ENZYME ELECTRODE PROBES

GEORGE G. GUILBAULT

Department of Chemistry, Louisiana State University in New Orleans, New Orleans, Louisiana 70122, U.S.A.

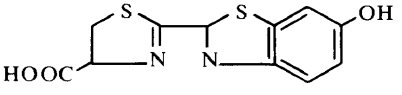
ABSTRACT

An electrical transducer for urea is described. The excellent stability of various types of urease enzyme electrodes developed in the author's laboratory is due to understanding the parameters which affect immobilized enzyme stability. Preliminary experiments have shown that a liquid layer of urease trapped in a double cellophane layer over a cation electrode may produce a useful enzyme electrode. Application of the apparatus for the determination of urea in blood and urine is described and some notes are given on an electrode for the determination of amino acids with application to the preparation of a L-amino electrode and comparison of some concentrations of enzymes in the immobilized layer.

GENERAL CONSIDERATIONS

Enzymes are biological catalysts which enable the many complex chemical reactions, upon which depends 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 catalysing 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 catalyses 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



$$+ \quad O_2$$

$$\xrightarrow[Mg^{2+}.\,ATP]{\text{Luciferase}} \text{Oxyluciferin (Green luminescence)} \qquad (1)$$

is glucose oxidase, which catalyses the oxidation of β-D-glucose to gluconic acid. A rather complete study of about 60 oxidizable sugars and their derivatives showed that only 2-deoxy-D-glucose is catalysed at a rate comparable to that of β-D-glucose. The anomer α-D-glucose is oxidized catalytically less than one per cent as rapidly as the β-anomer[2]. Urease, which catalyses 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 uncatalysed 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 neutral pH that the rates of the other thermodynamically possible reactions are negligible.

This specificity of enzymes, and their ability to catalyse reactions of substrates at low concentrations is of great use in chemical analysis. Enzyme-catalysed 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 labour 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 labour that have made enzyme-catalysed 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.

IMMOBILIZED ENZYME

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 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; (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 interference 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 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[3].

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 catalysed 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 versus glucose concentration are prepared, and from these the amount of glucose present in whole blood or plasma can be calculated[3].

We[4] 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 hydrolysed 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 of 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 later publications, we[5, 6] 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.

FACTORS THAT AFFECT THE RESPONSE OF THE UREASE ELECTRODE

Figure 1 shows the effect of substrate concentration on the response of the urease electrode. As the urea concentration is increased by a factor of ten, the steady-state response increases until at high substrate concentration the steady-state response is independent of the substrate concentration, as predicted from the Michaelis equation for enzymes in solution.

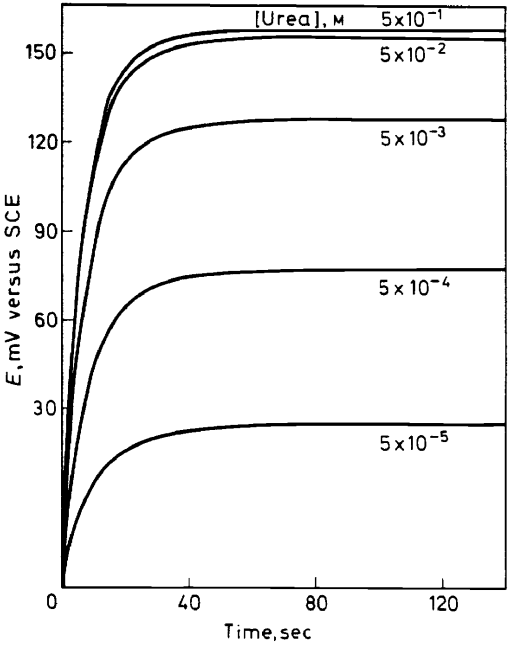


Figure 1. Urea response curves for a urease electrode containing 175 mg of urease/cm^3 of gel with a 350 μ netting; Type I electrode.

