Journal of Solid-Phase Biochemistry, Vol. 2, No. 4, 1977

USE OF IMMOBILIZED ENZYMES IN CHEMICAL ANALYSIS[1]

GEORGE G. GUILBAULT

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

Accepted May 1, 1978

Immobilized enzymes are becoming increasingly popular as analytical reagents because of their reusability, stability, and sensitivity to many inhibitors that would seriously interfere in assays using soluble enzymes. In this article, some of the kinetic and catalytic effects of immobilized enzymes in analysis will be discussed. The shift of the activity–pH profile curves on immobilization, the changes in temperature dependence, the inhibitor constants (K_i), Michaelis constants (K_m), and the maximum velocity (V_{max}), plus others, will be discussed. Finally, the use of these immobilized enzymes in fluorometric and electrochemical monitoring systems will be shown, and the future of these reagents in various areas will be discussed. A survey of enzyme electrodes will be presented as an example of the use of immobilized enzymes. Application of immobilized enzyme technology to the assay of BUN, glucose, uric acid, amino acids, ethanol, and other metabolites will be discussed.

INTRODUCTION

Excellent chemical analysis can be performed with enzymes, which are biological catalysts; the real advantages of immobilized enzymes are many in analyses using electrochemical probes or other methods of analysis. One advantage of the immobilized enzyme is a pH shift; i.e., the pH optimum can be shifted to that region at which one wants to make a measurement, by choosing the right support for immobilization. Take an enzyme with a narrow pH range of, say, 6–8; this can be shifted on immobilization down to the acidic side or, conversely, up to the basic side. The enzymes are furthermore much more stable. In some work at Edgewood Arsenal, Maryland, we actually heated our enzymes to 150°F and brought them back down to room temperature, with very little loss of activity. No soluble enzyme could be treated in this fashion.

One advantage often overlooked is that better selectivity can be realized with the enzyme when immobilized; this insolubilized reagent

[1]The review is taken, in part, from a presentation given at the US/USSR Conference on Enzyme Technology held in Talinn, Estonian SSR, November 26–December 2, 1977.

becomes much more selective for an inhibitor, and only the most powerful inhibitor can actually attack the enzyme. We demonstrated this several years ago in an immobilized cholinesterase alarm for the assay of organophosphorus compounds in air and water. No other common interferants disturbed the alarm—it responded only to organophosphorus compounds.

In 1961 at Edgewood Arsenal, I first experimented with some soluble enzymes, such as glucose oxidase, and developed an electrochemical assay for glucose. This led to the use of immobilized enzymes with a commercially available ion-selective electrode sensor to form one self-contained sensor that could be used to measure either organic or inorganic compounds which are primary or secondary substrates for the immobilized enzyme. The base sensor can be glass; i.e., the cation response can be measured (the ammonium ion, for example), or the pH change in a penicillin electrode can be measured, as done by Mosbach and Papariello and others. Or a gas membrane can be used as a base sensor, such as the ammonia or the CO_2 membrane. Next are the polarographic sensors which measure peroxide or oxygen, or any of the solid membrane electrodes, i.e., the cyanide electrode. For example, the enzyme can be placed on top of a flat glass electrode sensor; a membrane is then put over the outside of this sensor to hold the enzyme in and keep things like catalase and bacteria out. This protects the enzyme from bacterial spoilage, which is one of the primary reasons for loss of enzyme activity.

With potentiometric devices, we can measure the response either by a steady-state (i.e., equilibrium) method measuring millivolts or microamperes, or by a rate method which senses the change in millivolts or microamperes per minute. Measurements of substrate can be performed by either a steady-state or a rate method. But measurements of enzyme activity must be done by a rate method. This is a point often hazy in the literature—one can find many claiming that they are measuring enzymes by steady-state methods. This is impossible by basic definition of enzyme activity. Enzymes are catalysts and have to be measured by a rate method, but this may be either an interrupted or a continuous measurement of rate. In Table 1 is presented a compilation of enzyme electrodes—this list is not by any means complete. It is the first in a series of tables that were published in a recent book of mine, *Handbook of Enzymatic Analysis* (Dekker, New York, 1977). In this table are listed the enzymes that act on these various materials, and some of the base sensors that might be useful. Take as a typical example glucose, which can be assayed with glucose oxidase:

