal inactivation study at 60 °C. In this experiment segments of tubing with bound peroxidase or glucose oxidase were heated to 60 °C and their activities monitored at timed intervals. Under these conditions POD coupled to the nylon support lost 40 % of its initial activity over an 8 h period. The soluble enzyme lost 77 % of its activity within the same time frame (Figure 19).

Soluble POD lost approximately 80 % of its native activity within 3 hours while its immobilized counterpart decreased by only 50 % in the same time (Figure 20).

**pH Activity**

Dramatic perturbations in the enzyme’s apparent response to pH may take place upon immobilization. When the effect of pH variation on activity was monitored for soluble and immobilized POD a narrowing of the pH profile and a shift to a higher pH optimum was noted.

To a large extent, shifts such as that shown in Figure 21, are caused by a partitioning of protons between the enzyme’s microenvironment and the bulk phase. Residual charges at the support surface would result in a lower pH at the surface compared to the pH of the enzyme facing the bulk solution. Therefore, while the external pH may be, for example, 7, the internal pH at the surface will be less and the coupled enzyme will appear to shift its activity profile to the right. In the
FIGURE 19

THERMAL INACTIVATION STUDIES OF IMMOBILIZED AND SOLUBLE PEROXIDASE

Legend

Shown are inactivation curves when peroxidase coupled tubes and soluble peroxidase was heated to 60°C for 0-10 h.

Activity assays were completed with 0.3 mM H2D2, as described in the EXPERIMENTAL section. Initial rates were recorded on a Beckman 3S equipped with a 1 cm flow cell.

O—O = Immobilized Peroxidase
O—O = Soluble Peroxidase
The activity of peroxidase in the presence of buffers of pHs of 4 to 8.5 was monitored. Solutions of the native enzyme were prepared in the respective buffers. A 10 cm segment of immobilized POD was perfused with the buffer to bring it to the pH of interest.

Evaluation of activities was completed using 0.3 mM H$_2$O$_2$, 9.0 mM HDCBS and 2.4 mM 4-AAP. The closed circles represent the immobilized enzyme, the open circle indicate soluble peroxidase.

- - = Immobilized Peroxidase
- - = Soluble Peroxidase
FIGURE 21
systems studied, peroxidase bound to nylon tubing gave rise to a narrow pH optimum between 7.8-8.1.

In Figure 22, investigation of the effects of pH variation on activity measurements of GO were compared to its soluble analogue. A broadening of the pH profile utilizing immobilized GO was observed. In contrast, the soluble enzyme showed a sharp range between 5.3-5.7.

Broadening of both limbs of the graph may be in part due to diffusion limitations. The substrate may react only with enzyme at the outside limits and fail to penetrate the enzyme layer. When a restriction, such as pH variation, is introduced some inactivation of the enzyme can be expected. Therefore the substrate therefore moves deeper into the particle and more enzyme becomes available for reaction. As a result, pH changes which reduce the rate and enhancements in rates due to high enzyme loading work antagonistically to moderate the effect of pH variation.

These types of results have been described by other researchers in the field for glucose oxidase and peroxidase (3-8, 23). Ugarova et al. (104) observed little change in pH dependency when peroxidase was coupled to polyacrylamide gels.

2. Clinical Investigation

At this point it became important to document the clinical validity of this technology. Amino-substituted support was
FIGURE 22

pH PROFILES OF IMMobilIZED AND SOLUBLE GLUCOSE OXIDASE

Legend

Glucose oxidase activity was measured on soluble and nylon coupled GO preparations. Solutions of GO were prepared in buffers ranging from 3.5 to 8.0. The immobilized enzyme tube was washed with each buffer for 5 min prior to assay.

Activity measurements were completed as described in the methods using 30 mM glucose as substrate.

0-0 = Immobilized Glucose Oxidase
●-● = Soluble Glucose Oxidase
coupled with either peroxidase or glucose oxidase and incorporated into an AutoAnalyzer I manifold.

Preliminary studies of reactor length, sampling rate and sample to wash ratio were conducted to define those conditions that would allow optimal sensitivity but minimize sample carry over. Variation of sample transfer from 20 to 70 samples per hour showed that these criteria were satisfied using 60 samples per hour. A sampling rate of fifty per hour was employed using immobilized glucose oxidase. Similarly, comparison of sample to wash ratios of 1:1, 1:2 and 2:1 indicated that a 1:1 ratio gave the best improvement in wash characteristics to minimize sample carry over.

Peroxidase tubing from 0.30-1.0 m in length were incorporated into the POD manifold (Figure 9) and the effects on assay results monitored. It can be seen from Figure 23 that reactor lengths less than 60 cm suffer from sensitivity losses. Although shorter tubes demonstrated improved washing characteristics, substrate recoveries of 95 % were experienced using 80-100 cm of POD reactor and consequently a compromise in wash was taken and lengths of 1 m were routinely employed in serum assay quantitation. It should be noted that analyte measurements not requiring enhanced sensitivity could be easily conducted utilizing shorter reactor lengths.

For the determination of glucose it is suggested that GD activity, as the auxiliary reaction, exceed that of peroxidase, the indicator reaction, by several fold (146-148). Figure 24
FIGURE 23

EFFECT OF REACTOR LENGTH ON ABSORBANCE MEASUREMENTS

Legend

Varying lengths of peroxidase coupled to nylon support were used in the assay of hydrogen peroxide utilizing the immobilized POD manifold. The substrate concentrations were diluted 14 fold before passing through the 15 mm flow through cuvette.

Line "a" represents the effect on absorbance observed using 95 cm of bound POD. Similarly, b = 65 cm and c = 30 cm.
FIGURE 23
FIGURE 24

THE EFFECT OF GLUCOSE OXIDASE VARIATION ON ASSAY RESULTS

Legend

A 0.2 mM glucose standard was mixed with 9.0 mM HDCBS, 2.4 mM 4-AAP and 3 U.mL$^{-1}$ soluble peroxidase. The reaction was initiated with the addition of glucose oxidase solution varying from 15 U.mL$^{-1}$ to 120 U.mL$^{-1}$. The formation of product was monitored on the Beckman 35 at 510 nm.

Concentration of GO are represented by the following symbols:

- ■ = 120 U.mL$^{-1}$
- ▲▲ = 60 U.mL$^{-1}$
- ○○ = 45 U.mL$^{-1}$
- ●● = 15 U.mL$^{-1}$
FIGURE 24
represents the effect of varying the ratio of these enzymes from 5 to 40. In each case the concentration of peroxidase was maintained at 3 U.mL\(^{-1}\) as described by Artiss et al. (75,76). As a result of this study a concentration of 60 U.mL\(^{-1}\) of glucose oxidase was employed in measurements of serum glucose using immobilized peroxidase.

In the peroxidase and glucose oxidase manifolds, the analytical and indicator reactions were separated. This permitted the optimization of each reaction individually and also resulted in improved wash characteristics. It was observed that the quinoneimine dye produced by the enzyme catalyzed coupling of HDCBS and 4-AAP was strongly absorbed by the nylon tubing increasing sample carry over.

With the separation of these reactions the influence of catalase on the assay results became important. Addition of 0.3 mM NaN\(_3\) and the placement of the HDCBS line immediately after the immobilized enzyme coil minimized this effect.

Placement of the reagent lines in this order suggests that in the peroxidase catalyzed reaction peroxide initially interacts with 4-AAP forming an intermediate which can then couple to HDCBS in a non-enzymic step.

Applying these conditions, an evaluation of the nylon coupled peroxidase and glucose oxidase performance as an analytical tool was completed. In an attempt to avoid repetition data from POD and 60 immobilized tubes are presented together.
Blanking

In the POD and G0 manifolds blanking of the assay was achieved by pumping reagents through the system for at least 15 min prior to assay and zeroing the instrument against the reagent stream.

Interferences

Bilirubin is known to interfere with peroxidase coupled reactions by competing as a substrate in the POD-H2O2 reaction. It also contributes color to the final reaction mixture and thereby increases the concentration of the negatively affected chromogen (149-151). Concentrations of bilirubin from 25 - 200 µM had a negative effect on the values determined for serum glucose when measured using either immobilized POD or G0. This observation was, however, not significant at p > 0.05.

Hemoglobin has been shown to exhibit pseudoperoxidase activity and also adds its visible spectrum to the sample (150,151). To examine the effect of this compound on the proposed assay a glucose serum was treated with quantities of hemoglobin up to 10 g.L⁻¹ and assay results monitored. Under these circumstances a decrease of 4.6% apparent glucose was observed using nylon coupled POD and 5 g.L⁻¹ hemoglobin. In contrast, a decrease of only 2.68 % apparent glucose was found employing immobilized GO at the same level of interferent. Both results were found to be insignificant at the p >0.05 level of
certainty.

Lipemia is another possible interference to assays completed in a biological matrix. Concentrations of triglycerides up to 60 mM did not affect the results.

**Linearity**

The calibration curve obtained by measuring glucose standards utilizing immobilized peroxidase was linear up to 45 mM glucose with a slope of $5.14 \times 10^{-4}$, y-intercept of 0.026 and a correlation coefficient of 0.9961 (Figure 25). Linearity of measurements in a biological matrix was also shown by the assay of serial dilutions of both a high and low serum sample. Regression analysis of the relationship between fraction of sample and absorbance gave a good correlation where, $r = 0.9957$ and $r = 0.9995$ for the low and elevated samples, respectively.

A good correlation in the standard curve constructed for immobilized glucose oxidase can be seen from Figure 25. Linear regression gave the following data: slope = $2.62 \times 10^{-2}$, y-intercept = 0.0381 and $r = 0.9995$. Serial dilutions of a serum sample showed that the 60 manifold performed equally well in this media and correlation coefficients of 0.9987 and 0.9943 were found for the high and low samples, respectively.

Discrepancies in the slopes of the standard curves were expected due to differences in the manifold constructions illustrated in Figures 9 and 10.
STANDARD CURVES FOR GLUCOSE DETERMINATION
USING IMMOBILIZED PEROXIDASE AND
IMMOBILIZED GLUCOSE OXIDASE

Legend

Standard curves were constructed to relate concentration of glucose and absorbance. Each standard was assayed in duplicate.

The calibration curve derived using coupled POD and the manifold illustrated in Figure 9. Linear regression analysis indicated: \( m = 5.14 \times 10^{-4} \text{ mM}^{-1} \pm 5.09 \times 10^{-5}, \) y-intercept = 0.026 ± 0.003 and \( r = 0.9961. \)

The standard curve developed using GO immobilized manifold (Figure 10) has the following characteristics: \( m = 2.62 \times 10^{-2} \text{ mM}^{-1} \pm 3.9 \times 10^{-5}, \) y-intercept = 0.0381 ± 0.002 and \( r = 0.9995. \)

O-O = Glucose Oxidase Manifold
O-S = Peroxidase Manifold
Precision

The within-run reproducibility of the described method was determined by assaying fifteen replicates each of three serum pools within the same batch. Between-run precision was evaluated by analyzing duplicate samples over five analytical batches. For each pool assayed with bound POD both the within-run and between-run CVs were less than 5% (Table XVI).

