showed the presence of 4-methylimidazole but no ACN. The solvent was removed under reduced pressure, and the residue taken up in 2 ml of ether and treated with a saturated solution of oxalic acid in ether. The precipitated oxalate salt, recrystallized once from acetone–water (3:1) had mp 205–206° dec; lit.[38] 205–206° dec. The nmr spectrum in $D_2O$ of the salt obtained showed peaks at 1.45, 2.82, and 7.65 in the ratio 0.60:0.81:3.00. Authentic samples were prepared of oxalate salts of the following compounds and their nmr spectra in $D_2O$ then recorded: 4-methylimidazole oxalate, $\tau$ $H_2$ 1.45, $H_5$ 2.82, $CH_3$ 7.65; 2-methylimidazole oxalate, $\tau$ $H_4$, $H_5$ 2.67; $CH_3$ 7.35; 3-methylpyrazole oxalate, $\tau$ $H_4$ 3.30, $H_5$ 1.82; $CH_3$ 7.47. Thus, the only product of the irradiation of ACN is 4-methylimidazole, the product having a certain amount of deuterium incorporated at positions two and five.

In a similar experiment using a 100-fold molar excess of $D_2O$ the ratio of protons in the recovered oxalate salt of 4-methylimidazole was $H_2$:$H_5$:$CH_3$ = 0.21:0.77:3.00. Again tlc indicated the presence of 4-methylimidazole only.

**Deuterium Exchange on ACN in the Absence of Irradiation.** The nmr spectrum of a solution of ACN (0.1 *M*) in $D_2O$ was determined at intervals and the disappearance of the vinyl proton monitored. The amount of vinyl proton (compared to $CH_3$ = 3.00) remaining after various times was: 0 hr, 1.00; 2 hr, 0.82; 4 hr, 0.71; 8 hr, 0.24.

**Irradiation of 3-Methylpyrazole.** A solution of 3-methylpyrazole (0.041 g; Aldrich) in 250 ml of tetrahydrofuran (distilled from sodium) was irradiated in quartz at 254 nm for 24 hr. The solvent was removed under reduced pressure and the residue examined by tlc and by nmr. Only 3-methylpyrazole was detected.

In a second experiment, 3-methylpyrazole (0.041 g) plus benzophenone (0.009 g) in 250 ml of tetrahydrofuran was irradiated in Pyrex with 350-nm lamps for 48 hr. The solvent was removed under reduced pressure and the residue examined by tlc. Only 3-methylpyrazole and benzophenone were detected.

These results are somewhat surprising, since our conditions are not too much different from those reported by Tiefenthaler, *et al.*,[19] for the conversion of 3-methylpyrazole to 2- and 4-methylimidazole.

(38) A. Windaus and F. Knoop, *Ber.*, **338**, 1166 (1905).

**Attempted Detection of Intermediates in the Photolysis of ACN.** Five milliliters of a 5% (w/v) solution of ADN in acetonitrile was irradiated in quartz at 254 nm. Periodically, aliquots were withdrawn for examination by ir and tlc. After 155 hr of irradiation only bands due to ACN could be seen in the ir spectrum of the sample, these being somewhat diminished in intensity. Examination of the sample by tlc, however, indicated the presence of ACN and 4-methylimidazole (diazotized sulfanilic acid is about ten times as sensitive to imidazole as it is to ACN) as well as three other compounds whose $R_f$ values lay between the origin and 4-methylimidazole. These minor components were isolated by preparative tlc and each was irradiated at 254 nm for 2 hr. No 4-methylimidazole was detected by tlc or by uv absorption spectrum in any of the samples.

**Conversion of Cyanoacetaldehyde Dimer to 6-Aminonicotinonitrile.** Duplicate samples of cyanoacetaldehyde dimer ($5 \times 10^{-5}$ *M*)[29] in 1 *M* $NH_4OH$ were degassed with two freeze–pump–thaw cycles, irradiated in Pyrex and monitored spectrophotometrically. The disappearance of starting material (310 nm) and formation of product (263 nm) was complete after 12 days. Examination of the reaction mixture by paper chromatography in two solvent systems (BW and PA) showed that the $R_f$ value of the major component matched that of an authentic sample of 6-aminonicotinonitrile. This spot was eluted from the paper and its uv spectrum determined: neutral, $\lambda_{max}$ 263 nm; acid, 255 nm (lit.[29] for 6-aminonicotinonitrile; neutral, $\lambda_{max}$ 263 nm; acid, 255 nm).

