Analytical Applications of Immobilized Enzymes

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ABSTRACT

Several clinical laboratory methods using enzymes as reagents now may utilize immobilized enzymes. These are enzymes attached to solid surfaces by adsorption, covalent binding, cross linking or similar means. Immobilized enzymes are widely used presently in convenient tests for urine glucose and galactose or blood glucose and urea, and in serum glucose or urea determinations by automated methods. Advantages include enhanced stability, enzyme conservation, reuse and economy.

Limitations in clinical analysis include the requirement for proper handling of both the immobilized enzyme and the specimen with which it is used. Immobilized enzymes of the future should aid in new discoveries regarding sequential enzyme-catalyzed reactions in living cells and expand the utility of enzymes as reagents in analytical laboratory science.

Introduction

Enzymes have a critical role in the metabolic processes within all cells. The term enzyme was first suggested by Willy Kühne about one hundred years ago (1876) as an alternative for the noun "ferment," and derives from the Greek "in yeast." In only a relatively few instances in biological systems does an enzyme ever function other than in the specific cell where it was created. It is not surprising that the first understanding of enzymes in human physiology related to situations where the enzymes do leave the cells where they are formed. Over the centuries, studies of the enzymes of saliva, gastric juice and pancreatic juice have elucidated the role that these extracellular enzymes play in digestion. Their study has also been of utility in clinical laboratory science. The author has had an interest and involvement in this subject for many years.4,5 However, the natural habitat of the multitude of enzymes that are known to function in biological systems is within cells where they are immobilized not only within the cell but are closely attached to specific subcellular structures.

Enzymes have been used for analytical purposes for approximately one century but it is only within the past two decades that significant attention has been given to the employment of immobilized enzymes in analytical laboratory practice.7,11,12
Nature of Enzyme Immobilization

Enzymes as proteins or as functional groups within the proteins are readily linked or attached to solid surfaces by a variety of means. The physical adsorption of an enzyme on an insoluble matrix is one of the most common forms of immobilization. Cellulose mats have been employed by us quite extensively but many types of insoluble surfaces or matrices have been used, including controlled-pore glass beads, porous titania bodies and charcoal. Additionally, enzymes may be attached to synthetic ion exchange columns.

A second type of immobilization of enzymes is to establish the protein molecule inside the lattice of a gel which has pores too small to allow the entrapped protein to escape. A number of enzymes immobilized by this method have been prepared and studied. Acrylamide gels have been extensively used for this purpose.

A third means of immobilization of enzymes is to create a covalent binding of the protein to a water insoluble carrier. This can be established by binding functional groups of the enzyme which are not essential for its activity. Carboxymethyl cellulose azide, sepharose cyanogen bromide activated, and polystyrene tubing converted to poly-p-aminostyrene and diazotized are some examples.

A fourth approach is to create cross-linking of the protein using a suitable bifunctional reagent. The bifunctional reagents used quite commonly are those which have two identical functional groups such as glutaraldehyde and bisdiazobenzidine-2,2'-disulfonic acid.

A fifth method involves microencapsulation within semipermeable membranes which are either permanent or non-permanent. All of these methods have been discussed in detail by Zaborsky. These methods are listed in table I.

Examples of Analytical Applications

During the past two decades, solid state chemistry has been used with immobilized enzymes to establish several convenience test systems. The first of these was a test for glucose in urine which responds with a color change within 10 seconds when a dip-and-read test is moistened with a urine containing glucose. This test, which is called Clinitest* contains two immobilized enzymes (glucose oxidase and peroxidase) which are adsorbed onto a cellulose mat. In figure 1 are shown the chemical reactions involved. A somewhat comparable system which utilizes immobilized galactose oxidase provides a specific test for galactose in urine. In figure 2 are shown the equations for these reactions, and in figure 3 is shown a photograph of the urine galactose test called Galactostix.* Immobilized enzymes have also been used in creating rapid test systems using whole blood for the evaluation of blood sugar and blood urea.

