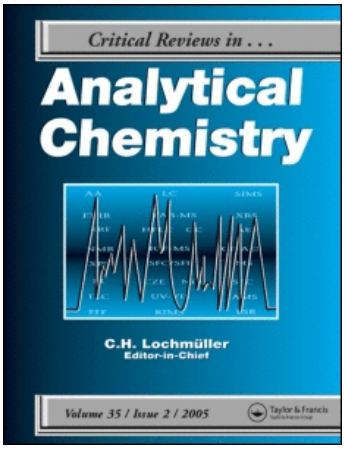

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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To cite this Article Guilbault, George G. and Mark Jr, Harry B.(1970) 'Enzymic Methods of Analysis', Critical Reviews in Analytical Chemistry, 1: 3, 376 — 413
To link to this Article: DOI: 10.1080/1040834ve08542739
URL: http://dx.doi.org/10.1080/1040834ve08542739

ENZYMIC METHODS OF ANALYSIS

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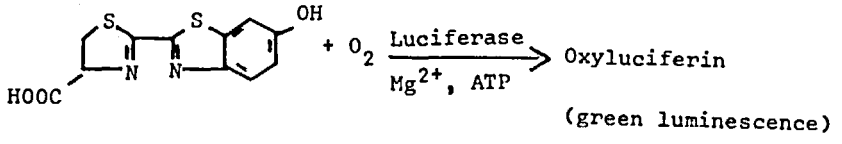
TABLE OF CONTENTS

I. INTRODUCTION

A. General Considerations

Enzymes are biological catalysts which enable the many complex chemical reactions, upon which depend the very existence of life as we know it, to take place at ordinary temperatures. Because enzymes work in complex living systems, one of their outstanding properties is specificity. An enzyme is capable of catalyzing a particular reaction of a particular substrate even though other isomers of that substrate or similar substrates may be present.

An example of the specificity of enzymes with respect to a particular substrate is found in luciferase, which catalyzes the oxidation of luciferin to oxyluciferin.[1] A rather complete study of many compounds similar in structure to luciferin showed that the catalytic oxidation resulting in the production of the green luminescence occurs only with luciferin. Substitution of an amino group for a hydroxyl group or addition of another hydroxyl group to the luciferin molecule alters the enzymic action, and the green luminescence is not produced. Another example of the



specificity of enzymes is glucose oxidase, which catalyzes the oxidation of β-D-glucose to gluconic acid. A rather complete study of about 60 oxidizable sugars and their derivatives showed that only the oxidation of 2-deoxy-D-glucose is catalyzed at a rate comparable to that of β-D-glucose. The anomer α-D-glucose is oxidized catalytically less than 1% as rapidly as the β-anomer.[2] Urease, which catalyzes the hydrolysis of urea, is even more specific.

Enzymes exhibit specificity with respect to a particular reaction. If one attempted to determine glucose by oxidation in an uncatalyzed way, for example by heating a solution of glucose and an oxidizing agent like ceric perchlorate, other side reactions would occur uncontrollably to yield products in addition to gluconic acid. With glucose oxidase, on the other hand, catalysis is so effective at room temperature and a pH near neutrality that the rates of the other thermodynamically possible reactions are negligible.

This specificity of enzymes and their ability to catalyze reactions of substrates at low concentrations are of great use in chemical analysis. Enzyme-catalyzed reactions have been used for analytical purposes for a long time for the determination of substrates, activators, inhibitors, and also of enzymes themselves. Until recently, however, the disadvantages associated with the use of enzymes have seriously limited their usefulness. Frequently cited objections to the use of enzymes for analytical purposes have been their unavailability, instability, poor precision, and the labor of performing the analyses. While these objections were valid earlier, numerous enzymes are now available in purified form, with high specific activity, at reasonable prices. The instability of enzymes is, of course, always a potential hazard; yet, if this instability is recognized and reasonable precautions are taken, the difficulty may be minimized. Again, the poor precision, slowness, and labor that have made enzyme-catalyzed reactions unappealing as a means of analysis may be more a consequence of the methods and techniques than the fault of the enzymes. With the advent of new techniques, fluorometric and electrochemical, many of the previous difficulties have been resolved. In addition, the automation of enzymic reactions has increased the speed, ease, and reproducibility of assays utilizing enzymes. Such automation will be discussed later in this review.

Another problem in the use of enzymes in analytical chemistry lies in the cost of using large amounts of these materials, especially in routine analysis. This problem has been solved to some extent by the development of an immobilized (insolubilized) enzyme technique which allows continuous use of the enzyme for up to one day. This aspect will likewise be discussed below.

The following is a Michaelis-Menten equation for an enzyme kinetics:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

In this mechanism, the substrate S combines with the enzyme E to form an intermediate complex ES, which subsequently breaks down into products P and liberates the enzyme. The equilibrium constant for the formation of the complex, K_m, the Michaelis constant, is defines as $(k_2 + k_{-1})/k_1$; the rate of reaction, V_0, is then some function of the enzyme and substrate (see Equation 1), as well as of activator and inhibitor concentration, if the latter two are present. At a fixed enzyme concentration

$$V_o = V_{max}[S]_o/(K_m + [S]_o)$$

The initial rate increases with substrate until a nonlimiting excess of substrate is reached, after which additional substrate causes no increase in rate.

The concentration of material participating in an enzymic reaction can be calculated in one of two ways: by measuring the total change that occurs by chemical, physical, or enzymatic analysis of the product or unreacted starting material; or from the rate of the enzyme reaction. In the first method, large amounts of enzyme and small amounts of substrate are used to ensure a relatively complete reaction. The reaction is allowed to reach equilibrium, and the amount of substrate S in the sample can be calculated from the amount of P formed ($S \xrightarrow{\epsilon} P$). P is chemically and physically distinguishable from S; e.g.,

$$\text{Ethanol} + \text{NAD} \xrightarrow{\text{Alcohol dehydrogenase}} \text{Acetaldehyde} + \text{NADH} \quad (1)$$

ϵ of NADH = 6.22×10^6 cm^2/mole at 340 nm).

Alternatively, a coupled reaction can be used to indicate how much substrate has been decomposed.

Enzyme reaction:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2 \quad (2)$$

Indicator reaction:

$$H_2O_2 + \text{leuco-dye} \xrightarrow{\text{Peroxidase}} H_2O + \text{dye} \quad (3)$$

The intensity of the dye produced is a measure of the concentration of glucose present.

In the second method, the kinetic method, the initial rate of reaction, V_0, is measured in one of many conventional ways, by following either the production of product or the disappearance of the substrate. The rate is a function of the concentrations of the substrate (S), enzyme (E), inhibitor (I), and activator (A). For example, the concentration of glucose can be determined by measuring the initial rate of production of dye in the example given above (Equations 2 and 3).

Because it is more reliable, the total change method is generally favored over the rate method. However, the former technique can only be used for substrate analysis and not for determinations of E, A, and I because the effects of these are catalytic in nature so that they affect only the rate and not the equilibrium. Also, the rate method is faster because the rate can be measured initially without having to wait for the reaction to go to completion. The conditions that affect the rate (pH, temperature, ionic strength) must be carefully controlled in the kinetic method for maximum sensitivity. The temperature coefficient of the enzyme reaction rate is roughly 10% per degree,[3] and a 10°C rise in temperature causes a 100% increase in the reaction rate. Hence, constant temperature is essential in the assay of enzyme activity. Recent work by Guilbault[4,5] and Pardue[6] has indicated that, with reasonable care, precision and accuracies of better than 1% can be obtained. Furthermore, some of the difficulties encountered because of side reactions are eliminated in rate methods, and greater sensitivities can be obtained in many cases. With the automated equipment now available for performing rate methods, such techniques will probably be the ones of choice in the future.

B. Books and Reviews

Guilbault[7] has written a book, *Enzymic Methods of Analysis*, that includes sections on immobilization of enzymes and automation of enzymic reactions, in addition to a discussion of the uses of enzymes in the analysis of substrates, activators, and inhibitors. Purdy[8] has written one on *Electroanalytical Methods in Biochemistry* that con-

tains material on enzymes, and a discussion of the use of fluorescence in the assay of enzymes and substrates may be found in books by Guilbault[9] and Udenfriend.[10] Ruyssen and Vadenriessche[11] have written a book on *Enzymes in Clinical Chemistry*, and Phillips and Elevitch[12] have written a chapter on Fluorometric Techniques in Clinical Pathology in Stefanini's book on *Progress in Clinical Pathology*. Both of these contain valuable procedures for enzyme analysis. Bergmeyer's *Methods of Enzymatic Analysis*[13] contains practical details of a vast range of enzymic methods. Blaedel and Hicks[14] have written a chapter on the Analytical Applications of Enzyme Catalyzed Reactions in Reilley's book.

Reviews on enzymic analysis have been written by Guilbault,[15] Bergmeyer,[16] Devlin,[17] and Roth.[18] Oldham[19] has reviewed radiochemical methods for enzyme assay.

C. Sources of Reagents

A comprehensive list of the source of all commercially available enzymes was compiled by Guilbault.[7] There are over 200 different enzymes available today, from several companies including: Boehringer Mannheim (Germany), Sigma (St. Louis), Calbiochem (Los Angeles), Mann Laboratories (New York), Worthington Biochemical (Freehold, N. J.), Nutritional Biochemical (Cleveland), General Biochemical (Chagrin Falls, Ohio), Gallard-Schlesinger (Long Island, New York), Merck (Darmstadt, Germany), and Miles Labs (Elkhart, Indiana). Chromogenic substrates are offered by many of the above; fluorogenic substrates are available from only a few companies such as Isolab, Inc. (Akron, Ohio), which offers a full line of these compounds.

II. DETERMINATION OF ENZYMES

A. General

Since the enzyme is a catalyst, theoretically one molecule of this material would eventually produce a sufficient change in the substrate to be measured. Hence, high sensitivities may be realized in enzyme analysis. Because the concentration of enzyme is so small, it always limits the rate of reaction, and the rate can be taken as a measure of the enzyme concentration. In Equation 2 the oxidation of glucose by oxygen to give peroxide and gluconic acid is catalyzed by glucose oxidase. The rate of production of peroxide is measured by a second coupled reaction, the oxidation of a leuco dye, such as *o*-dianisidine, to yield a highly colored dye. When glucose, leuco dye, and oxygen are non-rate limiting, the overall rate of reaction, as indicated by the rate of production of the dye, is proportional to the glucose oxidase activity.

Common techniques (spectrophotometric, change in pH, manometric) have been described for the assays of almost all enzymes[13-15] and these will not be discussed in the present review. Characteristics and assay procedures for most of the commercially available enzymes are likewise given in some manufacturers' catalogs.

Rather, some of the newer, more sensitive techniques for enzymic assay will be discussed. As was mentioned above, the use of enzymes as analytical reagents has received notoriety, mainly due to the poor precision and lengthiness of previous procedures. The recent trends in enzymic analysis have been in two main directions: to develop more sensitive procedures and to replace the long, tedious methods previously used for assay with rapid, easily instrumentable techniques.

B. Electrochemical Methods

Because of the simplicity of electrochemical techniques and their susceptibility to automation, such techniques have been used by a number of analysts to follow enzyme activity. Guilbault, Kramer, and Cannon[20] have developed a kinetic method for cholinesterase and thiocholine esters based on the electrochemical measurement of the rate of hydrolysis of the ester by the enzyme sample. Rates were measured by recording the difference in potential between two platinum electrodes polarized with a small constant current. The thiol produced upon enzymic hydrolysis is more electroactive than the substrate, so a decrease of potential results. The complete theory of this method has been worked out. Organophosphorus compounds (Sarin, Systox, parathion, malathion) inhibit the enzyme and may be determined at nanogram levels by this technique, with a deviation of about 1%.[21] The general applicability of this potentiometric method in following any enzymic reaction of the type $A \xrightarrow{B} C + D$, where the substrate A undergoes enzymolysis by B to form products C and D, has been demonstrated in procedures developed for glucose and glucose

oxidase,[22] xanthine oxidase,[23] and peroxidase and catalase.[24] In all cases, the time rate of change in potential with time, $\Delta E/\Delta t$, was found to be proportional to the concentration of the enzyme analyzed.

Pardue and Malmstadt have developed automatic electrochemical methods for the determination of glucose oxidase and glucose based on the oxidation of glucose to peroxide, followed by the oxidation of iodide to iodine by peroxide in the presence of molybdate as catalyst. The iodine produced, at a rate that is proportional to the rate of oxidation of glucose, is detected either potentiometrically[25-27] or amperometrically.[28,29] In either case, automatic control equipment provides a direct readout of the time required for a predetermined amount of iodine to be produced. The reciprocal of the time interval is proportional to the glucose oxidase activity or glucose concentration with relative standard deviations of about 2%. Blaedel and Olson[30] developed a method for the assay of glucose and glucose oxidase by an amperometric procedure similar to the one described except that the peroxide produced oxidized ferrocyanide to ferricyanide which is measured with a tubular platinum electrode. Pardue has extended the electrochemical techniques described to the assay of galactose and galactose oxidase.[31] The peroxide produced again reacts with iodide to form iodine, which is detected amperometrically. The reciprocal of the time interval required for a certain current to be produced is proportional to the materials analyzed with a deviation of about 2%. Purdy and coworkers have recently described amperometric and coulometric methods for the determination of enzymes and substrates. In one application, an amperometric method is used to follow the reaction of uric acid with the enzyme uricase.[32] The reaction forms hydrogen peroxide which reacts with iodide ion to form iodine. The formation of iodine is followed amperometrically, and the concentration of reacted uric acid is calculated from the amount of iodine formed. In another application, Purdy, Christian, and Knoblock described a method for the analysis of urease[33] based on the urease hydrolysis of urea to form ammonia. The resulting ammonia is then titrated with coulometrically generated hypobromite using a direct amperometric end point. In urine the ammonia could be titrated directly, but in blood samples the ammonia is first separated by the microdiffusion technique. Katz[34] has described a potentiometric method for urease. A Beckman cation-sensitive glass electrode that responds to $[NH_4^+]$ is used to follow the course of the reaction.

