cannot be correct, because many tumor cells have good respiration and oxidative phosphorylation. Subsequently the phenomenon was pushed aside and virtually no attempts were made to elucidate its mechanism. This negative posture was supported by observations that some normal cells, e.g. retinal cells, glycolyze rapidly under aerobic conditions. Yet high aerobic glycolysis is still the most generally accepted property shared by all rapidly growing cancer cells.

What is the reason for this rapid rate of fermentation? Our systematic studies over the past 20 years have revealed that a membrane lesion appears to be responsible. In some tumor cells the lesion is in the plasma membrane and affects the Na^+K^+-ATPase which generates an excessive amount of ADP and P_i. This, in turn, increases glycolysis because the regeneration of these cofactors of glycolysis is a rate-limiting step in the process of lactic acid formation. In some other tumor cells the lesion is in the mitochondrial membrane leading to an increased ATPase activity. In a third group of tumor cells there is also an increased rate of ATP hydrolysis, but the responsible catalyst has not been identified. We are attempting to analyze the lesion of the plasma membrane which appears to render the operation of the Na^+K^+ pump less efficient.

We are also trying to characterize the transport system which controls the excretion of lactate together with protons. What would happen to cells in which this transport is blocked by a specific inhibitor? Would they drown in their own acid? We have synthesized some reactive analogues of lactic acid and find that some interfere with lactate transport and some inhibit growth of cells in vitro and in vivo. However, thus far there seems to be no correlation between the effects on transport and on growth. We are intrigued and stimulated, and we continue the search for a solution to this puzzle.

The work carried out in our laboratory described in this review was supported by Grant No. CA-08964 and CA-14454 from the National Cancer Institute, Grant No. BC-156 from the American Cancer Society, and Grant No. BMS-75-17887 from the National Science Foundation.

Preparation and Analytical Uses of Immobilized Enzymes

George G. Guilbault*

Department of Chemistry, University of New Orleans, New Orleans, Louisiana 70122

Muhammed H. Sadar

Bureau of Drug Surveillance, Health Protection Branch, Health and Welfare Canada, Vanier, Ontario, Canada K1A0L2

Received August 18, 1978

Enzymes are polypeptides which serve as catalysts for chemical reactions in biological systems. These catalysts are usually very efficient, and are capable of increasing the rate of many complex chemical reactions, upon which depends the very existence of life as we know it, by several orders of magnitude at or near room temperature and at atmospheric pressure. Furthermore, enzymes are often very specific, and catalyze reactions of substates at low concentrations. Because of their specificity and sensitivity, enzymes continue to enjoy widespread use as analytical tools especially in biochemical and clinical laboratories. During the past few years alone, scores of books and review articles have been published describing the use of enzyme-catalyzed reactions for the trace analysis of substrates, activators, inhibitors, and enzymes themselves.[1-4]

The use of enzymes for analytical purposes, however, has been limited because of certain disadvantages, such as their instability, poor precision, and lack of availability. Moreover, aqueous solutions of enzymes often lose their catalytic ability fairly rapidly, and the enzymes can neither be recovered from such solutions, nor their activity regenerated. These difficulties have now been removed or minimized by the development of enzyme immobilization techniques. The free enzyme is immobilized (insolubilized) by trapping it in an inert matrix such that the immobilized enzyme retains its catalytic properties for a much longer time as compared to the free enzyme and can be used continuously for many more analyses.

The science and technology of immobilized enzymes have experienced phenomenal growth in the recent past. Consequently, there has been a very rapid accumulation of scientific literature describing various aspects of this subject.[5] Also, there are more specific reviews of the

George G. Guilbault is Professor of Chemistry at the University of New Orleans. He was born in New Orleans in 1936, and his undergraduate studies were completed there at Loyola University. After receiving the Ph.D. degree from Princeton University, he spent several years as a research chemist with Procter and Gamble and with the U.S. Army's Edgewood Arsenal. He was recipient of the 1977 ACS Award in Analytical Chemistry sponsored by Fisher Scientific Co.

Muhammed H. Sadar was born in Lahore, Pakistan, in 1939. He received the B.Sc. degree from Middle East Technical University in Ankara, Turkey, and the Ph.D. from the University of Saskatchewan, Regina. Following postdoctoral appointments at the University of Ottawa and the University of Manitoba and a year on the faculty at Middle East Technical University, he is now a research chemist in the Environmental Health Division of Health and Welfare Canada.

(1) G. G. Guilbault, "Enzymic Methods of Analysis", Pergamon Press, Oxford, England, 1970.
(2) G. G. Guilbault, "Handbook of Enzymic Analysis", Marcel Dekker, New York, 1977.
(3) G. G. Guilbault and M. H. Sadar, "Analytical Uses of Enzymes", Proceedings of the Analytical Division of the Chemical Society, London, 1978.
(4) M. M. Fishman, *Anal. Chem.*, **50**, 261R (1978).
(5) K. Mosbach, *Methods Enzymol.*, **44** (1976).

0001-4842/79/0112-0344$01.00/0 © 1979 American Chemical Society

applications of immobilized enzymes in analytical chemistry,[6-11] clinical chemistry,[12] automated analysis,[13,14] urinalysis,[15] and the analytical aspects of immobilized enzyme columns.[16]

There are several ways to immobilize an enzyme,[5,11,17] but most of these techniques fall into two categories: (1) the chemical modification of the polypeptide by binding it with an insoluble molecule and (2) the physical entrapment of the enzyme in an inert matrix such as starch or polyacrylamide gels. Detailed procedures on the preparation of physically entrapped and chemically attached enzymes can be found on p 600 of ref 5. The larger enzyme molecules remain trapped within the layers of the matrix, but relatively much smaller substrate and water molecules are able to diffuse in and out of this enzyme cage. The enzyme, although confined within certain fixed boundaries, is able to perform its catalytic function and can also be taken out of the reaction mixture when not needed.