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

One can measure the uptake of oxygen with a gas membrane electrode, a technique pioneered by Clark and perfected by Hicks and Updike, or

TABLE 1. **Some Enzyme Electrodes and Their Characteristics**

Type	Sensor	Stability	Response time (min)	Range (M)
1. Urea	Cation	>4 months	1–2	10^{-2} to 10^{-4}
	pH	3 weeks	5–10	5×10^{-3} to 5×10^{-5}
	Gas (NH_3)	>4 months	1–4	5×10^{-2} to 5×10^{-5}
2. Glucose	$Pt(O_2)$	>4 months	1	10^{-1} to 10^{-5}
	$Pt(H_2O_2)$	>14 months	1	2×10^{-2} to 10^{-4}
	Gas (O_2)	3 weeks	2–5	10^{-2} to 10^{-4}
	pH	1 week	5–10	10^{-1} to 10^{-3}
3. L-Amino acids	Pt	4–6 months	0.2	10^{-3} to 10^{-5}
General	NH_4^+	>1 month	1–3	10^{-2} to 10^{-4}
L-Tyrosine	Gas (CO_2)	3 weeks	1–2	10^{-1} to 10^{-4}
L-Asparagine	Cation	1 month	1	10^{-2} to 5×10^{-5}
4. D-Amino acids	Cation	1 month	1	10^{-2} to 5×10^{-5}
5. Lactic acid	Pt	<1 week	3–10	2×10^{-3} to 10^{-4}
6. Alcohols	$Pt(O_2)$	>4 months	0.5	0.5–100 mg/100 ml
7. Penicillin	pH	1–2 weeks	1–2	10^{-2} to 10^{-4}
8. Uric acid	$Pt(O_2)$	>4 months	0.5	10^{-2} to 10^{-4}
9. Amygdalin	CN^-	1 week	1–3	10^{-1} to 10^{-5}

record the peroxide or oxygen polarographically. There are other ways: one can measure the gluconic acid by a pH change, as Mosbach showed very nicely at low buffer capacity, or use an iodide membrane (the latter is much less recommended). The point I would like to make is that there are many ways to measure a particular substrate, and one should choose the one best for the application. For example, one would not choose to measure urea in biological fluid with an ammonium cation electrode, because of the interference of potassium and sodium. One would choose, preferentially, an ammonia gas membrane electrode in which there is no interference from sodium and potassium.

Among the basic characteristics of enzyme electrodes is the five-step process of their operation. First, the substrate must be transported to the surface of the electrode. Second, the substrate must diffuse through the membrane to the active site. Third, reaction occurs at the active site. Fourth, product formed in the enzymatic reaction is transported through the membrane to the surface of the electrode, where, fifth, it is measured. The first step—transport of the substrate—is most critically dependent on the stirring rate of the solution, so that rapid stirring will bring the substrate very rapidly to the electrode surface. If the membrane is kept very thin, using highly active enzyme, then steps 2 and 4 are eliminated or minimized; since

step 3 is very fast, the theoretical response of an enzyme electrode should approach the response time of the base sensor. Many researchers have shown with experimental data that one can approach this behavior by using a thin membrane and rapid stirring. Let us briefly discuss some of these factors. In Fig. 1 is shown a comparison of the characteristics of the amygdalin electrode using β-glucosidase; on the left-hand side are data obtained by Mascini and Liberti at the optimum pH of the enzyme reaction, which is about 7. The right-hand side shows the data of Rechnitz, as repeated by Mascini and Liberti, obtained at the optimum pH for the cyanide sensor, pH 9. Realizing that there is no free cyanide except at very high pH, say 9 or 10, Rechnitz reasoned that this optimum pH should be used in order to get the best sensitivity. But at this high pH the enzyme reaction is killed and the rate of conversion becomes very slow. If the optimum pH of the enzyme reaction is used, even though there is very little cyanide at this pH, since the enzyme layer is in such intimate contact with the base probe, the response is almost instantaneous. This has been shown in many cases; for example, Anfalt et al. in Sweden showed with the

