Articles

Amperometric Biosensor for Urea

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An enzyme electrode for the amperometric measurement of urea was prepared by co-immobilizing L-glutamate dehydrogenase and urease onto an Immobilon-AV affinity membrane attached to a glassy carbon electrode. The reduced nicotinamide adenine dinucleotide(NADH) was used as the electroactive species. The electrochemical oxidation of NADH was monitored at +1.0 volt *vs.* Ag/AgCl. The enzyme-immobilized electrode was linear over the range of 2.0×10^{-5} to 2×10^{-4} M. The response time of the electrode was approximately 3 min. and the optimum pH of the enzyme immobilized membrane was pH 7.4-7.6 (Dulbcco's buffer solution). It was stable for at least two weeks or 50 assays. There was no interference from other physiological species, except from high levels of ascorbic acid.

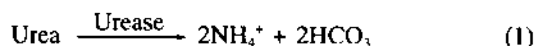
Introduction

Urea is widely distributed in nature, and its analysis is of considerable interest in clinical and agricultural chemistry since urea is used in fertilizers and plant food, environmental pollution testing, muscle and other applications. The most common enzymatic method for the determination of urea utilizes the catalyzed hydrolysis of urea.[1-11] The assay with urease can be followed by many different techniques such as colorimetry,[2,4] fluorometry[5,6] and electrochemistry.[8-12] For example, Naftalm *et al.*[2] used the absorbance change at 660 nm after the addition of hypobromide and phenol to measure the ammonia produced from urea in blood. Also, Wilson[4] described an automated method for the determination of urea using urease, hypochlorite and alkaline phenol. Kaltwasser and Schlegel[5] developed a coupled optical enzyme assay for urease, and Roch-Ramel[6] and Kuan *et al.*[7] assayed urea by measuring the NAD^+ formed from NADH by fluorometric method.

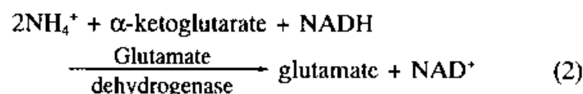
Many enzyme electrodes have been described for urea, using immobilized urease.[13-15] Keyes and Barabino[13] have described a continuous method for assay of urea using a column of chemically bound urease and an NH_3 gas electrode to measure the free NH_3 liberated in the urea-urease reaction.

But, potentiometric enzyme electrodes for urea have several disadvantages: they are slow to respond and even slower to attain the original base line. They suffer from interferences of Na^+ and K^+ in some base sensors (by the NH_4^+ ion electrodes). So Adams *et al.*[9] assayed urea coulometrically, using an immobilized enzyme reactor. Also, Kirsten *et al.*[16] developed an amperometric sensor for urea by measuring the current from the electrochemical oxidation of hydrazine. Seo *et al.*[17] determined NH_4^+ amperometrically, using immobilization of L-glutamate dehydrogenase on the Immobilon-AV membrane.

In this paper, an amperometric biosensor for urea is described. Concentration of urea was measured based on the urease catalyzed hydrolysis of urea; the α-ketoglutarate reacted with liberated NH_4^+ in the presence of reduced nicotinamide adenine dinucleotide (NADH) and L-glutamate dehydrogenase (GL-DH). The reaction scheme is as follows:



$$\text{Urea} \xrightarrow{\text{Urease}} 2NH_4^+ + 2HCO_3 \quad (1)$$



$$2NH_4^+ + \alpha\text{-ketoglutarate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{Glutamate}} \text{glutamate} + NAD^+ \quad (2)$$

Using this sequence reaction, we measured the current change as an excess NADH was oxidized to NAD^+. Also, we investigated the optimum conditions (effect of NADH, enzyme, pH, interfering species, reaction time, lifetime and calibration curve) for the determination of urea.

Experimental

Apparatus. The current was monitored with the Amperometric Biosensor Detector (Universal Sensors, Inc., USA) using a glassy carbon electrode (1.8 mm^2) poised at +1.0 volt *vs.* Ag/AgCl. A linear chart recorder (REC 80 servograph, Radiometer America, Inc.) was used to record the resulting current changes. The pH were measured with a pHM 84 Research pH meter (Radiometer America, Inc.). Adjustable-volume Finnpipettes were used to deliver solutions.

Reagents. All solutions were made with Dulbecco's buffer,[18] pH 7.4 which was prepared with double distilled water and contained the following salt concentration: 137 mM NaCl, 2.7 mM KCl, 3.0 mM Na_2HPO_4 and 1.5 mM KH_2PO_4.