To study the effect of enzyme concentration on the enzyme gel layer activity, gels were prepared with enzyme concentrations ranging from 8 to 292 mg of urease/cm^3 of gel. The steady-state response of each enzyme-coated electrode when dipped in urea solutions from 5×10^{-5} to 1.6×10^{-1} M was measured. The results are shown in *Figure 2.* The slope of each curve increases with the amount of enzyme in the gel layer on the electrode until with larger enzyme concentrations, only a small increase in activity of the gel membrane is obtained. *Figure 3* is a plot of the steady-state response for

8.33×10^{-2} M urea against the amount of urease in the gel membrane. There is a rapid increase in response or activity up to 20 mg of urease/cm^3 of gel. Above 20 mg of urease/cm^3 of gel a large increase in enzyme concentration gives only a small increase in activity of the enzyme gel membrane on the cation electrode. Optimum enzyme concentration in the gel, considering only the economy of enzyme, is obtained at about 20 mg of urease/cm^3 of gel.

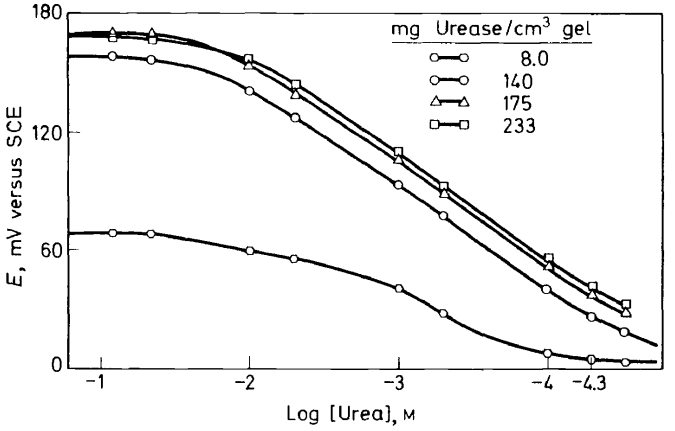


Figure 2. Effect of enzyme concentration on electrode response; type I electrode; 250 μ netting.

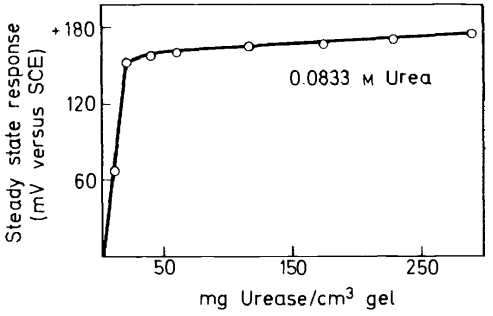


Figure 3. Dependence of gel-layer activity on enzyme concentration, type I electrode; 350 μ netting.

A series of urease electrodes was prepared with the same urease concentration (175 mg of urease/cm^3 of gel) but with different gel compositions to determine if the activity of the gel layer depends upon the gel composition. With a 350 μ gel layer over the cation electrode, variation of the gel per cent from 5 to 17.6 at constant monomer: crosslinking ratio gave less than a two per cent difference in response with 8.33×10^{-2} M urea. Variation of the per cent crosslinking material from 5 to 19 at constant gel concentration gave likewise a very small difference in response.

With a urease concentration of 175 mg/cm^3 of gel, the steady-state response to 8.33 $\times$ 10^{-2} M urea decreased by only two per cent upon decreasing the gel layer thickness from 350 to 60 μ.

Types I, II and III enzyme electrodes with a 350 μ netting and 175 mg of urease/cm^3 of gel gave essentially the same response to urea. The cellophane coatings had no effect on the response regardless of the urea concentration.