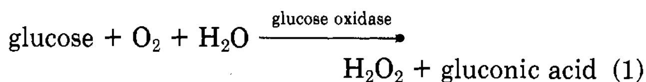
The enzyme, chemically bound to some other species acting as a support, may differ considerably in catalytic and other properties from the native enzyme.[18] Moreover, the insolubilizing groups can attach across the active site in the protein molecule and destroy the catalytic ability of the enzyme derivative.

Physical entrapment techniques, however, generally offer advantages of speed and ease of preparation over many chemical methods. The major difference between the entrapped and the chemically attached enzymes is that the former are isolated from large molecules which cannot diffuse into the matrix but 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. The chemical binding method produces a product that can be used for several thousand assays; the physically entrapped enzyme can only be used for a few hundred such analyses.

Natural and synthetic polymers, such as starch and polyacrylamide gels, are the materials most commonly used as an inert matrix for the physical entrapment of various enzymes. We[19] investigated the various parameters affecting the immobilization of cholinesterase and urease in starch gel, polyacrylamide, and silicone rubber. We found that the silicone rubber polymerization killed 80% of enzyme activity and that the starch gel was too weak to prevent loss of enzymic activity due to leaching. Polyacrylamide was found to be a better inert matrix for both the enzymes. Hicks and Updike[20] reported that lactate dehydrogenase and glucose oxidase, after immobilization in polyacrylamide gel, retained most of their activity up to 3 months. The use of polyurethane[21] and macroporous glasses involving maleimide[22] have been described for the immobilization of the enzymes cholinesterase and trypsin, respectively.

Enzyme electrodes represent the most recent advance in analytical chemistry. These devices combine the selectivity and sensitivity of enzymatic methods of analysis with the speed and simplicity of ion-selective electrode measurements. The result is a device that can be used to determine the concentration of a given compound in solution quickly and a method that requires a minimum of sample preparation. In constructing an enzyme electrode one need only (a) pick an enzyme that reacts with the substance to be determined, (b) obtain that enzyme from commercial sources or isolate it himself, (c) immobilize the enzyme by standard techniques or buy it already immobilized, if possible, and (d) place the immobilized enzyme around the appropriate electrode to monitor the reaction that occurs (note: this will probably be the limiting factor in the construction of an enzyme electrode since steps a–c are always possible).

The first report of an enzyme electrode was given by Clark and Lyons[23] who proposed that glucose could be determined amperometrically using soluble glucose oxidase held between cuprophane membranes. The oxygen uptake was measured using an O_2 electrode (eq 1). The term "enzyme electrode" was introduced by



$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid} \quad (1)$$

Updike and Hicks[24] who coated a layer of physically entrapped glucose oxidase in a polyacrylamide gel. The decrease in oxygen pressure was equivalent to the glucose content in blood and plasma. A response time of less than 1 min was observed. Since then the area has experienced a tremondous growth, and several excellent reviews have been written during the past few years alone.[25-28]

Immobilization Techniques

The selection of the most suitable method of immobilization depends upon the nature of each individual enzyme and the reaction it catalyzes.[5,29,30] The chemical method is a bit more difficult and time

(6) L. D. Bowers and P. W. Carr, *Anal. Chem.*, **48**, 544–559A (1976).
(7) I. V. Berezin and A. A. Klesov, *Zh. Anal. Khim.*, 31, 786 (1976).
(8) D. N. Gray, M. H. Keyes, and B. Watson, *Anal. Chem.*, **49**, 1067A (1977).
(9) D. N. Gray and M. H. Keyes, *CHEMTECH*, **7**, 642 (1977).
(10) A. H. Free, *Ann. Clin. Lab. Sci.*, 7, 679 (1977).
(11) G. G. Guilbault and M. H. Sadar, "Techniques in the Life Sciences Series", Elsevier/North Holland, Amsterdam, The Netherlands, 1978, Chapter 17.
(12) T. Murachi, *Hakko Kogyo*, **35**, 386 (1977).
(13) W. E. Hornby and G. A. Noy, *Methods Enzymol.*, **44** (1976).
(14) J. Campbell and W. E. Hornby in "Biomedical Applications of Immobilized Enzymes and Proteins 2", T. M. S. Chang, Ed., Plenum Press, New York, 1977.
(15) R. C. Boguslaski and R. S. Smith in ref 14.
(16) R. S. Schifreen, D. A. Hann, L. D. Bowers, and P. W. Carr, *Anal. Chem.*, **49**, 1929 (1977).
(17) K. J. Skinner, *Chem. Eng. News*, **53** (Aug 18, 1975).
(18) J. Lasch, L. Bessmertnaya, L. U. Kozlov, and U. K. Antonov, *Eur. J. Biochem.*, **63**, 591 (1976).
(19) G. G. Guilbault and J. Das, *Anal. Biochem.*, **33**, 341 (1970).
(20) S. J. Updike and G. P. Hicks, *Science*, **158**, 270 (1967).
(21) E. K. Bauman, L. H. Goodson, G. G. Guilbault, and D. N. Kramer, *Anal. Chem.*, **37**, 1378 (1965).
(22) J. Fischer, W. Heyer, F. Janowski, F. Wolf, and A. Schellenberger, *Acta Biol. Med. Ger.*, **36**, 999 (1977).
(23) L. C. Clark and C. Lyons, *Ann. N.Y. Acad. Sci.*, **102**, 29 (1962).
(24) S. Updike and G. Hicks, *Nature (London)*, **214**, 986 (1967).
(25) G. G. Guilbault and T. J. Rohm, *Int. J. Environ. Anal. Chem.*, 4, 51 (1975).
(26) I. V. Berezin and A. A. Klesov, *Usp. Khim.*, **45**, 180 (1976).
(27) G. G. Guilbault in ref 14.
(28) K. Lemke, E. M. Flak, H. Haker, and H. Seel, *Z. Med. Laboratoriumsdiazn*, **18**, 80 (1977).
(29) L. Goldstein and E. Katzir-Katachalski, *Appl. Biochem. Bioeng.*, 1, 1–22 (1976).
(30) L. Goldstein and G. Manecke, *Appl. Biochem. Bioeng.*, 1, 23–126 (1976).