NADH, α-ketoglutarate, L-glutamate dehydrogenase [EC 1.4.1.3] and urease [EC 3.5.1.5.] were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of 10^{-2} M α-ketoglutarate, 5.0×10^{-3} M NADH, and 10^{-2} M urea (EM Sci. Ind.) were prepared in Dulbecco's buffer solution. When not in use, stock solutions of NADH and α-ketoglutarate were stored at 4 °C.

Preparation of biosensor. 30 μL of 1,000 units/mL urease solution and 20 μL of 1,000 units/mL L-glutamate dehydrogenase solution were pipetted onto the membrane (Immobilon-AV Affinity membrane 0.65 μm pore size; Millipore Corporation, Bedford, MA) attached to an inverted electrode jacket.

The membranes were allowed to dry at room temperature for 2-3 hrs. Immobilized enzyme membranes were kept in Dulbecco's buffer solution at 0 °C when not in use. When currents were measured, the glassy carbon electrode was connected to an inverted electrode jacket onto which the immobilized enzyme membrane was attached.

Measurement procedure. A constant potential of +1.0 volt *vs.* Ag/AgCl[19] was applied to the glassy carbon working electrode onto which the enzyme immobilized membrane was attached. Responses of the electrode were measured for the test sample solution added to 5.0 mL of Dulbecco's buffer solution at 25 °C. Then, in order to obtain optimum conditions for the determination of urea, effect of NADH, amount of enzyme, pH, interfering species, reaction time, lifetime and calibration curve were investigated.

In the test sample, the reagent solution contained 4.0×10^{-4} M α-ketoglutarate, 2.0×10^{-4} M NADH and 2.0×10^{-4} M urea. When Peters fertilizers were determined, ammonia was vaporized in the alkaline solution and was identified by Nessler's reagent.

The analytical signal was displayed as a series of peaks on the recorder chart. The peak height was then related to the concentration of urea by means of a calibration curve. All data shown were the average of three measurements.

Results and Discussion

Completeness of the coupled enzymatic reaction. Urea is hydrolyzed by urease, yielding two equivalents of NH_4^+ ions per mole. In the presence of H^+ ions, reduced nicotinamide adenine dinucleotide (NADH), L-glutamate dehydrogenase (GL-DH), and NH_4^+ reacts with α-ketoglutarate to yield L-glutamate and oxidized NAD^+. This reaction is complete within a short time at the optimum concentration of NADH. For the 5 mL of sample solution containing 2.0×10^{-4} M NADH, 4.0×10^{-4} M α-ketoglutarate and 2.0×10^{-4} M urea, respectively, the enzymatic reaction is complete in approximately three minutes.

To determine the optimum concentration of NADH, the change in current with concentration of NADH was measured (Figure 1). The optimum limiting concentration of NADH was 2.0×10^{-4} M for 4.0×10^{-4} M α-ketoglutarate and 2.0×10^{-4} M urea, as shown in Figure 1. This result was due to chemical equivalent. So, concentration of NADH was needed at least equal to that of urea.

Effect of the composition of the membrane on the coupled enzymatic reaction. In order to determine the optimum amounts of the enzymes (urease/GL-DH), currents were measured with change of relative ratio of the enzymes (urease/GL-DH) pipetted onto the membrane, for 5 mL of sample solution containing 2.0×10^{-4} M urea, 2.0×10^{-4} M NADH and 4.0×10^{-4} M α-ketoglutarate, respectively (Figure 2). The enzymatic reaction takes place most effectively when the volume ratio of the enzymes (urease/GL-DH) was between 0.8 and 2.0 as shown in Figure 2. Hence, we used 30 μL of urease (1,000 units/mL) and 20 μL of GL-DH (1,000 units/mL) to construct the membranes.

Optimum pH. The effect of pH of the electrode response was investigated using Dulbecco's buffer solution (the pH changed with either 0.1 M HCl or 0.1 M NaOH) at concentrations of 2.0×10^{-4} M NADH, 2.0×10^{-4} M urea and 4.0×10^{-4} M α-ketoglutarate (Figure 3). The pH optimum for the urease catalyzed hydrolysis of urea is in the range of pH 6.0 to 7.5,[20] whereas GL-DH has a pH optimum of approximately pH 8.0.[21] But the coupled enzymatic catalyzed hydrolysis reaction of urea occurs at pH 7.4 to 7.6 using Dulbecco's buffer solution.