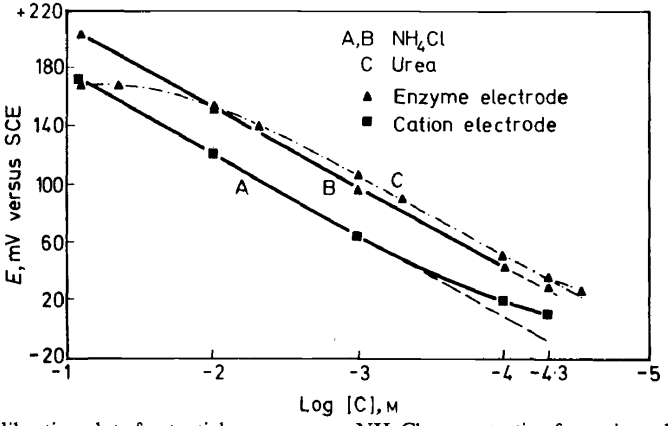


Figure 4. Calibration plot of potential versus urea or NH_4Cl concentration for various electrodes; 175 mg of urease/cm^3 gel: ▲, electrode coated with polymer containing 175 mg/cm^3 urease; ■, electrode uncoated or coated with polymer alone.

The potential resulting from changes in the urea concentration of the test solution should obey equation 2 within a certain range of urea concentrations as shown in *Figure 4*. Curves A and B of this figure show that the response

$$E_{obsd} = E^{0'} + (0.0591/n) \log [\text{urea}] \text{ (at } 25°) \qquad (2)$$

of the enzyme-coated electrode to ammonium chloride (the NH_4Cl was dissolved in the same buffer as the urea) is greater than that of the uncoated electrode. In addition, the response to NH_4^+ is linear down to much lower concentration as shown in Curve B. The higher sensitivity of the enzyme-coated electrode and more linear response at lower cation concentrations is due to the fact that at pH 7.0, the enzyme immobilized in the gel layer is negatively charged; the immobilized enzyme acts like a cation exchanger[5]. The plot of E versus log (urea), Curve C, gives a straight line with a slope of 50 mV at 25° in the range 10^{-3} to 5 $\times$ 10^{-5} M urea. Curve C lies above B since urea hydrolyses to give two NH_4^+ ions. The expected maximum separation of curves B and C (50 mV slope of the linear portions) is 50 log 2 = 15 mV. The observed separation was 9 mV. Below 5 $\times$ 10^{-5} M, the response was non-linear due to the poor response of the cation electrode to low ion concentrations.

The dynamic characteristics of the urease-coated cation electrode to urea were evaluated by exposing the electrode to a rapid change in urea concentration and recording the potential versus time curve. Typical response curves are shown in *Figure 1*, for type I electrode. The time required to reach the steady state is strongly dependent on the gel-layer thickness. The time interval for 98 per cent of the steady-state response was about 26 sec with the 60 μ netting and about 59 sec with the 350 μ netting for 8.33×10^{-2} M urea and an enzyme concentration of 175 mg/cm^3 of gel. The time interval for 98 per cent of the steady-state response for the uncoated cation electrode is 23 sec with 9.5×10^{-3} M NH_4Cl. The cellophane coatings for types II and III electrodes had little effect on the response time with the 350 μ netting.

After determination of a urea concentration, the reference and urease electrodes are removed from solution and the enzyme gel layer is rapidly flushed out in the automatic electrode washer[5]. For 10^{-3} M urea, a 4.35 ml/min wash rate, and with a 350 μ film over the glass electrode, the wash time is about 2 min. The washout time decreases with decrease in the urea concentration, increase in flowrate of buffer through the electrode washer, and decrease in enzyme gel-layer thickness.

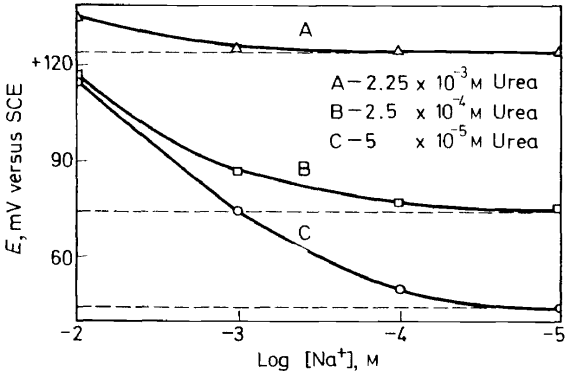


Figure 5. Effect of Na^+ ions on response of urea electrode to urea.