The results illustrated in Table XVII using immobilized GO, indicate that the coefficient of variation within a single run is only about 1.5%, whereas the between-run CV is about two times greater.

Recovery

Analytical recoveries were evaluated with the addition of known quantities of glucose to a serum sample. This data, summarized in Tables XVIII and XIX, show a mean recovery of 99.9 ± 2.4% for POD and 94.5 ± 1.8% when using the GO coupled manifold.

Stability Studies

Operational longevity and storage stabilities were evaluated on the enzyme coupled segments as a continual process. To ascertain the operational capabilities of the derivatized tubes, activity assays and response to an assayed control were monitored during routine use over a period of six
### TABLE XVI

**PRECISION STUDY FOR THE PROPOSED METHOD: IMMobilIZED PEROXIDASE**

<table>
<thead>
<tr>
<th></th>
<th>Within-run&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th>Between-run&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{X}$</td>
<td>SD</td>
<td>CV</td>
</tr>
<tr>
<td>5.10</td>
<td>0.04</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>6.19</td>
<td>0.05</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>16.00</td>
<td>0.71</td>
<td>4.40</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Each serum was assayed ten times using peroxidase immobilized on nylon tubing (1.77 U/mL). Soluble glucose oxidase was added to the system and assays completed as described in the Methods for POD manifold.

<sup>b</sup>Each sample was assayed in duplicate over five successive runs.
### TABLE XVII

**PRECISION STUDY FOR THE PROPOSED METHOD: IMMobilIZED GLUCOSE OXIDASE**

<table>
<thead>
<tr>
<th></th>
<th>Within-run&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th>Between-run&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} )</td>
<td>SD</td>
<td>CV</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>6.29</td>
<td>0.16</td>
<td>0.16</td>
<td>2.5</td>
</tr>
<tr>
<td>7.24</td>
<td>0.10</td>
<td>0.10</td>
<td>1.4</td>
</tr>
<tr>
<td>16.90</td>
<td>0.06</td>
<td>0.06</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each serum was measured ten times using immobilized glucose oxidase (2.61 U/m). The glucose oxidase manifold (Fig. 10) was employed using 3 U.mL<sup>-1</sup> soluble peroxidase as described.

<sup>b</sup>Serum samples were assayed in duplicate over five successive runs using immobilized glucose oxidase.
### TABLE XVIII

**RECOVERY OF GLUCOSE ADDED TO SERUM USING IMMOBILIZED PEROXIDASE**

<table>
<thead>
<tr>
<th>Glucose Added (mM)</th>
<th>Estimated Glucose (mM)</th>
<th>Recovery (mM)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>5.56</td>
<td>5.56</td>
<td></td>
</tr>
<tr>
<td>2.40</td>
<td>7.95</td>
<td>7.75</td>
<td>97.6</td>
</tr>
<tr>
<td>5.13</td>
<td>10.69</td>
<td>10.99</td>
<td>102.8</td>
</tr>
<tr>
<td>6.84</td>
<td>12.40</td>
<td>12.52</td>
<td>100.9</td>
</tr>
<tr>
<td>8.55</td>
<td>14.11</td>
<td>14.11</td>
<td>100.0</td>
</tr>
<tr>
<td>11.97</td>
<td>17.53</td>
<td>17.82</td>
<td>101.6</td>
</tr>
<tr>
<td>15.39</td>
<td>20.95</td>
<td>20.15</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Assays were completed in duplicate. The experiment was completed twice utilizing the peroxidase manifold illustrated in Figure 9 and 1.77 U/m POD labelled nylon.
### TABLE XIX

**RECOVERY OF GLUCOSE ADDED TO SERUM USING IMMobilIZED GLUCOSE OXIDASE**

<table>
<thead>
<tr>
<th>Glucose Added (mM)</th>
<th>Estimated Glucose (mM)</th>
<th>Recovery</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>7.95</td>
<td>10.14</td>
<td>96.5</td>
</tr>
<tr>
<td>2.56</td>
<td>10.51</td>
<td>13.10</td>
<td>100.1</td>
</tr>
<tr>
<td>5.13</td>
<td>13.08</td>
<td>14.76</td>
<td>99.8</td>
</tr>
<tr>
<td>6.84</td>
<td>14.79</td>
<td>16.09</td>
<td>97.5</td>
</tr>
<tr>
<td>8.55</td>
<td>16.50</td>
<td>20.10</td>
<td>100.9</td>
</tr>
<tr>
<td>11.97</td>
<td>19.92</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Assays were completed in duplicate. The experiment was completed twice utilizing the GO manifold shown in Fig. 10, and 2.61 U/m GO tube.
months.

As this investigation got under way it was observed that storage of the enzyme coupled carriers at room temperature caused an accelerated depreciation of the bound enzyme activity. Therefore, all enzyme coupled carriers were stored at 4°C, filled with 0.1 M phosphate buffer when not in use.

Peroxidase coupled to nylon tubing was found to be extremely stable and a 50 % loss in initial activity was recorded only after 6 months of use, during which approximately 1300 evaluations were completed.

Similar behavior has been observed with glucose oxidase tubes where, 600 assays conducted over a 6 week period demonstrated only a 20 % reduction to the initial activity of the support.

Correlation Studies

To document the clinical validity, results from the proposed technique were compared with those from a method in use (152). When plotted, the data collected with the POD manifold yielded a significant correlation coefficient ($r = 0.9928$) with the hexokinase:glucose-6-phosphate dehydrogenase (HK:G6PDH) reference method (Figure 26). The value of the t-test indicated that no significant difference between the means exists at the $p > 0.05$ level of certainty.

When 125 serum samples were assayed for glucose levels using immobilized GO and compared to calculated values using the
FIGURE 26

COMPARISON OF THE IMMOBILIZED PEROXIDASE METHOD WITH A REFERENCE TECHNIQUE

Legend

A correlation plot between the proposed method using immobilized peroxidase (1.77 U/m) and an accepted method utilizing hexokinase and glucose-6-phosphate dehydrogenase (152) is shown.

The equation of the regression line is $y = 0.9302x + 1.044$, the sample size taken was 100. A correlation coefficient of $r = 0.9923$ was calculated.
HK:G6PDH assay (152) a correlation coefficient of 0.9963 was observed (Figure 27). In addition, the value of the t-test indicates no significant difference between the means (p > 0.05).

Serum glucose levels were measured on 100 "normal" individuals as described in CHAPTER IIB, p.67. A gaussian distribution of the data was apparent from histograms of the data and from the K-S test for normality. A reference range, established by percentile estimate of the 95% confidence interval, was 5.1-7.9 mM utilizing immobilized peroxidase. Employing nylon coupled GO the normal range was established as 3.7-8.0 mM. These reference ranges reflect those obtained by other researchers using soluble PDD and GO (146-148).

B. COIMMOBILIZATION OF PEROXIDASE AND GLUCOSE OXIDASE

1. Analytical Investigation

The immobilization and application of multistep enzyme systems has generated great interest in the past few years (3,17). Immobilization techniques enable sequentially acting enzymes not naturally aggregated to be coimmobilized artificially forming tightly coupled enzyme systems.

There are basically two ways of creating an enzyme sequence: (a) by immobilizing different enzymes on separate particles which are either mixed in a column or arranged as sequential reactors in a flow apparatus and (b) different enzymes are coupled to the same segment of the support
FIGURE 27

COMPARISON OF THE IMMobilIZED GLUCOSE OXIDASE
WITH A REFERENCE TECHNIQUE

Legend

Linear regression analysis of the reference method (x-axis) against the proposed technique using immobilized glucose oxidase (2.61 U/m) indicated a correlation of 0.9963.

The line of best fit is,

\[ y = 1.06x + 0.861 \text{ and } n = 125 \]
FIGURE 27

![Graph showing a linear relationship between Reference Method (mM) and Immobilized GO (mM).]
particle. Examples of both approaches have appeared in the literature for coimmobilizations involving up to four different enzymes on the same segment of carrier. Reactors have been developed for the determination of creatine (17), creatinine (18), glucose (24,25) and 7α-hydroxy steroids (28).

Murachi et al. (25) studied the practicality of both mixed bed and sequential reactors in the immobilization of glucose oxidase and peroxidase to glutaraldehyde derivatized alkylamine glass beads. These researchers found that these enzymes, coimmobilized to a single bead were of superior sensitivity and could be employed quite successfully in the quantitation of serum glucose using a GO:POD ratio of 1.4:1 by concentration or 1:1 by activity.

In our investigation of the coimmobilization of GO and POD we began by mixing similar ratios of enzymes and observing the effect on the extent of immobilization. Solutions of oxidized peroxidase and oxidized glucose oxidase were prepared by treatment with a final concentration of 6 mM sodium periodate. The oxidation of POD and GO for coimmobilization was carried out in the manner described for their preparation in individual coupling experiments.

The oxidized solutions were, in all coimmobilization studies, coupled to nylon tubing derivatized with amine functionalities. The support was initially activated with 200 μM triethylxonium tetrafluoroborate (TEOTFB) and subsequently derivatized with diamine spacer arms of the ED-600 type shown in
Figure 2 to prepare a support which maintained a large surface area per spacer arm.

Quantitation of bound enzyme was effected by assay of the separate activities on the support. With coimmobilized glucose oxidase and peroxidase, the "total" activity expressed by the coimmobilized tube was assayed first with excess glucose as substrate plus HDCBS and 4-AAP. However, no soluble enzyme was added to the reaction vessel. This approach is illustrated in Figure 6C.

Peroxidase activity was measured next by adding an excess of substrate, hydrogen peroxide, to a solution containing the HDCBS and 4-AAP chromogen system. This technique is therefore exactly like that described for POD activity coupled individually to nylon tubing (Figure 6A).

Finally, glucose oxidase activity bound to the support was evaluated using the same substrate and chromogen concentrations employed in the "coupled" activity measurements, except that excess soluble peroxidase was present (Figure 6B.)

These measurements were accomplished employing the reactor apparatus illustrated in Figure 8 while maintaining a flow rate of 30 mL.min⁻¹ (pump scale = 8.5) as established previously. A reactor length of 5 - 10 cm was routinely used for all activity assays.

Multi-component analysis based upon the spectral differences of POD and GO provided the pre- and post-immobilization concentrations (µM) of the two enzymes. The
concentration of enzyme coupled was calculated as the difference between the levels available for immobilization and what appeared in the eluate after the coupling period. The concentration of enzyme coupled was corrected for non-covalently bound enzyme by adding the levels of enzyme eluted during washing procedures.

Using these methods of quantitation three approaches to the coimmobilization of peroxidase and glucose oxidase were evaluated. Briefly, these include:

1. Mix aliquots of periodate treated enzymes and couple directly.
2. Prepare a GO-POD conjugate, reoxidize and couple.
3. Prepare a GO-Dihydrazide-POD conjugate, reoxidize and couple.