A control reaction was carried out in parallel with the above runs which was identical in all respects except that a 40° water bath was used in place of the ultraviolet light. Only a slight decrease in the absorption at 310 nm was observed.

**Acknowledgments.** We thank Professor R. Strong for the flash photolysis results and for assistance in their interpretation, Professor H. Richtol for use of his luminescence apparatus and for assistance in interpreting the data, and Professor S. Wait for the HMO program and for helpful discussions concerning the calculations.

# An Enzyme Electrode for the Substrate Urea

**G. G. Guilbault and J. G. Montalvo**

*Contribution from the Department of Chemistry, Louisiana State University in New Orleans, Lakefront Campus, New Orleans, Louisiana 70122.* *Received August 13, 1969*

**Abstract:** The development of several types of a urea transducer suitable for rapid, continuous determination of urea is described. The transducer is called an enzyme electrode because it is made by placing a thin film of urease enzyme immobilized in acrylamide gel over the surface of a Beckman cationic electrode responsive to ammonium ions. The immobilized enzyme catalyzes the decomposition of urea to ammonium ion at the surface of the cationic electrode. The ammonium ion is sensed by the cation electrode; the steady-state potential developed is proportional to the logarithm of the urea concentration. The preparation of the various types of urease enzyme electrodes, the immobilization parameters that affect the response of the electrodes, factors that affect the stability of the immobilized enzyme, and the effect of foreign monovalent cations on electrode response are described. An electrode was made which could be used continuously at 25° for 14 days with no loss in activity. By placing a thin film of cellophane over the immobilized gel layer, an electrode could be used continuously at 25° for 3 weeks with no loss in activity.

Enzymes are finding increased use in analytical chemistry. Reviews on the use of these biological catalysts as analytical reagents have been published by Guilbault.[1,2] 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 assay using enzymes requires large amounts of these materials, quantities greater than can be reasonably supplied, and quantities that would represent a prohibitive expenditure in most instances. If, however,

(1) G. G. Guilbault, *Anal. Chem.*, **38**, 527R (1966).
(2) G. G. Guilbault, *ibid.*, **40**, 459R (1968).

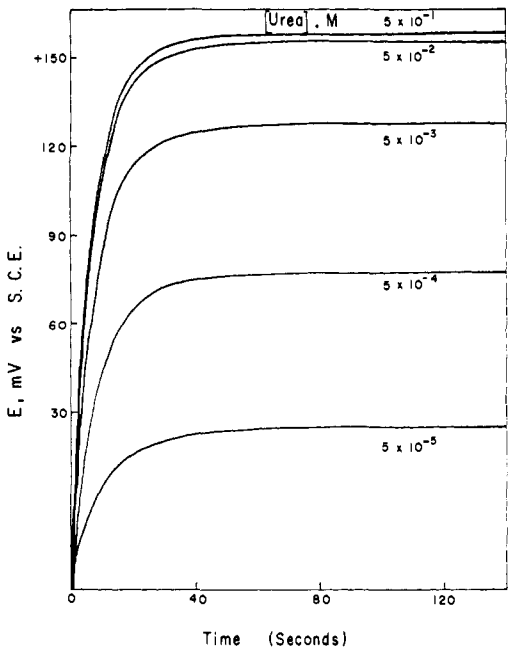


Figure 1. Urea response curves for a urease electrode containing 175 mg of urease/cc of gel with a 350-$\mu$ netting; type I electrode.

the enzyme could be prepared in a water insoluble matrix without loss of activity for at least a reasonable period of time, the same sample of immobilized (insolubilized) enzyme could be used over and over again. The immobilized enzyme could then be used in much the same way as 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 enzymatic activity.