Determination of urea. A typical calibration curve

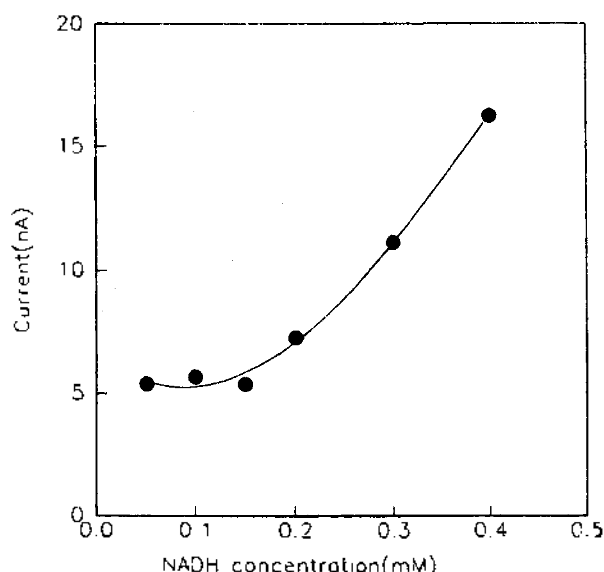


Figure 1. NADH-dependence of the coupled enzymatic reaction for 0.4 mM α-ketoglutarate and 0.2 mM urea.

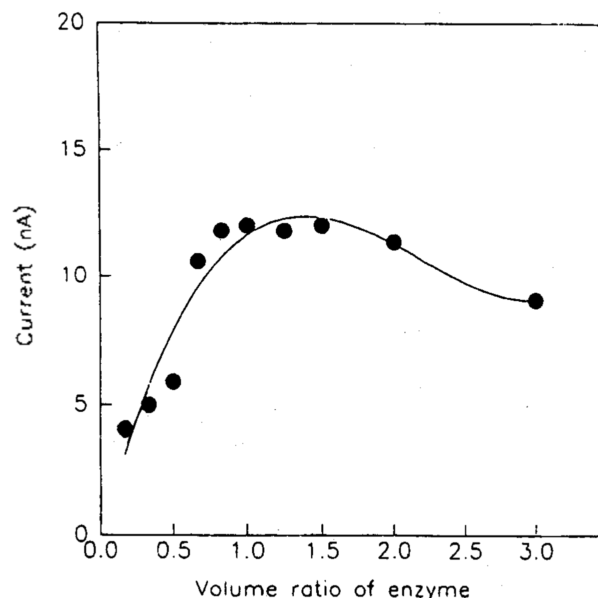


Figure 2. Effect of the ratio of immobilized coupled enzyme (urease/GL-DH) on the enzymatic reaction. [1,000 units/mL of urease and GL-DH, respectively].

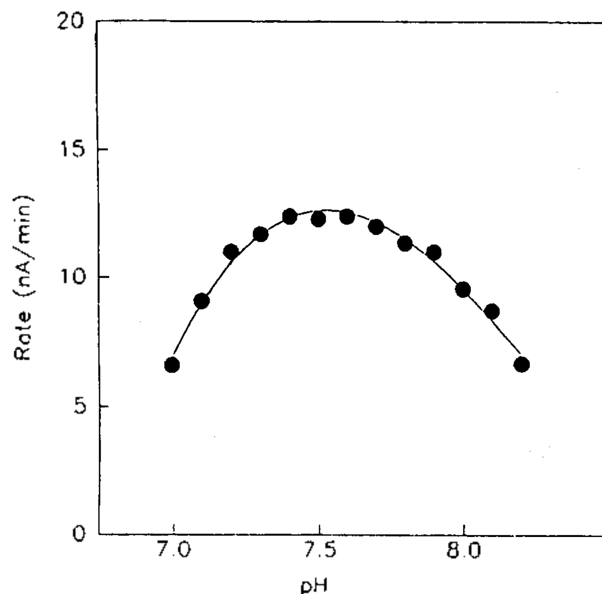


Figure 3. Effect of pH on the enzymatic reaction of immobilized coupled enzyme (urease/GL-DH) in Dulbecco's buffer solution.

of urea using electrode immobilized urease and GL-DH is shown in Figure 4. We determined urea by measuring the current change when the excess NADH is oxidized to NAD^+. Thus, currents were measured with change of urea for 5 mL of sample solution containing 2.0×10^{-4} M to 2.0×10^{-5} M urea (Figure 4). The detection limit was 5.0×10^{-6} M.

Biosensor results. Interference effects were investigated by measuring urea in the presence of 2.0×10^{-4} M and 2.0×10^{-3} M of L-asparagine, L-serine, L-glutamine, L-alanine, L-glutatione, L-phenylalanine, L-threonine, pyruvate, ascorbic acid, uric acid and cystine. At 1.0 volt *vs.* Ag/AgCl, there was no interference from other physiological species, except from high levels of ascorbic acid.[22]

Precision and accuracy. Precision was studied by repeating the urea analysis three times. Table 1 summarizes the repetitive measurement of urea concentration. The average standard deviation was ±0.3 (nA). These results are better than Lee's[23] for the continuous automated determination of urea by a potentiometric method and Adam's[9] using coulometric flow analysis.