Mix Aliquots of Periodate Treated Enzymes and Couple Directly

In this approach peroxidase and glucose oxidase were oxidized in separate vessels to yield aldehydic functionalities capable of coupling to distal amine groups on the support.

Solutions varying in ratio of GO to POD from 1:2 to 1:6 by concentration (or 1:1 to 2:1 by activity) were mixed and incubated with amino-substituted nylon for 18 h at 4°C. A summary of the pre- and post-immobilization activities is indicated in Table XX.
TABLE XX
COIMMOBILIZATION OF PEROXIDASE AND GLUCOSE OXIDASE

<table>
<thead>
<tr>
<th>Component Assayed</th>
<th>Enzyme in Starting Solution µM</th>
<th>Coupled Enzyme µM</th>
<th>Enzyme Activity Support U/m ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. GO</td>
<td>10.53</td>
<td>2.10</td>
<td>18.41</td>
</tr>
<tr>
<td>POD</td>
<td>19.83</td>
<td>4.12</td>
<td>22.22</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. GO</td>
<td>24.42</td>
<td>5.12</td>
<td>44.88</td>
</tr>
<tr>
<td>POD</td>
<td>22.37</td>
<td>3.93</td>
<td>21.14</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. GO</td>
<td>4.93</td>
<td>1.16</td>
<td>11.77</td>
</tr>
<tr>
<td>POD</td>
<td>29.40</td>
<td>5.42</td>
<td>28.85</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D. GO</td>
<td>20.87</td>
<td>6.57</td>
<td>57.59</td>
</tr>
<tr>
<td>POD</td>
<td>31.13</td>
<td>4.50</td>
<td>24.21</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Solutions of oxidized POD and GO were mixed and exposed to limited amino-substituted nylon. Immobilization was terminated after 18 h at 4°C.

\(^b\) Concentrations (µM) of POD and GO in the starting solution and post immobilization were calculated by spectral characteristics and multicomponent analysis. Assay of enzyme activity available in the starting solution was evaluated utilizing by enzyme assay with HDCBS and 4-AAP.

\(^c\) Enzyme bound to the support was determined by the difference between that present in the starting solution and the post immobilization eluate. U/m indicates the maximum activity that could be expected on the tube if all coupled enzyme activity was expressed as calculated by the concentration of enzyme bound (µM).

\(^d\) Concentrations of active enzyme bound were assayed by enzymic coupling of HDCBS and 4-AAP. POD activity was assayed using 0.3 mM H₂O₂ substrate; GO was evaluated in presence of excess soluble POD and 30 mM glucose substrate. Coupled activity represents the "total" activity on the tube in the absence of any added soluble enzyme and 30 mM glucose.
As increasing concentrations of peroxidase are employed greater levels were immobilized but a disproportionality between available enzyme and the concentration of enzymes coupled becomes obvious. This finding suggests a possible interference in the expression of activity due to steric hinderances. This observation was also pertinent to the glucose oxidase activities expressed compared to the concentrations (µM) of enzyme coupled. The significance of this interference is indicated by the 10-fold decrease in specific activities of enzymes in solution and their bound counterparts. This represents a more serious compromise in the activity of both glucose oxidase and peroxidase during their coimmobilization than was experienced in previous individual coupling experiments.

From Table XX, it would appear that GO:POD enzyme ratios of 1:2 by concentration yield the "best" nylon supported derivatives. In all cases studied, the conversion of substrate by the support was found to be dependent on the activity of the glucose oxidase coupled auxiliary reaction. Ideally, the linked enzyme activity of the coimmobilized carrier, labelled "coupled" activity in Table XX, should equal the activity expressed per m by glucose oxidase if the reaction were independent of POD activity. The efficiency of the tube, calculated as the ratio of coupled and GO activities permits an appraisal of the usefulness of the system as an analytical instrument. The efficiency of the system using tubes A and B are the greatest at 65% and 100%, while C and D are approximately 60% and 26%,
respectively.

The linked enzyme system prepared by direct coupling of a mixed solution of the two enzymes was therefore found to operate most satisfactorily when the concentration of peroxidase was equal to that of glucose oxidase (Tube B). This relationship may be alternatively expressed as 3:1 in terms of the initial activity of GO:POD provided in the pre-immobilization mixture. The relative activities of the two coupled enzymes found to be optimal was, however, much less than that used in soluble assay systems for glucose in the literature. In soluble systems GO:POD ratios of 25:1 (146), 20:1 (present study), 12:1 (147) and 5:1 (148) have been utilized.

In this discussion we have evaluated the usefulness of the coimmobilized supports by their efficiency ratios. This criterion for optimization would seem to suggest that the systems developed in which the ratio of coupled to GO activities was much less than 100 %, such as in tubes C and D, are unacceptable for use in kinetic determinations. However, even they can be readily utilized in an analytical system for endpoint determinations.

**Prepare a GO-POD Conjugate and Couple**

In another series of experiments, an oxidized preparation of glucose oxidase was incubated with native soluble peroxidase to form a Schiff base between the amine residues of POD and the aldehyde groups of oxidized GO. Ultrafiltration using a
membrane with an exclusion limit of 100,000 daltons permitted the removal of noncovalently bound peroxidase. The GO-POD derivative was subsequently reoxidized to produce additional aldehydic functionalities on peroxidase capable of binding to amine-substituted nylon. It was hoped that by adopting this approach a derivative may be produced that would permit greater concentrations of glucose oxidase to be bound by its association with peroxidase. Initial and final concentrations and bound activities were calculated by multicomponent analysis and enzyme activity assays as described previously to yield the results shown in Table XXI.

Again, a dramatic decrease (10 fold) in the specific activities of glucose oxidase and peroxidase added to the tube and the activities (U/m) expressed by the coupled enzyme was found. This suggests that a significant degree of inactivation has occurred upon immobilization.

This technique for enzyme coimmobilization did apparently result in the production of more active GO derivatives when compared to the concentration of enzyme coupled and the expressed activity (U/m) on the tube in Table XX. Unfortunately, the enzyme conjugate was not subjected to a purification process that would permit the removal of self-coupled GO aggregates or unbound GO. Therefore, the concentrations (µM) of GO bound may represent a generous estimate of that quantity that should realistically be coupled. The results in Table XXI indicate that, ratios of
TABLE XXI
COIMMOMLIZED PEROXIDASE AND GLUCOSE OXIDASE OXIDIZED GO COUPLED TO POD

<table>
<thead>
<tr>
<th>Component Assayed</th>
<th>a Enzyme in b Starting Solution</th>
<th>Coupled b Enzyme</th>
<th>Enzyme Activity c on Nylon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>U/m</td>
</tr>
<tr>
<td>A. GO</td>
<td>1.91</td>
<td>0.84</td>
<td>21.55</td>
</tr>
<tr>
<td>POD</td>
<td>24.69</td>
<td>4.93</td>
<td>49.30</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. GO</td>
<td>3.21</td>
<td>1.64</td>
<td>42.07</td>
</tr>
<tr>
<td>POD</td>
<td>8.75</td>
<td>3.48</td>
<td>34.80</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. GO</td>
<td>5.63</td>
<td>2.79</td>
<td>74.38</td>
</tr>
<tr>
<td>POD</td>
<td>20.17</td>
<td>4.18</td>
<td>41.80</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

aOxidized GO was incubated with peroxidase to form a GO-POD conjugate. The GO-POD derivative was reoxidized, filtered (XM100) and exposed to limited amino-substituted support.

bInitial concentrations of enzyme (µM) were calculated by multicomponent analysis immediately before immobilization. Post-immobilization eluates were measured after an 18 h incubation of the conjugate with nylon support. Enzyme coupled represents the difference of the pre- and post-immobilization solutions (µM). Calculation of the units/m of enzyme represents activities which could be expected and was calculated using the specific activity of pure enzyme.

cEnzyme activity was determined by the oxidative coupling of HDCBS and 4-AAP. Bound and free GO, activity was assayed using excess soluble POD and 30 mM glucose. POD activity was evaluated using 0.3 mM H₂O₂ substrate. Coupled measurements indicate the "total" activity bound to support and was assayed using 30 mM glucose substrate. No soluble POD was added to this reaction mixture.
GO:POD close to 1:3 by concentration (or 1:1 by initial activity), as in tubes B and C, produce the most efficient enzyme coupled supports. The efficiency of the support, calculated as the relationship of the coupled activity to the GO activity, were comparable to results obtained using the random approach to coimmobilization. At best, in tubes B and C efficiencies of 88% were observed, while that of A, where the concentration of POD exceeded that of GO by 20 fold, was only 56%.

Prepare a GO–Dihydrazide–POD Conjugate, Reoxidize and Couple

We then attempted to modify the soluble conjugate still further by the initial reaction of oxidized GO with adipic dihydrazide to yield a polyhydrazido derivative of this enzyme. This glucose oxidase–dihydrazide conjugate (GO–DH) was purified by ultrafiltration before coupling with mildly oxidized peroxidase.

Tijssen and Kurstak (109) observed that a recovery of 97% of the original peroxidase activity could be expected when the enzyme was treated with 1.0 mM sodium periodate. At this level of oxidizing reagent active conjugates of peroxidase could be formed in the presence of IgG molecules. Henceforth, "mild" oxidation of the glycoenzymes studied was accomplished utilizing a final concentration of 1.0 mM oxidizing reagent for 2 h.

After incubation of the GO–DH derivative and mildly oxidized POD the GO–Dihydrazide–POD conjugate formed was
cleaned up by ultrafiltration using 100,000 and 300,000 molecular weight exclusion membranes. This procedure permitted the removal of noncovalently bound peroxidase and self-coupled glucose oxidase aggregates. The purified conjugate was then incubated with arm amino-substituted nylon for 18 h at 4°C.

The results of this study are shown in Table XXII. Employing a ratio of 1:1.6 GO:POD by weight, or 1:1.5 by activity, an efficiency of 70 % for tube B could be realized using this technique for coimmobilization. With the exception of tube D, where the ratio of coupled to GO activity was 40 %, the supports were of equal efficiency (≈60 %). It is interesting to note that in D, the ratio of enzymes taken for immobilization was 2.3:1 by concentration or 6:1 by specific activity.

The reverse approach was also attempted. In this case oxidized peroxidase was coupled to dihydrazide first, then purified and incubated with a "mildly" oxidized preparation of glucose oxidase. This conjugate, POD-DH-GO, was reoxidized, filtered and coupled to nylon tubing modified with limited quantities of ED-600 diamine spacer arms. The results of this study for simultaneous immobilization (A) are indicated in Table XXIII.