Although the enzyme-coated electrode also responds[6] to monovalent cations such as Na^+, K^+, Ag^+ and Li^+, significant amounts of these foreign materials will not interfere in urea determinations, except for Ag^+ ion, which inactivates the enzyme. *Figures 5* and *6* show the effects of Na^+ and K^+ ions on several urea responses. The Na^+ ion concentration must be less than one-half the urea concentration and the K^+ ion concentration less than one-fifth the urea concentration, otherwise the urea response is not independent of the concentration of these ions. Because the Na^+ and/or K^+ ion concentration must be less than the urea concentration, it is probable that adequate buffer capacity could not be obtained using, for example, the phosphate salts. The necessity of using Tris buffer as a solvent may be a serious limitation of the electrode for certain analytical purposes.

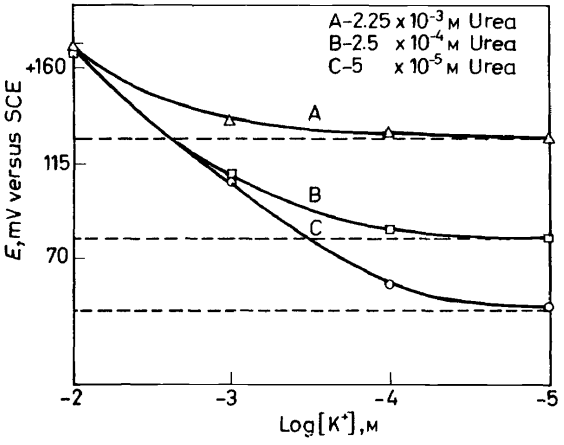


Figure 6. Effect of K^+ ions on response of urease electrode to urea.

FACTORS THAT AFFECT THE STABILITY OF THE UREASE ELECTRODE

Since it was experimentally easier and also more meaningful to study stability versus time of a hydrated electrode at an operational temperature of 25°, no studies have been performed at higher or lower temperatures or with a dehydrated electrode. To determine the effect of immobilization parameters on the stability of the enzyme electrode, a series of enzyme electrodes was prepared while varying one immobilization parameter and maintaining all of the other parameters constant. To determine the stability of the immobilized urease coating on the surface of the 39137 electrode, the steady-state potential was obtained for a given urea substrate concentration at periodic time intervals. If the steady-state potential is constant within a certain period of time, no loss of activity of the immobilized enzyme has occurred. All stability data reported were obtained with the electrode stored at 25° in Tris buffer between measurements.

The maximum stability that could be achieved with the type I enzyme electrode was obtained with the following immobilization parameters: photopolymerizing for one hour at 28° with a no. 1 photoflood lamp; a gel-layer thickness of 350 μ; and an enzyme concentration in the gel of 175 mg/cm^3 gel. The slope of the stability curve, $\Delta mV/\Delta t$, shows that the measured stability depends on the substrate concentration used in the stability measurements[6]. When the urea concentration is high enough so that the steady-state response is independent of the substrate concentration, $\Delta mV/\Delta t$ was 0.2 mV/day over a 14-day period. At lower substrate concentrations, as, for example, 1×10^{-3} M urea, the steady-state response is first order in urea concentration and a much smaller loss in activity was obtained, 0.05 mV/day over a 14-day period. Since 1×10^{-3} M urea represents the upper limit of substrate concentration which can be measured with the enzyme electrode, the steady-state response falls by only 0.7 mV during 14 days operation at

25°. After 14 days, the loss in activity was much greater for both substrate concentrations.

To study the effect of the activity of immobilized urease on enzyme gel stability, type I enzyme electrodes were prepared with activity of enzyme from 375 to 3500 Sumner units/gramme of enzyme. No appreciable change in stability occurred with this relatively large change in enzyme activity. On the other hand, highly purified urease is known to be very unstable in solution. A similar trend in stability would be expected with immobilized urease.