Polarographic techniques have been described for the determinations of cholinesterase,[35, 36] 3-hydroxy-anthranilic oxidase,[37] and catalase in microorganisms.[38] Rusznak et al.[39] determined starch by enzymic destruction using polarographic, chromatographic, and oscillopolarographic methods. Curtain[40] assayed the activity of cholinesterase using a silver thiol electrode and a thiocholine ester as substrate. Lipner et al.[41] have developed a galvanic cell for the measurement of oxygen-consuming enzyme systems using a bucking potential circuit, and Malmstadt and Piepmeier[42] have designed an inexpensive pH-stat with digital readout for quantitative enzyme determinations. A stability of ± 0.002 pH units is reported.

C. Fluorescence Methods

1. General

Because of limitations in molar absorptivities, measurements of gas volumes, or of changes in pH, most methods previously described for measuring components in enzyme reactions are limited to reactions of reagents present at concentrations greater than 10^{-6} *M*. Because fluorometric methods are generally several orders of magnitude more sensitive than chromogenic ones, a large increase in the sensitivity of measurement should result. Thus, much lower concentrations of reactants would be needed, and one could devise methods for substances at 10^{-9} *M* concentrations and lower. Moreover, fluorometric methods are quite useful in biochemical work, in the localization of enzymes and related substrates (activators), within organs, and even within individual cells.

Because of their sensitivity and specificity, fluorescent methods have found increasing usage in enzymology. For example, the reduced forms of nicotinamide adenine dinucleotide, NADH (DPNH), and nicotinamide adenine dinucleotide phosphate, NADPH (TPNH), are highly fluorescent. Thus, all NAD and NADP-dependent reactions involved in enzymatic analysis can be measured fluorometrically, with an increase of two to three orders of magnitude in sensitivity over colorimetric techniques.[43]

2. Hydrolytic Enzymes

Fluorescence methods have also been used extensively for the determination of hydrolytic enzymes, based on the enzyme-catalyzed hydrolysis of a nonfluorescent ester to a highly fluorescent alcohol or amine.

Guilbault and Kramer,[44, 45] for example, described a rapid, simple method for the determination of lipase based on its catalysis of the hydrolysis of the nonfluorescent dibutyryl ester of fluorescein. Fluorescein is produced upon enzymolysis and is highly fluorescent.

$$\underset{\text{(nonfluorescent)}}{\text{Dibutyryl fluorescein}} \xrightarrow{\text{Lipase}} \underset{\text{(fluorescent)}}{\text{Fluorescein}}$$

This reaction can be monitored by measurement of the rate of production of the highly fluorescent fluorescein with time, $\Delta F/\Delta t$. The concentration of enzyme can then be calculated from linear calibration plots of $\Delta F/\Delta t$ vs. enzyme concentration.

Guilbault, Sadar, Glazer, and Skou[46] prepared several esters as substrates for cholinesterase: the acetate, propionate, and butyrate esters of N-methylindoxyl, umbelliferone, and 4-methylumbelliferone. Comparison of these substrates with other fluorogenic esters, indoxyl acetate, indoxyl butyrate, resorufin acetate, β-carbonaphthoxycholine, and β-naphthyl acetate, indicated that N-methylindoxyl acetate and butyrate were the best substrates for true and pseudo cholinesterase, respectively. Analysis of as little as 5×10^{-5} unit per ml of cholinesterase can be performed by a direct initial reaction rate method in two to three minutes with an accuracy and precision of about 1.5%. All three N-methylindoxyl esters are very stable in solution and have a very low rate of spontaneous hydrolysis and a high rate of enzymic hydrolysis. All have good K_m values, and the N-methylindoxyl formed is not easily air-oxidized to indigo derivatives.

Guilbault and Heyn[47] tested several fluorogenic substrates for cellulase, namely fluorescein dibutyrate, α- and β-naphthyl acetates, indoxyl acetate, and resorufin butyrate. The latter is cleaved by cellulase to give the highly fluorescent resorufin (λ_{ex} =540 nm; λ_{em} = 580 nm). Using this substrate from 0.00010 to 0.060 units of cellulase can be determined in only two minutes with an accuracy and precision of 1.5%. This compares most favorably with other methods which take one to five hours and give precision and accuracies of only 5 to 10%.

Umbelliferone and 4-methylumbelliferone are highly fluorescent compounds which have been modified to form nonfluorescent substrates for the enzymes glucuronidase, glucosidase, and galactosidase. Robinson[48] used 4-methylumbelliferone-β-D-glucoside as a substrate for β-glucosidase. The substrate is split specificially by this enzyme to the highly fluorescent 4-methyl-umbelliferone.

Woolen and Walker[49] proposed 4-methylumbelliferone β-D-galactoside as a substrate for β-galactosidase, and Rotman, Zderic, and Edelstein[50] determined this enzyme fluorometrically in a similar manner. Woolen and Turner[51, 52] and Mead et al.[53] have proposed the use of the β-glucuronides of umbelliferone and 4-methylumbelliferone as substrates for glucuronidase. The rate of production of the fluorescent umbelliferone alcohols is a measure of the activity of the enzyme. An increase in sensitivity of about two orders of magnitude over the colorimetric procedure is obtained.

Still another example of a complex, tedious, and inaccurate enzyme assay procedure that can be easily replaced with a fast, highly sensitive, and accurate fluorometric method is that of hyaluronidase assay. Guilbault et al.[54] proposed the use of indoxyl acetate as a substrate for this enzyme. The highly fluorescent indigo white is produced upon hydrolysis, and as little as 10^{-3} μg of enzyme can be assayed in one to two minutes with an accuracy and precision of 1.8%.

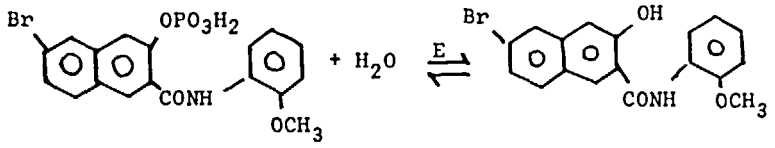
In a complete study of fluorometric substrates for lipase, Guilbault and Sadar[55] evaluated 12 different compounds from aspects of stability, spontaneous hydrolysis, enzymic hydrolysis, Michaelis constant for the enzyme-substrate complex, and total fluorescence of the final product. Optimum conditions of analysis were found for all substrates, and the lowest detectable enzyme concentration was found for each substrate. From all aspects, 4-methylumbelliferone heptanoate was found to be the best substrate for porcine pancrease lipase, and 4-methylumbelliferone octanoate was best for fungal lipase. As little as 2×10^{-5} units could be determined by a direct reaction rate method with an accuracy and precision of about 1.5%.

Guilbault and Hieserman[56] prepared several new fluorometric substrates for the assay of the

enzyme lipase. A study of 6 N-methylindoxyl esters as substrates for lipase indicated N-methylindoxyl myristate to be the best substrate for the analysis of this enzyme. Using this ester from 0.0002 to 4.0 unit per ml of porcine pancreas can be determined in the presence of several other esterases with an accuracy and precision of about 1.5%. Analysis is performed by a direct initial reaction rate method in two to three minutes.

Guilbault et al.[57] prepared umbelliferone phosphate as a fluorogenic substrate for acid and alkaline phosphatase and compared this substrate with other substrates listed in the literature. From aspects of stability, rate of enzymic hydrolysis, and fluorescence of product formed, umbelliferone phosphate appears to be an ideal substrate. As little as 10^{-6} unit of alkaline phosphatase and 10^{-5} unit of acid phosphatase is detectable. This represents an increase in sensitivity of two to four orders of magnitude over other techniques.

Guilbault and Vaughan[58,59] described the use of Naphthol AS derivatives as substrates for phosphatase. The fluorescent properties of a series of naphthol AS derivatives: Naphthol AS, AS-BI, AS-D, AS-GR, AS-LC, AS-MX, and AS-TR were compared (Table 1). The phosphate esters of these compounds were investigated as fluorogenic substrates for acid and alkaline phosphatase. The enzyme was determined by measuring the rate of formation of the fluorescent Naphthol AS:



Naphthol AS-BI Phosphate (Nonfluorescent)

Naphthol AS-BI (Fluorescent: λ_{ex} = 405 nm; λ_{em} = 515 nm)

TABLE 1

Comparison of Fluorescent Properties of Naphthol AS Derivatives at pH 9.0

Naphthol	Wavelength (nm) Excitation	Emission	Fluorescent[a] Coefficient	Blank Fluorescence[b] of Phosphate Ester
AS	388	516	2.2×10^5	0.133
AS-BI	405	515	2.5×10^5	0.11
AS-D	383	515	6.6×10^5	0.210
AS-GR	388[c]	488[c]		
AS-GR Phosphate	388	488	1.3×10^6	26.1[d]
AS-LC	388	522	2.8×10^5	0.09
AS-MX	388	512	2.8×10^5	0.076
AS-TR	388	512	3.1×10^5	0.014

[a] Fluorescence of hydrolyzed substrate divided by the concentration of original substrate in *M*. Value for quinine sulfate in 0.1 N sulfuric acid at λ_{ex} = 350 nm and λ_{em} = 450 nm was 2.5×10^6.

[b] The net increase in fluorescence of 3.2×10^{-4} *M* Naphthol AS phosphates at pH 9.0 after storage as a 10^{-2} *M* solution in methyl cellosolve at 3°C.

[c] In methyl Cellosolve.

[d] 3.5×10^{-5} *M*.

The amount of enzyme is calculated from calibration plots of initial rate against the concentration of alkaline phosphatase. In acid solution, Naphthol AS derivatives are nonfluorescent, but calibration curves for acid phosphatase can be obtained by quenching the enzyme reaction after a fixed time with alkali which develops the fluorescence of the liberated Naphthol AS. The amount of acid phosphatase is calculated from plots of change in fluorescence in the fixed reaction time against enzyme concentration.

Naphthol AS-BI phosphate was found to be the best substrate for both enzymes (Tables 2 and 3). It was found to give the highest rates of hydrolysis and the best K_m values of all the substrates investigated. Using this substrate, from 5×10^{-4} to 0.5 unit of alkaline phosphatase and 2×10^{-4} to 2×10^{-2} unit of acid phosphatase can be determined.

Guilbault and Hieserman[5,6] prepared five new fluorometric substrates for sulfatase: the sulfate esters of indoxyl, β-naphthol, 4-methylumbelliferone, fluorescein, and resorufin and compared these with each other and with other colorimetric substrates for sulfatase. Of these, β-naphthyl sulfate and 4-methylumbelliferone sulfate appear to be optimum for assay of various types of sulfatase. As little as 10^{-4} unit per ml of sulfatase can be

TABLE 2

Comparison of Naphthol AS Phosphates as Substrates for Acid Phosphatase

Naphthol Phosphate	Rate[a]	Lowest Detectable[b] Amount (Units)	K_m
AS	0.01	2×10^{-3}	–
AS-BI	0.204	2×10^{-4}	1.5×10^{-4}
AS-D	Very Slow Reaction		–
AS-LC	0.03	8×10^{-4}	1.45×10^{-4}
AS-MX	0.078	2×10^{-4}	1.8×10^{-4}
AS-TR	0.033	8×10^{-4}	2.5×10^{-4}

[a] Net change in fluorescence with 2×10^{-3} units in three minutes.

[b] Lowest detectable amount obtained using a reaction time of ten minutes.

TABLE 3

Comparison of Naphthol AS Phosphates as Substrates for Alkaline Phosphatase

Naphthol Phosphate	Rate[a]	Lowest Detectable Conc. (Units)	K_m
AS	0.13	8×10^{-4}	5×10^{-4}
AS-BI	0.155	5×10^{-4}	2.2×10^{-5}
AS-D	0.25	8×10^{-4}	2.3×10^{-4}
AS-G	0.075	5×10^{-3}	–
AS-LC	0.086	7×10^{-4}	1.8×10^{-5}
AS-MX	0.069	5×10^{-4}	2.8×10^{-4}
AS-TR	0.057	5×10^{-4}	6×10^{-4}

[a] Rate with 5×10^{-3} units alkaline phosphatase expressed as $\Delta F\ min^{-1}$. Blank rate of all esters = 0.

determined by a direct reaction rate method in two to three minutes with a precision and accuracy of about 1.5%.