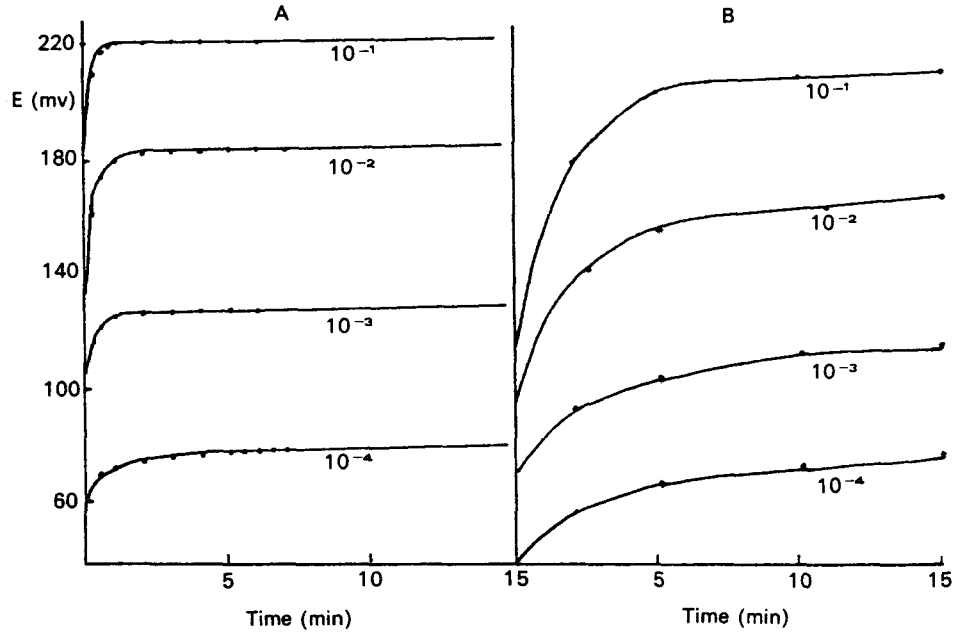

FIG. 1. Amygdalin response–time curves for an electrode containing 1 mg of β-glucosidase immobilized by a dialysis paper. A: at pH 7; B: at pH 10. [M. Mascini and A. Liberti, Anal. Chim. Acta 68, 177 (1974); reprinted with permission of author.

urea–urease reaction that the ammonia liberated from this reaction could be measured more effectively at pH 7 or 7.5 than at pH 9 or 10, because the enzyme reaction was functioning much better at this low pH. Another factor often overlooked in the use of ion-selective electrodes is that the stirring rate not only will promote a faster response at the enzyme electrode or at any probe but also will affect the equilibrium potential or the equilibrium pH that is measured. This becomes very critical: if one is going to stir, one has to stir at a constant rate; otherwise, a different value will be obtained every time.

The stability of the electrode depends on the type of entrapment. Here again there is much ambiguous reporting of immobilization data in the literature. Some individuals use dry storage for a long period of time and then report a fantastically long lifetime. Realistically, the immobilization characteristics and the stability of the enzyme should be defined in terms of dry storage *and* use storage. The lifetime of most soluble enzymes, except perhaps in the case of some types of glucose oxidase which are quite stable in the crude form, is generally about 1 week or 25–50 assays. However, one must realize that there are potential interferences that arise in the use of soluble enzymes which are not found in the use of an entrapped enzyme. The physically entrapped enzyme lasts about 3–4 weeks or 50–200 assays. For the chemically bound enzyme, 200–1000 assays is a good range. In many cases, we and others have achieved at least this, and furthermore there are many enzymes available—bound onto nylon tubes, for example, such as the ones Technicon is producing for use on SMAC or the AutoAnalyzer, the ones Boehringer has been experimenting with, and the ones Miles is selling under the trade name Catalink—which are very stable. These tubes have been demonstrated for 10,000 assays.

The stability is dependent also on the content of enzyme in the gel, on the optimum conditions, as was mentioned, and finally on the stability of the base sensor itself. Figure 2 shows the stability of some electrodes prepared using some entrapment methods for glucose oxidase: entrapment of a solubilized enzyme on an electrode surface and two types of immobilization, physical entrapment in a gel and covalent bonding. The type of chemical bonding serves two purposes: (a) it selects the pH range and (b) it provides the best immobilization method for each enzyme. This will be shown later for some studies of cholesterol.