The effect of reduction upon the coupled activities are also shown in this table. Similar quantities (µM) of each enzyme were bound in this procedure and the loss of activity (U/m) for the enzyme aggregate in solution compared to that
TABLE XXII

COIMMOBILIZATION OF PEROXIDASE AND GLUCOSE OXIDASE: DIHYDRAZIDE CONJUGATED GO COUPLED TO POD

<table>
<thead>
<tr>
<th>Component Assayed</th>
<th>Enzyme in Starting Solution</th>
<th>Coupled Enzyme</th>
<th>Enzyme Activity on Support</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>U/m</td>
</tr>
<tr>
<td>A. GO</td>
<td>1.95</td>
<td>1.45</td>
<td>37.50</td>
</tr>
<tr>
<td>POD</td>
<td>2.27</td>
<td>1.37</td>
<td>13.70</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. GO</td>
<td>2.63</td>
<td>1.21</td>
<td>31.03</td>
</tr>
<tr>
<td>POD</td>
<td>4.22</td>
<td>1.12</td>
<td>11.12</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. GO</td>
<td>2.35</td>
<td>1.44</td>
<td>36.94</td>
</tr>
<tr>
<td>POD</td>
<td>6.00</td>
<td>2.50</td>
<td>25.00</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D. GO</td>
<td>3.60</td>
<td>1.38</td>
<td>46.17</td>
</tr>
<tr>
<td>POD</td>
<td>1.60</td>
<td>0.90</td>
<td>9.00</td>
</tr>
<tr>
<td>Coupled</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Oxidized GO was derivatized with 10 mM dihydrazide and reduced with 10 mM NaCNBH₃. The conjugate was purified by ultrafiltration (XM-300) and (XM-100) and then incubated with mildly oxidized POD. The GO-DH-POD derivative was ultrafiltered (XM-50) reoxidized and coupled to amino-substituted nylon.

b Enzyme concentrations (µM) in the starting solution after ultrafiltration and post immobilization eluates were calculated by multicomponent analysis. The difference between the initial and final concentrations was taken as the concentration of enzyme coupled (µM). Calculation of the units/m coupled represents the activity of enzyme that can be expected if all bound activity was expressed and was calculated from the specific activities of the native enzymes.

c Enzyme activities (U/m) were calculated with the oxidative coupling of HDCBS and 4-AAP. POD assays were completed in the presence of 0.3 mM H₂O₂; GO activity was determined in the presence of excess soluble POD and 30 mM glucose. The coupled measurement represents the "total" activity on the tube using glucose substrate, no soluble enzymes were added in this assay.
**TABLE XXIII**

COIMMOBILIZATION OF PEROXIDASE AND GLUCOSE OXIDASE DIHYDRAZIDE CONJUGATED POD COUPLED TO GO

<table>
<thead>
<tr>
<th>Component Assayed</th>
<th>Not Reduced</th>
<th></th>
<th></th>
<th>Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Enzyme Concentration</td>
<td>Coupled Enzyme</td>
<td>Enzyme Activity on Nylon</td>
<td>Initial Enzyme Concentration</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>U/m</td>
<td>U/m</td>
</tr>
<tr>
<td>POD-DH-GO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>11.5</td>
<td>2.60</td>
<td>8.48</td>
<td>2.45</td>
</tr>
<tr>
<td>POD</td>
<td>14.2</td>
<td>4.40</td>
<td>44.00</td>
<td>3.65</td>
</tr>
<tr>
<td>Coupled</td>
<td></td>
<td></td>
<td></td>
<td>1.33</td>
</tr>
<tr>
<td>POD-DH-GO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>11.80</td>
<td>6.90</td>
<td>22.50</td>
<td>7.58</td>
</tr>
<tr>
<td>POD</td>
<td>23.50</td>
<td>4.50</td>
<td>45.00</td>
<td>4.08</td>
</tr>
<tr>
<td>Coupled</td>
<td></td>
<td></td>
<td></td>
<td>2.05</td>
</tr>
</tbody>
</table>

---

A. Simultaneous Immobilization\(^a,c\)

B. Sequential Immobilization\(^b,c\)

\(^a\) An oxidized solution of peroxidase was incubated with 10 mM dihydrazide, pH 7.5 phosphate buffer, 0.1 M. Uncoupled POD was removed by ultrafiltration with 50,000 and 30,000 membranes. The POD-DH conjugate was then coupled with oxidized GO and purified by ultrafiltration with XH300 membranes. Coupling of the POD-DH-GO derivative with the support was accomplished in the presence or absence of 10 mM NaCNBH\(_3\) in 0.1 M phosphate buffer.

\(^b\) A POD-DH conjugate was prepared as indicated above and the coupled to amino-substituted nylon. Unbound POD was collected in the post immobilization eluate. The POD-DH derivatized tube was then perfused with oxidized GO and incubated for 18 h at 4°C.

\(^c\) Initial and post immobilization eluates were analyzed by multicomponent analysis. Coupled enzyme was calculated by difference. Enzyme activities were evaluated as follows: POD utilizing 0.3 mM H\(_2\)O\(_2\) substrate; GO, with 30 mM glucose and soluble POD; coupled activity with 30 mM glucose; in the absence of added soluble POD.
expressed on the support was comparable in the presence and absence of NaCNBH₃. Leaching of bound material from the support was determined as the quantity of coupled enzyme units lost from the support after stopping the pump. Coupled enzyme leaching was minimal and corresponded to 0.010 ± 0.003 U/m disappearing from the immobilized support.

In this study, the "total" coupled activity does not reflect the activity of glucose oxidase and an efficiency of 36% was all that was realized. The ratio of concentrations utilized in the pre-immobilization solution (after ultrafiltration) was 1:1.2 GO:POD, however, this corresponds to an activity ratio of 2:1 and was therefore inappropriate.

An attempt at sequential coimmobilization of POD and GO was made. Mildly oxidized POD was initially reacted with adipic dihydrazide and reoxidized to generate additional aldehydeic sites. Self coupled POD were removed by ultrafiltration through 50,000 and 30,000 molecular weight ultrafiltration membranes before incubation with the diamine activated support. Unbound peroxidase was subsequently removed in the post-immobilization eluate and a solution of oxidized GO pumped into the now POD-DH modified support. Immobilization then proceeded as described for glucose oxidase coupling.

By creating a "peroxidase floor" this approach produced highly active GO derivatives, unfortunately however, the overall efficiency (27%) of the tube was compromised presumably by the inaccessibility of H₂O₂ to the peroxidase molecules. The ratio
of GO:POD in the starting solution was 1:2 by concentration (1:1.3 by activity) and was in the range that produced acceptable results previously. It would seem that once liberated by GO, the hydrogen peroxide produced could only interact with a peroxidase molecule if it moved toward the support surface accounting for some degree of the inefficiency of this model. It might be, therefore, worthwhile to study the reverse approach, that is, couple glucose oxidase first and then peroxidase.

The data evaluating the effect of reduction on the sequential coimmobilization characteristics is incomplete but it would appear that the results obtained in the presence and absence of reducing reagent are comparable.

In the preparation of all of the coimmobilized carriers a significant degree of inactivation of each enzyme has been observed. The results shown earlier in Tables VII and VIII demonstrate the relationship between specific activity and oxidation for soluble POD and GO. It was indicated here that even after two successive oxidations inactivation due to this treatment was almost negligible and can not be considered an explanation for this phenomena. In addition, the results in Tables VII and VIII show that limited inactivation occurs upon coupling to ethanolamine in solution. This leads to the suggestion that initial binding of peroxidase and glucose oxidase in coimmobilization experiments could be blocking access to the active site of GO and/or POD to such a degree that only
partial expression of the catalytic activity is possible.

A study of the effect of each modification necessary in
the preparation of GO-Dihydrazide-POD conjugates was
undertaken. The results in Table XXIV demonstrate the effect of
reduction on the development of the GO-DH-POD derivative. It
was observed that with either peroxidase or glucose oxidase
coupled with adipic dihydrazide, the loss in activity of the
enzyme was more substantial in the presence of NaCNBH₃.
Therefore, reduction was not included in the development of such
conjugates.

In Table XXV the results of the combination of GO, adipic
dihydrazide and POD upon their activities in solution is shown.
The minimum recovery of the original activity using oxidized
preparations found in this experiment was 82% GO and 87% POD.
In addition, the final GO-DH-POD aggregate demonstrated 90-95% of
the initial activity for both enzymes. The effect of
reduction of this conjugate was similar to previous results, in
this case an 11% decrease in POD activity but minimal losses in
GO specific activity were observed. These data suggest that the
characteristics of the support itself may be the cause of the
observed loss in specific activity. Since the losses are much
more substantial than those seen during the individual
immobilization of POD and GO, it would seem reasonable to
suggest that binding of the long arms with the conjugate forces
the enzyme into a conformation that blocks access to the
catalytic site or alters the nature of the site itself. The
TABLE XXIV
THE EFFECT OF REDUCTION ON GLUCOSE OXIDASE AND PEROXIDASE DIHYDROSIDE CONJUGATE PREPARATION

<table>
<thead>
<tr>
<th>Component Assayed</th>
<th>Protein Concentration mg/mL</th>
<th>Spectral Concentration μM</th>
<th>Specific Activity U/mg</th>
<th>With Reduction</th>
<th>Protein Concentration mg/mL</th>
<th>Spectral Concentration μM</th>
<th>Specific Activity U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. GO$_{ox}$</td>
<td>2.25</td>
<td>18.2</td>
<td>204</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>POD$_{ox}$</td>
<td>1.92</td>
<td>36.8</td>
<td>274</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. GO-DH$^a$</td>
<td>1.72</td>
<td>17.0</td>
<td>178</td>
<td>1.18</td>
<td>19.3</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>POD-DH</td>
<td>1.53</td>
<td>29.8</td>
<td>266</td>
<td></td>
<td>1.18</td>
<td>19.3</td>
<td>148</td>
</tr>
<tr>
<td>C. GO-DH-POD$^a$</td>
<td>0.24</td>
<td>2.35</td>
<td>230</td>
<td>0.30</td>
<td>2.60</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>0.36</td>
<td>8.00</td>
<td>237</td>
<td>0.46</td>
<td>8.68</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>POD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ A solution of oxidized glucose oxidase and peroxidase was initially incubated with 10 mM adipic dihydrazide. The complex formed was purified by ultrafiltration through an XM300 membrane before further modification with an oxidized peroxidase preparation to form a GO-DH-POD conjugate. Purification of this conjugate was by XM60 ultrafiltration.