Guilbault and Zimmerman[60] have developed a new technique for monitoring the concentration of an enzyme directly on a solid surface. In this technique the reagents for the assay of the enzyme are placed in the form of a film on silicone rubber on a solid surface (i.e., a glass slide). Provision for insertion of the slide into the cell compartment of an Aminco filter fluorometer allows a direct monitoring of the rate of reaction after addition of the enzyme. The technique was applied to the assay of cholinesterase in concentrations as low as 20 μg per ml using an initial rate method with an accuracy and precision better than 5.0%. N-methyl-indoxyl acetate was employed as the substrate.

3. Oxidative Enzymes

Fluorescence methods can likewise be used in the assay of oxidative and dehydrogenative enzyme systems with similar increases in sensitivity.

Guilbault et al. reported homovanillic acid[61,62] and *p*-hydroxyphenylacetic acid[63] to be ideal substrates for the determination of oxidative enzymes. As little as 10^{-5} unit of amino acid oxidase,[61] peroxidase,[62]

$$\text{Substrate} \xrightarrow{\text{Oxidase}} H_2O_2$$

$$H_2O_2 + p\text{-Hydroxyphenylacetic acid} \xrightarrow{\text{Peroxidase}} \text{Oxidized product (fluorescent)}$$

glucose oxidase[62,63] and xanthine oxidase[62] can be determined fluorometrically.

The rate of formation of the fluorescent oxidized product is followed and equated to the concentration of enzyme. In attempting to develop methods for galactose oxidase and invertase, Guilbault, Brignac, and Juneau[63] tried 25 different substrates for use in the assay of oxidative enzymes. *p*-Hydroxyphenylacetic acid was chosen as the best substrate for measuring oxidative enzymes because of its stability, low cost, and the high fluorescence coefficient of its oxidized form.[63]

4. Dehydrogenase Enzymes

An important class of enzymes are the dehydrogenases which, in the presence of a hydrogen acceptor such as nicotinamide adenine dinucleotide (NAD) (DPN) or nicotinamide adenine dinucleotide phosphate (NADP) (TPN), effect the dehydrogenation of hydroxy compounds. It has been stated that almost every enzyme of biological interest can be assayed with the aid of auxiliary enzymes and the coenzymes NAD and NADP.[64] Reduced NADH has maximum absorbance at 340 nm, while NAD has little absorbance at this wavelength. Hence, a spectrophotometric measurement of NADH or NADPH indicates the progress of the enzymic reaction:

$$\text{Substrate} + \text{NAD or NADP} \xrightarrow{\text{dehydrogenase}} \text{NADH} + \text{oxidized substrate (or NADPH)}$$

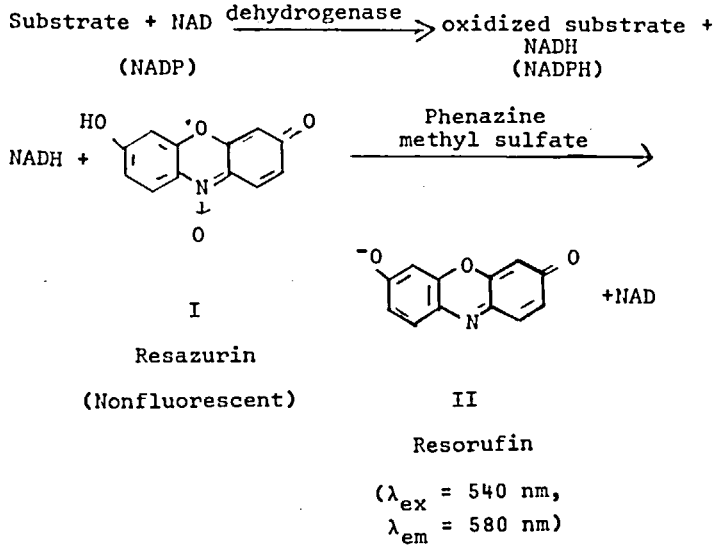
Likewise, all NAD- and NADP-dependent dehydrogenases can be monitored fluorometrically since NADH has a high fluorescence (λ_{ex} = 340 nm, λ_{em} = 450 nm). Lowery et al.[64] have described procedures for using the fluorescence of NADH and NADPH for the assay of enzymes and have discussed the effect of solvents, pH, and trace metals on the fluorescence. The greater sensitivity of fluorescence permits the use of smaller samples and smaller amounts of expensive substrates and cofactors. At least two orders of magnitude increase in sensitivity is achieved over colorimetric methods. The appearance of fluorescence or the loss of fluorescence may be measured directly in the reaction mixture either kinetically or after a predetermined period, in a manner completely analogous to the measurements of absorbance at 340 nm. The upper limit of concentration in the final solution to be measured, for which the fluorescence of NADH or NADPH would be strictly linear with concentration, is of the order of 2.5 μg/ml. Ideally, conditions in kinetic methods should be arranged such that the concentration of NADH or NADPH does not exceed this concentration.

Kaplan et al.[65] have found that NAD and NADP are converted to highly fluorescent products when heated with alkali, and Lowery et al.[64] stabilized the fluorophor and have reported its fluorescence characteristics. Huff and Perlzweig[66] have shown that NAD and NADP condense with acetone in alkaline solution to form highly fluorescent products.

Thus, the activity of a dehydrogenase can be determined fluorometrically, either by monitoring

the fluorescence of NADH or NADPH directly or by a measurement of the NAD or NADP after treatment with alkali or acetone.

A simple, rapid fluorometric method was described by Guilbault and Kramer[67,68] for measuring the activity of dehydrogenases. The method is based on the conversion of the nonfluorescent material resazurin (I) to the highly fluorescent resorufin (II) in conjunction with the NAD-NADH or NADP-NADPH system. As little as 10^{-4} unit per ml of the enzymes lactic dehydrogenase, alcohol dehydrogenase, malic dehydrogenase, glutamate



I

Resazurin

(Nonfluorescent)

II

Resorufin

(λ_{ex} = 540 nm, λ_{em} = 580 nm)

dehydrogenase, glucose-6-phosphate dehydrogenase, L-α-glycerophosphate dehydrogenase, and glycerol dehydrogenase could be determined with standard deviations of less than 1%. Likewise, NADH (10^{-7} to $10^{-5}M$) may be determined with a standard deviation of about 0.5%.

Because of the intense fluorescence of resorufin (as little as $10^{-9}M$ can be detected), an increase in sensitivity of one to two orders of magnitude over the NADH fluorescence method is achieved. Resazurin has been successfully used as a substrate for dehydrogenase in automated systems developed by Technicon.[69] The resorufin formed is continually monitored.

III. DETERMINATION OF SUBSTRATES

A. General

At a fixed enzyme concentration, the initial rate of an enzymatic reaction increases with increasing substrate concentration until a nonlimiting excess of substrate is reached, after which additional substrate causes no increase in rate. The region in which linearity is achieved, and in which an analytical determination of substrate concentration can be made based on the rate of reaction, lies below 0.1 K_m. The most important advantage of an enzymatic assay is its specificity. Frequently, only one member of a homologous series is active in the enzyme catalyzed reaction; other members are totally inactive or react at much slower rates. Most enzymes are also specific for one optical isomer of the substrate. Thus, in the enzymatic assay of amino acids, bacterial amino acid decarboxylase is specific for L-amino acid only.[70] Another advantage in the use of enzymes for substrate analysis lies in the great sensitivity obtained. Glucose, for example, is oxidized at the rate of a few percent per minute, regardless of concentration. Thus, a $10^{-7}M$ solution can be analyzed as easily as a $10^{-4}M$ one.

A complete compilation of enzymic methods for the assay of carbohydrates, amines, amino acids, organic acids, hydroxy compounds, esters, aldehydes, and inorganic substances has been prepared by Guilbault[7,15] and Bergmeyer.[13] In this review only some of the more unusual examples of the use of enzymes for analysis of substrates will be discussed.

B. Carbohydrates

Enzymes offer advantages of specificity and sensitivity over other nonenzymic methods for the

determination of carbohydrates. Furthermore, by the use of several enzymes, each selective for one carbohydrate, a complex mixture of carbohydrates could be assayed. Guilbault, Sadar,[80] and Peres[71] described fluorometric methods for the assay of mixtures of the carbohydrates, glucose, fructose, maltose, cellobiose, lactose, glycogen, and salicin. Glucose and fructose are determined fluorometrically using hexokinase and the resazurin-resorufin indicator reaction.

$$\text{Glucose} + \text{ATP} + MgCl_2 \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{Glucose-6-phosphate dehydrogenase}} \text{NADPH} + \text{glucono phosphate}$$

$$\underset{\text{(nonfluorescent)}}{\text{NADPH} + \text{resazurin}} \xrightarrow{\text{Phenazine methosulfate}} \underset{\text{(fluorescent)}}{\text{NADP} + \textit{resorufin}}$$

The rate of formation of resorufin is measured and is proportional to the glucose present (or to fructose using phosphohexose isomerase).

$$\text{Fructose} + \text{ATP} + MgCl_2 \xrightarrow{\text{hexokinase}} \text{fructose-6-phosphate}$$

Maltose, cellobiose, lactose, glycogen, and salicin are enzymatically hydrolyzed to glucose, which is then determined fluorometrically using the respective enzyme, glucose oxidase, together with *p*-hydroxyphenylacetic acid and peroxidase:

$$\text{Glycogen} \xrightarrow{\text{Amyloglucosidase}} \text{glucose}$$

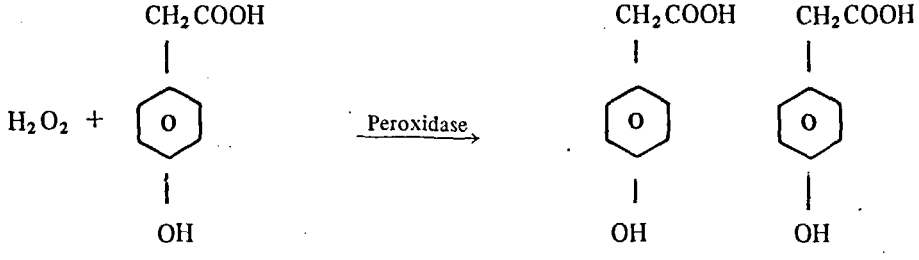
$$\text{Maltose} \xrightarrow{\text{Maltase}} \text{glucose}$$

$$\text{Cellobiose} \xrightarrow{\beta\text{-Glucosidase}} \text{glucose}$$

$$\text{Salicin} \xrightarrow{\beta\text{-Glucosidase}} \text{glucose}$$

$$\text{Lactose} \xrightarrow{\text{Lactase}} \text{glucose}$$

$$\text{Glucose} \xrightarrow{\text{Glucose oxidase}} H_2O_2$$



(fluorescent)

Some of the enzyme systems, together with the range of concentrations and interferences in the determination of various sugars, are listed in Table 4. All the enzyme systems are highly selective: only cellobiose and salicin, two substrates seldom found together, are substrates for β-glucosidase; maltase acts specifically on maltose; β-galactosidase (lactase) acts specifically on lactose and amyloglucosidase specifically on glycogen.

The analysis of a mixture of lactose, maltose, fructose, and glycogen was attempted using the four enzyme systems, β-galactosidase, maltase, hexokinase, and amyloglucosidase, with no prior separation. The sample was split into four aliquots, and each was assayed for one of the four components. Table 5 indicates the excellent results obtained for the simultaneous determination of these four carbohydrates.

TABLE 4

Enzyme Systems, Range of Concentrations, and Interferences in the Determination of Various Sugars

Substrate	Enzyme	Range, μg/ml	Interferences
Glucose	Hexokinase	0.05 – 50	None
Fructose	Hexokinase	0.05 – 52	Glucose
Cellobiose	β-Glucosidase	0.3 – 35	Glucose, Salicin
Maltose	Maltase	3 – 35	Glucose
Lactose	β-Galactosidase	3 – 35	Glucose
Salicin	β-Glucosidase	0.3 – 35	Glucose, cellobiose
Glycogen	Amyloglucosidase	3 – 35	Glucose

TABLE 5

Determination of a Mixture of Sugars

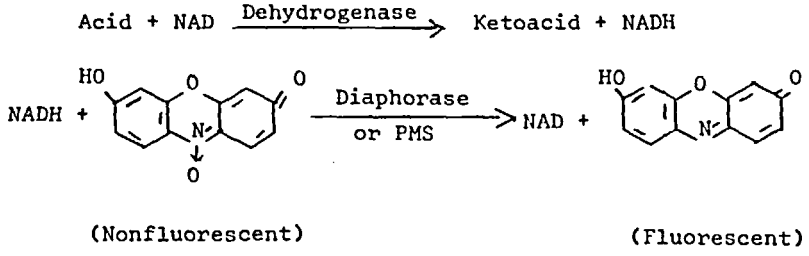
Lactose[a]		Maltose[a]		Fructose[a]		Glycogen[a]	
Added	Found[b]	Added	Found[b]	Added	Found[b]	Added	Found[b]
13.3	13.0	3.30	3.30	0.100	0.102	3.30	3.40
10.0	10.0	26.7	27.0	0.100	0.100	10.0	9.70
26.7	27.3	13.3	13.7	1.00	0.970	20.0	20.3
33.3	33.3	16.7	16.7	1.00	1.02	26.7	26.7
Av. Error	1.2%		1.0%		1.7%		1.6%

[a] Concentrations in μg/ml of total solution.

[b] Represents an average of three or more results with precision of ± 1.2%.