After the enzymes and chromogens have been absorbed on the cellulose fibers with each of these systems, the fibers are coated with ethyl cellulose. Glucose or urea readily and rapidly diffuse through the ethyl cellulose membrane, but the presence of the coating prevents the cells from adsorbing on the cellulose and allows them to be washed away. In figure 4 are shown the reaction equations of Azostix,* the rapid blood urea test. All

<table>
<thead>
<tr>
<th>Methods for Immobilization of Enzymes</th>
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<tbody>
<tr>
<td>Physical adsorption</td>
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<tr>
<td>Occlusion in cross-linked polymeric matrices</td>
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<tr>
<td>Covalent binding to water insoluble carrier</td>
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<tr>
<td>Intermolecular cross-linking using bifunctional reagents</td>
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<tr>
<td>Microencapsulation</td>
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*Registered trademark of Miles Laboratories, Inc., Elkhart, IN.
of these systems are employed as discrete testing methods and are completely disposable.

Research Products, Miles Laboratories, Inc., has developed analytical systems for automated instruments utilizing immobilized enzymes. These products, which are based on the work of Hornby and his associates,1,6 are called Catalinks. Catalinks Glucose Oxidase is a nylon tube with a 1 mm internal diameter. Glucose oxidase is covalently bound to the inner surface of the tube. The Catalinks tube is established in the flow pattern of a continuous analyzer and as the fluid passes through the tube, the immobilized enzyme catalyzes the oxidation of glucose to d-gluconic acid and hydrogen peroxide. The equations in the measurement of glucose are shown in figure 5. In figure 6 is shown a photograph of a Catalinks unit.

A second form of Catalinks utilizes immobilized urease which is covalently bound to the inner surface of a 1 mm inner diameter nylon tube. In this case, the immobilized enzyme urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide. The ammonia in turn reacts with sodium hypochlorite to form chloramine, which then reacts at the para-position of salicylic acid yield-
ing 2-hydroxy-5-aminobenzoic acid. Subsequently, the latter product reacts with another molecule of salicylate to produce an indophenol derivative which, when oxidized by excess hypochlorite, finally produces a greenish-blue indophenol of salicylic acid. The overall rate and reproducibility of the reaction is enhanced by nitroprusside.

Kunz and Stastny\(^9\) have described a procedure for measuring glucose in serum utilizing immobilized glucose oxidase in which the enzyme is attached to controlled-pore glass beads. Good stability and performance were reported.

Leon et al\(^10\) have described a continuous flow tubular reactor containing immobilized glucose oxidase. This was used with an automated system which performed as many as 25,000 measurements with a single enzyme tube.

Kiang et al\(^8\) have employed immobilized glucose oxidase and peroxidase in an analytical system in which the enzymes are attached to a cylindrical magnetic stirrer so that the enzymes are exposed to the reaction mixture quite rapidly. This approach is in contrast to most methods in which the immobilized enzymes have been stationary. In this procedure, relatively high concentrations of ascorbic acid, uric acid and creatinine did not interfere.

A review of current clinical laboratory practice indicates some 15 enzymes which receive significant usage as analytical reagents. These enzymes are listed in table II. Nine of these are commercially available in one or more immobilized forms, and these are identified in the table. Additionally, research studies have described immobilized models of several of the other enzymes listed in table II.

**Advantages of Immobilized Enzymes as Reagents**

A number of advantages which immobilized enzymes may provide over

\[
\begin{align*}
\text{NH}_2 &\xrightarrow{\text{C} = \text{O} + 3\text{H}_2\text{O} + \text{BROMTHYMOL BLUE}} \text{IMMOBILIZED UREASE} \\
\text{NH}_2 \quad \text{YELLOW COLOR} &\xrightarrow{\text{CO}_2 + 2\text{NH}_4\text{OH} + \text{BROMTHYMOL BLUE}} \text{GREEN to BLUE COLOR} \\
\text{UREA} &\quad \text{(due to higher pH)}
\end{align*}
\]
soluble enzymes includes enzyme conservation, enzyme reuse, enhanced stability, economy, convenience, reproducible results, ready-to-use, space-saving, adaptability to sequential reactions and adaptability to various systems. Every application of an immobilized enzyme does not necessarily result in the achievement of each advantage; however, in the majority of the applications which have been established, most of the advantages are manifested.