$^b$ The conjugates were prepared in the presence or absence of 10 mM NaCNBH$_4$. Protein data was collected by Lowry determination (141), spectral concentrations were measured by multicomponent analysis and specific activities of peroxidase and glucose oxidase by enzyme assay. POD assay was completed utilizing 0.3 mM H$_2$O$_2$, GO with 30 mM glucose in the presence of 3 U/mL $^1$ soluble POD.
<table>
<thead>
<tr>
<th>Component Assayed</th>
<th>Protein(^d) mg/mL</th>
<th>Spectra(^e) Concentration (\mu)M</th>
<th>Specific Activity(^f) U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. GO(_{ox}) &amp; 2.25</td>
<td>18.20</td>
<td>204</td>
<td></td>
</tr>
<tr>
<td>POD(_{ox}) &amp; 0.48</td>
<td>35.00</td>
<td>273</td>
<td></td>
</tr>
<tr>
<td>GO-DH &amp; 1.72</td>
<td>17.00</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>POD-DH &amp; 0.51</td>
<td>29.80</td>
<td>266</td>
<td></td>
</tr>
<tr>
<td>B. GO-DH-POD(^b) &amp; 0.24</td>
<td>2.35</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>GO &amp; 0.36</td>
<td>2.00</td>
<td>237</td>
<td></td>
</tr>
<tr>
<td>C. GO-DH-POD, 2nd oxidation(^c) &amp; 0.19</td>
<td>2.98</td>
<td>202</td>
<td></td>
</tr>
<tr>
<td>GO &amp; 0.29</td>
<td>11.20</td>
<td>335</td>
<td></td>
</tr>
<tr>
<td>D. GO-DH-POD, Ethanolamine(^c) &amp; 0.23</td>
<td>0.90</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td>GO &amp; 0.34</td>
<td>7.80</td>
<td>258</td>
<td></td>
</tr>
<tr>
<td>E. GO-DH-POD, Ethanolamine &amp; 0.25</td>
<td>0.98</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>POD &amp; 0.31</td>
<td>8.54</td>
<td>254</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Glucose oxidase of peroxidase was treated with 1.0 mM periodate and subsequently exposed to 10 mM adipic dihydrazide solution and filtered through a XM300 ultrafiltration membrane.

\(^b\) The GO-DH solution was then incubated with an oxidized POD preparation for 18 h in the absence of reducing agent followed by ultrafiltration using a XM50 membrane.

\(^c\) A second successive oxidation of the conjugate yielded a derivative that could be coupled, in solution with an amine functionality. The GO-DH-POD conjugate was therefore incubated with 10 mM ethanolamine for 18 h at 4°C to permit Schiff base formation. In D, no reducing agent was added. The results of E are from the conjugate coupled to ethanolamine in the presence of 10 mM NaCNBH\(_3\). Excess reagent was isolated by ultrafiltration (YM30).

\(^d\) Protein concentrations were calculated by the method of Lowry et al. (141).

\(^e\) Spectral concentrations were determined by multicomponent analysis.

\(^f\) Specific activities were measured by the oxidative coupling of 9.0 mM HDCBS and 2.4 mM 4-AAP, using the gradientless reactor assembly and 0.3 mM \(H_2O_2\) for POD determination. GO assay was completed using 30 mM glucose and 3 U/mL\(^{-1}\) soluble POD.
proximity of the enzymes to each other could aggravate this situation further.

2. Clinical Investigation

A determination of the clinical features of this assay was then undertaken. The data that follows were obtained using coimmobilized POD:GO prepared by mixing oxidized solutions of peroxidase and glucose oxidase and incubating this preparation with amino-substituted nylon for 18 h at 4°C. Evaluation of this derivative in end-point mode glucose determinations permits some documentation of the usefulness of coimmobilized derivatives, however, further study of the clinical applicability utilizing the other coimmobilized conjugates conjugates is warranted by this work.

As with the manifolds derived for peroxidase and glucose oxidase a sampling rate of 50 per hour at a 1:1 sample:wash was observed to provide optimal conditions. Utilizing the conditions described in Figure 11 a carry over coefficient of 1.6 % was calculated from a low-high-low sample sequence.

Blanking

Reagent blanking was completed by washing the manifold with reagents for 15 min prior to assay and zeroing the instrument against this solution to correct the absorbance for reagent interferences.
Interferences

Interfering reagents were added to serum samples in duplicate and the effect on apparent glucose monitored.

Bilirubin up to 100 μM, hemoglobin concentrations up to 5 g.L\(^{-1}\) and triglyceride levels of 60 mM did not significantly (p > 0.05) interfere with assay results.

Linearity

A standard curve, indicating the relationship between glucose and absorbance gave a linear correlation of \(r = 0.9987\) (Figure 28). The slope of the line of best fit was \(1.15 \times 10^{-2}\) and the y-intercept, \(-0.026\).

When a serum sample possessing an elevated concentration of glucose was serially diluted a correlation coefficient of \(r = 0.9986\) was observed.

Precision

Within-run reproducibility was evaluated using ten replicates of three different serum samples. The mean coefficient of variation calculated from this study was 2.8 %.

Serum samples assayed in duplicate over five successive runs provided an estimate of the between-run precision shown in Table XXVI. The between-run CV was found to be approximately 3 fold greater than the within-assay value.

Recovery
FIGURE 28

STANDARD CURVE FOR GLUCOSE DETERMINATION
USING COIMMOBILIZED PEROXIDASE
AND GLUCOSE OXIDASE

Legend

The standard curve developed using the coimmobilized POD:GO manifold (Figure 11) is illustrated. Each standard was assayed in duplicate using a coimmobilized tube possessing 1.92 U.m⁻¹ GO, 2.50 U.m⁻¹ POD and 1.25 U.m⁻¹ coupled activity (Table XX, system A).

Linear regression analysis of the line gave the following characteristics: \( m = 1.15 \times 10^{-2} \pm 1.52 \times 10^{-4} \) mM⁻¹, \( y \)-intercept = 0.026 ± 0.0022 and \( r = 0.95 \pm 0.7 \).
FIGURE 28

![Graph showing the relationship between absorbance at 505 nm and glucose concentration in mM. The graph is a straight line with data points.]
TABLE XXVI

PRECISION STUDY FOR THE PROPOSED METHOD:
COIMMOBILIZED ENZYMES

<table>
<thead>
<tr>
<th></th>
<th>Within-run&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th>Between-run&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>SD</td>
<td>CV</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>5.74</td>
<td>0.13</td>
<td>2.3</td>
<td>5.28</td>
</tr>
<tr>
<td>11.86</td>
<td>0.39</td>
<td>3.8</td>
<td>11.95</td>
</tr>
<tr>
<td>15.34</td>
<td>0.34</td>
<td>2.2</td>
<td>15.55</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each serum sample was measured ten times.

<sup>b</sup>Serum samples were assayed in duplicate over five successive runs utilizing a coimmobilized derivative possessing 1.25 U/m coupled activity.
When known quantities of glucose were added to a serum sample a mean recovery of 99.5 ± 2.7 % was observed. The data in Table XXVII were from duplicate assays from two successive experiments.

**Stability**

All coimmobilized tubes were filled with 0.1 M phosphate buffer, pH 7.0 and stored at 4°C when not in use.

Operational stability was evaluated with the weekly assay of coupled enzyme activity remaining on the support (U/m) and routine evaluation of serum controls.

After 450 assays carried out over a six week period 97.1 ± 1.5 % of the coupled activity could be accounted for by colorimetric assay of enzyme activity.

**Correlation Studies**

The results from serum glucose quantitation using coimmobilized PDD:BJ were compared with the data from an established technique (152). Linear regression of the line of best fit through the points in Figure 29 indicated a good correlation between the data from the proposed method and the reference method (r = 0.9941). The value of the t-test was not significant at the p > 0.05 level.

A normal range, calculated by percentile estimates from 110 "normal" individuals was established as 3.6-7.3 mM. This reference range was comparable to that found in the literature
### TABLE XXVII

RECOVERY STUDY EMPLOYING COIMMOBILIZED GO:POD

<table>
<thead>
<tr>
<th>Glucose Added (mM)</th>
<th>Observed (mM)</th>
<th>Expected (mM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>7.09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.56</td>
<td>9.61</td>
<td>9.65</td>
<td>99.5</td>
</tr>
<tr>
<td>5.13</td>
<td>12.64</td>
<td>12.24</td>
<td>103.1</td>
</tr>
<tr>
<td>6.85</td>
<td>13.74</td>
<td>13.94</td>
<td>98.6</td>
</tr>
<tr>
<td>8.55</td>
<td>15.25</td>
<td>15.64</td>
<td>97.5</td>
</tr>
<tr>
<td>11.97</td>
<td>19.62</td>
<td>19.06</td>
<td>102.9</td>
</tr>
<tr>
<td>15.39</td>
<td>21.40</td>
<td>22.48</td>
<td>95.2</td>
</tr>
</tbody>
</table>

Mean of duplicate samples in two consecutive repetitions of experiment. Assays were completed utilizing the coimmobilized manifold (Fig. 11) and a 1 m length of coimmobilized derivative having 1.25 U/m activity.
FIGURE 29

COMPARISON OF COIMMOBILIZATION ASSAY
RESULTS WITH A REFERENCE TECHNIQUE

Legend

Serum samples were assayed for glucose using coimmobilized POD:GO coupled to amino substituted nylon (coupled activity of the tube was 1.25 U/m). Linear regression analysis of the line of best fit gave the following characteristics: \( m = 1.06 \), y-intercept = 0.792 and \( r = 0.9941 \).

A sample population of 125 was used.
for GO:POD coupled reactions (146-148).

C. IMMOBILIZATION OF OXALATE OXIDASE

In attempt to demonstrate the diversity of enzyme immobilization through nylon tubing modified with limited amino spacer arms, a study of its applicability to a third enzyme, oxalate oxidase was undertaken.

1. Analytical Investigation

Activity assays were completed using the enzyme reactor described previously in Figure 8. A flow rate of 30 mL.min⁻¹ was maintained throughout the study.

The activity of oxalate oxidase (OOx) was investigated with the peroxidase catalyzed coupling of 3-methyl-2-benzo-thiazolinone hydrazone (MBTH) with its formaldehyde azine. Coupling of a second molecule of MBTH to the azine forms a blue dye in a peroxidase catalyzed reaction (Figures 4 and 7). This reaction sequence was developed in this laboratory as a method for quantitation of peroxide in peroxide generating reactions (132) and later for the determination of erythrocyte membrane sialic acid (133). The application of this reaction was extended, in this work, to the determination of oxalate oxidase activity.

The initial phase of the study consisted of the reconstruction of the assay system as developed by Capaldi and Taylor (132). Horseradish peroxidase maintains optimal activity
at pHs close to 7.5, however, an isoenzyme of POD exhibits a pH optima at much more acidic values and can, therefore, be used to determine analytes at pHs of 3 or 4. The pH optimum of oxalate oxidase in solution has been reported as 3.50 (111). It was verified that for the coupled reaction, the pH optimum was in the range 3.0–3.8 (Figure 30) and a value of 3.60, was maintained throughout the study.

Oxalate oxidase has been observed to be very sensitive to the type of buffer employed (111,112). Comparison of 0.05 M succinate (pKa 4.1), acetate (pKa 4.7) and citrate (pKa 3.1) buffers, prepared to pH 3.60, showed that optimal activity could be expected using a 0.05 M succinate buffer. This finding concurs with the result of Chiriboga (112,115).

Suiguria (111) and Chiriboga (112,115) demonstrated the activation of oxalate oxidase by flavin compounds. Limited information is available regarding the mechanism of this reaction and no evidence has been presented to indicate that riboflavin is a native cofactor or that it binds to the enzyme itself. The accumulated data shows very low binding affinity of the flavins with OOX suggesting that these compounds may act free in the system.