Interferences can be in the sensor itself or from other substrates for the enzyme. For example, alcohol oxidase can be used for an excellent acetic acid electrode; an interference would be the native substrate, ethanol. Inhibitors of the enzyme are also interferences, and here the immobilized enzyme makes the enzyme much less susceptible to environmental factors. To gain an overall view of what has been done and how broad the field really

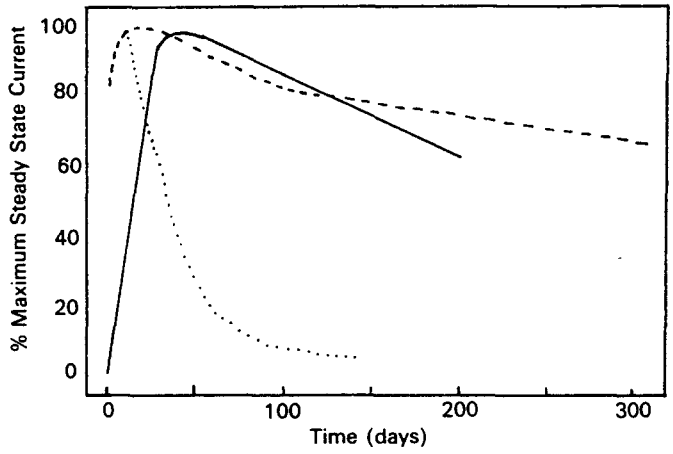

FIG. 2. Long-term stability of glucose electrodes by the steadystate method. ---, Type 1 electrode; —— Type 2 electrode; · · · Type 3 electrode. [From G. G. Guilbault and G. J. Lubrano, Anal. Chim. Acta 64, 439 (1973); reprinted with permission of author.]

is we shall first discuss inorganic ion determinations. Nonenzymatic electrodes have been designed for phosphate, sulfate, and nitrate, but these have undesirable characteristics. The phosphate and sulfate electrodes are totally useless except for titrations of sulfate or phosphate, and other commercial electrodes such as the nitrate electrode suffer serious interferences. An enzyme-based phosphate electrode has been formulated, using alkaline phosphatase and glucose oxidase immobilized in a membrane, with measurement of the oxygen uptake by the glucose reaction:

$$\text{Glucose phosphate} \underset{\text{phosphatase}}{\overset{\text{alkaline}}{\rightleftharpoons}} \text{phosphate} + \text{glucose} \tag{2}$$

$$\text{Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2 \tag{3}$$

The amount of glucose is controlled by the phosphate, which is reacting with glucose in this reversible reaction, forming glucose phosphate. So, by a measurement of the rate of oxygen uptake, one can measure the phosphate concentration, since the rate is proportional to the concentration of this anion. The curve is similar to that of an inhibitor of an enzyme reaction and is extremely reproducible, but what is remarkable, is the specificity. We ran about 50 anions and cations, and very few interfered. Tungstate and arsenate are the only materials that gave an appreciable interference, and fortunately these are almost never found in blood or estuaries. Molybdate responds somewhat; the selectivity is about 10:1. Borate and EDTA give a slight interference. The selectivity of the electrode for phosphate over

sulfate ion is about 500 : 1. Other ions, chloride, acetate, bromide, etc., did not respond at all. So, indeed, the phosphate electrode is selective. Another electrode we built was a sulfate electrode based on the hydrolysis of 4-nitrocatechol sulfate, catalyzed by arylsulfatase, to produce 4-nitrocatechol and sulfate. We looked at the polarographic wave for oxidation of the product of the enzymatic reaction, 4-nitrocatechol, to the corresponding quinone. Hence the rate of oxidation to the quinone is proportional to the sulfate concentration. Such an enzymatic sulfate electrode is extremely difficult to fabricate. Many people have dabbled in this with no success, for one primary reason: the measuring technique is critically dependent on both the type of substrate (some substrates do not work at all) and the concentration of substrate. So all these factors have to be considered in formulating the enzyme electrode. However, the system does work very nicely. Figure 3 shows some curves demonstrating the specificity of the electrode. The response to sulfate is quite good. Note the line for phosphate, which gives a slight response at 10^{-4} M to a very moderate response at 10^{-2} M. Normally,

