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Ideal Hydrogen Peroxide-Based Glucose Sensor

GIUSEPPE PALLESCHI,[1,3] MOHAMMAD H. FARIDNIA,[1] GLENN J. LUBRANO,[2] AND GEORGE G. GUILBAULT*,[1]

[1]*Department of Chemistry, University of New Orleans, Lakefront, New Orleans, LA 70148;* [2]*Universal Sensors, Inc., 5258 Veterans Blvd., Suite D, Metairie, LA 70006; and* [3]*Present address: Dipartimento di Scienze e Technologie Chimiche, Universitä di Roma, "Tor Vergata" Via O. Raimondo 00173, Rome, Italy*

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ABSTRACT

Amperometric glucose sensors have been assembled by immobilizing glucose oxidase on Nucleopore polycarbonate membranes via glutaraldehyde crosslinking. To eliminate electrochemical interferences, novel blocking membranes were used that are permeable to hydrogen peroxide, making them interference free, highly stable, reproducible, and with extended linearity for glucose up to 50 m*M*.

Index Entries: Glucose oxidase; blocking membrane; glucose; hydrogen peroxide; glutaraldehyde; extended linearity.

INTRODUCTION

Glucose measurements based on hydrogen peroxide electrodes coupled with the enzyme glucose oxidase (GOD) have been extensively studied and reported in several papers (*1–9*). As described in the literature, glucose probes based on hydrogen peroxide electrodes are the most successful produced to date (*10*) and are being used in clinical, industrial, environmental, and food analyses. Glucose analyzers assembled with hydrogen peroxide-based glucose probes have been successfully commercialized by

*Author to whom all correspondence and reprint requests should be addressed.

many analytical companies (*11*). Miniaturization of glucose sensors for in vivo implantation is, at present, a tremendously important subject of study (*12*).

Although these probes have sufficient stability and precision, they lack accuracy when in the presence of electrochemical interferences at the platinum electrode. Extensive studies have been carried out to develop an interference-free hydrogen peroxide sensor (*1,2,5,13–15*). Palleschi et al. analyzed the main blood constituents that are possible interferors in clinical glucose measurements using a hydrogen peroxide-based glucose sensor (*16*). Although a nominal 100 mol wt cut-off membrane was used as an electrochemical interference blocking membrane, acetaminophen, MW 151.2, interfered and even, ascorbic acid slightly interfered when the probe was left in the measuring solution for a long time. This clearly demonstrated that this probe cannot be used for continuous monitoring in the presence of, or during variations of, these drugs.

Another problem of glucose sensors is limited upper linear range (30 mg/dL) and the required dilution procedures in many practical applications. Glucose probes with extended linearity is another subject that is attracting the attention of many scientists who measure glucose in whole blood in diabetes, in urgent care, at the bedside, in industrial processes, and generally in all cases where a glucose dilution step is not possible.

A glucose probe operates according to the following reaction

$$\text{Glucose} + O_2 \xrightarrow{\text{GLUCOSE OXIDASE}} \text{Gluconic acid} + H_2O_2$$

This reaction is catalyzed by the enzyme glucose oxidase and is oxygen-dependent. When the concentration of glucose is high, the reaction rate is controlled at a certain steady state by the oxygen availability. There are two ways to avoid this: One is to increase the oxygen concentration at the enzyme reaction sites; another is to decrease the glucose diffusion to the enzyme reaction site by incorporating a glucose diffusion limiting membrane. In the latter case, the enzyme "senses" a much lower glucose concentration, consequently resulting in an apparent increase in the K_m and extended linearity.

Approaches to improve the glucose sensor's linearity have been carried out during the last few years (*7,8,17–20*). These methods require membrane pretreatment and often lack stability and reproducibility. Oxygen independent glucose probes have been developed to extend the linearity of the glucose response (*21*). They are mainly used as disposable systems, but require further improvements if used for continuous analysis, because of poor mediator stability and electrochemical interferences, oxygen included. Attempts to increase oxygen diffusion to the probe have been carried out mainly by using oxygen electrodes (*7,22,23*). These

probes have advantages in terms of selectivity and response time, but they require expensive and more complex instrumentation.

According to Geise et al. (24), ideally, a hydrogen peroxide-based glucose electrode should have a good response to 5 mmol/L glucose (90 mg/dL), and should not respond to acetaminophen, the drug chosen as test-interferor for its stability, low mol wt, and fast reaction on platinum electrodes. We would like to add "should have good response to 1 mmol/L glucose (18 mg/dL, which is below the hypoglycemic range) and an extended linearity up to 50 m*M* glucose (900 mg/dL, above hyperglycemic range)." A probe with the above characteristics has been developed in our laboratory by assembling an interference-free hydrogen peroxide-based glucose probe, with a linear range of 1–1200 mg/dL and a detection limit of 0.5 mg/dL glucose.

