Pergamon

0039-9140(94)00197-9

Talanta, Vol. 41, No. 12, pp. 2195–2200, 1994

0039-9140/94 $7.00 + 0.00

DETERMINATION OF UREA IN URINE USING A CONDUCTIVITY CELL WITH SURFACE ACOUSTIC WAVE RESONATOR-BASED MEASUREMENT CIRCUIT

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(*Received* 1 *November* 1993. *Revised* 4 *March* 1994. *Accepted* 16 *June* 1994)

Summary—A conductivity cell employing a 61 MHz surface acoustic wave resonator-based measurement circuit was applied to the detection of the urea/urease reaction. The kinetic enzymatic parameters of the urease were estimated from the frequency shifts. The effects of pH, temperature and inhibitor on the response of the enzyme conductivity measurement system were investigated. The system was applied to rapid determination of urea in small urine samples. The lowest detection limit of urea was 30 ng/ml.

Biosensors, coupling the biochemical selectivity of enzymes, antibodies and chemoreceptors with the sensitivity of optical, electrochemical, thermal or mass-sensitive transducers, are presently finding increasing applications in chemical and biomedical analysis.[1] The conductivity cell as a simple biosensing device has been applied recently to a wide variety of enzyme/substrate systems especially hydrolytic ones. The principle is very simple: the conductance of the substrate solution varies when the enzymatic reaction occurs with changes in charge distribution.[2,3] Although the technique is generally useful, its applications are limited because the detection limit is ultimately controlled by the ratio $\Delta G/G$, where G is the conductance of the medium and ΔG is the conductance change that results from the enzymatic process.[4] Thus the sensitivity and the accuracy of the conductometric methods become poor in solution systems of high conductance background. In addition, the temperature must also be carefully controlled. It is reported that a temperature change of 1°C leads to an average conductivity variation of about 2%, greater than the amplitude of the hydrodynamic and electronic background noise.[5]

Urea is the chief nitrogenous end-product of protein catabolism in humans and other mammals, and its concentration in serum and urine is important in the diagnosis of liver and kidney diseases. There are several methods for urea determination based on the use of urease: conductometry,[5,6] amperometry,[7] potentiometry,[8,9] fiber-optic sensor,[10] *etc*. In the urea/urease reaction

$$H_2NCONH_2 + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^-$$

the initially uncharged substrate is hydrolyzed to yield four charge-bearing species. So the sensitivity of the conductometry allows the determination of urea at the 30 nM level in 0.1 mM Tris–HCl buffer.[11] However, the medium composition (especially the choice of buffer and its ionic strength) must be carefully controlled and buffers of low ionic strength must be used for the detection of low levels of substrate.

In previous papers,[12,13] a new conductivity cell with a surface acoustic wave (SAW) resonator-based measurement circuit was developed and used to detect total salt content in serum and phase transitions in lipid multibilayers. Recently, the circuit network theory was applied to investigate the effects of solution conductance and capacitance on the frequency characteristics of the system. It was found that in a certain conductance region, the higher the conductance, the higher the sensitivity of the conductivity measurement system. This advantage makes the system more attractive than the normal conductometric methods.[14]

In this work, the conductivity cell, with the surface acoustic wave (SAW) resonator operating at 61 MHz, was used to detect the urea/urease reaction. The kinetic enzymatic par-

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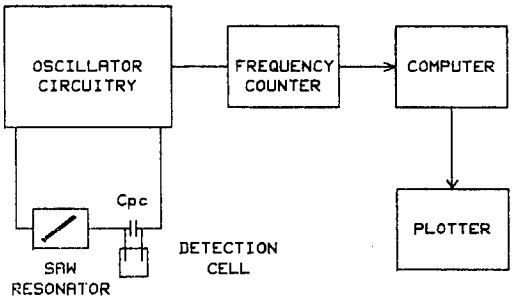

Fig. 1. Schematic diagram of the experimental set-up.

ameters of the urease were estimated from the frequency shifts. The effects of pH, temperature and inhibitors on the responses of the urease conductivity cell were investigated. Results of the determination of urea in urine samples are also presented.