consuming, but the products so formed are usually more stable and can be stored at room temperature for up to several months. Both the organic and inorganic matrices can be used to immobilize an enzyme through chemical binding, but in our own experience, the polyacrylamide diazo coupling[31,32] and glutaraldehyde[33,34] methods have yielded very satisfactory results. Wilson[35] 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[36] and to polyaminostyrene beads,[37] and also immobilized on polytyrosyl polypeptides[38] and on a collodion matrix[39] and encapsulated in semipermeable microcapsules made of synthetic polymers.[40] Habeeb[41] synthesized water-insoluble derivatives of trypsin using glutaraldehyde to conjugate trypsin to aminoethylcellulose. Weetall and Weliky[42] have described the synthesis and continual operation of a carboxymethycellulose enzyme column and the manufacture of a similar enzyme paper which still retains its activity after 2-month's storage without refrigeration.[43] Reese and Mandels[44] 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.

Insolubilized papain can be 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 taking place at 4 °C over a 20-h period.[45,46] 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 reduction.[46]

Katchalski[47] prepared water-insoluble derivatives of papain by adsorption of papain chemical derivatives on a collodion column. The acetyl-papain, succinylpoly-(L-ornithyl)-papain, and (4-amino-4′-(*N*-ethylamino)-starch-papain derivatives were prepared for investigation.

Inorganic carriers usually 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,[48] urease,[49] trypsin, and papain[50] were covalently coupled to porous 96% silica glass with a silane coupling agent. The glass (100-mesh particle size containing pores of 790 Å diameter) was cleaned and then coupled to (α-aminopropyl)uriethoxysilane in toluene solution. The aminoalkyl group was then converted to an aminoaryl group by coupling with *p*-nitrobenzoic acid. The product was reduced, diazotized, and added to a solution containing the enzyme. The final product was washed and stored at 4 °C.

The alkaline phosphatase immobilized on glass contained the equivalent of 0.74 mg of active enzyme/g of glass,[48] the urease 1.0 mg/g of glass,[49] and the trypsin 0.12 to 25 mg/g of glass.[50] All products were used continuously in columns for long periods of time with no loss of activity.

In a later paper, Weetall and Hersh[51] 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 the soluble enzyme in the range of 10–40 °C. No change in pH optimum 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 chemically bound enzyme, Hersh et al.[52] ionically bound heparin to glass. This heparin coating resisted fluid shear stresses as high as 10^4 dyn/cm^2 at 30 °C for 300 h.

A large number of enzymes have already been successfully immobilized, and quite a few of them are commercially available for use in biochemical and clinical analyses. For example, several recent reports have described different methods for the quantitative determination of various sugars in complex biological systems using immobilized enzymes.[10,12,53,54] Most of these methods provide a highly desirable dual combination of a higher degree of sensitivity and specificity. Immobilized glucose oxidase has been used for the quantitative determination of glucose in serum,[55] plasma,[56] and blood,[57] for the automation of glucose analysis,[58,59] and for the quantitative determination of fructose in glucose-containing aqueous solution.[60]

A new technique in immobilized enzyme technology is the cross-linking of enzymes to nylon tubes. Such