C. Organic Acids

This same philosophy outlined above could be applied to the assay of any complex mixture of organic substances, provided an enzyme was available for the specific assay of each component of the mixture. Guilbault, Sadar, and McQueen[72] described an assay procedure using six enzyme systems for the determination of mixtures of 21 organic acids. Lactate (Types II and IV), malate, glutamate, isocitrate, and β-hydroxybutyrate dehydrogenases are used, coupled with NAD, phenazine methosulfate, and resazurin, in a fluorometric procedure for the determination of acetic, adipic, benzilic, butyric, D-α- and D-β-hydroxybutyric, chloroacetic, DL-citric, formic, L-glutamic, glutaric, glycolic, *threo*-D-isocitric, L-lactic, L-malic, malonic, oxalic, phthalic, DL-succinic, and L-tartaric acids in the approximate range of 0.1 - 500 μg with an accuracy and precision of about 2%. The rate of production of the highly fluorescent resorufin is proportional to the concentration of the acid.



Some typical results for the analysis of acids are shown in Table 6. With β-hydroxybutyrate dehydrogenase, 1-75 μg/ml of D-β-hydroxybutyrate were analyzed in the presence of L-glutamic, L-lactic, L-malic, and D-α-hydroxybutyric acids at concentrations of 1 mg-ml with an accuracy of ± 1.3% and a precision of 2%. DL-Citric acid is the only interference being determinable in the range 10-110 μg/ml with a precision and accuracy of about 2%.

A four-component acid mixture of DL-citric acid, D-isocitric acid, L-lactic acid, and L-glutamic acid was analyzed and the results are given in Table 7. The sample was divided into four equal parts, and analysis of each acid performed with a different dehydrogenase: β-hydroxybutyrate dehydrogenase for DL-citric acid, isocitric dehydrogenase for D-isocitric acid, lactate dehydrogenase Type II for L-lactic acid, and glutamate dehydrogenase for L-glutamic acid. A phosphate buffer, (pH 6.5), was used for ICDH, a glycine-hydrazine buffer (pH 9.5) for β-OH-BuDH, and tris buffer (pH 9.5) for LDH and GDH. Essentially, the same precisions and accuracies were obtained for the analysis of the mixture (Table 7) as were obtained in the determination of the acid alone (Table 6).

D. Amines and Amino Acids

1. General Electrochemical Methods

Guilbault and co-workers have recently described several simple, direct electrochemical probes for the assay of amines and amino acids.

In one paper, Guilbault, Smith, and Montalvo[73] described the use of a cationic electrode that responds to NH_4^+ for the electrochemical assay of amines (urea, glutamine, asparagine, etc.) and amino acids:

Urea	$\xrightarrow{\text{Urease}}$	NH_4^+
Glutamine	$\xrightarrow{\text{Glutaminase}}$	NH_4^+
Asparagine	$\xrightarrow{\text{Asparaginase}}$	NH_4^+
Amino acid	$\xrightarrow{\text{Oxidase}}$	NH_4^+
Amine	$\xrightarrow{\text{Oxidase}}$	NH_4^+
Glutamic acid	$\xrightarrow{\text{Dehydrogenase}}$	NH_4^+

TABLE 6

Typical Determinations of Acids

D-β-Hydroxybutyric Acid[a]			L-Glutamic Acid[b]			L-Lactic Acid[c]		
Added (μg/ml)	Found (μg/ml)	Rel. Error (%)	Added (μg/ml)	Found (μg/ml)	Rel. Error (%)	Added (μg/ml)	Found (μg/ml)	Rel. Error (%)
1.00	1.01	+ 1.0	5.00	4.95	− 1.0	0.500	0.510	+ 2.0
5.00	4.90	− 2.0	15.0	14.7	− 2.0	1.50	1.47	− 2.0
10.00	10.0	0.0	25.0	25.0	0.0	3.0	3.08	+ 2.7
50.0	51.0	+ 2.0	75.0	77.0	+ 2.2	5.00	4.85	− 3.0
75.0	74.0	− 1.5	100.0	102.0	+ 2.0	10.0	10.0	0.0
Av. Rel. Error		± 1.3			± 1.4			± 1.9

[a] Analysis with β-hydroxybutyric dehydrogenase in the presence of 1 mg/ml each of L-glutamic, L-lactic, L-malic, and D-α-hydroxybutyric acids.

[b] Analysis with glutamate dehydrogenase in the presence of 1 mg/ml each of acetic, D-tartaric, and D-β-hydroxybutyric acids.

[c] Analysis with lactate dehydrogenase Type IV in the presence of 1 mg/ml each of L-malic, L-glutamic, and D-β-hydroxybutyric acids.

TABLE 7

Analysis of a Mixture of Citric, Isocitric, Lactic, and Glutamic Acids

Added (μg/ml)			
DL-Citric	D-Isocitric	L-Lactic	L-Glutamic
10.00	1.00	10.0	10.0
10.0	1.00	5.00	50.0
10.0	1.00	10.0	50.0
50.0	0.500	10.0	50.0
Found (μg/ml)			
DL-Citric	D-Isocitric	L-Lactic	L-Glutamic
10.1	1.00	9.90	10.2
10.0	1.01	5.10	49.2
9.9	0.980	10.1	51.0
49.2	0.495	10.0	51.0
Rel. Error (%)			
DL-Citric	D-Isocitric	L-Lactic	L-Glutamic
+ 1.0	0.0	− 1.0	+ 2.0
0.0	+ 1.0	+ 2.0	− 1.6
− 1.0	− 2.0	+ 1.0	+ 2.0
− 1.6	− 1.0	0.0	+ 2.0

Some results obtained in the use of this method for the analysis of various enzymes and substrates are indicated in Table 8. The urease, glutaminase, asparaginase and D- and L-amino acid oxidase systems worked well. Attempts to get the glutamic acid dehydrogenase system to work failed, probably because of the presence of ions in the enzyme. This was indicated by the large potentials observed before initiation of the enzymic reaction and the serious drift problems encountered. All attempts to remove the ionic interferences failed. Similar problems were sometimes encountered in assay of the other enzyme systems, but such problems were generally eliminated by pretreatment of the enzyme and/or substrate with an ion exchange resin.

2. Enzyme Electrode Probes for the Assay of Substrates

Carrying the concept of an electrochemical probe one step further, Guilbault and Montalvo[74] described a urea enzyme electrode suitable for the continuous assay of urea in body fluids.

The urea transducer is called a urease electrode because it is made by polymerizing a gelatinous membrane of immobilized enzyme over a Beckman cationic glass electrode which is responsive to ammonium ions. Specificity for urea is obtained by immobilizing the enzyme urease in a layer of acrylamide gel 60–350 μ thick on the surface of the glass electrode. When the urease electrode is placed in contact with a solution containing urea, the substrate diffuses into the gel layer of immobilized enzyme. The enzyme catalyzes the decomposition of urea to ammonium ion as shown in the following equation.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2\,NH_3 + CO_2$$

The ammonium ion produced at the surface of the electrode is sensed by the specially formulated glass which measures the activity of this monovalent cation in a manner analogous to pH determination with a glass electrode.

TABLE 8

Substrates and Enzymes Determined Electrochemically with the NH_4^+-Sensitive Electrode

Substance	Range	Rel. Error %
Urea	0.5 – 100 μg/ml	2.0
Urease	0.0010 – 0.04 units	2.5
Glutamine	0.5 – 10 μg/ml	5.0
Glutaminase	0.0010 – 0.40 units	5.0
Asparagine	0.5 – 10.0 μg/ml	3.0
Asparaginase	0.0010 – 0.40 units	3.0
L-Tyrosine	1 – 40 μg/ml	2.0
L-Leucine	1 – 40 μg/ml	2.5
D-Tyrosine	10 – 100 μg/ml	3.5
D-Methionine	10 – 100 μg/ml	4.0
L-Amino acid oxidase	0.010 – 0.2 units	2.5
D-Amino acid oxidase	0.05 – 1.0 units	3.5

The potential of this electrode is measured after allowing sufficient time for the diffusion process to reach the steady state. This interval varies from about 25 to 60 sec for 98% of the steady state response, depending on the thickness of the gel membrane.

When the urea concentration is below the apparent K_m for the immobilized enzyme, but above 0.6 mg of urea/100 ml of solution, the potential of the electrode varies linearly with the logarithm of the urea concentration. Also, the response curve goes from first order at low urea concentrations to zero order at high substrate concentrations.

In order to improve the stability of the urea electrode a thin film of cellophane was placed around the enzyme gel layer to prevent leaching of the urease into the surrounding solution.[75] In this way an electrode could be used continuously for over 21 days at 25°C with no loss of activity.

In a later paper, Guilbault and Montalvo[76] discussed the preparation of various types of urease enzyme electrodes, the immobilization parameters that affect the response of the electrodes, factors that affect the stability of the immobilized enzyme, and the effect of foreign monovalent cations on the enzyme response.

Guilbault and Hrabankova[77] described a specific electrode for the direct assay of urea in body fluids (blood and urine). It was found that, by using a glass electrode (Beckman 39137) as the reference electrode and an ion exchange resin to remove interferences from Na^+ and K^+, urea could be assayed in blood (Table 9) or urine (Table 10). The precision obtainable is about 1% averaging three or more samples. The difference between the results obtained with the urea electrode and the standard spectrophotometric method is about 2 to 3%, but it is believed that the electrode method is more accurate due to the low reliability factor of the spectrophotometric method.

The stability of the urea electrode is illustrated in Figure 1. With many electrodes no loss in activity occurred over the first 21 days regardless of the urea concentration; excellent reproducible (within 1 to 2%) voltages were obtained over a three-week period. With other electrodes a small drift in potential from day to day was observed. The same standard urea solution used above ($5 \times 10^{-4} M$) was also used to control the electrode stability. If the potential drifted more than 1 mV, a new calibration plot was made.

The variation between electrodes was also compared, and it was found that of ten urea electrodes prepared as described above (polyacrylamide layer of urease over a Beckman cation electrode covered with a cellophane sheet), at least nine had electrode responses that agreed within 3 to 5% of each other. This indicates that it is not difficult to make an urea electrode. For maximum precision, however, a separate calibration plot was made for each electrode, as is usually done with any selective ion electrode.

Guilbault and Hrabankova[78,79] described the preparation of an amino acid electrode and described its electrode properties.

The L-amino acid electrode was made by placing a thin layer of L-amino acid oxidase (L-AAO) over a Beckman monovalent cation electrode. The enzyme catalyzes the decomposition of amino acid to NH_4^+ ions by the reaction:

$$RCHNH_3{}^+COO^- + H_2O + O_2 \xrightarrow{\text{L-AAO}} RCOCOO^- + NH_4^+ + H_2O_2 \quad (4)$$

The H_2O_2 formed reacts nonenzymatically with the α-keto acid product:

$$RCOCOO^- + H_2O_2 \longrightarrow RCOO^- + CO_2 + H_2O \quad (5)$$

If H_2O_2 is destroyed by catalase, the overall reaction is described by Equation 6:

$$2RCHNH_3{}^+COO^- + O_2 \longrightarrow 2RCOCOO^- + 2NH_4^+ \quad (6)$$

TABLE 9

Determination of Urea in Blood Serum (mg%)

Sample No.	Spectrophotometric Method	Urea Electrode Method	Difference %
1.	11	11	0
2	37	38	2.7
3	28	30	7
4	21	20	4.1
5	32	32	0
6	58	59	1.7
7	82	81	1.2
8	28	30	7
9	54	55	1.8
10	80	82	2.4
		Av. Difference	2.8

TABLE 10

Determination of Urea in Urine (g/100 ml)

Sample No.	Spectrophotometric Method	Urea Electrode Method	Difference %
1	1.24	1.20	3.3
2	1.14	1.16	1.7
3	1.21	1.28	5.8
4	1.10	1.03	2.7
5	0.73	0.72	1.3
6	1.18	1.18	0
7	2.40	2.30	4.2
8	3.46	3.50	1.1
9	1.60	1.62	1.2
10	0.54	0.55	1.8
		Av. Difference	2.3