An enhancement in the activity of oxalate oxidase by chelation of metallic inhibitors has been exhibited in the presence of synthetic chelates such as EDTA and 8-hydroxyquinoline (112). Table XXVIII, shows the results of a study conducted to evaluate the effect of these reagents on the
TABLE XXVIII
THE EFFECT OF SYNTHETIC CHELATES ON
OXALATE OXIDASE ACTIVITY

<table>
<thead>
<tr>
<th>Final Concentration M</th>
<th>Increase in Activity $%$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Activity</td>
<td>100</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td></td>
</tr>
<tr>
<td>$2 \times 10^{-4}$</td>
<td>400</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>547</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>562</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>275</td>
</tr>
<tr>
<td>$2 \times 10^{-4}$</td>
<td>422</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>463</td>
</tr>
</tbody>
</table>

$^a$Results are the mean of data collected from two experiments. Activity assays were completed using a 4.0 mM oxalate standard in the presence of 0.57 mM MBTH and 0.55 mM formaldehyde.
FIGURE 30

pH PROFILE FOR OXALATE OXIDASE CATALYZED
CHROMOPHORE PRODUCTION

Legend

The influence of pH on the formation of
tetraazapentamethine from MBTH and its formaldehyde azine
is illustrated.

The reaction was carried out using the standard
protocol with a 0.3 mM oxalate standard. Reactions were
determined at $\lambda_{\text{max}} = 630$ nm after a 7 min incubation at
35°C.

The enzyme solutions were prepared in 0.05 M succinate
buffer varying in pH from 3 to 6.
activity of this enzyme. It was observed that oxalate oxidase was significantly activated by both reagents, however, the effect of 8-hydroxyquinoline was more dramatic and a level of $1 \times 10^{-5}$ M was routinely added to the succinate buffered enzyme solution.

The overall system was initially constructed so that only the concentration of oxalate oxidase would be rate determining. This necessitates adjusting the concentration of substrate to approximately 100 times the $K_m$ of the enzyme. Table XXIX shows the effect of oxalate concentration on the initial rates of reaction. Above 4.2 mM oxalate substrate inhibitory effect becomes important and as a result this concentration was considered a sufficient excess to permit accurate determination of the enzyme activity.

Figure 31 shows that this reaction scheme for measuring oxalate oxidase maintains a good correlation ($r = 0.9917$) with initial rates and can be used in the quantitation of enzyme levels in the nmolar range.

In this study a decay rate for the tetraazapentamethine product of 0.30 % per min (0.056 pmoles per min) using a 0.30 mM standard was observed. Quenching of the reaction with 100 % acetone or 1 N HCl has been reported to alleviate this difficulty and also increase the extinction coefficient from 52.7 mM$^{-1}$cm$^{-1}$ to 68.7 mM$^{-1}$cm$^{-1}$ in aqueous organic medium and 55.1 mM$^{-1}$cm$^{-1}$ in acidic media. As a result of our observations quenching of the reaction mixture was not incorporated as a
TABLE XXIX

OPTIMIZATION OF OXALATE CONCENTRATION

<table>
<thead>
<tr>
<th>Oxalate mM</th>
<th>Absorbance Change $\Delta A_{630}^{nm.min^{-1}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.64</td>
<td>1.57 ± 0.25</td>
</tr>
<tr>
<td>8.32</td>
<td>1.20 ± 0.17</td>
</tr>
<tr>
<td>4.16</td>
<td>2.33 ± 0.13</td>
</tr>
<tr>
<td>1.67</td>
<td>1.75 ± 0.20</td>
</tr>
<tr>
<td>1.25</td>
<td>1.64 ± 0.21</td>
</tr>
<tr>
<td>0.83</td>
<td>1.59 ± 0.20</td>
</tr>
<tr>
<td>0.50</td>
<td>1.50 ± 0.15</td>
</tr>
</tbody>
</table>

$^a$Initial rates were measured in the presence of 0.57 mM MBTH, 0.57 mM formaldehyde and 1.67 U.mL$^{-1}$ POD with varying cuvette concentrations of oxalate. The reaction was monitored at room temperature utilizing a 0.05 M succinate buffer, pH 3.60.

$^b$Errors shown are mean uncertainties due to duplicate assays.
Duplicate rate measurements were completed using a solution containing final concentrations of 4.0 mM oxalate, 0.57 mM MBTH, 0.57 mM formaldehyde and 1.67 U.mL$^{-1}$ soluble peroxidase in 0.05 M succinate buffer, pH 3.60.

The change in absorbance at 630 nm was recorded on the Beckman 35 utilizing varying concentrations of oxalate oxidase.

Linear regression analysis of the line of best fit gave the following characteristics: $m = 0.1181$ μM$^{-1}$.min$^{-1}$, y-intercept $= -0.005$ and $r = 0.9917$. 
FIGURE 31

\[ \Delta A_{630} \cdot \text{min}^{-1} \]

\[ \text{COx} \quad \mu \text{M} \]

- The graph shows a linear relationship between the concentration of COx (in \( \mu \text{M} \)) and the change in absorbance at 630 nm (\( \Delta A_{630} \cdot \text{min}^{-1} \)).
- The points on the graph indicate a linear trend with increasing COx concentration.

routine step in the measurement of oxalate oxidase activity or oxalate standards.

**Enzyme Immobilization**

Structural information for oxalate oxidase is limited and the presence of a carbohydrate component is unknown, however, an evaluation of the effects of periodate oxidation on enzyme activity was undertaken. In a control experiment oxalate oxidase preparations were treated with 6 mM sodium periodate in the manner described for the glycoenzymes. This treatment caused a 50% decrease in the specific activity of the enzyme and enzyme immobilization was, therefore, directed through amine residues contained in the protein moiety of the enzyme.

Both, Rose et al. (134,135) and Kasidas and Rose (136,137) employed O-alkylation with TEOTFB to activate the nylon support. These authors utilized levels of activating reagent up to 0.6 M which are sufficient to saturate the tube with amino groups. The reagents used by these authors in the preparation of amino-substituted tubing included diaminooethane (134) and polyethyleneimine (136).

In the present study, limited activation of the carrier was affected by a 50 min incubation with a 200 μM solution of TEOTFB. An amino-substituted derivative formed using the ED-600 arms described previously was subsequently coupled with glutaraldehyde to yield a support capable of reacting with amine functionalities on the enzyme. A general model for this
approach to enzyme immobilization was illustrated in Figure 5. All coupling experiments for this enzyme were concluded after an 18 h incubation of OOX solution with the glutaraldehyde modified nylon tubing.

Three approaches to oxalate oxidase immobilization were evaluated.

1. Direct coupling, no reduction.
2. Initial reduction of the glutaraldehyde linkage before coupling (no reduction).
3. Simultaneously reduce and immobilize.

**Direct Coupling, No Reduction**

Initial studies involved direct coupling of the enzyme without any further modification to the support. The results in Table XXX show that this approach permitted coupling of approximately 70% of the starting solution.

**Reduction of Glutaraldehyde Linkage Before Coupling**

Initial treatment of the glutaraldehyde functions with 10 mM NaCNBH₃ effected reductive amination of the imine linkage to primary amino residues demonstrated no remarkable advantages in the immobilization of OOX. Comparable concentrations of enzyme were immobilized and the effect on the leaching of enzyme from the support was negligible. In the presence and absence of reducing agent similar levels of enzyme were released from a 1 m length of tubing.
<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>Enzyme Available in Starting Solutions</th>
<th>Enzyme Coupled</th>
<th>Leaching</th>
<th>Immobilization Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration mg/mL</td>
<td>Activity U/mL</td>
<td>Concentration mg/mL</td>
<td>Activity U/mL</td>
</tr>
<tr>
<td>A. Direct Immobilization&lt;sup&gt;a&lt;/sup&gt; O0x</td>
<td>0.0659</td>
<td>1.94</td>
<td>0.0449</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>0.0879</td>
<td>4.06</td>
<td>0.0515</td>
<td>2.01</td>
</tr>
<tr>
<td>B. Reduced After Glutaraldehyde&lt;sup&gt;b&lt;/sup&gt; O0x</td>
<td>0.0659</td>
<td>1.94</td>
<td>0.0356</td>
<td>0.98</td>
</tr>
<tr>
<td>C. Reduced Simultaneously with Immobilization</td>
<td>0.0659</td>
<td>1.94</td>
<td>0.0115</td>
<td>1.55</td>
</tr>
</tbody>
</table>

<sup>a</sup>Solutions of oxalate oxidase (O0x) in 0.25 M phosphate buffer, pH 7.0 were coupled to glutaraldehyde modified amino-substituted nylon for 18 h at 4°C.

<sup>b</sup>In this experiment the imine bonds of the glutaraldehyde modified tube were reduced within pH 7.0, 10 mM NaCNBH₃, for 2 h at 4°C, prior to coupling with oxalate oxidase. Incubation of the latter with the support was concluded after 18 h at 4°C.

<sup>c</sup>Simultaneous reduction and immobilization of the imine linkages of the coupled oxalate oxidase was performed in the presence of 10 mM NaCNBH₃. Enzyme coupling was concluded after 18 h at 4°C.

<sup>d</sup>Concentrations of protein in the starting solution and that present after immobilization were determined by the Lowry method (141). Coupled concentrations represent the difference between initial and post coupling levels. Enzyme activity before and after immobilization was determined using a 4.0 mM oxalate standard according to the protocol described in Methods.

<sup>e</sup>Leaching was determined by enzymic assay of O0x in the flow cell after the pump was stopped. Rates observed were calculated as the units of enzyme disappearing from a m length of tubing.
Simultaneous Reduction And Immobilization

Similar results were found when immobilization and reduction of the imine linkages to the support were completed simultaneously. Furthermore, washing the unreduced tubing with 5, 10 mL volumes of succinate buffer (0.05 M, pH 3.60) showed enzymic activity in the 0.1 U.mL\(^{-1}\) range in the first two eluates, but the losses became constant thereafter and represented only 0.02 U.mL\(^{-1}\) in the fifth wash.

These data do not support the incorporation of a reduction step in the immobilization of oxalate oxidase.

The immobilization factor, IF, indicates the relationship of the activities of the support bound enzyme and the enzyme free in solution. Generally, the IF values calculated for DOX using the more conventional technique for immobilization, were comparable to those observed for POD and GO coupling experiments. Experiment B, showed a much higher IF indicating that minimal inactivation of DOX has occurred utilizing this approach for coupling. Whether this is a true representation of the potential for this technique or just plain luck is unclear at this point.

Thermal Stability

When immobilized DOX prepared without reduction and its soluble analogue were assayed for activity at temperatures from 20 - 50 °C, no dramatic perturbations in thermal stability as a
result of immobilization were apparent.

Data for a thermal inactivation study conducted at 60°C is incomplete, however, it is obvious from this work and that of Suigura (129) that the soluble enzyme possesses remarkable stability at elevated temperatures. We have found that more than 60% of the activity from the soluble analogue remained after heating the solution at 60°C for 30 min.