Enzymes have been immobilized by chemical bonding to a water insoluble carrier and by physical entrapment of the enzyme in the insoluble carrier. A review on the preparation of insoluble enzymes has been prepared by Chibata and Tosa.[3] A more recent review was presented by Guilbault.[2]

Guilbault and Montalvo[4,5] have recently reported the development of several types of a urea transducer suitable for rapid, continuous determination of the substrate urea. This urea transducer is called a urease electrode because it is made by placing a thin film of immobilized urease over a Beckman cationic glass electrode which is responsive to ammonium ions. Specificity for the urea substrate is obtained by immobilizing the enzyme in a layer of acrylamide gel on the surface of the glass electrode. When the urease electrode is placed in a solution containing urea, in an appropriate solvent, the substrate diffuses into the gel layer of immobilized enzyme. The enzyme catalyzes the decomposition of urea to ammonium ion. The ammonium ion produced at the surface of the electrode is sensed by the monovalent cation electrode in a manner analogous to pH determination with a glass electrode. The potential of this electrode is measured after allowing sufficient time for the diffusion process to reach the steady state.

In this paper are reported a detailed preparation of the urease electrode, the results of a thorough investigation of the factors that affect the response of this electrode, and the factors that affect the stability of the urease immobilized on the electrode. A careful reading of the literature on immobilized enzymes has revealed to us that no one had considered the possibility that the enzymatic stability of an enzyme depends on the parameters involved in its immobilization. We, however, felt otherwise, and, in fact, could control the stability of our immobilized urease by simple variation of the immobilization parameters. We believe that this represents a breakthrough in the field of immobilized enzymes and will show that by careful control of the immobilization parameters, it is possible to obtain immobilized enzymes with very high stability. An electrode could be made which could be used continuously for 3 weeks at 25° with no loss of activity.

## Experimental Section

**Preparation of Urease Electrodes (Types I, II, and III).** The preparation of the acrylamide gel solution and various types of urease electrodes has been reported elsewhere.[5,6] Type I electrode consisted of an enzyme gel (urease–acrylamide) coating over the glass sensing bulb of the 39137 Beckman electrode. For Type II electrode, a cellophane film was added over the enzyme gel layer (*i.e.*, glass, enzyme gel, cellophane). Two cellophane layers were used with Type III electrode (glass, cellophane, enzyme gel, cellophane).

The cellophane membrane was donated by the Gulf South Research Institute (New Orleans, La. 70122).[1] This membrane was made by GSRI under Contract No. 14-01-0001-1271, Office of Saline Water, U.S. Department of the Interior. At the present time, the properties of the membrane are classified. The membrane, however, has a molecular weight exclusion of 1500.

**Apparatus.** The enzyme electrode was connected to the indicating electrode terminal of a Beckman Zeromatic Model II pH meter. A standard fiber-junction saturated calomel electrode (SCE) served as reference electrode. Millivolt measurements were displayed on a recorder. All measurements were carried out in a cell thermostated at 25 ± 0.01°.

**Chemicals.** A stock solution of urea, 0.5 *M*, was prepared in Tris buffer, 0.1 *M*, pH 7.0, and made fresh every 7 days. The water used in making all solutions was triply distilled. The urease enzyme used to make the urease electrode was obtained from the California Corporation for Biochemical Research, Grade B (activity, 375 Sumner Units per gram of enzyme).

**Procedure for Potentiometric Measurements.** Potentiometric measurements of the steady-state response for the determination of enzymatic activity and determination of stability of the various types of enzyme electrodes were carried out in the usual manner. An aliquot of the 0.5 *M* urea stock solution is pipetted into a 100-cc beaker containing 50 cc of Tris buffer. The solution is magnetically stirred and the steady-state potential recorded. After a measurement, the urease coating on the electrode was washed free of residual $NH_4^+$ ion by placing both the enzyme electrode and reference electrode in an automatic electrode washed[6] and monitoring the potential until a low level of ion concentration is obtained in the enzyme gel layer. The enzyme electrode was stored in Tris buffer at 25° between measurements.

## 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 10, the steady-state response increases until at high substrate concentrations the steady-state response is independent of the

(3) I. Chibata and T. Tosa, *Tampakushitsu Kakusan Koso*, 11, 23 (1966).

(4) G. G. Guilbault and J. G. Montalvo, Jr., *J. Amer. Chem. Soc.*, 91, 2164 (1969).

(5) G. G. Guilbault and J. G. Montalvo, Jr., *Anal. Lett.*, 2, 283 (1969).

(6) J. G. Montalvo, Jr., and G. G. Guilbault, *Anal. Chem.*, 41, 1897 (1969).