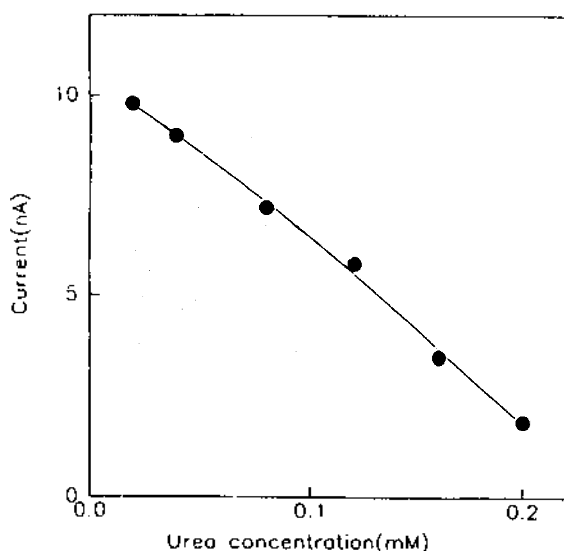


Figure 4. Calibration curve for the determination of urea.

Table 1. Precision test for amperometric determination of urea[a]

Concentration (M)	Current (nA) (x±s)	Rel. Std. Dev. (%)
4.0×10^{-5}	9.0±0.3	3.2
8.0×10^{-5}	6.0±0.3	4.2
1.2×10^{-4}	5.5±0.3	5.2
1.6×10^{-4}	3.4±0.2	5.6

x: average of current, s: std. dev. [a] three assays.

Table 2. Accuracy test for amperometric determination of urea

Urea (M)		Recovery	Rel. error
Added	Found[a]	(%)	(%)
4.0×10^{-5}	4.10 ± 10^{-5}	102.5	2.5
8.0×10^{-5}	7.80 ± 10^{-5}	97.5	-2.5
1.2×10^{-4}	1.25 ± 10^{-4}	104.2	4.2
1.6×10^{-4}	1.66 ± 10^{-4}	103.8	3.8

[a] $x\pm s=102\pm7$. x: average of recovery, s: std. dev.

Also, Table 2 shows the accuracy for the determination of urea by the amperometric method. The average recovery of urea was 102±1.7% comparable to data of Lee (99.6%) and Adams (100%).

Stability of biosensor. The immobilized enzyme offers various advantages (reusability, stability, and less interference) over the soluble enzyme as an analytical reagent. Hence, in order to investigate the stability and the reusability of immobilized enzyme, the activity of the immobilized enzyme membrane was measured with change of time for a 5 mL sample solution containing 2.0×10^{-4} M

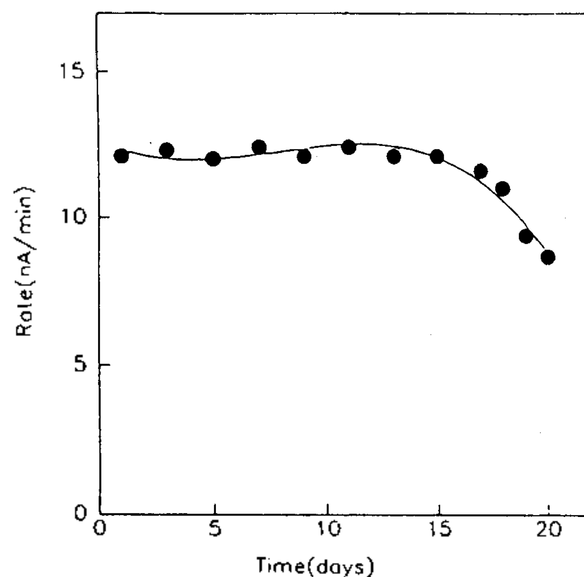


Figure 5. Life time of the immobilized coupled enzyme membrane (30 μL of 1,000 units/mL of urease and 20 μL of 1,000 units/mL of GL-DH).

Table 3. Comparison study of the present method with AOAC standard method

Sample of Fertilizer	Urea (%)	
	Present method	AOAC method
African violet	1.43	1.25
House plant	5.50	5.10
All Purpose	10.40	10.20

urea, 2.0×10^{-4} M NADH and 4.0×10^{-4} M α-ketoglutarate (Figure 5).