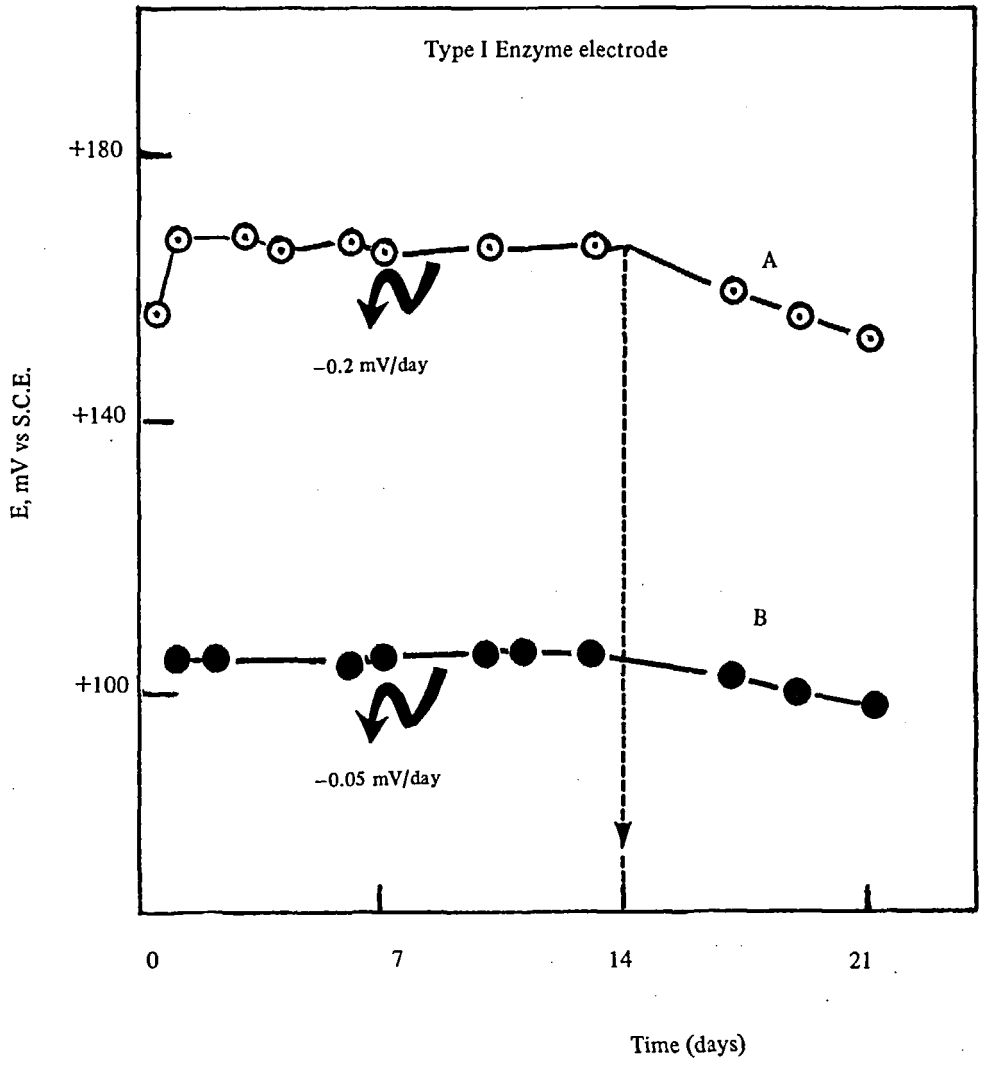


FIGURE 1. Stability of the urea enzyme electrode in tris buffer at 25°C. A. Response to 8.33 × 10^{-2} *M* Urea. B. Response to 1 × 10^{-3} *M* Urea.

Ammonium ions formed in Equations 4 or 6, respectively, are sensed by the electrode described, the steady state potential of which is proportional to the activity of NH_4^+ ions in the enzyme layer, i.e., to the concentration of amino acid in the solution.

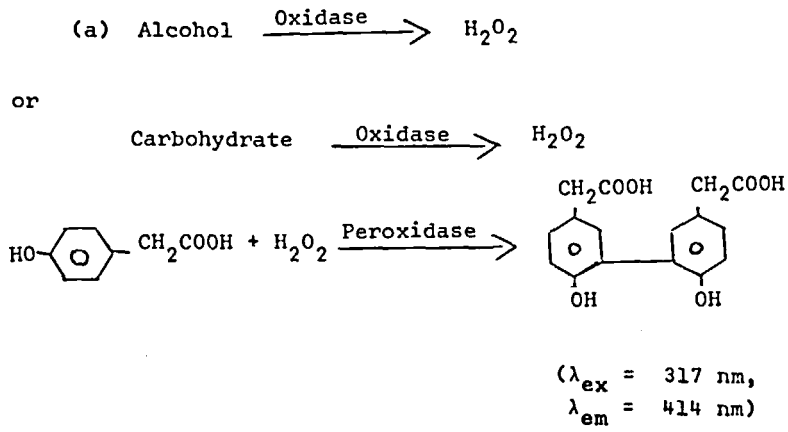
The stability of electrodes containing 20 and 100 mg of L-AAO per ml of solution was compared. Electrodes with the higher enzyme concentration were more stable. The results obtained are summarized in Table 11. Each value is taken from three independent stability measurements carried out under the same conditions.

An attempt was also made to prepare a similar electrode for the determination of D-amino acids, i.e., using D- amino acid oxidase. The electrode gives a response to D-amino acids, but at the present time we have not found a convenient way to stabilize the enzyme. This problem continues to be a subject of study.

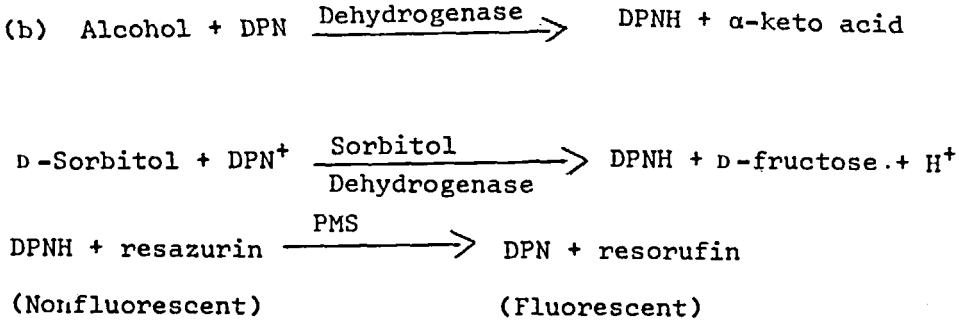
E. Hydroxy Compounds

Guilbault and Sader[80] have developed enzymic methods for the assay of mixtures of hydroxy compounds. Four enzyme systems were used, each for the assay of one alcohol in the mixture: alco-

hol dehydrogenase, alcohol oxidase, carbohydrate oxidase, and sorbitol dehydrogenase. In each case a fluorometric monitoring system was used tc follow the reaction



The rate of formation of the fluorescent product is followed and is proportional to the alcohol or carbohydrate content.



The formation of resorufin is measured and is proportional to the concentration of sorbitol or alcohol present. Sorbitol, methanol, ethanol, allyl alcohol, cyclohexanol, and *sec*-butyl alcohol did not interfere in the determination of glucose, xylose, or sorbose using carbohydrate oxidase. Likewise, sugars like glucose, xylose, or sorbose and alcohols like methanol, ethanol, allyl alcohol, etc. did not interfere in the assay of sorbitol using sorbitol dehydrogenase, nor did sorbitol or any

TABLE 11

Stability of Four Types of L-Amino Acid Electrodes

Electrode[a]	1	2	3	4
2×10^{-4} *M* L-phe	0.32 mV/day[b]	2.5 mV/day[b]	0.13 mV/day[b]	0.05 mV/day[b]
2×10^{-4} *M* L-phe	2.6 mV/day[b]	4.2 mV/day[b]	2.1 mV/day[b]	1.5 mV/day[b]

[a] Electrode 1: liquid membrane, Type II, 20 mg L-AAO/ml.
Electrode 2: polymerized Type I electrode, 100 mg L-AAO/mg gel solution.
Electrode 3: liquid membrane, Type II, 100 mg L-AAO/ml.
Electrode 4: same as 3, stored in phosphate buffer, pH 5.5.

[b] Decrease in the response of the electrode to a solution of L-phenylalanine in mV/day.

Liquid membrane electrodes were stable for about two weeks; after that time more rapid decrease of responses was observed.

sugars interfere in the determination of alcohols using alcohol oxidase.

Some typical results of the analysis of a mixture of glucose and sorbitol using carbohydrate oxidase and sorbitol dehydrogenase, respectively, are shown in Table 12. An average error of about 1.4% and a precision of about 1.5% were obtained.

Finally, a three-component mixture of methanol, xylose, and sorbitol was assayed using three different enzymes: alcohol oxidase, carbohydrate oxidase, and sorbitol dehydrogenase. The results, given in Table 13, indicated a precision of about 1.5% and an average error of 1 - 1.5%.

TABLE 12
Assay of a Mixture of Glucose and Sorbitol (Glucose assayed with carbohydrate oxidase, sorbitol with sorbitol dehydrogenase)

Glucose[a]		Sorbitol[b]	
Added	Found[b]	Added	Found[b]
79.9	79.0	17.2	17.2
70.0	70.0	36.3	36.0
83.6	84.2	29.6	30.2
99.2	99.2	16.8	16.8
Av. Error	1.5%		1.4%

[a] Concentrations in μg/ml of total solution.

[b] Represents an average of three or more results with precision of ± 1.5%.

F. Inorganic Substances

Although enzymic methods have been described for the assay of many inorganic substances, two very sensitive procedures that are worthy of special note are those for phosphate and ammonium ions. The enzymatic determination of ammonia in tissue body fluids was described by Faway and Dahl,[81] in body fluids by Mondzac, Ehrlich, and Seegmiller,[82] in blood and tissue by Reichelt, Kvamme, and Tveit,[83] and in blood by Kirsten, Gerez, and Kirsten.[84] All used the oxoglutarate-glutamic dehydrogenase-NADH system, measuring the change in absorbance at 340 mμ.

$$NH_4^+ + H^+ + NADH + \text{oxoglutarate} \xrightarrow{\text{dehydrogenase}} \text{glutamate} + NAD^+ + H_2O$$

The enzymatic procedure eliminates all the disadvantages of previous methods and is specific for ammonia in the presence of amines. Roch-Ramel[85] and Rubin and Knott[86] described fluorometric methods for ammonia using this reaction sequence and following the disappearance of the fluorescent NADH ($\lambda_{ex} = 340$nm, $\lambda_{em} = 460$ nm). From 4×10^{-11} to 2×10^{-10} equivalents of NH_4^+ were determinable.

Schulz, Passonneau, and Lowry[87] have described a fluorometric enzymic method for the measurement of inorganic phosphate based on the following enzyme sequence.

TABLE 13
Assay of a Mixture of Methanol, Xylose, and Sorbitol (Methanol assayed with alcohol oxidase, xylose with carbohydrate oxidase, sorbitol with sorbitol dehydrogenase)

Methanol[a]		Xylose[a]		Sorbitol[a]	
Added	Found[b]	Added	Found[b]	Added	Found[b]
10.3	10.3	226.7	226.7	17.2	17.2
15.6	15.8	230.0	232.1	36.3	36.0
25.1	25.1	245.2	245.2	29.6	30.2
33.2	33.8	210.5	218.0	16.8	16.8
Av. Error	1.5%		1%		1.4%

[a] Concentration in μg/ml of total solution.

[b] Average of three or more results with a precision of ± 1.5%.

$$\text{Glycogen} + \text{phosphate} \xrightarrow{\text{Phosphorylase a}} \text{Glucose-1-phosphate}$$

$$\text{Glucose-1-phosphate} \xrightarrow{\text{Phosphoglucomutase}} \text{Glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{dehydrogenase}} \text{6-Phosphogluconolactone} + \text{NADPH}$$

NADPH is fluorescent, and the rate of its formation indicates the phosphate present at concentrations of $0.02-5 \times 10^{-10}$ mole. Faway, Roth, and Faway[88] assayed inorganic phosphate in tissue and serum using this same reaction sequence except for a spectrophotometric measurement of NADPH.

IV. DETERMINATION OF ACTIVATORS

An enzyme activator is a substance which is required for an enzyme to be an active catalyst: E(inactive) + Activator $\rightleftharpoons$ E(active). The activity of the enzyme will increase until enough activator has been used to fully activate the enzyme. The initial rate of the enzyme reaction is proportional to the activator concentration at low concentrations, thus providing a method for its determination. Very little has been done in the analytical determination of activators. A method for magnesium in plasma is described by Baum and Czok,[89] based on the activation of isocitric dehydrogenase. With constant amounts of enzyme, the rate is dependent on magnesium concentration down to $10^{-6}M$. A thorough study of this reaction was made by Adler, Gunther, and Plass,[90] and by Blaedel and Hicks,[91] who found that only Mg^{2+} and Mn^{2+} efficiently activate this enzyme. The useful analytical range extends up to about 100 parts per billion for Mn^{2+} and $2 \times 10^{-4}M$ for Mg^{2+}; Hg^{2+} and Ag^{+} at $10^{-5}M$, and Ca^{+2} at 10^{-4} M inhibited the Mn^{+2} activation completely.