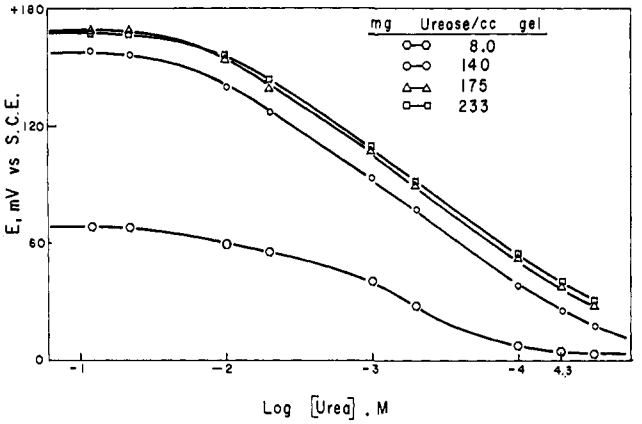


Figure 2. Effect of enzyme concentration on electrode response; type I electrode; 250-$\mu$ netting.

substrate concentration, as predicted from the Michaelis equation for enzymes in solution.[7]

To study the effect of enzyme concentration of the enzyme gel layer activity, gels were prepared with enzyme concentrations ranging from 8 to 292 mg of urease/cc of gel. The steady-state response of each enzyme-coated electrode when dipped in urea solutions from $5 \times 10^{-5}$ to $1.6 \times 10^{-1}$ $M$ 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 $8.33 \times 10^{-2}$ $M$ 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/cc of gel. Above 20 mg of urease/cc 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/cc of gel.

A series of urease electrodes were prepared with the same urease concentration (175 mg of urease/cc of gel) but with different gel compositions to determine if the activity of the gel layer depends upon the gel composition. With a 350-$\mu$ 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 2 per cent difference in response with $8.33 \times 10^{-2}$ $M$ 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/cc of gel, the steady-state response to $8.33 \times 10^{-2}$ $M$ urea decreased by only 2 per cent upon decreasing the gel layer thickness from 350 to 60 $\mu$.

Types I, II, and III enzyme electrodes with a 350-$\mu$ netting and 175 mg of urease/cc of gel gave essentially the same response to urea. The cellophane coatings had no effect on the response regardless of the urea concentration.

The potential resulting from changes in the urea concentration of the test solution should obey eq 1 within a certain range of urea concentrations as shown

(7) M. Dixon and E. C. Webb, "Enzymes," 1st ed, Academic Press, New York, N. Y., 1964.

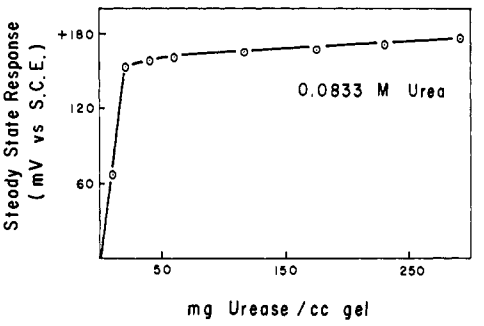


Figure 3. Dependence of gel-layer activity on enzyme concentration; type I electrode; 350-$\mu$ netting.

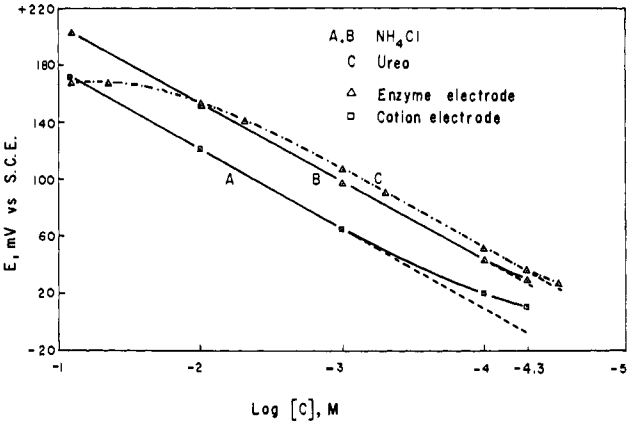


Figure 4. Calibration plot of potential *vs.* urea or $NH_4Cl$ concentration for various electrodes; 175 mg of urease/cc gel: △, electrode coated with polymer containing 175 mg/cc urease; □, electrode uncoated or coated with polymer alone.