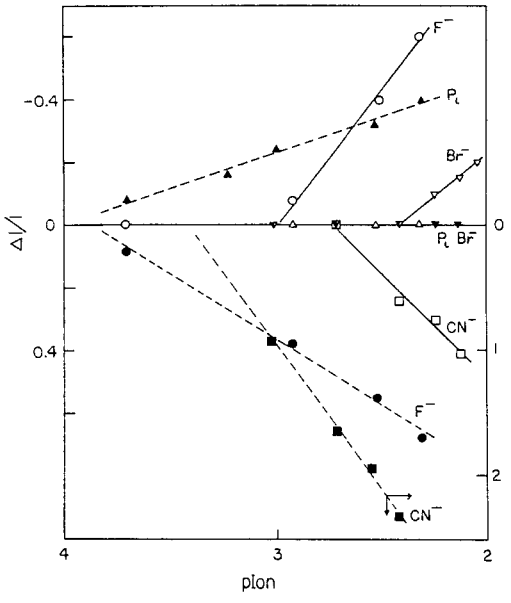

FIG. 3. Anion effects on the sulfatase electrode. $\Delta I/I$ is plotted against the p Ion. Electrode type IV; 10% (wt/wt) enzyme in the active layer; pH 4.10; $E = +0.8$ V vs. SCE. Open marks with the solid lines are at a 2×10^{-4} M NCS concentration; closed (solid) marks with dashed lines are at 3.8×10^{-5} M NCS concentration. △ Phosphate. □ Cyanide. ○ Fluoride. ▽ Bromide. [G. G. Guilbault and T. Cserfalvi, Anal. Chim. Acta 84, 259 (1976); reprinted with permission of authors.]

the response to sulfate would be at least 10 times as great. The response to cyanide depends on the concentration of nitrocatechol sulfate. By using a low concentration of nitrocatechol sulfate, we could eliminate much of the interference of ions like cyanide.

We shall now discuss one more inorganic system before going to the organics: an assay of nitrate–nitrite ions in foodstuff. Both of these ions are important in food technology. Recent studies have shown that, besides being the cause of methemoglobinemia, nitrate and nitrite can react with secondary and tertiary amines to form a carcinogenic reagent, *N*-nitrosamine. Thus the analysis of nitrate and nitrite in the environment has become increasingly important. A specific and sensitive method has been developed by using an enzymatic approach and newly developed electrochemical and fluorometric approaches. MVH (methyl viologen, reduced form)–nitrate reductase (E.C. 1.9.6.1), induced from *Escherichia coli*, and MVH-nitrite reductase (E.C. 1.6.6.4), isolated from spinach leaves, were purified and immobilized. A newly developed air gap electrode was adapted to monitor the ammonia formed by reduction of nitrate and nitrite with MVH-nitrate and -nitrite reductase. The analytical characteristics of both methods have been thoroughly studied. The detection limit using the air gap electrode is 5×10^{-5} M for nitrite or nitrate, and using the fluorometric monitoring method the limit is 1×10^{-6} M nitrate. Meat samples and water samples have been analyzed. The results obtained are very satisfactory, especially those obtained by the fluorometric method (Tables 2–4).

Most recently an NADH (nicotinamide adenine dinucleotide, reduced form)–nitrate reductase (E.C. 1.6.6.1) induced from *Chlorella vulgaris* was highly purified by affinity chromatography and was used for a highly specific assay of NO_3^-:

$$NO_3^- + NADH \xrightarrow{E} NO_2^- + NAD + H_2O \tag{4}$$

TABLE 2. **Determination of Nitrite in Meat Samples**

Sample	Griess method[a] (ppm)	Proposed method (ppm)	Difference (%)
1	33.50	31.50	−6
2	31.18	28.80	−7.7
3	10.90	8.60	−21.1
4	11.90	9.50	−20.2
5	19.30	16.50	−14.5
6	30.48	29.90	−1.9
7	28.70	26.90	−6.3

[a]Data obtained from Frey Meat Co.

TABLE 3. **Determination of Nitrate in Water Samples**

Sample	UV method[a] (ppm)	Proposed method[b] (ppm)	SD (ppm)	CV (%)
Tap water				
1	3.72	3.77	0.09	2.3
2	5.28	5.41	0.07	1.3
River water				
1	4.37	4.92	0.12	2.4
2	6.23	6.05	0.13	2.1

[a]Data obtained from Jefferson Water Works. Average of three measurements.
[b]Average of five measurements.