(31) G. G. Guilbault and G. J. Lubrano, *Anal. Chim. Acta*, **64**, 439 (1973).
(32) G. G. Guilbault and G. J. Lubrano, *Anal. Chim. Acta*, **69**, 183 (1974).
(33) G. G. Guilbault and M. Nanjo, *Anal. Chem.*, **46**, 1769 (1974).
(34) G. G. Guilbault and M. Nanjo, *Anal. Chim. Acta*, **73**, 367 (1974).
(35) C. Wilson, *Anal. Biochem.*, **31**, 506 (1969).
(36) M. A. Mitz and L. J. Summaria, *Nature (London)*, **189**, 576 (1961).
(37) N. Grubhofer and L. Schleith, *Naturwissenschaften*, **40**, 508 (1953).
(38) A. Bar-Eli and E. Katchalski, *J. Biol. Chem.*, **238**, 1690 (1963).
(39) R. Goldman, H. I. Silman, S. Caplan, O. Kadern, and E. Katchalski, *Science*, **150**, 758 (1965).
(40) T. M. Chang, *Science*, **146**, 524 (1964).
(41) A. Habeeb, *Arch. Biochim. Biophys.*, **119**, 264 (1967).
(42) H. H. Weetall and N. Weliky, *Jet Propulsion Lab. Space Programs Summ. No. 37-26*, 4, 160 (1965).
(43) H. H. Weetall and N. Weliky, *Anal. Biochem.*, **14**, 160 (1966).
(44) E. T. Reese and M. Mandels, *J. Am. Chem. Soc.*, **80**, 4625 (1958).
(45) I. H. Silman, M. Albu-Weissenberg, and E. Katchalski, *Biopolymers*, **4**, 441 (1966).
(46) J. J. Cebra, D. Givol, H. I. Silman, and E. Katchalski, *J. Biol. Chem.*, **236**, 1720 (1961).
(47) E. Katchalski, Technical Report, AFOSR Grant 67-2025, June 30, 1967.
(48) H. H. Weetall, *Nature (London)*, **223** (Aug 30, 1969).
(49) H. H. Weetall and L. S. Hersh, *Biochim. Biophys. Acta*, **185**, 464 (1969).
(50) H. H. Weetall, *Science*, **166**, 615 (1969).
(51) H. H. Weetall and L. S. Hersh, *Biochim. Biophys. Acta*, **206**, 54 (1970).
(52) L. S. Hersh, H. H. Weetall, and I. W. Brown, *J. Biomed. Mater. Res.*, **3**, 471 (1969).
(53) H. L. Schmidt, G. Drisam, and G. Grenner, *Biochim. Biophys. Acta*, **429**, 283 (1976).
(54) B. Mattiasson, B. Danielsson, and K. Mosbach, *Anal. Lett.*, **9**, 217 (1976).
(55) B. Danielsson, K. Gadd, B. Mattiasson, and K. Mosbach, *Clin. Chim. Acta*, 81, 163 (1977).
(56) S. W. Kiang, J. W. Kuan, S. S. Kuan, and G. G. Guilbault, *Clin. Chem.* **22**, 1378 (1976).
(57) K. F. O'Driscoll, A. Kapoulas, A. M. Albisser, and R. Gander, *Polym. Prepr., Am. Chem. Soc., Div. Polym. Chem.*, **16**, 372 (1975).
(58) W. E. Hoehne, P. Heitmann, C. Flemming, and A. Reichert, *Z. Med. Labortech.*, **17**, 79 (1976).
(59) J. Campbell, A. S. Chawla, and T. M. S. Chang, *Anal. Biochem.*, **83**, 330 (1977).
(60) R. Fresenius, W. Flemming, and J. Mondani, German Patent 263-0043, 1978.

bound enzymes are quite stable, and have been reused for up to 10 000 assays.[61] The combined use of insolubilized glucose oxidase and peroxidase in a nylon tube has been described for the analysis of glucose.[62] Weibel and Humphrey[63] have reported the determination of serum galactose and milk lactose using similarly immobilized galactose oxidase. Immobilized urease has been used for the analysis of serum and plasma urea in blood.[64,65]

Enzyme thermistors based on specific reversible immobilization of enzymes have been developed for the assay of hydrogen peroxide, penicillin, sucrose, glucose, phenol, and tyrosine[66] and for cyanide ion in the environment.[67]

An immobilized tryptophanase–lactate dehydrogenase coupled system has been used for the assay of tryptophan,[68] and a combination of an ion-selective electrode-immobilized enzyme was applied for the determination of L-asparagine and L-arginine.[69] The combined use of immobilized enzymes and mass spectrometry for biochemical assay of urea has been reported by Weaver et al.[70]

Besides being valuable analytical and clinical reagents, immobilized enzymes are finding many other diversified roles, such as in porphyrin biosynthesis[71] and in neuronal cell dissociation.[72]

Analytical Uses of Immobilized Enzymes

Our first efforts in the analytical uses of immobilized enzymes dates back to the early 1960s, when we demonstrated that horse serum cholinesterase could be successfully entrapped in a starch gel matrix and then used for the assay of organophosphorus pesticides in the environment.[73] A small air-monitoring detector was prepared, using two platinum electrodes to sense the products of the enzymatic reaction, as inhibited by the pesticide. A field instrument based on this concept was designed by L. H. Goodson and E. V. Bauman of Midwest Research Institute, Kansis City, and is still sold today as a water–air pollution monitor.

Enthused by this fantastic success, we then built a system for assay of glucose using glucose oxidase.[74] The H_2O_2 produced enzymatically was detected using a Pt electrode. Similar "enzyme electrodes" were designed for many other substances.

In 1969 we built the first simple, very stable urea electrode by polymerizing urease in a polyacrylamide matrix on 100-μm dacron and nylon nets.[75] These nets were placed over the Beckman 39137 cation selective electrode (which responds to NH_4^+). The resulting "enzyme electrode" responds only to urea. The urea diffuses to the urease membrane where it is hydrolyzed to NH_4^+. This NH_4^+ is monitored by the ammonium ion selective electrode, the potential observed being proportional to the urea content of the sample in the range 1.0–30 mg of urea/100 mL of solution. This enzyme electrode appears to possess excellent 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 s after initiation of the test, and the electrode can be used for individual samples or in continuous operation.

In a later publication, we[76] 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 the stability of four types of urease electrode were discussed.[76]

In subsequent studies we reported the preparation of a sensitized cation-selective electrode.[77] By placing a film of urease over the outside of an ordinary cation-selective glass electrode we obtained an electrode with increased sensitivity. Adams and Carr have developed a flow coulometric detector immobilized enzyme analyzer for urea.[78]

Because sodium and potassium ions interfered with measurements of serum urea we[79] used an uncoated NH_4^+ electrode as the reference electrode to the urease-coated NH_4^+ electrode and added ion-exchange resin in attempts to develop a urea electrode useful for assaying blood and urine. Good precision and accuracy were obtained.