**pH Stability**

The effect of pH on the soluble and immobilized preparations of Don is illustrated in Figure 32.

Similar profiles were obtained for both although the pH optimum for the bound enzyme was sharper at 3.75 than the 3.0 - 4.0 range found for soluble oxalate oxidase.

**Kinetic Study**

An investigation of the effect of immobilization on the Michaelis-Menten constants for oxalate oxidase was completed. An apparent Km of 0.572 ± 0.025 mM and V_max 0.481 ± 0.097 mM.min⁻¹ was obtained from the data shown in Figure 33. The Eadie-Hofstee replot was linear indicating that diffusion limitations were minimized. By comparison the soluble analogue indicated a Km of 0.114 in our laboratory. A value of 0.42 mM was obtained by Chiriboga's group for soluble oxalate oxidase (115).
Oxalate oxidase activity was measured on soluble and nylon coupled 00x preparations. Solutions containing soluble 00x were prepared in succinate buffers ranging from pH 2.0 to 6.0. The immobilized enzyme tube was washed with each buffer for 5 min prior to assay.

Activity measurements were completed as described in the Methods section using a 3.0 mM oxalate standard.

□ = Soluble Oxalate Oxidase
■ = Immobilized Oxalate Oxidase
FIGURE 32

% ACTIVITY

pH

2 4 6
FIGURE 33

LINEWEAVER-BURK PLOTS OF KINETIC DATA
FOR SOLUBLE AND IMMOBILIZED
OXALATE OXIDASE

Legend

Double reciprocal plots of the data collected for oxalate oxidase immobilized to nylon tubing is shown. Duplicate assays were completed as described in the Methods section for O0x activity measurements. Substrate concentrations between 0.17 - 4.20 mM were employed.

K_M^app for immobilized O0x (closed rectangles) was calculated by linear regression to be 0.572 ± 0.025 mM and V_max 0.482 ± 0.097 mM.min^-1 (r = 0.9969).

The Michaelis constant for the soluble enzyme (open rectangles) was calculated to be 0.114 ± 0.040 mM and V_max 0.694 ± 0.063 mM.min^-1 (r=0.900).
2. Clinical Investigation

Because of the specificity and sensitivity possible this reaction scheme was also investigated as a method for the determination of serum or urinary oxalate concentrations. Boer et al. (125) described a method for the quantitation of this analyte in plasma involving the determination of CO$_2$ produced from initial reaction with oxalate oxidase. This procedure requires long reaction times and is limited by the presence of endogeneous CO$_2$. Other methods include the reaction of MBTH with HALPS (123) and DMA (125,129).

Optimization of a manifold utilizing oxalate oxidase coupled to nylon tubing is incomplete at this point and the data obtained will therefore not be presented here. As optimization studies progressed it became obvious that nylon supported OOX did not express an operational stability comparable to either, the bound glycoenzymes evaluated or its soluble analogue and all activity was lost after ten days.

The following data was derived from studies completed using soluble oxalate oxidase and were completed on the Beckman 35.

**Linearity**

To effect the measurement of oxalate in this study the concentration of oxalate oxidase was adjusted to achieve maximum rates, while at the same time, for economy, a large excess of enzyme was avoided. Figure 34 demonstrates the effect of OOX
FIGURE 34

THE EFFECTS OF OXALATE OXIDASE VARIATION ON ASSAY RESULTS

Legend

The effects of oxalate oxidase concentrations on an oxalate standard are demonstrated. A 0.25 mM oxalate standard was mixed with 0.57 mM MBTH, 0.55 mM formaldehyde and 1.67 U.mL⁻¹ peroxidase according to the standard protocol. The reaction was initiated with the addition of 00x solution with cuvette concentrations varying from 0.10 to 0.40 U.mL⁻¹. Formation of the blue dye was monitored on the Beckman 35 at 630 nm.

Oxalate oxidase concentrations are represented by the following symbols:

●● = 0.40 U.mL⁻¹
○○ = 0.25 U.mL⁻¹
△△ = 0.15 U.mL⁻¹
△△ = 0.10 U.mL⁻¹
concentrations on the assay results. This study indicated that use of 0.25 U.mL⁻¹ soluble oxalate oxidase was sufficient to permit endpoint determination in a reasonable time interval (7 min). This conclusion was verified from the growth curves in Figure 35 for oxalate standards ranging from 0.025 - 0.085 mM.

A linear correlation between oxalate concentration and absorbance was observed. The standard curve illustrated in Figure 36 gave rise to a correlation coefficient of $r = 0.9968$.

Recovery

Measurement of oxalate quantities in biological matrices has received considerable attention of late. Numerous articles describing the optimization of the steps necessary to perform this assay have appeared in the literature (119,125,131,142,154,-)

155). In a study conducted by Hodgkinson (155) an evaluation of recoveries of oxalate in urine showed that acidification with 1 M HCl to a pH < 1.6 was sufficient to stabilize and preserve the solubility of oxalate. These researchers also noted the importance of collection of the sample over acid. Ascorbic acid present in urine is spontaneously converted to oxalate on standing at pHs greater than 7. The presence of this interferent has been reported to cause a 200% increase in apparent oxalate at neutral pH. Therefore, all samples to be studied were collected over acid and the pH adjusted to less than 2.

Measurements completed utilizing D0x present another
CHROMOPHORE FORMATION AND STABILITY IN
THE OXALATE OXIDASE-PEROXIDASE SYSTEM

Legend

Oxalate standards were incubated at room temperature
with 0.25 U.mL⁻¹ ODx and 1.67 U.mL⁻¹ POD in the presence of
0.55 mM MBTH and its formaldehyde azine. Absorbance at 630
nm was monitored as a function of time using the Beckman 35.
Cuvette levels of standards are indicated.
FIGURE 36

STANDARD CURVE FOR OXALATE DETERMINATION USING SOLUBLE OXALATE OXIDASE

Legend

A standard curve prepared using soluble ODX was constructed to relate concentration of oxalate and absorbance using 0.25 U.mL⁻¹ ODX, 1.67 U.mL⁻¹ POD with 0.57 mM MBTH and 0.55 mM formaldehyde. Each standard was measured in duplicate on the Beckman 35. Linear regression analysis indicated the following characteristics: \( m = 1.01 \pm 0.0068 \text{ mM}^{-1} \), \( y\)-intercept = 0.0116 \( \pm 2.62 \times 10^{-4} \) and \( r = 0.9968 \).
problem to the analyst in that urine contains an inhibitor to
oxalate oxidase. Studies completed by Potenzy (30) have shown
that the inhibitor is of low molecular mass (< 500 Daltons) and
is soluble in water, methanol and acetone however further
identification of the compound is, to date, incomplete.

Several alternatives for isolation of the oxalate have been
suggested, including simple dilution (122,123), the adsorption
to a mixed resin column (128) and precipitation with calcium
chloride. Sigma recently introduced another absorption
technique (130).

Initial attempts at the quantitation of urine oxalate using
the soluble assay sequence were completed using a kit introduced
by Sigma to effect the isolation of oxalate by absorption
(130). These, unfortunately, failed miserably and a recovery
of, at most, 48 % was all that was realized. Furthermore,
significant variability in recoveries was noted using this
technique (25 - 48 %). Dilution with 1 N HCl produced equally
impressive data with a mean recovery of 50 ± 15 %.

Precipitation of oxalate with calcium chloride and then
redissolving the pellet in ethanol according to the method of
Mazzachi et al. (142) indicated a mean recovery of 77.2 ± 1.5
%. Although this technique is both long and tedious and an
overnight precipitation was neccessary, this method was adopted
for isolation of oxalate. Data for this recovery study is
indicated in Table XXXI.
<table>
<thead>
<tr>
<th>Oxalate Added (mM)</th>
<th>Estimated Oxalate (mM)</th>
<th>Recovery (mM)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.1680</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>0.0446</td>
<td>0.2126</td>
<td>0.1760</td>
<td>82.7</td>
</tr>
<tr>
<td>0.0893</td>
<td>0.2523</td>
<td>0.2056</td>
<td>81.5</td>
</tr>
<tr>
<td>0.1859</td>
<td>0.3539</td>
<td>0.2647</td>
<td>74.8</td>
</tr>
<tr>
<td>0.3719</td>
<td>0.5399</td>
<td>0.3779</td>
<td>69.9</td>
</tr>
</tbody>
</table>

Assays were completed in duplicate utilizing 0.25 U.mL⁻¹ soluble oxalate oxidase and the standard protocol for oxalate estimation.
Precision

The within-run reproducibility of the proposed method using soluble oxalate oxidase was determined by assaying ten replicates each of three 24-h urines prepared as described previously. Between-run precision was evaluated by analyzing triplicate samples over a three day period (Table XXXII).

The within-run CV was shown to be less than 1 % while the between-run coefficient was less than 6.5 %.
### TABLE XXXII

**PRECISION STUDY OF THE PROPOSED METHOD USING SOLUBLE OXALATE OXIDASE**

<table>
<thead>
<tr>
<th>Within-run&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Between-run&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$\bar{X}$</strong></td>
<td><strong>SD</strong></td>
</tr>
<tr>
<td>$\mu M$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>77</td>
<td>0.5</td>
</tr>
<tr>
<td>272</td>
<td>1.8</td>
</tr>
<tr>
<td>465</td>
<td>4.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each sample was assayed ten times.

<sup>b</sup>Samples were measured in triplicate over three days.
CHAPTER IV

SUMMARY AND CONCLUSIONS

A. IMMOBILIZATION OF PEROXIDASE AND GLUCOSE OXIDASE

Our study was primarily concerned with the development of a method for the immobilization of POD and GO to nylon tubing modified with \( \alpha, \omega \)-poly(oxyethylene)diamine spacer arms.

An investigation of the degree and period of support alkylation on the activities of peroxidase indicated that levels of 350 fold less than that routinely utilized in the literature permitted expression of comparable intrinsic activities. In addition, the disproportionality between activity expressed on the tube and the concentration that disappeared from the starting solution suggests that an enhanced degree of enzyme inactivation occurs at greater levels of alkylation (or smaller cross-sectional area per arm). This could be attributed to multiple bond formation and steric interactions which hinder the approach of substrate to the active site.

A study of the effect of arm length on bound peroxidase activity showed that when using longer spacers a decrease in the apparent \( K_m \) to approach that calculated for the soluble enzyme could be expected. This observation also suggests that, at greater distances from the support, steric interactions are
minimized to permit an enhanced expression of the bound activity. Immobilization of periodate treated POD or GO was effected through Schiff base formation with amino residues on the nylon support. Coupling of the enzymes was optimized with respect to pH, time and the presence of reducing agent. Under the conditions described in the Methods section coupling yields of 35% and 15% for peroxidase and glucose oxidase were obtained, respectively.