EXPERIMENTAL

Apparatus

The 61-MHz one-port resonator used in this study was originally manufactured by Zhuzhou Radio Factory (Hunan, China), in y, z-cut $LiNbO_3$ crystal with aluminum metallization and mounted on round 2-pin-to-5 headers with epoxy and gold wirebonds. The aluminum interdigital transducers (IDTs) were 13,000 Å in thickness and the resonator was designed with an acoustic aperture and a path length of several wavelengths. At the center of the $LiNbO_3$ crystal chip, there are 20 pairs of IDTs with 500 reflectors placed on two sides. Each IDT electrode was 14 μm wide and spaced 14 μm from the next electrode. The nominal insertion loss of the resonator was 6.8 dB. The whole device was epoxied by a lid to seal from the atmosphere.

A schematic diagram of the experimental set-up used is shown in Fig. 1. In most experiments, solutions were placed in a magnetically stirred and water-jacketed detection cell. The temperature of circulating water was monitored and controlled by a model WMZK-01 Temperature Controller (Medical Instrument Co., Shanghai, China) and during the experiment the stirring speed was kept constant. Two platinum electrodes of dimensions 0.5 × 0.5 cm were selected, ground with sand paper, and placed parallel into a detection cell at a fixed distance. The oscillation frequency of the SAW resonator was measured using a model SC7201 Iwatsu Universal Counter at a resolution of 1 Hz and data were collected at 6 points/min. The pH values of the working buffer was measured by a model EA940 Orion IonAnalyzer using a calibrated glass electrode. 12 V DC power was supplied by an adjustable dual track DC power supply.

Reagents

Urease (from jack beans, 1.7 E.U. per mg, EC 3.5.1.5) was obtained from BDH (Poole, U.K.) and used without further purification. Stock solution of enzyme was prepared in 50% glycerine solution (5 mg/ml) and stored in a refrigerator before experiment. Human seroalbumin was from Biochemical Products Institute, Shanghai, China. Control urines of different urea concentrations were from the clinic containing 5 mg/ml glucose and 1 mg/ml human seroalbumin. Human urine samples were also obtained fresh from the clinic and were detected without any further treatment. The working buffer was 1 m*M* glycine–NaOH containing 1 m*M* EDTA, pH 7.0. All other chemicals were of analytical reagent grade. Double distilled water was used throughout.

Procedures

Thirty min was required to stabilize the whole setup including the frequency counter and power supply before the experiment. The detection cell was thermostatted at 30°C ± 0.1°C. The baseline drift was determined using frequency data collected for 5 min prior to the experiment. A typical experiment procedure was done as follows. The cell was filled with working buffer (9.5 ml) and enzyme solution (0.5 ml). The enzymatic reaction was started by addition of the substrate solution and the frequency change recorded with time. Added substrates volume was in the microliter range to allow the dilution to be neglected. The procedure was carried out for different urea concentrations, different temperatures, different pH values and different inhibitors. The calibration curve was constructed by measurement of the conductivity cell responses to control urines in different concentrations. Only 5 μl urine sample were needed for urea measurement and the urea concentration was determined by the calibration method.

RESULTS AND DISCUSSION

Kinetic enzymatic parameters

Kinetic enzymatic parameters depend on the type and concentration of buffer, sometimes very markedly. For jack bean urease, the value

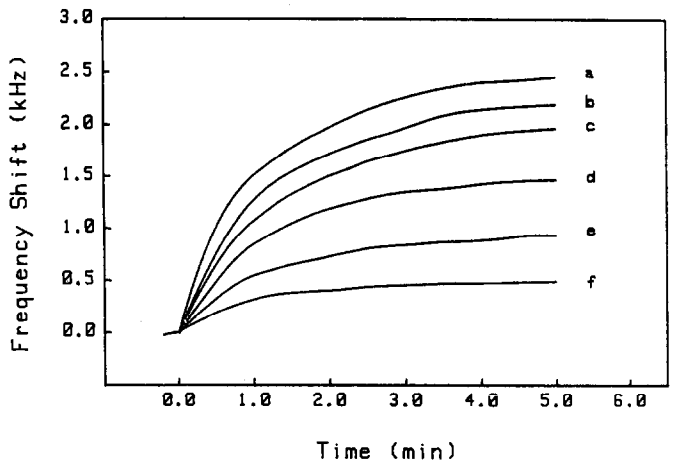

Fig. 2. Frequency responses of the urease conductivity measurement system to standard urea solutions of concentration (a) 25, (b) 20, (c) 15, (d) 10, (e) 5 and (f) 2.5 μg/ml.