In attempting to improve the selectivity of the urea determination, we next used a silicone rubber based nonactin ammonium ion selective electrode as the sensor for the NH_4^+ liberated in the urease reaction.[80] The selectivity coefficients of this electrode were 6.5 for NH_4^+/K^+, 7.50×10^2 for NH_4^+/Na^+, and much higher for other cations. The electrode's reaction layer was made of urease enzyme chemically immobilized on polyacrylic gel. A still further improvement was described by us using a three-electrode system, which allowed dilution to a constant interference level. Analysis of blood serum showed good agreement with spectrophotometric methods, and the enzyme electrode was stable for 4 months at 4 °C.[81]

Still further improvement in the selectivity of this type of electrode was obtained by Anfalt et al.,[82] who polymerized urease directly onto the surface of an Orion ammonia gas electrode probe by means of glutaraldehyde. Sufficient NH_3 was produced in the enzyme reaction layer even at pH's as low as 7 or 8 to allow

(61) L. P. Leon, M. Sansur, L. R. Snyder, and C. Horvath, *Clin. Chem.*, **23**, 1556 (1977).
(62) J. N. Miller, B. F. Rocks, and D. T. Burns, *Anal. Chim. Acta*, **93**, 353 (1977).
(63) M. K. Weibel and A. E. Humphrey, Enzyme Technology Grantees-Users Conference, NSF/RA-760032, PB-265,548, 116-23, National Science Foundation, Washington, DC, 1975.
(64) B. Watson and M. H. Keyes, *Anal. Lett.*, **9**, 713 (1976).
(65) B. Danielsson, K. Gadd, B. Mattiasson, and K. Mosbach, *Anal. Lett.*, **9**, 987 (1976).
(66) B. Mattiasson, *FEBS Lett.*, **77**, 107 (1977).
(67) B. Mattiasson, K. Mosbach, and A. Svenson, *Biotechnol. Bioeng.*, **19**, 1643 (1977).
(68) S. Ikeda and S. Fukui, *FEBS Lett.*, **41**, 216 (1974).
(69) T. T. Ngo, *Can. J. Biochem.*, **54**, 62 (1976).
(70) J. C. Weaver, M. K. Mason, J. A. Jarrell, and J. W. Peterson, *Biochim. Biophys. Acta*, **438**, 296 (1976).
(71) A. M. Stella and A. M. D. C. Batlee, *Int. J. Biochem.*, **8**, 353 (1977).
(72) E. Ginns and M. Guarnieri, *Exp. Cell Res.*, **97**, 42 (1976).
(73) G. G. Guilbault, D. N. Kramer, E. V. Bauman, and L. H. Goodson, *Anal. Chem.*, **34**, 1437 (1962); **37**, 1378 (1965).
(74) G. G. Guilbault, B. Tyson, and D. N. Kramer, *Anal. Chem.*, **35**, 582 (1963).
(75) G. G. Guilbault and J. Montalvo, *J. Am. Chem. Soc.*, **91**, 2164 (1969).
(76) G. G. Guilbault and J. Montalvo, *J. Am. Chem. Soc.*, **92**, 2533 (1970).
(77) G. G. Guilbault and J. Montalvo, *Anal. Chem.*, **41**, 1897 (1969).
(78) R. E. Adams and P. W. Carr, *Anal. Chem.*, **50**, 944 (1978).
(79) G. G. Guilbault and E. Hrabankova, *Anal. Chim. Acta*, **52**, 287 (1970).
(80) G. G. Guilbault and G. Nagy, *Anal. Chem.*, **45**, 417 (1973).
(81) G. G. Guilbault and G. Nagy, *Anal. Lett.*, **6**, 301 (1973).
(82) T. Anfalt, A. Granelli, and D. Jagner, *Anal. Lett.*, **6**, 969 (1973).

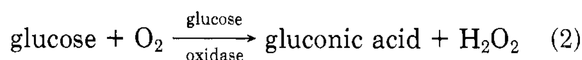
direct assay of urea in the presence of large amounts of Na^+ and K^+. A response time of 2–4 min was observed.

In a dramatic improvement over previous urea electrodes, we[83] described a total interference-free, direct-reading electrode for urea, using the air gap electrode of Ruzicka and Hansen.[84] A thin layer of urease chemically bound to polyacrylic acid was used at a solution pH of 8.5, where good enzyme activity was still obtained, yet where sufficient NH_3 is liberated to yield a sensitive measurement with the air gap NH_3 electrode. The urea diffuses into the gel; the NH_3 produced diffuses out of solution to the surface of the air gap electrode, where it is measured. A linear range of 3×10^{-2} to 5×10^{-5} M was obtained with a slope of 0.75 pH unit/decade. The electrode could be used continuously for blood serum analysis for up to 1 month (at least 500 samples) with an accuracy and precision of less than 2%. The response time is 2–4 min at pH 8.5, and the electrode is washed under a water tap for 5–10 s after each measurement. Absolutely no interference from any level of substances commonly present in blood was observed (Na^+, K^+, NH_4^+, ascorbic acid, etc.).