Hornby and Inman described the use of immobilized enzymes in continuous flow analyzers. In one example, during the course of approximately one month, some 3,500 glucose measurements were made on serum with one coil of tubing containing immobilized glucose oxidase. In another experiment of about the same duration, approximately 5,000 serum urea measurements were made. In either instance, no changes in the sensitivity adjustments of the instrument were made. With sensitivity adjustments, several times as many measurements can be made with a single tube. The amount of enzyme which is required is very much...
TABLE II
Enzymes Used in Clinical Analyses

<table>
<thead>
<tr>
<th>Name</th>
<th>Measurement</th>
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<tbody>
<tr>
<td>Alcohol dehydrogenase*</td>
<td>ethyl alcohol</td>
</tr>
<tr>
<td>Catalase*</td>
<td>in coupled systems</td>
</tr>
<tr>
<td>Catechol methyltransferase</td>
<td>epinephrine, norepinephrine</td>
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<tr>
<td>Cholesterol esterase</td>
<td>coupled system for cholesterol</td>
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<tr>
<td>Cholesterol oxidase</td>
<td>cholesterol</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>createine</td>
</tr>
<tr>
<td>Galactose oxidase*</td>
<td>galactose</td>
</tr>
<tr>
<td>Glucose oxidase*</td>
<td>glucose</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>coupled system-urinary steroids</td>
</tr>
<tr>
<td>Hexokinase*</td>
<td>lactic acid</td>
</tr>
<tr>
<td>Lactic dehydrogenase*</td>
<td>triglycerides</td>
</tr>
<tr>
<td>Lipase*</td>
<td>coupled system-urea</td>
</tr>
<tr>
<td>Peroxidase*</td>
<td>uric acid</td>
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</tbody>
</table>

*Commercially available in immobilized form.

less when it is immobilized with the quantity being from 1/3 to 1/10 or 1/20 as great.

In general, immobilized enzymes are more stable than their counterparts which are free in solution. The reason for this is not understood, although it may be due to the fact that the insoluble enzyme has its stability enhanced by its physical or chemical bonds to the insoluble matrix. Increased stability of the enzyme in a system provides a strengthening of what, in many cases, is the weakest link.

Limitations of Immobilized Enzymes as Reagents

Immobilized enzymes present a form of analytical tool which requires care and consideration on the part of the user if good results are to be obtained. Proper storage is of great importance with all enzymes but is of particular significance with immobilized enzymes.

The reagent dip-and-read systems for glucose, galactose and urea, which involve enzymes absorbed onto cellulose mats, require storage in an atmosphere with a very low humidity. This is readily achieved by the use of a closed, glass bottle and desiccant packets. However, if the bottle top is not replaced promptly after removing a reagent strip, moisture will enter and cause deterioration of the product.

In contrast, the immobilized enzymes in Catalinks need to be kept moist to provide for optimum storage. Enzyme poisons and inhibitors are limiting factors, although in some immobilized enzyme systems there is much less influence of inhibitors.

In systems involving immobilized enzymes within tubes, it is necessary to use small bore tubing in order to have the solution being analyzed come in contact with the enzyme. However, such tubes are prone to becoming obstructed if care is not taken to eliminate any particulate matter or fibrin clots.

In summary, immobilized enzymes have limitations. In many instances the limitations are related to the requirement for proper handling and appropriately following directions. Immobilized enzymes may be subject to inhibitors and poisons. Fine bore tubes may become plugged. In some instances, the use of immobilized enzymes may not be practical.

Future of Immobilized Enzymes

Speculating about the future presents interesting opportunities to interrelate the past and the present. Enzymes are amazing catalysts. Within living cells, enzymes catalyze sequential chemical reactions which biochemists are just beginning to understand. Perhaps, in the future, sequences which occur within cells
can be achieved through the use of immobilized enzymes. Then we can anticipate the discovery of new knowledge of a myriad of reaction systems which may reveal defects and inadequate functioning within cells and tissues which have heretofore only been suspected. Perhaps, in the future, immobilized enzymes can be transplanted into those unfortunate individuals whose genetic backgrounds cause them to be deficient in a necessary enzyme.

The expanded use of enzymes in analytical clinical analysis is quite probable. Enzymes provide a high degree of specificity and sensitivity which should supersede the many non-specific empirical methods which have been used in the past. It is quite likely that the relatively high cost of high purity enzymes will have a marked effect in encouraging the use of immobilized enzymes which are able to function effectively with markedly lower quantities of enzymes.

The capability of being able to locate an enzyme at a discrete, specific place provides an opportunity to place the enzyme where it will not be exposed to inhibitors or to enzyme poisons. Correspondingly, the enzyme can be located at a spot within the system where it will be possible to have it function and then cease to function because the reacting mixture has moved away from the immobilized enzyme.

Quite definitely, immobilized enzymes present interesting and exciting challenges to the clinical laboratory scientist.

References