Greater stability with type I enzyme electrode was always obtained when the gel solution was less than two days old. Gel solutions were stored without added polymerization catalysts when the storage period was greater than two days. The solutions were always stored in the dark at room temperature. The stability of the urease type I electrode was studied as a function of enzyme gel-layer thickness in the range 30 to 350 μ. The stability increased with increased thickness of the enzyme gel layer.

The effect of urease concentration in the gel layer on the stability of the enzyme electrode was also studied. Below 20 mg of urease/cm^3 of gel, the response of the enzyme electrode varied greatly with urease concentration, *Figure 3*. Above 20 mg of urease/cm^3 gel, the response is much less dependent on the urease concentration. The measured stability of the enzyme electrode was always less with a urease concentration below 20 mg of urease/cm^3 gel. In all of the stability work 175 mg of urease/cm^3 of gel was employed unless the stability parameter studied was urease concentration in the gel.

Several experiments were run to determine quantitatively the effect of photopolymerization light intensity and photopolymerization time on type I enzyme electrode stability. When the high intensity photoflood lamp is substituted with a 60 W domestic lamp, the loss in activity rises from 0.2 to 4.2 mV/day for 8.33×10^{-2} M urea. A similar loss in activity for type I electrode was obtained when only the photopolymerization time was reduced from one hour to 15 min.

To study the effect of photopolymerization temperature and water content of the gel layer during photopolymerization on type I electrode stability, a series of enzyme electrodes was prepared with photopolymerization temperature ranging from 4° to 43°. The water content of the gel layer over the electrode surface was also varied when the photopolymerization temperature was changed; this is because the rate of evaporation of water for the thin enzyme gel layer varies directly with temperature. When the photopolymerization temperature and water content of the gel were varied to study type I electrode stability, the other immobilization parameters were adjusted to give maximum stability. The stability, measured with 8.33×10^{-2} M urea, showed a loss of only 0.2 mV/day at 28° photopolymerization temperature; upon lowering the immobilization or photopolymerization temperature to 6°, the loss in electrode activity is much higher, 3.7 mV/day. At 6° the rate of evaporation of water from the enzyme gel layer during photopolymerization is so slow that the gel layer is still damp to the touch after immobilization is complete. This large loss in activity is due to leaching of enzyme from the gel layer. Enzyme which had leached out of the gel layer could easily be detected in the buffer solution used to store the electrode. At 28° the rate of evaporation of water from the gel layer is sufficiently rapid so that when the polymerization is

complete, the electrode is dry to the touch. The enzyme electrode is now more stable because a less porous polymer is formed. At higher polymerization temperature, such as 43°, the resulting electrode is again less stable than when the polymerization temperature is 28°. Therefore, maximum stability is obtained with type I enzyme electrode when the photopolymerization temperature is 25° to 28°.

To determine the effect of a film of cellophane on enzyme electrode stability, a type II electrode was made by placing a thin film of cellophane over the enzyme gel layer. The cellophane was permeable to the urea substrate but not the high molecular weight enzyme. Polymerization parameters were the same as those used to obtain the maximum stability for type I electrode. Enzyme electrode type II stability, measured with either 8.33×10^{-2} or 1×10^{-3} M urea, showed no measurable loss in activity for 21 days (electrode stored between measurements in Tris buffer at 25°). After 21 days, the electrode began to lose activity. The increased stability of type II electrode over type I electrode is apparently due to the cellophane which prevents any enzyme from leaching out of the enzyme gel layer. The stability of type III electrode was identical with that of type II.

An attempt was made to determine if the enzyme activity in the gel layer was actually more stable than enzyme in free solution. When 60 mg of urease/100 cm^3 solution was suspended in the buffer solution at 25°, the activity of the enzyme increased over the first eight days. On the other hand, if the urease suspension was allowed to dissolve for only 30 min, then filtered, the stability of the filtered solution was less than that of the unfiltered solution. In the unfiltered solution, the increase in activity with time was due to more of the active enzyme dissolving. The loss in activity (0.4 mV/day) of the filtered solution was greater than with the optimum stability obtained with type II enzyme electrode. However, the break in the stability curve with the type II electrode occurred after 21 days, whereas with the filtered free enzyme solution, no break occurred even after 30 days. The loss in activity of the filtered solution should be even greater at lower enzyme concentrations, because the lower the enzyme concentration in solution, the greater the danger of enzyme inactivation by impurities in solution. Taking into account the actual amount of enzyme immobilized over the cation electrode and the amount of buffer which came in contact with type I and type II enzyme electrodes to obtain the stability data, the stability of the immobilized enzyme may in reality be greater than free solution stability.