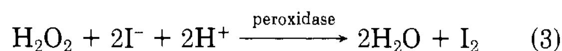
Still another urea electrode possibility is the use of a CO_2 sensor to measure the second product of the urea–urease reaction, HCO_3^-. We evaluated the use of the CO_2 sensor and found that a urea electrode, prepared by coupling a CO_2 electrode with a layer of urease covered with a dialysis net, had a linear range of 10^{-4} to 10^{-1} M, a response time of about 1–3 min, and a slight response to only acetic acid. Na^+ and K^+ ions had no interference.[85]

Glucose Electrodes. Following our successes with electrodes for the assay of urea, we next turned our attention to probes for glucose—an important metabolite indicating diabetes. In 1972–1973 we described a simple, stable, rapid-reading electrode for glucose. The electrode consists of a metallic sensing layer (Pt or Pt glass)[86,87] covered by a thin film of immobilized glucose oxidase held in place by cellophane. When poised at the correct potential, the resulting current is proportional to the glucose concentration. The time of measurement with this amperometric approach is less than 12 s using a kinetic method. Stored at room temperature, the electrode is stable for more than 1 year with only a 0.1% response change from maximum per day. The enzyme electrode determination of blood sugar compares favorably with commonly used methods with respect to accuracy, precision, and stability, and the only reagent needed for assay is a buffer solution.

Another probe was developed[88] for glucose based on an iodide membrane sensor:



$$\text{glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2 \quad (2)$$



$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{peroxidase}} 2H_2O + I_2 \quad (3)$$

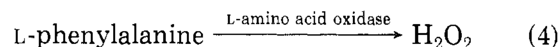
The highly selective iodide sensor monitors the local decrease in iodide activity at the electrode surface. The glucose assay was performed in a flow stream and at a stationary electrode. Pretreatment of the blood sample was required to remove interfering reducing agents, such as ascorbic acid, tyrosine, and uric acid.

Nillsson et al.[89] described the use of conventional hydrogen on glass electrodes for preparing enzyme–pH electrodes either by entrapping the enzymes within polyacrylamide gels around the glass electrode or as a liquid layer trapped within a cellophane membrane. In an assay of glucose based on a measurement of the gluconic acid produced, the pH response was linear from almost 10^{-1} to 10^{-3} M with ΔpH of about 0.85/decade. Electrodes of this type were also constructed for urea and penicillin. The ionic strength and pH were controlled using a weak (10^{-3} M) phosphate buffer, pH 6.9, and 0.1 M Na_2SO_4.

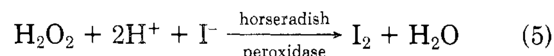
Amino Acid Electrodes. Another very important area of analysis is that of amino acids. Two key amino acids, L-lysine and L-methionine, are indicative of protein quality. Others, like L-tyrosine and L-phenylalanine, are useful in clinical analysis. We evaluated the CO_2 sensor for response to tyrosine when coupled with tyrosine decarboxylase held in an immobilized form by a dialysis membrane.[85] A linear range of 2.5×10^{-4} to 10^{-2} M was observed with a slightly faster response time than with the urea electrode mentioned earlier. A slope of 55 mV/decade was obtained, compared to 57 mV/decade for the urea electrode.

Enzyme electrodes for determining L-amino acids were developed by our group[90] by placing an immobilized layer of L-amino acid oxidase over a monovalent cation electrode to detect the ammonium ion formed in the enzyme-catalyzed oxidation of the amino acid. These electrodes are stable for about 2 weeks, and have a 1–2-min response time.

Two different kinds of enzyme electrodes were prepared by us[81] for determining L-phenylalanine. One of the electrodes used a dual enzyme reaction layer—L-amino acid oxidase with horseradish peroxide—in a polyacrylamide gel over an iodide-selective electrode. The electrode responds to decreasing iodide activity at the electrode surface, owing to the enzymatic reaction and subsequent oxidation of iodide (eq 4 and 5). The other electrode used was a silicone rubber



$$\text{L-phenylalanine} \xrightarrow{\text{L-amino acid oxidase}} H_2O_2 \quad (4)$$

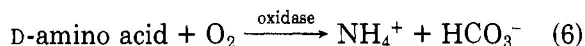


$$H_2O_2 + 2H^+ + I^- \xrightarrow[\text{peroxidase}]{\text{horseradish}} I_2 + H_2O \quad (5)$$

based, nonactin-type ammonium ion selective electrode covered with L-amino acid oxidase in a polyacrylic gel. The same principle of substrate diffusion into the gel layer, enzymatic reaction, and detection of the released ammonium applied to this system. Linear calibration plots were also obtained for L-leucine and L-methionine in the range 10^{-4}–10^{-3} M.

Electrodes specific for D-amino acids, which are oxidatively catalyzed by D-amino acid oxidase, were reported by us.[91] The NH_4^+ produced is monitored with a cation electrode (eq 6). The stability of these

(83) G. G. Guilbault and M. Tarp, *Anal. Chim. Acta*, **73**, 355 (1974).
(84) J. Ruzicka and E. H. Hansen, *Anal. Chim. Acta*, **69**, 129 (1974).
(85) G. G. Guilbault and F. Shu, *Anal. Chem.*, 44, 2161 (1972).
(86) G. G. Guilbault and G. J. Lubrano, *Anal. Chim. Acta*, **60**, 254 (1972).
(87) G. G. Guilbault and G. J. Lubrano, *Anal. Chim. Acta*, **64**, 439 (1973).
(88) G. Nagy, L. H. von Storp, and G. G. Guilbault, *Anal. Chim. Acta*, **66**, 443 (1973).
(89) H. Nillsson, A. Åkerlund, and K. Mosbach, *Biochim. Biophys. Acta*, **320**, 529 (1973).
(90) G. G. Guilbault and E. Hrabankova, *Anal. Lett.*, **3**, 53 (1970).
(91) G. G. Guilbault and E. Hrabankova, *Anal. Chim. Acta*, **56**, 285 (1971).