in Figure 4. Curves A and B of this figure show that

$$E_{obsd} = E^{0\prime} + \frac{0.0591}{n}\log[\text{urea}]\,(\text{at } 25°) \qquad (1)$$

the response 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 $NH_4^+$ in 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.[6] The plot of $E$ *vs.* log [urea], curve C, gives a straight line with a slope of 50 mV at 25° in the range $10^{-3.5} \times 10^{-5}$ $M$ urea. Curve C lies above B since urea hydrolyzes 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 nonlinear due to the poor response of the cation electrode to low ion concentrations.

The dynamic response 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 *vs.* 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

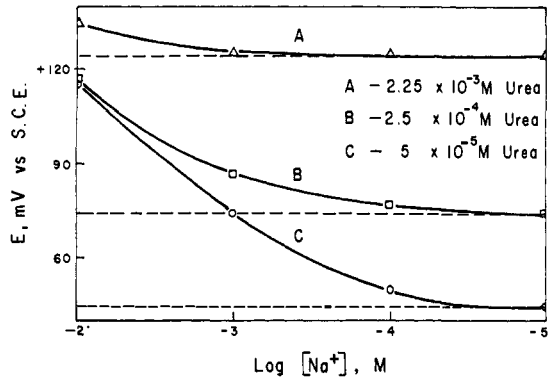


Figure 5. Effect of $Na^+$ ions on response of urease electrode to urea.

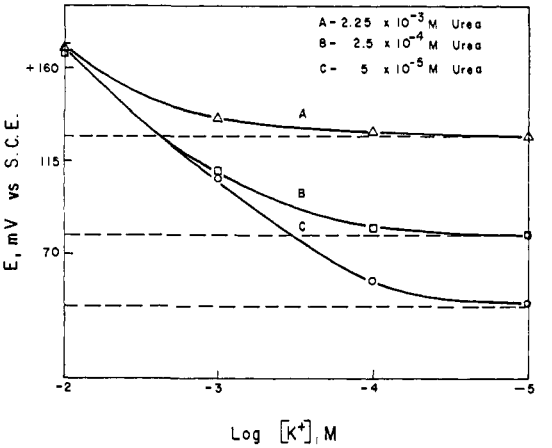


Figure 6. Effect of $K^+$ ions on response of urease electrode to urea.

thickness. The time interval for 98% of the steady-state response was about 26 sec with the 60-$\mu$ netting and about 59 sec with the 350-$\mu$ netting for $8.33 \times 10^{-2}$ $M$ urea and an enzyme concentration of 175 mg/cc of gel. The time interval for 98% of the steady-state response for the uncoated cation electrode is 23 sec with $9.5 \times 10^{-3}$ $M$ $NH_4Cl$. The cellophane coatings for types II and III electrodes had little effect on the response time with the 350-$\mu$ 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.[6] For $10^{-3}$ $M$ urea, 4.35 cc/min wash rate, and with a 350-$\mu$ film over the glass electrode, the wash time is about 2 min. The washout time decreases with decrease in the urea concentration, increase in flow rate of buffer through the electrode washer, and decrease in enzyme gel-layer thickness.

Although the enzyme-coated electrode responds also to monovalent cations such as $Na^+$, $K^+$, $H^+$, $Ag^+$, and $Li^+$,[6] 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.

### Factors That Affect the Stability of the Urease Electrode

Since it was experimentally easier and also more meaningful to study stability *vs.* 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.

Table I shows that the measured stability of enzymatic activity for the urease electrode depends on many parameters. The maximum stability that could be achieved with the type I enzyme electrode was obtained with the following immobilization parameters: photopolymerizing for 1 hr at 28° with a no. 1 photoflood lamp; gel-layer thickness of 350 $\mu$; and the enzyme concentration in the gel 175 mg/cc 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.[5] 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, $1 \times 10^{-3}$ $M$ 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 $1 \times 10^{-3}$ $M$ urea represents the upper limit of substrate