In summary, an electrical transducer for urea is described. The design of the analytical device takes advantage of the high sensitivity of the Beckman 39137 çation electrode and the specificity associated with enzyme analysis. The excellent stability of the various types of urease enzyme electrodes developed here is due to an understanding of the parameters that affect immobilized enzyme stability. A review of the literature shows that enzymes have been immobilized for many years, yet heretofore an immobilized enzyme could only be used continuously for 10 to 12 hours at or above room temperature without loss of activity[7]. Since the response of the urease electrode is not affected by cellophane coatings around the enzyme gel layer, it should be possible to trap a liquid enzyme layer in cellophane over the surface of an electrode sensor. Such enzymes containing membrane electrodes

were first described by Clark and Lyons[8], but no data were given with regard to response time and stability. Preliminary experiments have shown that a liquid layer of urease trapped in a double cellophane layer over a cation electrode may produce a useful enzyme electrode[9].

DETERMINATION OF UREA IN BLOOD AND URINE

For the determination of urea in biological materials such as blood or urine, it is not possible to use the simple method described above, because some components of these liquids influence the electrode response. This effect is probably partly caused by a change of liquid junction potential with changing composition of solution and partly by changing ionic strength.

A cell using a glass electrode (Beckman Electrode 39137 or 39047) as the reference electrode was tried in an attempt to eliminate the effect described above. Calibration curves for urea were found to be the same when the cell with the SCE reference electrode was used. Also, the interferences of monovalent cations in solution are smaller in this case because both electrodes are sensitive to these ions. However, concentrations of Na^+ and K^+ higher than 10^{-4} M, considerably decreased the electrode response. This effect could be explained as a decrease of activity coefficients in the presence of other ions or as decrease of enzymic reaction rate caused by a higher ionic strength.

Combining the cell with the uncoated glass reference electrode with ion exchanger, the determination of urea in blood and urine is possible.

Results obtained for the determination of urea in blood using the described procedure are summarized in *Tables 1* and *2*. For the determination of urea in serum, the calibration curve obtained in the presence of protein was used.

Although the slope of the calibration curve remains constant during the course of a day, the entire standard curve may shift by several millivolts. Therefore, the electrode potential of a standard solution of urea (5×10^{-4} M)

Table 1. 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 2. 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

was determined between each pair of measurements and this potential was used as the standard reference value in calculation of the true potential of an unknown urea solution:

$$E_{unk}(\text{True}) = E_{unk}(\text{Observed}) - (E_{std} - \text{constant}) \quad (3)$$

This constant (the potential of a 5×10^{-4} M urea solution in buffer minus the potential of Tris buffer, 0.5 M, pH 8.0) is determined once a day. The true potential of the unknown urea solution is then calculated via equation 3. This method of comparison to a standard has been described for ion-selective electrodes and gives more reproducible results compared to a method which uses a standard reference potential obtained from a buffer solution.

If Beckman microelectrodes are used, ten times smaller amounts of sample (0.1 ml), ion-exchanger (0.2 g), and buffer solution (5 ml) can be used with roughly the same accuracy.

The results obtained with the urea electrode are compared with values obtained by the standard spectrometric method in *Tables 1* and *2*[10]. The precision obtainable is about one per cent averaging three or more samples. The difference between the results obtained with the urea electrode and the standard spectrophotometric method is about two or three per cent; however, it is believed that the electrode method is more accurate due to the low reliability factor of the spectrophotometric method[11].