A number of enzymes require for their activity a specific coenzyme which participates in the enzymic reaction. By measuring the amount of activation of such an enzyme by the coenzyme, a plot of initial rate of reaction against coenzyme concentration may be constructed. At low concentrations of coenzyme, the degree of activation will be proportional to the concentration of coenzyme added. For example, flavine adenine dinucleotide (FAD), which is the coenzyme of D-amino acid oxidase, can be determined by this method:

$$\text{D-Alanine} + \text{FAD} \xrightarrow{\text{D-Amino acid oxidase}} \text{Pyruvic acid} + \text{FADH} + NH_3$$

$$\text{FADH} + O_2 \longrightarrow \text{FAD} + H_2O_2$$

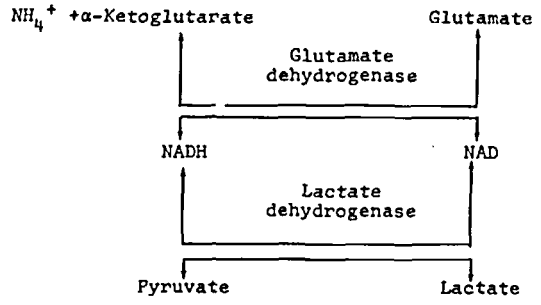
Warburg and Christian[92] and Straub[93] were the first to describe this method. Unknown samples of FAD containing 0 to 0.3 μg of FAD can be determined by measuring the increase in the rate of oxidation of alanine by D-amino acid oxidase.[94]

The firefly reaction has been shown to require ATP and Mg^{+2} in addition to luciferin, luciferase, and oxygen:[95]

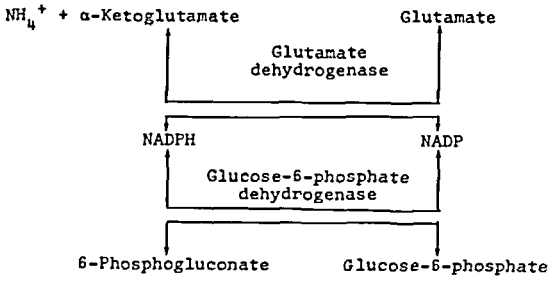
$$\text{Luciferin} + O_2 + \text{ATP} \xrightarrow[Mg^{+2}]{\text{Luciferase}} \text{Oxyluciferin} + \text{ADP} + \text{Pi}$$

This reaction has been used as the basis for the most sensitive method for ATP that is know.[96] This reaction may also be used to assay oxygen at partial pressures below 10^{-3} mm,[97] when the gas is passed through a bacterial emulsion containing all requirements for the luminescent reaction except oxygen.

Recently, Lowry[98] described an enzymatic cycling method for the measurement of pyridine nucleotides. The nucleotide to be determined is made to catalyze an enzymatic reaction between two substrates, which are transformed in amounts far greater (10^3—to 10^4-fold) than the nucleotide. NAD is determined by its catalysis of the dismutation of α-ketoglutarate, NH_4^+, and lactate to glutamate and pyruvate. The pyruvate produced is measured in a second step with lactic dehydrogenase and an added excess of NADH.



In the determination of NADP, glucose-6-phosphate and 6-phosphogluconate dehydrogenase are used instead of lactate and lactic dehydrogenase. As little as 10^{-15} mole of NAD or NADP can be determined by repeating the cycling process with an overall yield of 10^7 to 10^8.

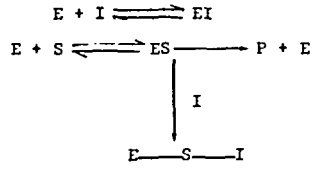


This theoretically is sufficient to measure one molecule of any enzyme which forms a product that can lead to a pyridine nucleotide system.

V. DETERMINATION OF INHIBITORS

A. General

An inhibitor is a compound that causes a decrease in the rate of enzyme reaction, either by reacting with the enzyme to form an enzyme-inhibitor complex or by reacting with the enzyme-substrate intermediate to form a complex:



In general, the initial rate of an enzymic reaction will decrease with increasing inhibitor concentration, linearly at low inhibitor concentrations, then will gradually approach zero.

Analytical working curves for inhibitor assay are generally constructed by plotting % inhibition against concentration of inhibitor. The % inhibition is calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{Rate})_{\text{No Inhibitor}} - (\text{Rate})_{\text{Inhibitor}}}{(\text{Rate})_{\text{No Inhibitor}}} \times 100$$

A control rate is recorded with no inhibitor present but with the same volume of the solvent used to contain the inhibitor added. This is especially critical in studies of inhibitors added in nonaqueous solution since most nonaqueous solvents will inhibit the enzyme in concentrations greater than 3%. In addition to the control (non-inhibited) rate, the rate of spontaneous (non-enzymic) hydrolysis or oxidation of the substrate should be measured and all rates corrected for such non-enzymic effects, if necessary.

Generally, a plot of % inhibition against concentration is a typical exponential type curve with a linear range extending from 0 to 60 or 70% inhibition. This linear region is the most analytically useful range. The concentration (M) of inhibitor that causes a 50% inhibition of enzymic activity is the I_{50} and is a measure of the strength of an inhibitor.

B. Assay of Inorganic Substances

Enzymic methods have been described for the determinations of many common inorganic cations and anions: Ag^+, Al^{3+}, Be^{2+}, Bi^{3+}, Ce^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, In^{3+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, CN^-, $Cr_2O_7^=$, F^-, and $S^=$. A complete listing of these procedures was compiled by Guilbault.[7]

C. Assay of Pesticides

One of the most interesting newer aspects of enzymic methods of analysis has been the selective assay of pesticides.

Several analytical methods have been proposed based on the inhibition of an enzyme reaction. Keller[99] has reported a fairly specific method for the determination of microgram concentrations of DDT, based on the inhibition of carbonic anhydrase. DDT inhibits this enzyme at concentrations at which other inhibitors (except the sulfonami-

des) are inactive. The most specific and sensitive method for some organophosphorus compounds is based on the inhibition of cholinesterase, and numerous papers have been published on this system. Kitz[100] has published a review with 158 references on the chemistry of anticholinesterase compounds. Giang and Hall[101] assayed TEPP, paraoxan, and other insecticides that inhibit cholinesterase in vitro, and Kramer and Gamson[102] have developed a colorimetric procedure with compounds related to the indophenyl acetates for the determination of 1 to 10 μg of various organophosphorus compounds.

Guilbault, Kramer, and Cannon[21] described an electrochemical method for the analysis of Sarin, Systox, parathion, and malathion. The decrease in the rate of the cholinesterase catalyzed hydrolysis of butyrylthiocholine iodide, as measured by dual polarized platinum indicator electrodes, is linearly related to concentrations of the organophosphorus compounds.

Guilbault and Sadar[103] have developed sensitive methods, based on the use of enzyme systems for the determinations of chlorinated insecticides, carbamate insecticides, and herbicides. Fluorescence methods were used because whenever these have been tried they have been shown to allow the determination of much lower concentrations of enzymic inhibitors (for lower enzymic activities can be measured). The substrate 4-methylumbelliferone heptanoate was used; it is cleaved by as little as 10^{-5} unit of lipase to 4-methylumbelliferone. The chlorinated insecticides, aldrin, heptachlor, lindane, and DDT, the carbamate Sevin, and the herbicide 2,4-D, were found to inhibit lipolytic activity and could be determined in the concentration range 0.1 - 30 μg/ml.

The results reported indicate that the method of lipase inhibition is the most sensitive enzymatic method yet reported for heptachlor, aldrin, lindane, and 2,4-D. The proposed inhibition scheme is likewise a good one for DDT. Although carbonic anhydrase is inhibited by lower concentrations of DDT[99] the lipase procedure described is an easier and more convenient one to carry out.

A fluorometric procedure for the assay of aldrin, heptachlor, and methyl parathion was developed by Guilbault, Sadar, and Zimmer.[104] The method was based on the inhibition of acid and alkaline phosphatase by these pesticides.

The use of cholinesterase for the detection of pesticides has received the most interest from analysts. Reviews on cholinesterase and cholinesterase inhibitors have been prepared by Cohen et al.[105] and Delga and Foulhoux.[106]

Matousek, Fischer, and Cerman[107] proposed a highly sensitive method for the determination of organophosphorus compounds by their inhibition of cholinesterase; indoxyl acetate was used as substrate. Butygin et al.[108] evaluated the toxicity of Sevin by its effect on cholinesterase activity in blood, and Davis and Maloney[109] used anticholinesterase inhibition to study water polluted by organic pesticides. Zweig and Devine[110] analyzed 25 pesticides and their residues in water by an enzymic cholinesterase method.

Abou-Donia and Menzel[111] studied the inhibition of fish brain cholinesterase by carbamates and developed an automatic method for the assay of this pesticide. Winteringham and Fowler,[112] in a survey of the carbamate inhibition of acetylcholinesterase, suggested that the Sevin inhibition is caused by an enzymic destruction of the carbamates.

Weiss and Galstatter detected pesticides in water by the inhibition of brain cholinesterase in fish. Bluegill sunfish were found to be most sensitive.[113] Matousek and Cerman[114] have reported a highly sensitive and simple method for detecting cholinesterase inhibitors. Paper impregnated with butyrylthiocholine iodide and bromthymol blue as a pH indicator were used. The presence of an inhibitor was indicated by the inability of the cholinesterase used to effect the hydrolysis of the substrate.

Beyman and Stoydin[115] reported a rapid screening test for cholinesterase inhibition by pesticides making use of agar-agar plates. As little as 10^{-3} μg of DDVP (Vapona) and other inhibitors could be detected. Several workers described the automation of cholinesterase-inhibition determinations using the AutoAnalyzer. Voss[116] and Ott and Gunther[117] proposed procedures that required prior extraction and cleaning, and Ott and Gunther in a later publication[118] used the spots scraped off a TLC plate as input sample for the Auto-Analyzer. A test for the detection of organophosphorus pesticides on TLC plates, using cholinesterase and either 2-azobenzene-1-naphthyl acetate or indoxyl acetate, was described by Ortloff and Franz.[119] Ackerman[120] used silica-gel TLC plates for the semi-quantitative determination of organophosphorus and carbamate pesticides.

A thin-layer chromatographic enzymic inhibition procedure for pesticides in plant extracts without elaborate clean-up was described by Mendoza et al.,[121] and Mendoza, Wales, McLeod, and McKinley[122] evaluated indoxyl, substituted indoxyl, and naphthyl acetates as substrates for esterases in a thin-layer enzymic detection system. Wales, McLeod, and McKinley[123] extended these procedures of Mendoza et al.[121, 122] to the separation and detection of carbamates. Winterlin, Walker, and Frank[124] described a thin-layer chromatographic procedure for the separation and detection of 17 anticholinesterase pesticides on alumina and silica gel, and a thin-layer enzymic detection scheme for insecticides was outlined by Ackerman.[125]

Because various animals and insects are known to be adversely affected by low concentrations of different pesticides, cholinesterases from these sources have been isolated and used for assay of pesticides. Guilbault, Kuan, and Sadar[126] purified cholinesterases from honey bees and boll weevils and studied the effect of 12 different pesticides on these enzymes. Boll weevil was used for the specific assay of Vapona (DDVP) and bee cholinesterase for Vapona and Paraoxan. Zhuravskaya and Bobyreva[127] found the esterase of the cotton aphid was most sensitive to methylmercaptophos while Laphygma moth was most sensitive to Sevin.

Soliman[128] and Goszezynska and Stycyznska[129] studied the inhibition of housefly head cholinesterase by organophosphorus compounds, and Voss[130] found peacock plasma to be a useful cholinesterase source for the analysis of insecticidal carbamates.

The effect of 12 different pesticides on liver enzymes isolated from rabbit, pigeon, chicken, sheep, and pig was studied by Guilbault, Sadar, and Kuan.[131] The cholinesterases from these sources were found to be inhibited at very low concentrations by the organophosphorus pesticides DDVP, parathion, and methyl parathion. None of these enzymes was inhibited by any of the chlorinated pesticides, and pigeon and sheep liver cholinesterase were not inhibited by Sevin.

Guilbault et al.[132] studied the effect of 15 different pesticides including carbamates, chlorinated hydrocarbons, and organophosphorus compounds on cholinesterases from insects such as the housefly, sugar boll weevil, fire ant, and German cockroach. Some of the results obtained are shown in Table 14.

Fire ant cholinesterase is specifically inhibited by DDVP and methyl parathion. Even parathion had little effect on this enzyme. None of the other twelve inhibitors – Paraoxan, Aldrin, Captan, Dalapon, DDT, Dieldrin, 2,4-D-Acid, Heptachlor, Lindane, Mirex, Methoxychlor, and Sevin – inhibits it at all. Cholinesterase from both types of housefly (NAIDM and DDT-resistant strains) are specific for DDVP and Sevin, and others have little or no effect up to $10^{-3}M$ concentration of the inhibitor. Although not as sensitive as other cholinesterases, the sugar boll weevil is totally specific for DDVP, below $10^{-6}M$ concentration. Parathion interferes at concentrations greater than $10^{-3}M$; although paraoxan was not tried, it is expected to interfere also. The enzyme from American cockroaches is the most sensitive of all but lacks specificity. Although it is most sensitive to DDVP, Paraoxan, Parathion, and Methyl Parathion, a good number of chlorinated insecticides, carbamates, and herbicides inhibit it also.

From the results of this study it can be concluded that in addition to the sensitivity – see I_{50} chart (Table 14) – a further specificity and selectivity in the assay of pesticides using enzymic methods has been achieved.

Guilbault et al.[133] have discussed a specific enzymic method for the assay of chlorinated pesticides in the presence of herbicides, organophosphorus pesticides, carbamates, and fungicides. The method is based on the selective inhibition of hexokinase by aldrin, chlorodane, DDT, and heptachlor (Table 15). Parts per million concentrations of these four pesticides have been assayed in the presence of large amounts of other chlorinated pesticides (DDD, DDE, dieldrin, lindane, methoxychlor, etc.), as well as other types of pesticides, with an accuracy of about 2%.