of the Michaelis constant K_m, ranges from 1 to 5 mM, depending on the experimental conditions.[15,16] In general, the amino buffers such as glycylglycine, tricine, *etc.*, result in a greater enzyme activity than do the simple inorganic buffers such as phosphate and carbonate. In this experiment, a 1 mM glycine–NaOH buffer was selected. Typical responses of the urease

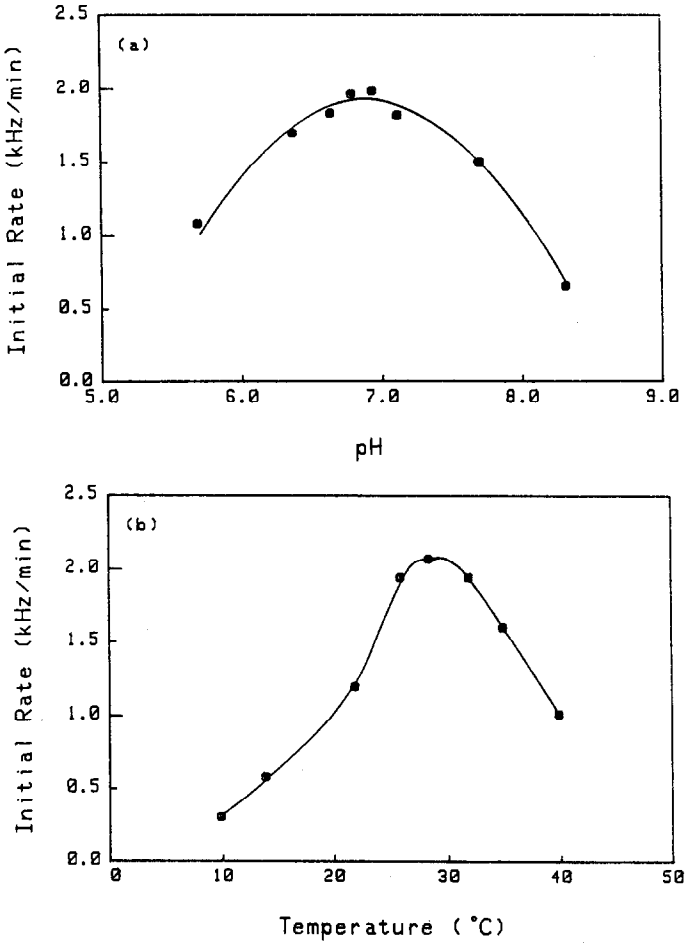

Fig. 3. Optimization of the experimental conditions for the urease conductivity cell. Effect of pH (a) and temperature (b) on the initial rate detected by the measurement system in glycine buffer (1 mM). Urea concentration, 20 μg/ml.

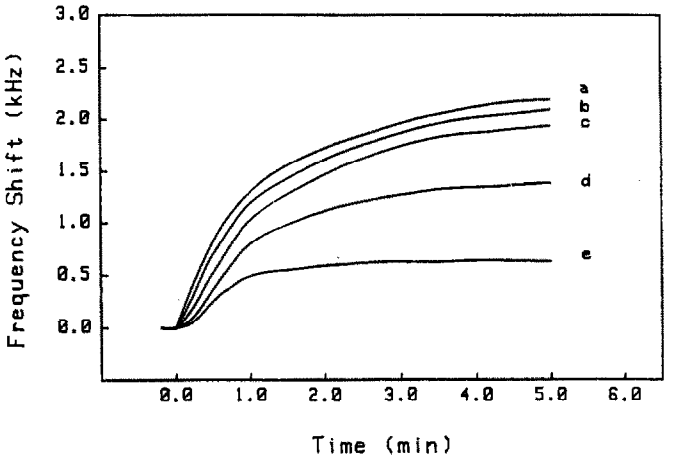

Fig. 4. Responses of the urease conductivity measurement system to standard urea solutions (20 μg/ml) with inhibitor Pb^{2+} in different concentrations: (a) 0, (b) 0.05, (c) 0.1, (d) 0.3 and (e) 0.5 m*M*. EDTA concentration, 1 m*M*.