The decrease in the absorbance, fluorescence, or diffusion current of NADH is measured and related to the concentration of nitrate.

Another enzyme was isolated from *Azotobacter crococcum* and will be used for the assay of NO_2^-:

$$NO_2^- + NADH_2 \xrightarrow{E} NO + NAD + H_2O \tag{5}$$

No other anions respond in this enzyme sequence. At least 10^{-6} M concentrations should be assayable.

Electrodes for amino acids using the nonselective enzyme L-amino acid oxidase, which reacts with L-amino acids to produce peroxide or ammonia:

$$\text{L-Amino acids} \xrightarrow{\text{oxidase}} H_2O_2 + NH_3 \tag{6}$$

Our interest in the same food technology program is to induce and isolate specific amino acid enzymes which would act on only one of these key amino acids, such as lysine, methionine, or tyrosine, and to use a base probe which is a CO_2 or an ammonia electrode in order to measure the product of the enzymatic reaction. We have used phenylalanine ammonia lyase, isolated from Wisconsin potatoes; following irradiation the enzyme is isolated and immobilized, which makes it useful for the very rapid assay of phenylalanine. The enzyme is totally specific for L-phenylalanine. L-Arginine and L-lysine can be measured with an enzyme from *E. coli* which has been purified to get the decarboxylase and thus to obtain total specificity. Histidine and tyrosine have been assayed using enzymes from various strains. To fabricate the enzyme electrode, a glutaraldehyde solution of the amino acid decarboxylase or lyase is smeared directly on the tip of a CO_2 or NH_3 membrane in a very thin film, so that no problems with a slow return to baseline occur. Thus the amino acid electrode is simply a base CO_2 or NH_3

TABLE 4. **Determination of Nitrate and/or Nitrite at a 1:1 Ratio**[a]

$(NO_3^- + NO_2^-)$ (M)		Relative error	(NO_2^-) (M)	Relative error	(NO_3^-)[b] (M)	Relative error
Added	Found	(%)	found	(%)	calculated	(%)
1×10^{-4}	1.06×10^{-4}	6.0	4.55×10^{-5}	9.0	6.05×10^{-5}	21.0
5×10^{-4}	5.22×10^{-4}	4.4	2.38×10^{-4}	4.8	2.84×10^{-4}	13.6
1×10^{-3}	0.96×10^{-3}	4.0	4.88×10^{-4}	2.4	4.72×10^{-4}	5.6
5×10^{-5}	5.12×10^{-3}	2.4	2.51×10^{-3}	0.4	2.61×10^{-3}	5.2
1×10^{-2}	1.03×10^{-2}	3.0	4.93×10^{-3}	1.4	5.37×10^{-3}	7.4
	Average	3.96	Average	3.6	Average	10.56

[a] Assay using immobilized nitrate and nitrite reductases. A 1.0-ml sample solution is used for a mixture of nitrite and nitrate. The reaction solution is passed through both columns A and B, and the eluent is measured as described above. In this way, total nitrate plus nitrite, and nitrite, can be obtained, respectively, and nitrate can be calculated by subtracting nitrite from the total nitrate plus nitrite.

[b] $(NO_3^-) = (NO_3^- + NO_2^-) - (NO_2^-)$.

sensor; on top of this CO_2 or NH_3 membrane is placed an enzyme layer. Typical response times are of the order of 1–3 min, and the return to baseline is about 10 min. The main problem with any electrode is that if the enzyme layer is too thick the return to baseline is slow. We, and others, have shown that it is not necessary to wait for the complete return to baseline. One can inject the next sample at three-fourths or even two-thirds of the time necessary for return to baseline; this is a matter of only a couple of minutes. However, this can be done only when high substrate concentrations are being measured. If low concentrations are to be assayed, then, of course, it is necessary to wait for a complete return to baseline. Typical calibration curves are linear from 10^{-1} to 5×10^{-5} M for most of the amino acid electrodes; the coefficient of variation is about 2.5%. The arginine electrode is completely specific for L-arginine. Of some 50-odd amino acids tested at first, only L-glutamate interfered. This is a native impurity (enzyme impurity) in the arginine decarboxylase, i.e., the glutamate decarboxylase, which can then be separated out by affinity chromatography, making the arginine electrode totally specific. In the L-lysine electrode, arginine was an interference; this is because both enzymes are present in *E. coli*. But this impurity can be removed by purification of the enzyme. Histidine decarboxylase, because it comes from a different source from which no other amino acid decarboxylase is found, is totally specific for histidine. Likewise, the L-tyrosine electrode produced is totally specific for this essential amino acid.