An apparent loss of specific activity for the coupled enzyme was manifested by calculation of the immobilization factor. Typical IF values for POD and GO were 0.62 and 0.75, respectively. Despite this finding the nylon supported enzymes were observed to maintain a degree of activity and operational stability that could be useful analytically. The immobilized conjugates were, therefore, evaluated as clinical tools. Stability to pH variation and thermal inactivation was superior for the bound enzymes. When incorporated into an AutoAnalyzer I system the proposed methods were found to be precise and recoveries quantitative. Interferences due to lipemia, bilirubin and hemoglobin were minimal. Comparison of the proposed methods with a method in use indicated a significant correlation. The data passed the t-test for differences in the means.

B. COIMMOBILIZATION OF PEROXIDASE AND GLUCOSE OXIDASE
Three approaches to coimmobilization of peroxidase and glucose oxidase to nylon tubing were investigated. Examination of the data with respect to the "efficiency" of the reactors showed that the methods studied were comparable. This factor, which describes the relationship between the coupled activity to GO activity, was somewhat improved in the experiments conducted by simply mixing and allowing random binding of POD and GO to the tube. The preparation of ratios of GO:POD in the range of 1:3 by weight was difficult because of the manipulations involved for GO-POD and GO-Dihydrazide-POD conjugates, however, their synthesis may alter this conclusion. Further examination of these approaches is warranted by the data available.

When a coimmobilized derivative prepared by mixing aliquots of oxidized POD and GO and coupling directly was evaluated as a clinical tool, it was found to be both precise and sensitive. Recoveries of glucose added to a serum pool were quantitative and a significant correlation between the proposed technique and a method in use was observed.

C. IMMOBILIZATION OF OXALATE OXIDASE

Oxalate oxidase was determined by a colorimetric method employing the peroxidase catalyzed coupling of MBTH and its formaldehyde azine. This system was used to advantage for the
quantitation of this enzyme which functions at an optimal pH close to that necessary for use of this technique. The assay was optimized with respect to pH, substrate concentration, temperature and the level of 8-hydroxyquinoline. Application of the proposed method for quantitation of oxalate levels was demonstrated after investigation of the effects of time and oxalate oxidase concentration on chromophore production.

Immobilization of this non-glycosylated enzyme was effected through coupling of amine residues on the enzyme to aldehyde functionalities on the support. In this approach, the nylon support was initially derivatized with a limited number of \( \alpha, \omega \)-poly(oxyethylene)diamine spacer arms. The carrier was activated with TEOTFB using the technique developed for glycoenzyme coupling. The bound enzyme was found to maintain a pH optima close to its soluble counterpart. Kinetic studies on the immobilized enzyme were conducted indicating an apparent \( K_m \) comparable to that described elsewhere for soluble oxalate oxidase (112).

Evaluation of the proposed method utilizing immobilized O0x for determination of urinary oxalate is incomplete to date, however, when using the soluble enzyme the system was found to be precise and better recoveries of oxalate could be realized using initial calcium chloride precipitation as developed by Mazzachi et al. (142). The method optimized by these researchers was found to avoid the difficulties caused by an inhibitor to oxalate oxidase found in the urine. Further
study of this method as a system for the determination of serum or urinary oxalate levels using immobilized COx or its soluble analogue is warranted by these results and should include more extensive interference and correlation studies.
APPENDIX A

Calculation of Functional Group Density on Nylon Surfaces:

1. Surface area available on support = \(2\pi rL\)
   
   if, Length, \(L = 1.0\) m 
   
   Radius, \(r = 0.5\) mm
   
   then, Surface Area = \(3.14 \times 10^{17}\) \(\AA^2\)

   Assuming that CaCl\(_2\) etching increases the surface area ten fold then, the support surface area becomes
   
   \(3.14 \times 10^{18}\) \(\AA^2\).

2. Molecules of Activating Reagent/ m Support:
   
   if, 100 nmoles / m carrier are employed
   
   then, Molecules /m = \(6.02 \times 10^{16}\)

3. Therefore the Surface Area / Site = \(\frac{3.14 \times 10^{18}}{6.02 \times 10^{16}}\)
   
   = 52.13 \(\AA^2\) per site

Therefore, spacer arms are arranged in an area of 52.13 \(\AA^2\) or patches of 7.22 \(\times\) 7.22 \(\AA\).
APPENDIX B

DATA FROM KINETIC STUDIES

Uncertainties represent the error due to duplicate determinations.

A. SOLUBLE PEROXIDASE

1. Native Peroxidase

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.029</td>
<td>0.1620 ± 0.025</td>
</tr>
<tr>
<td>0.049</td>
<td>0.2560 ± 0.010</td>
</tr>
<tr>
<td>0.059</td>
<td>0.3257 ± 0.015</td>
</tr>
<tr>
<td>0.089</td>
<td>0.3846 ± 0.016</td>
</tr>
<tr>
<td>0.149</td>
<td>0.4800 ± 0.013</td>
</tr>
<tr>
<td>0.209</td>
<td>0.4958 ± 0.014</td>
</tr>
</tbody>
</table>

2. Oxidized Peroxidase

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.029</td>
<td>0.1680 ± 0.020</td>
</tr>
<tr>
<td>0.039</td>
<td>0.2150 ± 0.015</td>
</tr>
<tr>
<td>0.059</td>
<td>0.3067 ± 0.019</td>
</tr>
<tr>
<td>0.089</td>
<td>0.3378 ± 0.013</td>
</tr>
<tr>
<td>0.149</td>
<td>0.4693 ± 0.010</td>
</tr>
<tr>
<td>0.209</td>
<td>0.4813 ± 0.014</td>
</tr>
</tbody>
</table>

B. IMMOBILIZED PEROXIDASE

1. Ethylenediamine Arms

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.032</td>
<td>0.0666 ± 0.015</td>
</tr>
<tr>
<td>0.048</td>
<td>0.1005 ± 0.011</td>
</tr>
<tr>
<td>0.079</td>
<td>0.1523 ± 0.009</td>
</tr>
<tr>
<td>0.159</td>
<td>0.4888 ± 0.010</td>
</tr>
<tr>
<td>0.317</td>
<td>0.5333 ± 0.025</td>
</tr>
</tbody>
</table>
2. **ED-600 Arms**

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.032</td>
<td>0.0666 ± 0.025</td>
</tr>
<tr>
<td>0.048</td>
<td>0.1386 ± 0.035</td>
</tr>
<tr>
<td>0.079</td>
<td>0.1507 ± 0.013</td>
</tr>
<tr>
<td>0.159</td>
<td>0.2221 ± 0.020</td>
</tr>
<tr>
<td>0.225</td>
<td>0.3251 ± 0.015</td>
</tr>
<tr>
<td>0.317</td>
<td>0.3550 ± 0.017</td>
</tr>
</tbody>
</table>

3. **ED-2000 Arms**

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.032</td>
<td>0.0668 ± 0.008</td>
</tr>
<tr>
<td>0.048</td>
<td>0.0981 ± 0.020</td>
</tr>
<tr>
<td>0.079</td>
<td>0.1482 ± 0.013</td>
</tr>
<tr>
<td>0.159</td>
<td>0.4333 ± 0.025</td>
</tr>
<tr>
<td>0.317</td>
<td>0.3250 ± 0.019</td>
</tr>
<tr>
<td>0.634</td>
<td>0.4150 ± 0.020</td>
</tr>
</tbody>
</table>

4. **ED-6000 Arms**

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.032</td>
<td>0.1250 ± 0.035</td>
</tr>
<tr>
<td>0.048</td>
<td>0.1166 ± 0.030</td>
</tr>
<tr>
<td>0.079</td>
<td>0.2461 ± 0.023</td>
</tr>
<tr>
<td>0.159</td>
<td>0.3922 ± 0.021</td>
</tr>
<tr>
<td>0.317</td>
<td>0.4237 ± 0.021</td>
</tr>
</tbody>
</table>

C. **SOLUBLE GLUCOSE OXIDASE**

1. **NAtive Glucose Oxidase**

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.77</td>
<td>0.0666 ± 0.020</td>
</tr>
<tr>
<td>2.66</td>
<td>0.0902 ± 0.017</td>
</tr>
<tr>
<td>3.55</td>
<td>0.1121 ± 0.019</td>
</tr>
<tr>
<td>5.33</td>
<td>0.1459 ± 0.015</td>
</tr>
<tr>
<td>7.39</td>
<td>0.1831 ± 0.010</td>
</tr>
<tr>
<td>14.79</td>
<td>0.3413 ± 0.010</td>
</tr>
<tr>
<td>29.59</td>
<td>0.3690 ± 0.017</td>
</tr>
</tbody>
</table>
2. Oxidized Glucose Oxidase

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.77</td>
<td>0.0501 ± 0.007</td>
</tr>
<tr>
<td>2.66</td>
<td>0.0505 ± 0.009</td>
</tr>
<tr>
<td>3.55</td>
<td>0.0711 ± 0.010</td>
</tr>
<tr>
<td>5.33</td>
<td>0.0939 ± 0.011</td>
</tr>
<tr>
<td>7.39</td>
<td>0.1004 ± 0.010</td>
</tr>
<tr>
<td>14.79</td>
<td>0.1684 ± 0.009</td>
</tr>
<tr>
<td>29.59</td>
<td>0.2267 ± 0.010</td>
</tr>
</tbody>
</table>

D. IMMOBILIZED GLUCOSE OXIDASE

1. ED-600 Arms

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.19</td>
<td>0.8018 ± 0.040</td>
</tr>
<tr>
<td>2.52</td>
<td>1.5310 ± 0.010</td>
</tr>
<tr>
<td>3.36</td>
<td>2.3390 ± 0.015</td>
</tr>
<tr>
<td>5.04</td>
<td>2.8070 ± 0.015</td>
</tr>
<tr>
<td>14.00</td>
<td>3.7300 ± 0.013</td>
</tr>
<tr>
<td>28.00</td>
<td>5.1800 ± 0.012</td>
</tr>
</tbody>
</table>

E. IMMOBILIZED OXALATE OXIDASE

1. ED-600 Arms

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Rate (mM⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.177</td>
<td>0.1085 ± 0.050</td>
</tr>
<tr>
<td>0.250</td>
<td>0.1492 ± 0.035</td>
</tr>
<tr>
<td>0.500</td>
<td>0.2852 ± 0.033</td>
</tr>
<tr>
<td>0.830</td>
<td>0.3025 ± 0.025</td>
</tr>
<tr>
<td>1.250</td>
<td>0.3102 ± 0.023</td>
</tr>
<tr>
<td>1.67</td>
<td>0.3327 ± 0.025</td>
</tr>
<tr>
<td>4.16</td>
<td>0.4429 ± 0.034</td>
</tr>
</tbody>
</table>
REFERENCES


88. Ugarova, N. and Labedeva, O. (1978) Biokhimiya 43,
1731-1742.


130. Sigma Chemical Co. Package Insert, Oxalate Assay, St. Louis, MI 63178.


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  "Enzyme Immobilization on Modified Nylon Tubes".

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