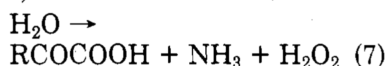


$$\text{D-amino acid} + O_2 \xrightarrow{\text{oxidase}} NH_4^+ + HCO_3^- \quad (6)$$

electrodes could be maintained for 21 days stored in a buffered flavin adenine dinucleotide (FAD) solution because the FAD is weakly bound to the active site of the enzyme and is needed for its activity. Electrode probes suitable for the assay of D-phenylalanine, D-alanine, D-valine, D-methionine, D-leucine, D-norleucine, and D-isoleucine were developed. An electrode for asparagine was also developed using asparaginase as the catalyst. No cofactor was necessary.[91]

We also described an enzyme electrode for glutamine, prepared by entrapping glutaminase on a nylon net between a layer of cellophane and a cation electrode. The electrode responds to glutamine over the concentration range 10^{-1}–10^{-4} M, with a response time of only 1–2 min.[92]

An electrode for L-amino acids was prepared by us[93] by coupling chemically bound L-amino acid oxidase to a Pt electrode to sense the peroxide produced in the enzyme reaction (eq 7). The time of measurement

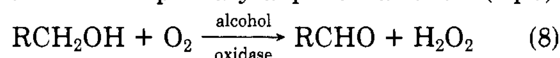


$$\text{L-amino acid} + O_2 + H_2O \rightarrow RCOCOOH + NH_3 + H_2O_2 \quad (7)$$

using a kinetic measurement of the rate increase in current per unit time is less than 12 s, and the only reagent required is a phosphate buffer. The L-amino acids cysteine, leucine, tyrosine, phenylalanine, tryptophan, and methionine were assayed.

In a remarkable demonstration of the ultimate specificity and sensitivity of enzyme probes, we demonstrated the development of totally specific electrodes for L-lysine[94] and L-methionine,[95] using the enzymes L-lysine decarboxylase and L-methionine ammonia-lyase. These two probes are being used in the food industry for protein quality assays.

Other Electrodes. Alcohol oxidase catalyzes the oxidation of lower primary aliphatic alcohols (eq 8).



$$RCH_2OH + O_2 \xrightarrow[\text{oxidase}]{\text{alcohol}} RCHO + H_2O_2 \quad (8)$$

The hydrogen peroxide produced in these reactions may be determined amperometrically with a platinum electrode as described earlier for determining glucose. We[96] used the alcohol oxidase obtained from Basidiomycete to determine the ethanol concentration of 1-mL samples over the range 0–10 mg/100 mL, with an average relative error of 3.2% in the range 0.5–7.5 mg/100 mL. This procedure should be adequate for clinical determinations of blood ethanol, since normal blood from individuals who have not ingested ethanol ranges from 40 to 50 mg/100 mL. Methanol seriously interferes with the procedure because the alcohol oxidase is more active for methanol than ethanol. However, the concentration of methanol in blood is negligible compared to that of ethanol.

A self-contained rapid reading electrode for uric acid was described by us.[97] The electrode was prepared by placing a layer of glutaraldehyde-bound uricase over the tip of a Beckman Pt electrode. The enzyme was then covered for support with a thin layer of dialysis membrane. The decrease in the level of dissolved oxygen in solution due to the enzymic reaction, given by eq 9, was maintained at an applied potential of −0.6

$$\text{uric acid} + O_2 \xrightarrow{\text{uricase}} \text{allantoin}\cdot H_2O_2 + H_2O \quad (9)$$

V vs. SCE. The current observed was proportional to the level of uric acid at concentrations of 10^{-5}–10^{-1} M. By measuring the initial rate of change in current, an assay can be performed in less than 30 s. Further studies indicated that the electrode could be used to assay glucose, amino acids, and alcohols.[98]

It was found that the peroxide produced in the reaction could not be monitored at +0.6 V vs. SCE, as described in methods we developed for glucose, amino acids, and alcohols, because the polarographic curves for uric acid and hydrogen peroxide are too close to be separated at any pH useful for the enzyme reaction and because an allantoin–peroxide complex is the product of uric acid oxidation, not free peroxide. Additionally, the oxygen uptake method was found to be more sensitive, enabling the assay of lower substrate concentrations.

The use of a Pt electrode rather than a Clark-type oxygen electrode eliminates the problems associated with gas membrane electrodes (i.e., slow response and blockage of the membrane by substances present in blood).

Williams et al.[99] prepared electrodes for analyzing glucose and lactate by entrapping the enzymes glucose oxidase and lactate dehydrogenase, respectively, between an electrochemical sensor (a platinum electrode) and a dialysis membrane. Enzyme electrodes have also been used for the assay of sucrose[100] and for the amperometric determination of lactate.[101] Rechnitz and Riechel[102] used a nucleotide selective enzyme electrode to record D-fructose-1,6-diphosphatase–AMP binding measurements. Highly selective enzyme electrodes for the assay of 5′-adenosine monophosphate,[103] creatinine,[104] and penicillin[105] have also been developed. Enzyme electrodes have been used for the measurement of cholesterol and its esters in blood using soluble enzyme,[106,107] and for total cholesterol in serum using chemical bound enzyme.[108] An electrochemical system with immobilized tyrosinase has been used to determine phenol concentrations in industrial effluents and surface waters.[109]

A novel application of the enzyme electrodes has been for the selective assay of several anions and heavy metal ions. Ogren and Johansson[110] have reported the de-