As shown in Figure 5, the lifetime of the immobilized enzyme electrode was about two weeks when stored in Dulbecco's buffer solution at 0 °C. Also, an immobilized enzyme membrane electrode could be used for at least 50 assays.

Assay of Peters fertilizer. The immobilized enzyme electrode was applied to the determination of the urea content in some Peters fertilizers. After ammonia was vaporized in an alkaline solution, urea was determined in the Peters fertilizer under optimal conditions.

Urea was also determined in the Peters fertilizer by the standard AOAC method.[24] The method was carried out as follows: weigh 10 ± 0.01 g sample and transfer to 15 cm Whatman No. 12 fluted paper. Leach with 300 mL H_2O into 500 mL volume flask. Add 75-100 mL saturated $Ba(OH)_2$ solution to phosphates. Let settle and add 20 mL 10% Na_2CO_3 solution to excess $Ba(OH)_2$. Let settle and filter to 15 cm Whatman No. 12 fluted paper. Transfer 25 mL and add 1-2 drops of methyl purple. Acidify with 2 N HCl and add 2-3 drops excess. Neutze solution with 0.1 N NaOH to first change in color of indicator. Add 20 mL neutral urease solution and close flask with rubber stopper, and let stand 1 hr. at 20-25 °C. Cool flask in ice-H_2O slurry and titration at once with 0.1 N HCl to full purple; then add 5 mL excess. Record total volume added. Back titration excess HCl with 0.1 N NaOH to end point.

The results obtained from the two methods are shown in Table 3. Very good agreement between the two methods was observed, indicating the amperometric urea biosensor offers considerable promise.

References

1. Katz, S. A. *Anal. Chem.* **1964**, *36*, 2500.
2. Naftalm, L.; Whitaker, J. A.; Stephens, B. *Clin. Chim. Acta* **1966**, *14*, 771.
3. Guilbault, G. G.; Nagy, G. *Anal. Chem.* **1973**, *45*, 417.
4. Wilson, R. W. *Clin. Chem.* **1966**, *12*, 360.
5. Kaltwasser, H.; Schiegel, H. G. *Anal. Biochem.* **1966**, *16*, 132.
6. Roch-Ramel, A. *Anal. Biochem.* **1967**, *21*, 372.
7. Kuan, J.; Lau, H.; Guilbault, G. G. *Clin. Chem.* **1975**, *21*, 67.
8. Guilbault, G. G.; Montalvo, J. G. *J. Amer. Chem. Soc.* **1970**, *92*, 2533.
9. Adams, R. E.; W. Carr, P. W. *Anal. Chem.* **1978**, *50*, 944.
10. Guilbault, G. G.; Shu, H. *Anal. Chem.* **1972**, *44*, 2161.
11. Nikolelis, D.; Papastathopoul, D. S.; Hadjioannok, J. P. *Anal. Chim. Acta* **1981**, *126*, 42.
12. Llenado, R.; Rechnitz, G. *Anal. Chem.* **1974**, *46*, 1109.
13. Keyes, M.; Barabino, R. *3rd International Conference on Enzyme Engineering*; Plenum press: New York, U.S.A., 1975; Vol. 3.
14. Kunz, H. J.; Stastny, M. *Clin. Chem.* **1974**, *20*, 1018.
15. Dritschilo, W.; Weibi, M. *Biochem. Med.* **1974**, *11*, 242.
16. Kirstein, Dr.; Kirstein, L.; Scheller, F. *Biosensors* **1985**, *1*, 117.
17. Seo, M. L.; Kim, J. S.; Lee, S. S.; Bae, Z. U.; Lee, H. L.; Park, T. M. *J. Korean Chem. Soc.* **1993**, *37*(10), 937.
18. Palleschi, G.; Nabi Rahni, M. A.; Lubrano, G. J.; Ngwainbi, J. N.; Guilbault, G. G. *Anal. Biochem.* **1986**, *159*, 114.
19. Burnett, J. N.; Underwood, A. L. *Biochemistry* **1965**, *4*, 2060.
20. Montalvo, J. G. *Anal. Biochem.* **1970**, *38*, 357.
21. Guilbault, G. G. *Handbook of Enzymatic Methods of Analysis*; Marcel Dekker, Inc.: New York, U.S.A. 1976, Vol. 4, p 223.
22. Zuman, P. *Coll. Czech. Commun.* **1957**, *15*, 1188.
23. Lee, H. L.; Yang, S. T. *J. Korean Chem. Soc.* **1992**, *36*, 393.
24. *AOAC Official Methods of Analysis*; 1990, p 21.