**Table I.** Measured Stability of Enzymatic Activity for the Immobilized Urease in a Function of Many Parameters

| Resolved |
|---|
| 1. Urea concentration |
| 2. Activity of immobilized urease |
| 3. Gel solution age |
| 4. Enzyme gel-layer thickness |
| 5. Photopolymerization time |
| 6. Urease concentration in gel |
| 7. Photopolymerization light intensity |
| 8. Photopolymerization temperature and water content of gel during photopolymerization |
| 9. Enzyme electrode type |
| **Unresolved** |
| 1. Gel composition (per cent gel and monomer:crosslinking ratio) |
| 2. Immobilized stability *vs.* free solution stability |

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/gram 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.[8] 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 2 days old. Gel solutions were stored without added polymerization catalysts when the storage period was greater than 2 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 60–350 $\mu$. 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/cc of gel, the response of the enzyme electrode varied greatly with urease concentration, Figure 3. Above 20 mg of urease/cc 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/cc gel. In all of the stability work 175 mg of urease/cc 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 house lamp, the loss in activity rises from 0.2 to 4.2 mV/day for $8.33 \times 10^{-2}$ $M$ urea. A similar loss in activity for type I electrode was obtained when only the photopolymerization time was reduced from 1 hr 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 also varied when the photopolymerization temperature was changed; this is because the rate of evaporation of water from 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 $8.33 \times 10^{-2}$ $M$ 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 temperatures, 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 25–28°.

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 $8.33 \times 10^{-2}$ or $1 \times 10^{-3}$ $M$ 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 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 cc solution was suspended in the buffer solution at 25°, the activity of the enzyme increased over the first 8 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 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 cation 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

(8) G. Gorin and C. Chin, *Anal. Biochem.*, 17, 49 (1966).

immobilized enzyme stability. A review of the literature shows that enzymes have been immobilized for about 4 years, yet heretofore an immobilized enzyme could only be used continuously for 10–12 hr at or above room temperature without loss of activity.[9,10] Since the response of the urease electrode is not effected 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,[11] 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.

Finally, it is hoped that the primary disadvantage of the present electrode, namely the interference by $Na^+$ and $K^+$, can be overcome by the use of ion-exchange resins. Two approaches appear feasible: (1) the addition of 1 g of cation exchange to the solution, with stirring, before introduction of the electrode and (2) the placing of a cation-exchange resin membrane over the outside of the urease membrane in place of the cellophane. The latter would serve to both remove cations and help retain the enzyme urease.

(9) G. P. Hicks and S. J. Updike, *Anal. Chem.*, **38**, 726 (1966).
(10) E. K. Bauman, L. H. Goodson, G. G. Guilbault, and D. N. Kramer, *ibid.*, **37**, 1378 (1965).
(11) L. C. Clark, Jr., and C. Lyons, *Ann. N. Y. Acad. Sci.*, **102**, 29 (1962).

**Acknowledgment.** The financial assistance of the National Science Foundation (Grant No. GM-12669) is gratefully acknowledged.

# *Communications to the Editor*

## Solvent Assistance in the Solvolysis of Secondary Substrates. I. The 2-Adamantyl System, a Standard for Limiting Solvolysis in a Secondary Substrate

*Sir:*

The exact definition of the mechanistic details of the solvolysis of simple substrates has long presented difficulties, especially with regard to secondary systems.[1-7] Whereas primary substrates usually react by nucleophilic displacement (SN2,[2] **N**[3]) and tertiary substrates *via* carbonium ions (SN1,[2] **Lim**[3]), secondary systems often exhibit "borderline" behavior which has traditionally been described either by considering intermediate mechanisms or by assuming concurrent unimolecular and bimolecular pathways.[1-5]

More recently, several authors have presented data which were interpreted to show that intimate[8] and solvent-separated ion pairs intervene in the solvolysis of simple secondary substrates.[3,6,7,9] We feel that the degree to which solvent participates nucleophilically in the overall solvolytic process or in any of the component stages has not been clearly established for such substrates. Solvent must be closely involved at the backside, at least in the product-forming step, because the substitution product is now known to be virtually completely inverted.[5-7] In this initial set of communications, we are concerned solely with the degree to which the overall solvolysis rate ($k_t$) is affected by nucleophilic participation of the solvent.