VI. IMMOBILIZED ENZYME

A. General

One of the primary objections to the use of enzymes in chemical analysis is the high cost of these materials. A continuous or semicontinuous routine analysis using enzymes would require large amounts of these materials, quantities greater than can be reasonably supplied, and quantities that would represent a prohibitive expenditure in many cases. If, however, the enzyme could be prepared in an immobilized (insolubilized) form without

TABLE 14

I_{50} Values for Various Cholinesterases

Enzyme Source	Paraoxan	Parathion	Methyl Parathion	DDVP	Sevin	Aldrin	Heptachlor
Housefly (DDT Resist)	x	–	–	$6.3 \times 10^{-8} M$	$8.2 \times 10^{-7} M$	–	–
Housefly (NAIDM)	x	–	–	$6.5 \times 10^{-8} M$	$8.8 \times 10^{-7} M$	–	–
Sugar Boll Weevil	x	–	–	$5.3 \times 10^{-6} M$	–	–	–
Fire Ant	–	–	$1.5 \times 10^{-6} M$	$9.6 \times 10^{-8} M$	–	–	–
Cockroaches	$3.2 \times 10^{-8} M$	$2.0 \times 10^{-7} M$	$7.3 \times 10^{-7} M$	$4.8 \times 10^{-8} M$	$9.3 \times 10^{-7} M$	$5 \times 10^{-6} M$	$6.0 \times 10^{-6} M$

– indicates that the I_{50} is greater than $1 \times 10^{-3} M$ or that inhibition does not reach 50%.

x not investigated.

TABLE 15

Inhibition of Hexokinase by Pesticides

Pesticide	I_{50}, M
Aldrin	6.8×10^{-6}
Chlordane	1.22×10^{-5}
DDT	1.08×10^{-5}
Heptachlor	1.075×10^{-5}
Dieldrin	x
Lindane	–
2,4-D	x
Paraoxan	–
Parathion	–
DDVP	–
Sevin	–

x Inhibition does not exceed 20 to 30% at high concentrations.

– No inhibition at any concentration.

loss of activity so that one sample could be used continuously for many hours, a considerable advantage would be realized. The immobilized enzyme can be used analytically in much the same way that the soluble enzyme is used, that is, to determine the concentration of a substrate that is acted upon by the enzyme, an inhibitor that inactivates the enzyme, or an activator that provides an acceleration in enzyme activity.

Two major techniques can be used to immobilize an enzyme: (1) the chemical modification of the molecule by the introduction of insolubilizing groups. This technique, resulting in a chemical "tying down" of the enzyme, is in practice sometimes difficult to achieve because the insolubilizing groups can attach across the active site destroying the activity of the enzyme; and (2) the physical entrapment of the enzyme in an inert matrix, such as starch or polyacrylamide gels. Physical entrapment techniques offer advantages of speed and ease of preparation. The major difference between the entrapped and the attached enzymes is that the former is isolated from large molecules which cannot diffuse into its matrix. The attached enzyme may be exposed to molecules of all sizes. Hence, the two types of immobilized enzymes will differ in the form of the kinetics observed and in the kinds of interferences observed. Thus, for the assay of large substrates as proteins with proteolytic enzymes, an attached enzyme must be used and not an entrapped enzyme. Either enzyme could be used for the assay of small substrates such as urea.

A review on the preparation of insoluble enzymes has been prepared by Chibata and Tosa[134] and by Guilbault.[7] The techniques of combining active enzymes with some insoluble carrier with covalent bonds, ionic combination, or physical adsorption are discussed.

B. Physical Entrapment

Vasta and Usdin[135] have shown that cholinesterase could be insolubilized by entrapment in a starch gel. The preparation of immobilized cholinesterase for use in analytical chemistry was described by Bauman, Goodson, Guilbault, and Kramer.[136] The enzyme, immobilized by the use of a starch matrix and placed on a polyurethane foam pad, was found to be stable and active for 12 hours under continuous use. The activity of the enzyme was monitored electrochemically, using two platinum electrodes and an applied current of 2 μA. This immobilized enzyme was used to determine the substrates acetyl- and butyrylthiocholine iodide, both in individual samples and continuously. A fluorometric system for the assay of anticholinesterase compounds using this immobilized cholinesterase was described by Guilbault and Kramer.[137] As long as the enzyme is active a fluorescence is produced because of the hydrolysis of the 2-naphthyl acetate to 2-naphthol. Upon inhibition, the fluorescence drops to a value approaching zero. Hicks and Updike[138] have demonstrated the immobilization of enzyme activity in polyacrylamide gel. The preparation is stable and can be lyopholized and conveniently stored. Several enzyme systems were trapped in the gels, namely, glucose oxidase, catalase, lactic dehydrogenase, amino acid oxidase, glutamic dehydrogenase, and enzyme activity in human serum.

The polyacrylamide enzyme gels were found to show little loss of activity after three months of storage at 0 to 4° C. Lactate dehydrogenase (LDH) lost about 30% of its activity in three months while glucose oxidase (GO) showed no loss in activity. Hydration of a LDH gel caused a loss of all activity in three months at 4° C, whereas a hydrated GO gel exhibited a loss of only 5% at 0° C in three months. The gels are very resistant to flow loss and were used for the assay of the substrates glucose and lactic acid.[138]

To test the stability of the immobilized enzyme

as compared to the soluble enzyme, the activity of a series of identical glucose oxidase columns and glucose oxidase solutions were compared after heating for ten minutes at temperatures of 37 to 70° C. About half of the activity of GO in both gel and solution was destroyed in ten minutes at 60° C and all of the activity of both was lost at 70° C.

Updike and Hicks have coupled the immobilized glucose oxidase system with an electrochemical sensor for the determination of glucose in blood.[139] The oxygen electrode was used to monitor the oxygen uptake:

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} H_2O_2 + \text{Gluconic Acid}$$

The apparatus used to monitor the reaction is indicated in ref. 139. Immobilized glucose oxidase is placed in a miniature chromatographic column, and samples containing glucose to be analyzed are pumped over the column at a rate of 0.4 ml/min with a peristaltic pump. Using an immobilized enzyme and an oxygen electrode as the sensing device, a "reagentless" analyzer was achieved.

Wieland, Determan, and Buennig[140] also prepared insoluble enzymes in polyacrylamide gels. The enzymes, alcohol dehydrogenase, trypsin, and lactate dehydrogenases, were immobilized by physical entrapment using a procedure similar to that described above.

McLaren and Peterson,[141] Nikolaev and Mardashev,[142] and Barnett and Bull[143] have attempted the physical entrapment of the enzymes, asparaginase, ribonuclease, and chymotrypsin by adsorption, absorption, or ion exchange, and enzymes have been encapsulated in semipermeable micro capsules made of synthetic polymers.[144] Bernfeld and Wan[145] used polyacrylamide gels for entrapping enzyme activity.

The lattice entrapment of hexokinase, phosphoglucoisomerase, phosphofructokinase, and aldose in polyacrylamide gel was described by Brown et al.[146] Van Duijn et al.[147] discussed the theoretical and experimental aspects of an enzyme determination in a cytochemical model system of polyacrylamide films containing alkaline phosphatase. Lojda, van der Ploeg, and van Duijn[148] used the phosphates of the Naphthol AS series in the quantitative determination of alkaline and acid phosphatase activities in polyacrylamide membrane model systems.

Guilbault and Das[149] conducted a thorough investigation of various parameters affecting the immobilization of the enzymes cholinesterase and urease. The immobilization of these enzymes in starch gel, polyacrylamide, and silicone rubber was investigated, and the stability of the insolubilized enzymes in storage and in use was reported.

It was found that the optimum method for immobilization of either cholinesterase or urease appears to be by physical entrapment in a polyacrylamide gel. The silicone rubber polymerization is too rough on these enzyme, and about 80% of the activity is lost. The starch gel entrapped enzyme is too weakly held, and much of the enzymic activity is lost due to washing out. The stability of both enzymes in polyacrylamide is longer, both on storage dry and wet between use, than the corresponding starch gel pads.

However, the preparation of the starch gel pad is simpler, and very little enzyme is lost during preparation. Some enzyme is lost during the acrylamide polymerization, and the amount lost depends on the experimental conditions of the polymerization (range 10 to 25% loss). Another disadvantage in the use of polyacrylamide lies in the fact that riboflavin and $K_2S_2O_8$ must be used as catalysts for the polymerization. Riboflavin, being fluorescent, interferes in the fluorometric monitoring of the enzyme. One has to wait for the fluorescence baseline to become steady (most of the riboflavin is washed out of the pad) before starting the actual experiments. In cases in which potentiometric measurements are made, $K_2S_2O_8$ should not be used. Consideration of all factors indicates that polyacrylamide is the best of all the three gel materials tried, and starch gel should be used only when long-term use of the pad is not desired.

Tris buffer should be used in all studies for maximum pad stability. The pads may be stored dry for up to 80 days with little appreciable loss of activity. The urease pads can be used for up to 80 days even when used and stored wet. The cholinesterase pads can be reproducibly used for about 40 hours when stored wet between use.

C. Chemical Bonding of Enzymes to Organic and Inorganic Matrices

1. Organic Matrices

a. General

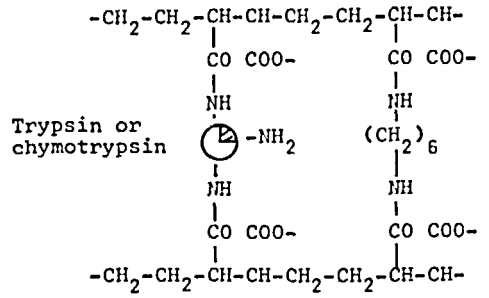
Wilson[150] attached lactate dehydrogenase to anion exchange cellulose sheets; the stability and kinetic properties of the immobilized enzyme were reported. Enzymes have been diazotized to cellulose particles[151] and to polyaminostyrene beads.[152] Enzymes have also been immobilized on polytyrosyl polypeptides,[153] on a colloidin matrix[154] and encapsulated in semipermeable micro capsules made of synthetic polymers.[155] Habeeb[156] manufactured water-insoluble derivatives of trypsin using glutaraldehyde to conjugate trypsin to aminoethyl cellulose. Weetall and Weliky[157] have described the synthesis and continual operation of a carboxymethylcellulose enzyme column and the manufacture of a similar enzyme paper preparation which still retains its activity after two months' storage without refrigeration.[158] Reese and Mandels[159] described a method of obtaining an essentially continuous enzyme reaction on a two-phase column utilizing partition chromatography. The enzyme was retained as the stationary phase on a column of the hydrophilic solid, cellulose. Enzymes immobilized by binding to carboxymethyl cellulose are available from Serevac (Maidenhead, England).

b. Covalent Bonding to Polymeric Lattices

Scientists of the Department of Biophysics of the Weismann Institute (Reharoth, Israel) have pioneered in the preparation of enzymes insolubilized by covalent bonding to polymeric lattices. These modified enzymes retain significant fractions of their native activities while, according to initial studies, certain other properties have in fact been altered.[160-162] Three of the insolubilized covalently-bound enzymes (trypsin, chymotrypsin, and papain) are available as lyophilized powders from Miles (Elkhart, Indiana).

i. Insolubilized Trypsin and Chymotrypsin

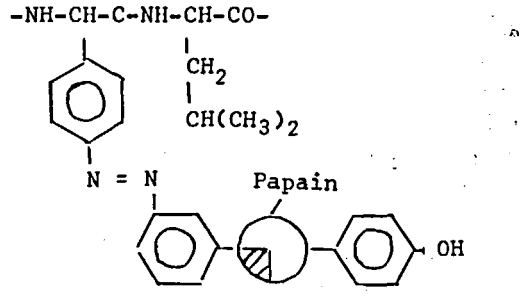
An appropriate amount of trypsin or chymotrypsin is added to a copolymer of maleic anhydride and ethylene, previously cross-linked with hexamethylenediamine to decrease its water solubility. The reaction occurs in buffer solution overnight at 4° C.



By altering the ratio of enzyme to carrier, derivatives of differing characteristics are produced.[160]

ii. Insolubilized Papain

The form provided is prepared by coupling native papain to a water-soluble diazonium salt derived from a copolymer of *p*-amino-DL-phenylalanine and L-leucine, the reaction occurring at 4° C over a 20-hour period.[162,163] The product is a stable water-insoluble papain derivative retaining up to 70% of the original papain activity on low molecular weight substrates and up to 30% on high molecular weight substrates:



This insolubilized papain preparation has been used to study the structure of rabbit γ-globulin. Because this product is active in the hydrolysis of protein in the absence of added reducing agents, it is possible to differentiate protein fragments produced by proteolysis from those produced by reduction.[163]

Katchalski[164] prepared water insoluble derivatives of papain by adsorption of papain chemical derivatives on a collodion column. The acetyl-, succinyl-, poly-L-ornithyl-, poly-γ-benzyl-L-glutamyl-, water-insoluble (maleic acid-ethylene)-, and (4-amino-biphenyl-4 -N -aminoethyl)-starch-papain derivatives were prepared for investigation.

2. Inorganic Matrices

Weetall and Hersh have described procedures for the covalent coupling of enzymes to inorganic

materials with the aid of an intermediate coupling agent.[165-169] Inorganic carriers are not subject to microbial attack, they do not change configuration over an extensive pH range or under various solvent conditions, and with their greater rigidity, they immobilize enzymes to a greater degree than do organic polymers.

Alkaline phosphatase,[165] urease,[166] trypsin, and papain[167] were covalently coupled to porous 96% silica glass with a silane coupling agent. The glass (100 mesh particle size containing pores of 79 nm diameter) was cleaned and then coupled to α-amino-propyltriethoxysilane in toluene solution. The amino alkyl group was then converted to an aminoaryl group by coupling p-nitrobenzoic acid. This was reduced, diazotized, and added to a solution containing the enzyme. The product was washed and stored at 4° C.