AN ELECTRODE FOR DETERMINATION OF AMINO ACIDS

An electrode suitable for the determination of L-amino acids is described. The liquid membrane electrode is made by covering the Beckman cation sensitive glass electrode with a layer of L-AAO solution. The stability of the electrode is good for the first two weeks. The sensitivity of the electrode, and the influence of pH, temperature, concentration of substrate, oxygen and

enzyme, are reported. The addition of catalase to the enzyme solution yields an improved electrode.

An electrode for the determination of amino acids is described which is made with immobilized L-amino acid-oxidase. The electrode detects NH_4^+ ions formed in the enzyme-catalysed oxidation of the amino acid.

An L-amino acid electrode was made by placing a thin layer of L-amino acid oxidase (L-AAO) over a Beckman monovalent cation electrode[12]. The enzyme catalyses 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 hydrogen peroxide formed reacts non-enzymatically with the α-keto acid product

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

If hydrogen peroxide is destroyed by catalase, the overall reaction is described by equation 6

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

Ammonium ions formed in equation 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 PREPARATION OF L-AMINO ACID ELECTRODE

The Beckman monovalent cationic electrode 39137 was used as the sensor of ammonium ions. A nylon netting was placed over the glass bulb and was fixed with rubber rings. An enzyme gel solution (0.58 g *N,N'*-methylene bisacrylamide + 5 g acrylamide per 25 ml of solution. 3 mg of $K_2S_2O_2$ and riboflavin added as catalyst) was dropped on the netting and polymerized for one hour (type I electrode). The type II electrode, 'liquid membrane electrode', was prepared by soaking a nylon netting with buffer solution containing a definite amount of L-AAO (optimum amount 100 mg/ml). Both electrodes (types I and II) were covered with a film of dialysis paper. The electrodes were stored in buffer solutions when not in use.

CONCENTRATION OF ENZYME IN THE IMMOBILIZED LAYER

The stability of electrodes containing 20 and 100 mg of L-AAO per ml of solution, were compared. Electrodes with the higher enzyme concentration were more stable. The results obtained are summarized in *Table 3*. Each value is taken from three independent stability measurements carried out under the same conditions.

Table 3. Stability of four types of L-amino acid electrodes

Electrode[a]	1	2	3	4
2×10^{-4} M L-phe	0.35 mV/day[b]	2.5 mV/day[b]	0.13 mV/day[b]	0.05 mV/day[b]
2×10^{-3} 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 a more rapid decrease of response was observed.

ACKNOWLEDGEMENT

The financial assistance of the National Science Foundation (GB 12669) and the National Institutes of Health (GM 17268) is gratefully acknowledged.

REFERENCES

1 E. W. White, F. McCapra and G. F. Field, *J. Am. Chem. Soc.* **85**, 337 (1963).
2 S. P. Colowick and N. O. Kaplan (eds.), *Methods of Enzymology*, p 107, Academic Press: New York (1957).
3 S. Updike and G. Hicks, *Nature, London*, **214**, 986 (1967).
4 G. G. Guilbault and J. Montalvo, *J. Am. Chem. Soc.* **91**, 2164 (1969).
5 J. G. Montalvo and G. G. Guilbault, *Analyt. Chem.* **41**, 1897 (1969).
6 G. G. Guilbault and J. Montalvo, *Anal. Letters*, **2**, 283 (1969).
7 E. K. Bauman, L. H. Goodson, G. G. Guilbault and D. N. Kramer, *Analyt. Chem.* **37**, 1378 (1965).
8 L. C. Clark and C. Lyons, *Ann. N.Y. Acad. Sci.* **102**, 29 (1962).
9 G. G. Guilbault and J. Montalvo, *J. Am. Chem. Soc.* **92**, 2533 (1970).
10 G. G. Guilbault and E. Hrabankova, *Analyt. Chim. Acta*, **52**, 287 (1970).
11 HYCEL, *Colorimetric Determination of Urea*, Hycel, Inc., Houston, Texas (1969).
12 G. G. Guilbault and E. Hrabankova, *Analyt. Chem.* **42**, 1779 (1970).