We have recently provided experimental evidence[10] to support the thesis[11] that secondary $\beta$-arylalkyl systems can react by two discrete pathways: neighboring aryl assisted ($k_\Delta$)[12] and solvent assisted ($k_s$).[12] This separation of pathways requires, if no crossover is to be observed, that *both* pathways, $k_\Delta$ and $k_s$, in competition

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(2) (a) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," 2nd ed. Cornell University Press, Ithaca, N. Y., 1969, Chapter VII, p 418 ff; (b) M. L. Bird, E. D. Hughes, and C. K. Ingold, *J. Chem. Soc.*, 634 (1954).
(3) (a) S. Winstein, E. Grunwald, and H. W. Jones, *J. Amer. Chem. Soc.*, **73**, 2700 (1951); (b) A. F. Díaz, I. Lazdins, and S. Winstein, *ibid.*, **90**, 1904 (1968), and papers therein cited.
(4) V. Gold, *J. Chem. Soc.*, 4633 (1956).
(5) M. C. Whiting, *Chem. Brit.*, **2**, 482 (1966); N. C. G. Campbell, D. M. Muir, R. R. Hill, J. H. Parish, R. M. Southam, and M. C. Whiting, *J. Chem. Soc., B*. 355 (1968); M. Pánková, J. Sicher, M. Tichý, and M. C. Whiting, *ibid.*, 365 (1968).
(6) H. Weiner and R. A. Sneen, *J. Amer. Chem. Soc.*, **87**, 287, 292 (1965); R. A. Sneen and J. W. Larsen, *ibid.*, **88**, 2593 (1966); **91**, 362 (1969); **91**, 6031 (1969).
(7) A. Streitwieser, Jr., T. D. Walsh, and J. R. Wolfe, Jr., *ibid.*, **87**, 3682 (1965); A. Streitwieser, Jr., and T. D. Walsh, *ibid.*, **87**, 3686 (1965). Also see M. P. Balfe, W. H. F. Jackman, and J. Kenyon. *J. Chem. Soc.*, 965 (1954).
(8) Intimate carbonium ion pairs in such simple secondary systems should not be likened to free carbonium ions. In such ion pairs, significant bonding probably still exists between the charged fragments with the anion providing a great deal of specific or nucleophilic solvation. For this reason, it would probably be better not to represent such an ion pair in the conventional manner, $R^+X^-$, but as $R^{\delta+}\cdots X^{\delta-}$. $R^+X^-$ suggests a similarity with $R^+$, but the two species can behave quite differently. The positive fragment of such ion pairs at best has only *some* "carbonium ion character"; it would perhaps be better to describe as "cationoid" the reactions of such species (H. C. Brown, private communication). That considerable bonding between partners in an ion pair exists is shown by the incomplete oxygen scrambling observed during ion-pair return (H. L. Goering and E. C. Linsay, *J. Amer. Chem. Soc.*, **91**, 7435 (1969), and references cited therein).
(9) V. J. Shiner, Jr., and W. Dowd, *ibid.*, **91**, 6528 (1969); V. J. Shiner, Jr., R. D. Fisher, and W. Dowd, *ibid.*, **91**, 7748 (1969).
(10) C. J. Lancelot and P. v. R. Schleyer, *ibid.*, **91**, 4291, 4296, (1969); C. J. Lancelot, J. J. Harper, and P. v. R. Schleyer, *ibid.*, **91**, 4294 (1969); P. v. R. Schleyer and C. J. Lancelot, *ibid.*, **91**, 4297 (1969); *cf.* J. M. Harris, F. L. Schadt, P. v. R. Schleyer, and C. J. Lancelot, *ibid.*, **91**, 7508 (1969).
(11) (a) J. A. Thompson and D. J. Cram, *ibid.*, **91**, 1778 (1969); (b) A. F. Diaz and S. Winstein, *ibid.*, **91**, 4300 (1969), and references cited in both these papers. In general, the earlier workers *assumed* discreteness of pathways and used *product data* to partition the overall rate constants, *e.g.*, $k_t$, into $Fk_\Delta$ and $k_s$.[11b] We[10] provided independent methods to evaluate $Fk_\Delta$ and $k_s$ kinetically and then showed that rate and product data were in agreement with one another.
(12) Definitions of these rate constants are found in paper III: P. v. R. Schleyer, J. L. Fry, L. K. M. Lam, and C. J. Lancelot, *ibid.*, **92**, 2542 (1970).