(92) G. G. Guilbault and F. R. Shu, *Anal. Chim. Acta*, **56**, 333 (1971).
(93) G. G. Guilbault and G. J. Lubrano, *Anal. Chim. Acta*, **69**, 183 (1974).
(94) G. G. Guilbault and C. White, *Anal. Chem.*, **50**, 1481 (1978).
(95) G. G. Guilbault, F. Fung, and S. S. Kuan, *Anal. Chem.*, in press.
(96) G. G. Guilbault and G. J. Lubrano, *Anal. Chim. Acta*, **69**, 189 (1974).
(97) G. G. Guilbault and M. Nanjo, *Anal. Chem.*, **46**, 1769 (1974).
(98) G. G. Guilbault and M. Nanjo, *Anal. Chim. Acta*, **73**, 367 (1974).
(99) D. L. Williams, A. R. Doig, and A. Korosi, *Anal. Chem.*, **42**, 118 (1970).
(100) I. Satoh, I. Karube, and S. Suzuki, *Biotechnol. Bioeng.*, **18**, 269 (1976).
(101) H. Durliat, M. Comtat, J. Mahenc, and A. Baudras, *J. Electroanal. Chem. Interfac. Electrochem.*, **66**, 73 (1975).
(102) T. L. Riechel and G. A. Rechnitz, *Biochem. Biophys. Res. Commun.*, **74**, 1377 (1977).
(103) D. S. Papastathopoulos and G. A. Rechnitz, *Anal. Chem.*, **48**, 862 (1976).
(104) M. Meyerhoff and G. A. Rechnitz, *Anal. Chim. Acta*, **85**, 277 (1976).
(105) S. O. Enfors, *Proc. Anal. Div. Chem. Soc.*, **14**, 106 (1977).
(106) L. C. Clark and C. R. Emory in "Ion Enzyme Electrodes in Biology and Medicine", M. Kessler and L. C. Clark, Eds., International Workshop Proceedings, University Park Press, Baltimore, MD, 1976.
(107) I. Satoh, I. Karube, and S. Suzuki, *Biotechnol. Bioeng.*, **19**, 1095 (1977).
(108) H. S. Huang, S. S. Kuan, and G. G. Guilbault, *Clin. Chem.*, **23**, 671 (1977).
(109) J. G. Schiller, A. K. Chen, and C. C. Liu, *Anal. Biochem.*, **85**, 25 (1978).

termination of traces of mercury(II) by inhibition of an enzyme reactor electrode loaded with immobilized urease. Enzyme electrode based on immobilized arylsulfatase and nitrite reductase have been developed by Guilbault et al. for the analysis of sulfate[111] and nitrate and nitrite ions,[112-113] respectively.

The Future of Immobilized Enzymes

Several recent attempts have been devoted to design of stable, self-contained enzyme electrode probes that can be easily fabricated in large scale. Guilbault and Lubrano[114] described the production of such electrodes for glucose and L-amino acids, using various membrane films. A generally useful mild coupling method for enzyme or collagen membranes, using acyl azide activation, has been reported by Coulet et al.[115-119] Stable, very sensitive, glucose sensors have been described[116,120] that can measure as little as 1×10^{-8} M concentrations.[120]

Finally, it should be mentioned that immobilized enzymes, together with electrochemical sensors, are used in several instruments available commercially. Owens-Illinois (Kimble) has designed a urea instrument using immobilized urease and an ammonia electrode probe. Yellowsprings Instrument Co. developed a glucose instrument using insolubilized glucose oxidase and a Pt electrode, and has other instruments available for triglycerides, lipase, and amylase. Leeds and Northrup (North Wales, PA) has instruments for glucose, galactose, maltose, and lactose using glucose oxidase, galactase oxidase, invertase–mutarotase–glucose oxidase, maltase–glucose oxidase, and lactase–glucose oxidase, respectively.

In the next decade the reader can expect both immobilized enzyme instruments as well as self-contained enzyme electrode probes to be available for the assay of most substrates of analytical interest. Also immobilized enzyme tubes (nylon tubes with immobilized enzymes) are already available from two companies: Miles Laboratories, Stoke Poges, England, "Catalinks", and Technicon Inc., Tarrytown, NY, for glucose (hexokinase–glucose phosphate dehydrogenase) and uric acid (uricase). Many more can be expected from these companies, as well as the advent of more industrial concerns into this market.

(110) L. Ogren and G. Johansson, *Anal. Chim. Acta*, **96**, 1 (1978).
(111) T. Cserfalvi and G. G. Guilbault, *Anal. Chim. Acta*, **84**, 259 (1976).
(112) C. H. Kiang, S. S. Kuan, and G. G. Guilbault, *Anal. Chim. Acta*, **80**, 209 (1975).
(113) C. H. Kiang, S. S. Kuan, and G. G. Guilbault, *Anal. Chem.*, **50**, 1319, 1323 (1978).
(114) G. G. Guilbault and G. Lubrano, *Anal. Chim. Acta*, **97**, 229 (1978).
(115) P. R. Coulet, J. Julkard, and D. Gautheron, *Biotechnol. Bioeng.*, **16**, 1055 (1974).
(116) D. R. Thevenot, P. R. Coulet, R. Sternberg, and D. C. Gautheron, *Enzyme Eng.*, 4, 221 (1978).
(117) P. R. Coulet, C. Godinot, and D. Gautheron, *Biochim. Biophys. Acta*, **391**, 272 (1975).
(118) J. M. Engasser, P. R. Coulet, and D. C. Gautheron, *J. Biol. Chem.*, **252**, 7919 (1977).
(119) J. M. Brillouet, P. R. Coulet, and D. C. Gautheron, *Biotechnol. Bioeng.*, **18**, 1821 (1976); **19**, 125 (1977).
(120) D. R. Thevenot, P. R. Coulet, R. Sternberg, J. Laurnet, and D. C. Gautheron, *Anal. Chem.*, **51**, 96 (1978).