The L-lysine electrodes have been used to determine the lysine content in standard mixtures containing 20 common amino acids (Table 5) and also in flour samples spiked with lysine (Table 6). Excellent results are obtained.

After 50 days of use, a L-lysine electrode retained approximately 84% of its initial response; a L-tyrosine electrode retained 57% of its initial response after 60 days of use.

TABLE 5. **Analysis of L-Lysine in Mixtures of Amino Acids**

Electrode	L-Lysine (M) (and pLys)		Percent error
	Calculated	Found	
LS1	2.90×10^{-4} (3.54)	2.94×10^{-4} (3.53 ± 0.02)	1.7
	1.45×10^{-3} (2.84)	1.37×10^{-3} (2.87 ± 0.08)	5.8
	8.10×10^{-4} (3.06)	7.61×10^{-4} (3.12 ± 0.04)	14.3
LS2	8.10×10^{-4} (3.06)	8.00×10^{-4} (3.10 ± 0.01)	8.8
	1.16×10^{-3} (2.94)	1.14×10^{-3} (2.95 ± 0.04)	1.8

[a] A standard mixture of amino acids was prepared by weighting out known amounts of 20 common amino acids and dissolving in buffer. The solution was diluted and assayed for L-lysine content using the specific electrode.

TABLE 6. **Recovery Studies in the Analysis of L-Lysine in Spiked Flour**[a]

Sample	Flour (mg)	Lys (added) (mg)	Lys (found) (mg)	Percent recovery
F	201.5	0	0 (no response)	106
1	204.0	4.8	5.1	106
2	222.0	7.9	8.4	106
3	247.5	12.1	11.6	96.4
4	206.3	24.6	28.3	115

Sample	Lys (found) (mg)	CV	Percent recovery
1 (×4)	5.4±0.4	7.4	112
2 (×3)	8.6±0.1	1.1	108
3 (×3)	13.0±0.4	3.1	107
4 (×3)	26.1±0.6	2.3	106
1 (×3)	5.8±0.3	5.1	120
		3.8	

[a]To several samples of approximately 200 mg each was added a known amount of L-lysine. The mixture was suspended in 0.5 M acetate buffer, pH 5.7, and the L-lysine content was determined.

The use of L-methionine racemase and D-amino acid oxidase as catalysts in forming a totally specific L-methionine electrode has been investigated. The H_2O_2 formed is then assayed:

$$\text{L-Methionine} \xrightarrow{\text{racemase}} \text{D-methionine} \xrightarrow[\text{oxidase}]{\text{D-amino acid}} NH_3 + H_2O_2 \qquad (7)$$

The base electrodes used were Pt (for H_2O_2) and gas (NH_3).

Preliminary runs have shown the feasibility of this system, but more work is to be done in the future. Furthermore, the use of L-methionine ammonia lyase from various sources is under investigation; the NH_3 liberated can be measured with an ammonia electrode.

$$\text{L-Methionine} \xrightarrow{\text{lyase}} NH_3 \qquad (8)$$

An enzymatic system for cholesterol uses the enzymes cholesterol esterase and cholesterol oxidase:

$$\text{Cholesterol esters} \xrightarrow[\text{esterase}]{\text{cholesterol}} \text{cholesterol} \qquad (9)$$

$$\text{Cholesterol} \xrightarrow[\text{oxidase}]{\text{cholesterol}} H_2O_2 \qquad (10)$$