The alkaline phosphatase immobilized on glass contained the equivalent of 0.74 mg of active enzyme per g of glass,[165] the urease 1.0 mg per g of glass,[166] and the trypsin 0.12 to 25 mg per g of glass.[167] All products were used continuously in a column for long periods of time with no loss of activity.

In a later paper Weetall and Hersh[168] insolubilized glucose oxidase by covalently binding the enzyme to NiO on a Ni screen through a silane coupling agent. The stability of the chemically bonded enzyme appears to have been increased over that of the soluble enzyme in the range of 10 to 40° C. No change in the optimum pH and only a slight change in K_m were observed with the insolubilized glucose oxidase. This would indicate that no charge-charge interaction is involved.

In still another application of bound enzymes, Hersh, Weetall, and Brown[169] ionically bound heparin to glass. The heparin coating resisted fluid shear stresses as high as 10^4 dynes/cm^2 at 30° C for 300 hours.

D. The Enzyme Electrode

One of the most interesting uses of the immobilized enzyme has been as the active element of an electrochemical probe or sensor. Such enzyme electrodes possess the properties of the enzyme, namely specificity and sensitivity, and are adaptable to automation. The first enzyme electrode was described by Updike and Hicks.[170]

The electrode was a miniature chemical transducer which is prepared by polymerizing a gelatinous membrane over a polarographic oxygen electrode. When the enzyme electrode is placed in contact with a biological solution or tissue, glucose and oxygen diffuse into the gel layer of immobilized enzyme. The rate of diffusion of oxygen through the plastic membrane to the electrode is reduced in the presence of glucose and glucose oxidase by the enzyme-catalyzed oxidation of glucose.

When the glucose concentration is well below the K_m for insolubilized glucose oxidase and the oxygen is in non-rate-limiting excess, there is a linear relationship between the reduction in oxygen content and the glucose concentration. Calibration curves of electrode response against glucose concentration are prepared, and from these the amount of glucose present in whole blood or plasma can be calculated.[170]

Guilbault and Montalvo[74] have prepared a urea electrode by polymerizing urease in a polyacrylamide matrix on 100-micron Dacron and nylon nets. These nets were placed over the Beckman 39137 cation-selective electrode (which responds to NH_4^+ ion). The resulting "enzyme" electrode responds only to urea. The urea diffuses to the urease membrane where it is hydrolyzed to NH_4^+ ion. This NH_4^+ ion is monitored by the ammonium ion-selective electrode, the potential observed being proportional to the urea content of the sample in the range 1.0 to 30 mg or urea/100 ml of solution. This enzyme electrode appears to possess stability (the same electrode has been used for weeks with little change in potential readings or drift), sensitivity (as little as 10^{-4} *M* urea is determinable), and specificity. Results are available to the analyst in less than 100 seconds after initiation of the test, and the electrode can be used for individual samples or in continuous operation.

In a later publication, Guilbault and Montalvo[75] described an improved urea-specific enzyme electrode that was prepared by placing a thin film of cellophane around the enzyme gel layer to prevent leaching of urease into the surrounding solution. The electrode could be used continuously for 21 days with no loss of activity. A full discussion of the parameters that affect the polymerization of urease as well as of the stability of four types of urease electrodes was published by Guilbault and Montalvo.[76]

Guilbault and Montalvo[171] described the preparation of a sensitized cation-selective electrode. By placing a film of urease over the outside of an ordinary cation-selective glass electrode these

workers obtained an electrode with increased sensitivity.

Williams, Doig, and Korosi[172] prepared electrodes for glucose and lactate by entrapping the enzymes, glucose oxidase and lactate dehydrogenase, respectively, between an electrochemical sensor (a platinum electrode) and a dialysis membrane.

E. Commercial Availability of Immobilized Enzymes

It is likely that immobilization methods will soon be developed for all enzymes. Several companies (Miles, Mann, and Serevac) already offer some immobilized enzymes and many more will probably be commercially available soon. Polysciences (Harrington, Pa.) supplies polymer substrates for enzyme immobilization as described by Katchalski and co-workers. Corning Glass Works has offered to supply over 60 different enzymes covalently bound to glass.

As the commercial availability of immobilized enzymes increases, so will the number of analytical applications.

F. Future Applications

The immobilized enzyme will likely bring a new future to enzymic analysis and to biochemistry in general. Enzyme electrodes (transducers with immobilized enzymes) would allow simple, direct, continuous in vivo analysis of important body chemicals. A glucose electrode, for example, would permit a continuous analysis of blood glucose levels in patients or the analysis of glucose in blood or urine samples in a hospital or clinical laboratory in as simple a manner as a pH measurement. Similarly, implanted transducers using immobilized enzymes could be used for patient therapy. The uses of immobilized enzymes in synthesis and therapy would be limitless.

VII. AUTOMATION OF ENZYME REACTIONS

Many of the experimental difficulties of using enzymes in analysis by reaction rate methods could be eliminated or lessened by the use of automation. Ideally, all the steps in an enzymic procedure would be automated: the addition of reagents, the measurement of the reaction rate, and the calculation of results. Excellent reviews have been written by Schwartz and Bodansky,[173] Blaedel and Hicks,[174] and Guilbault.[7]

In order to automate an enzyme procedure, the rate of reaction must generally be calculated. From this the amount of substance being analyzed in solution can be determined. To do this, a pseudo-first-order condition is established by adding excess quantities of all reactants except the one to be assayed. The measurement of the initial rate is made, and this is proportional to the concentration of the substance being determined (i.e., A): $A + B \xrightarrow{E} X + Y$.

At excess B and E, $\frac{dX}{dt}$ k(A)

where (A) represents the concentration of A at time t and k is a pseudo-first-order rate constant with magnitude depending upon many factors including pH, temperature, enzyme activity, and concentrations of B and E.

The Analytical Instrument Division of American Optical Corp. (Richmond, California) markets an instrument that completely automates routine enzymic analysis procedures. The instrument, the Robot Chemist, can handle 120 samples per hour and carries out all the steps of the enzymic procedure: addition of sample and reagent, mixing, incubation of the reaction, spectrophotometric measurement, calculation of results, and direct printout. The instrument uses the fixed time procedure, measuring the absorbance change resulting from the enzymic reaction, which is then proportional to the enzyme or substrate to be assayed.

Bausch and Lomb markets an instrument for unattended automatic enzymic analysis. The Zymat 340 does all the pipetting, measuring, mixing, stirring, and heat controlling normally done by the laboratory technician. As many as 47 samples can be handled in each loading, and highly precise answers are printed out directly in International Enzyme Units with identifying serial numbers. The instrument is intended for determinations of lactate dehydrogenase (LDH), glutamate oxalate transaminase (SGOT), and glutamate pyruvate transaminase (SGPT) but should be adaptable for other determinations.[175] The instrument uses a spectrophotometric monitoring of the enzymic reaction.

The DuPont Co. (Instrument Products Division, Wilmington) has developed an instrument, the Automatic Clinical Analyzer (ACA), that is de-

signed to reduce to a minimum the time between sampling and transmittal of precise laboratory data. A separate pack is provided for each test performed on a sample in an ACA. Each pack contains both the test name for convenient operator identification and binary code to instruct the instrument. The technician programs the analyzer by inserting the appropriate pack or packs behind each sample cup in the ACA input tray.

The analyzer automatically injects the exact amount of sample and diluent into each pack in succession, mixes the reagents, waits a preset amount of time, forms a precise optical cell within the transparent pack walls, and measures the reaction photometrically. These operations are controlled and monitored by a built-in solid-state special-purpose computer and are performed under precisely regulated conditions within the instrument. The computer calculates the concentration value for each test and prints out the results on a separate report sheet for each sample. This report contains all the test results on that sample along with the patient identification. The used test packs are automatically discarded into a waste container.

The instrument has a coefficient of variation of 1 to 3%, and the first result is obtained in less than seven minutes after sample injection. In continuous operation successive test results are obtained every 35 to 70 seconds.

Some of the enzyme tests for which the ACA is programmed include glucose (using glucose oxidaseperoxidase), urea (with urease and glutamate dehydrogenase), alkaline phosphatase (with p-nitrophenyl-phosphate), pseudocholinesterase, lactate dehydrogenase, hydroxybutyric dehydrogenase, and aspartate aminotransferase.

The assay of urea nitrogen (BUN) illustrates the test operation. Urease specifically hydrolyzes urea to form ammonia and carbon dioxide. This ammonia is utilized by the enzyme glutamic dehydrogenase (GDH) to aminate α-keto-glutarate. Since NADH is required for the amination, the reaction rate is measured by observing its decrease in absorbance at 340 nm.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 - + \text{NADH} + H^+$$

$$+ \alpha\text{-ketoglutarate} \xrightarrow{\text{GDH}} \text{NAD} + \text{L-glutamate}$$

DuPont expects to start marketing the ACA following additional field evaluations and estimates the price to be $65,000.

Another continuous system that carries out all the manipulations is the AutoAnalyzer, originally described by Skeggs[176] and available from Technicon Co., Inc. (Tarrytown, New York). The AutoAnalyzer uses continuously flowing streams metered proportionally by a single multichannel peristaltic pump and uses either a colorimetric or fluorometric readout of concentration changes at a fixed time. In Auto-Analysis chemical reactions take place in continuously flowing air-segmented systems.

Briefly, operation is as follows: the samples to be analyzed are loaded into the cups on the sampler, and a multiple-channel proportioning pump, operating continuously, moves the samples, one following another, and a number of streams of reagents, into the system. Sample and reagents are brought together under controlled conditions, causing a chemical reaction and color development. Color intensity of the analytical stream is measured in a colorimeter, and the results of any analysis are presented as a series of peaks on a recorder chart.

Fundamental to AutoAnalyzer techniques is the exposure of a known standard to exactly the same reaction steps as the unknown samples. The concentrations of the unknowns are continuously plotted against the known concentrations. Hence, reactions need not be carried to completion as in conventional procedures.

A variety of analyses can be performed with the AutoAnalyzer using detection methods that include colorimetry, spectrophotometry, flame photometry, fluorometry, and atomic absorption spectrometry. A bibliography of 1825 papers describing automated analysis with the AutoAnalyzer in the last 10 years is available from Technicon.[177] Guilbault in his review articles on the Use of Enzymes in Analytical Chemistry[15] has likewise listed many enzymic analyses that can easily be performed using the AutoAnalyzer. Typical analyses include: acid and alkaline phosphatase, amylase, cholinesterase, glucose oxidase, LDH, SGOT, SGPT, lactic acid, glucose, uric acid, and triglycerides, to name but a few.

As methods have become available which take advantage of the inherent high sensitivity and specificity of fluorometry, researchers have adapted these to the AutoAnalyzer. In a number of recent applications the fluorescence of NADH,

when excited at 340 nm, provides a common method for the measurement of all enzyme systems that involve NAD or NADP. Methods have been developed for the enzymes LDH,[178] SGOT, and SGPT[179] using a fluorometric readout. Technicon has developed a new, highly sensitive, very stable fluorometer for use with these new methods. Built to form an integral part of automated continuous flow systems, the fluorometric AutoAnalyzer can handle up to 60 samples per hour.

Aminco and Turner Instrument Companies also market fluorometers that can be used with the AutoAnalyzer in flow systems.

Another recent development in automation is a multichannel analyzer developed by Skeggs[180] which determines 12 substances in a single 2-ml sample of blood. Technicon has marketed this concept in the SMA 12/60 Analyzer which can run 60 such samples per hour. The time from aspiration of a given sample to finished chart is only nine minutes. Results are automatically recorded on a precalibrated strip chart, the Serum Chemistry Graph. The final product is a comprehensive chemical profile of each patient available quickly and at less cost than the few tests he now receives. The enzymes SGOT, alkaline phosphatase, and LDH, and glucose and urea are determined by enzymic methods, in addition to other important biochemical substances determined non-enzymatically.

The final step in automating the clinical lab is represented by the Technicon On Line computer system. It will monitor, calculate, store, and report results, printed out in concentration units with associated sample identification numbers from any combination of AutoAnalyzer and/or other instrument systems. This unit will be marketed by Technicon in the near future.

In this section an attempt has been made to briefly review what has been done recently in the automation of enzymic analysis. In the next decade an upsurge in attempts towards complete automation will undoubtedly result. Many new instruments will be marketed, and the present instruments will unquestionably be improved. The clinical or hospital lab of the 1970s will be one that routinely performs many enzymic analyses, all with complete automation and with computer assisted storage and evaluation of data.

VIII. CONCLUSIONS

Enzymes possess a great potential usefulness in analytical chemistry. The specificity of enzymes can solve the primary problem of most analytical chemists, the analysis of one substance in the presence of many similar compounds that interfere in the analysis. The sensitivity of enzymes allows the determination of as little as 10^{-10} g of material. With the advent of new techniques, electrochemical and fluorometric, many of the previous difficulties of enzymic analysis have been eliminated. The advent of the immobilized enzyme has alleviated the problem of the cost and supply of enzymes. Finally, significant progress has been made in the direction of complete automation of enzymic methods for rapid, accurate analysis.

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