conductivity measurement system to standard urea solution in different concentrations are depicted in Fig. 2. A linear correlation of the total frequency shift (5 min) *vs.* urea concentration was observed

$$\Delta f = 1.89 \times 10^5[\text{urea}] + 13$$

$$n = 8 \quad r = 0.9995,$$

where the urea concentration is expressed in mg/ml and the Δf in Hz. The linearity ranges up to 25 μg/ml. With the lowest noise level of 2 Hz and a signal-to-noise ratio of three, the lowest detection limit of the conductivity cell was 30 ng/ml ($5 \times 10^{-7}M$). The K_m value for the enzyme was calculated from a Lineweaver–Burke plot with the substrate concentrations ranging from 0.25 to 25 μg/ml. A value of 2.4 m*M* was obtained. The maximum velocity V_{max} was 26 kHz/min.

For a first order reaction ([urea]$\ll K_m$), the initial rate of the enzyme reaction is proportional to the urea concentration. So the urea concentration can be detected using the kinetic method. A correlation of the initial rate *vs.* urea concentration was presented

$$V_0 = 1.66 \times 10^5[\text{urea}] + 7$$

$$n = 8 \quad r = 0.9989,$$

where the urea concentration in mg/ml and V_0 in Hz/min. The linearity ranges up to 20 μg/ml. The kinetic method can decrease the time of measurement but it has a lower sensitivity and a narrower linearity than the end-point method. Thus for determination of urea concentration, the end-point method was employed.

Effects of pH and temperature

Enzymes are very sensitive to changes in pH and function best over a limited range with a

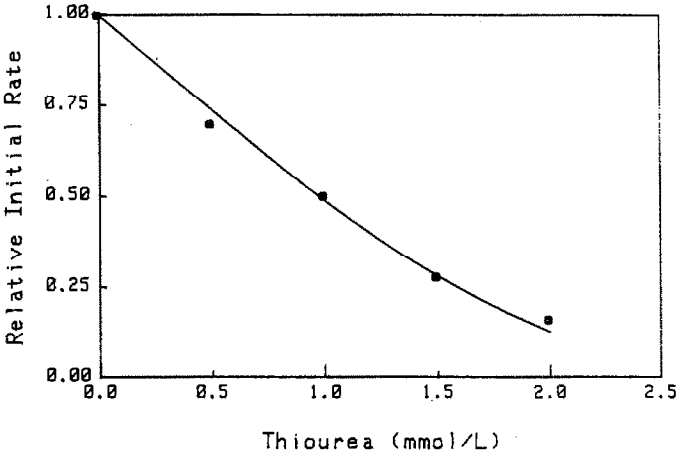

Fig. 5. Relative initial rate as a function of the concentration of inhibitor thiourea.

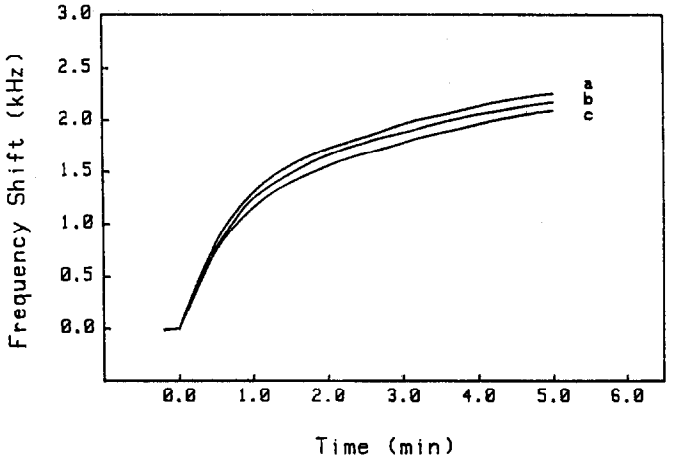

Fig. 6. Comparison of responses of the urease conductivity measurement system to (a) standard urea solution 20 μg/ml, (b) urea solution 20 μg/ml + human seroalbumin 1 mg/ml and (c) urea solution 20 μg/ml + glucose 1 mg/ml.