Using this system, we attempted to develop an immobilized enzyme electrode for cholesterol. The "self-contained" electrode did not work because both cholesterol and the insolubilized enzymes are heterogeneous. After about $2\frac{1}{2}$ years of research, we discovered that the only feasible approach was the use of an enzyme stirrer. This stirrer is about the size of a small piece of chalk ($1\frac{1}{2}$ cm high). It has a tiny bore magnet, inserted in the bottom section, that will stir the system automatically at a constant rate. In the top part there is an enzyme chamber, which has nylon cloth portholes—many of these—to allow free passage of the cholesterol esters (and cholesterol) into the enzyme layer where they can react with the enzyme. By stirring very rapidly, we could get a very fast introduction of the substrate into the chamber with fast diffusion of the product out. A platinum electrode is placed very closely adjacent to the stirrer, so that as the stirrer is moving the enzymes first catalyze the hydrolysis of cholesterol esters and then the oxidation of cholesterol to peroxide. The peroxide is then sensed by the polarographic sensor. Sodium cholate, Triton X-100, and other solubilizing agents are added in order to facilitate a transport of the insoluble cholesterol into the enzyme layer. In fact, in the body itself the reason some people are much more susceptible to cholesterol and its buildup in the blood-stream, with increased possible danger of a heart attack, is that these individuals have the necessary lipoproteins present which effect the transport of cholesterol across the gastrointestinal trace from the internal lining of the stomach into the bloodstream. We are doing the same sort of thing here, by placing the cholesterol in a solubilized form where it can be transported into the enzyme layer for conversion to product, which is then measured. Although the cholesterol oxidase is difficult to immobilize, the esterase is much more difficult, and its stability is not nearly so good as that of the oxidase. But we have succeeded in getting both enzymes stabilized. Those data are presented in Fig. 4, which shows the stability of the enzyme cholesterol oxidase. We have tried many different immobilization procedures in this long period of trying to immobilize the enzymes, and a paper describing these attempts appeared in *Clinical Chemistry* in May 1977. We tried glutaraldehyde but found it unsatisfactory for long-term use. Then we tried binding to cellulose; this works very well but the activity falls off after about 50 assays. It was only the binding to alkylamine glass beads by glutaraldehyde that gave any measure of stability that we found acceptable. Our criterion was stability for at least 200–1000 assays for both oxidase and the esterase. And finally there is the cost factor: the estimated cost of the soluble enzymes is about $1.03 (conservatively) per enzyme assay, whereas with the dual immobilized enzymes, figuring on only 200 assays, it is 4¢ per assay. If we can get 1000 assays, the former figure would reduce to 8¢ per assay.

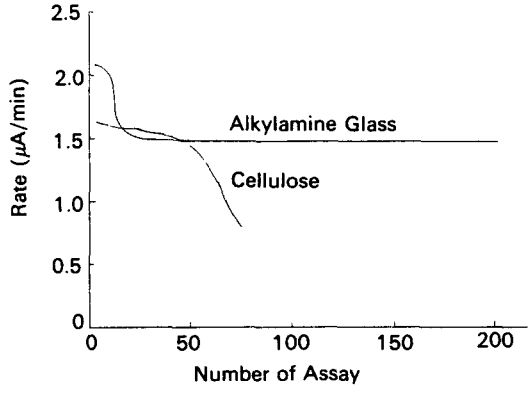

FIG. 4. Long-term stability of immobilized C.O. [G. G. Guilbault and N. Huang, Clin. Chem. 23:671 (1977); reprinted with permission of authors.]

A comparison was quite satisfactory of results obtained by the accepted clinical procedure—the Abel–Levy method, a modification of the Liberman–Burchart reaction—with those obtained by the steady-state method and the rate electrochemical methods for assay of cholesterol. In fact, in the results we obtained using the "electrode" the coefficient of variation is much better than those obtained by the accepted spectrophotometric method.

Another electrode we are developing is a triglyceride probe using a high-purity lipase which effects a rapid hydrolysis to glycerol. Then glycerol, in one-step glycerol dehydrogenase catalyzed reaction, is oxidized, with concomitant reduction of NAD to NADH. The electroactive NADH is measured. This approach appears promising.

The field of analytical uses of immobilized electrodes is extremely broad. It is impossible to do justice to it in a brief review because there is so much that has been done, and can be done. The future is wide open. Enzymes can be grown to act on anything. We have even induced enzymes that act on pesticides known to be very slightly biodegradable. Such enzymes can be grown by induction and genetic microbiology, to act on any substance. So any enzyme electrode can be built provided that the response can be measured electrochemically. Otherwise, one can use a thermistor (as in the approach of Carr, Mosbach, and others) or a spectrophotometric approach (as in the AutoAnalyzer).