definite optimum pH. The effects of pH are due to changes in the ionic state of the amino acid residues of the enzyme and the substrate molecules, causing varying efficiency in the binding of a substrate and affecting the rate of reaction.[17] Over a narrow pH range, these effects will be reversible. As variations in the pH and temperature of the buffer solution also produce ion strength variations. Calculated amounts of KCl were also added into the buffer, to maintain a constant ionic strength. Figure 3(a) shows the effect of the pH of the glycine buffer on the response of the urease conductivity measurement system over the pH range 5.7–8.3. As can be seen there is no significant difference in frequency sensitivity over a certain pH range and at pH value 7 the system gives the maximum sensitivity; at pH values less than 4 no response was observed. Temperature drift of the conductivity measurement system was very small, only 10 Hz/°C in water.[12] However, temperature affects the activity of the enzyme as well as the solution conductivity. So the temperature effect on the response of the conductivity cell to urea/urease reaction was studied. The frequency change, as a function of the temperature (Fig. 3b), showed the temperature had a considerable effect on the response. In the range of 27–31°C the temperature influence can be neglected and the urease conductivity cell yields the greatest sensitivity. Therefore, a temperature of 30°C was selected in our experiment.

Table 1. Determination of urea in urine samples

Sample	Δf (*Hz*)	[Urea] (*mg/ml*)	RSD (%)
1	1675	14.6	3.0
2	1220	10.5	4.8
3	2035	17.8	1.9
4	1443	12.5	3.8

Effects of inhibitors

Inhibitor can decrease the rate of an enzyme-catalyzed reaction. Heavy metals such as Pb^{2+}, Ag^{+}, Cu^{+}, *etc.*, inhibit the activity by destroying the function group of the enzyme. The inhibition of Pb^{2+} on the urea/urease reaction is shown in Fig. 4. If the concentration of Pb^{2+} is less than 0.1 m*M*, the reaction rate does not decrease significantly in the presence of EDTA (EDTA was added according to literature[18]). An inhibitor like thiourea structurally resembles urea and may be bound by the urease but cannot be converted to the products. The influence of thiourea on the initial rate of the reaction is shown in Fig. 5. The plot is nearly linear when the concentration of thiourea is small. The influences of glucose and human seroalbumin on the urea/urease reaction were also investigated (Fig. 6). It can be seen that their influences on the reaction rate can be neglected.

Determination of urea in urine

The sensitivity of the urease conductivity measurement system response to control urine was higher than that to standard urea solution because of the higher ionic strength of the control urine. For this reason, in the analysis of authentic samples of different ion strength with the conductivity cell, standardization must be

done with a solution of the same ionic strength. From curves like that in Fig. 2, the total frequency shift for eight control urines, ranging from 5 to 40 mg/ml, was measured. A linear relationship between the frequency response of the urease conductivity cell and the urea concentration was obtained

$$[\text{urea}] = 4.5 \times 10^{-6}\Delta f - 2.3 \times 10^{-4}$$

$$n = 8 \quad r = 0.9964,$$

where the urea concentration is expressed in mg/ml and Δf in Hz. The recovery was found to be 97.7–102.3%. Urea concentrations of four urine samples were then determined based on the above calibration equation and the urine samples were 5 μl for all the assays. The results are shown in Table 1. Precisions (as RSD) were calculated from five determinations. The urea concentrations coincide with the literature range of healthy adults.[19]

From these results, it can be concluded that the new urease conductivity measurement system is well-adapted for routine urea assays on small urine samples.

Acknowledgements—This work was supported by funds from the National Science Foundation and Education Commission Fund of China.

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