Calibration curves, pH, and temperature studies have been carried out for the novel hydrogen peroxide electrode, as well as for the glucose probe. Both demonstrated excellent stability and reproducibility, and good response time and reusability, making these electrodes useful analytical tools for practical analyses.

EXPERIMENTAL

Apparatus

A Tacussel PRG-GLUC electrochemical HPLC detector was used for constant potential studies performed anodically at +650 mV. For rate measurements, a Gould differentiator amplifier model 13-4615-71 and a Gould medium gain preamplifier, model 13-4615-10 were used. Alternatively, the Universal Sensors Amperometric Biosensor Detector (ABD, cat. no. 3001, Universal Sensors Inc. Metairie, LA) was used for both constant potential and rate measurements. The steady-state current and rate were recorded simultaneously with a dual pen Houston Microscribe strip chart recorder. The base sensor was a Universal Sensors Hydrogen Peroxide Electrode (Cat. no. 4106) that has a combined working electrode (Pt) and reference electrode (silver/silver chloride), contained in the same electrode jacket.

For temperature studies, a jacketed glass wall beaker was thermostated by an MS-20 LAUDA Heating Circulator from Brinkmann Instruments Inc.

Chemicals

Glucose oxidase, E.C. 1.1.3.4, from *Aspergillus Niger*, type VII was obtained from Sigma Chemical Co. All other chemicals were reagent grade.

Materials

Microporous polycarbonate membranes (0.015, 0.03, 0.05, and 0.08 μm) were obtained from Nuclepore Corporation. The new blocking membrane, hydrophobic, Polytetrafluoroethylene with 3 mil thickness and 0.5 μm pore size (cat. no. 4106), was obtained from Universal Sensors.

Procedure

Hydrogen Peroxide Electrode

Assembly and Measurements

The hydrogen peroxide base sensor was assembled by securing a 1 cm^2 piece of the blocking membrane on the tip of an inverted electrode jacket with an O-ring. The jacket was then filled with a physiological phosphate buffer, pH 7.4. The combined working/reference probe was inserted into the jacket and screwed down until the tip of the platinum electrode was pressed against the blocking membrane, resulting in formation of a thin layer of electrolytic solution between the working electrode and the inner membrane while the reference electrode was exposed to the bulk of the filling solution. Because both electrodes are on the same side of the hydrophobic membrane, there is no conduction problem between the two electrodes. The sensor was then immersed in 10 mL of the physiological buffer and allowed to equilibrate. Aliquots of hydrogen peroxide were then injected and the current and rate of current change were recorded.

Glucose Sensor Assembly and Measurements

Enzyme Membrane Preparations

Polycarbonate membrane (1 cm^2) was secured to the tip of an electrode jacket with an O-ring. Glucose oxidase was immobilized on the polycarbonate membranes and a number of preactivated membranes (Immunodyne,, Immobilon, Ultrabind, and Biodyne) with a modified BSA/glutaraldehyde procedure.

The BSA/glutaraldehyde procedure is well known and described in the literature (*25*). However, since the glucose oxidase from Sigma has a high amount of protein, in order to make the enzyme membrane as thin as possible, we did not use the BSA as protein support. The enzyme membranes were prepared by mixing 20 U of glucose oxidase with 10 μL of phosphate buffer, pH 7.5, containing 2.5% glutaraldehyde on the polycarbonate membrane. This resulted in a very thin enzyme membrane approx 5–10 μm thick.

The preactivated membranes are suitable for direct protein immobilization, consequently, their immobilization procedure are simple. For each

membrane, approx 20 U of the enzyme was mixed with 10 μL of 0.1*M* phosphate buffer (except for Immobilon, where a 0.5*M* phosphate buffer was used) and the enzyme solution was injected onto each membrane secured on an inverted electrode jacket. The membranes were allowed to dry at room temperature for 2 h, then washed and stored in the physiological phosphate buffer. The membranes were sequentially tested by assembling the jackets with the same platinum electrode.

Glucose electrodes were assembled using the same procedure as for the hydrogen peroxide sensor, with the following membrane configuration

INNER	MIDDLE	OUTER
Hydrogen peroxide memb.	Enzyme	Polycarbonate

The procedure for glucose measurements was the same as for the hydrogen peroxide probe, but aliquots of glucose were injected.

RESULTS AND DISCUSSION

Hydrogen Peroxide Sensor

A good hydrogen peroxide sensor should have the following characteristics: reproducibility, stability, sensitivity, response time, reusability, large linear range, and high selectivity. Since the novel hydrogen peroxide membrane is hydrophobic, we would expect no response for hydrogen peroxide. Despite this, the membrane displayed a good response to hydrogen peroxide presumably owing to its somewhat high vapor pressure. The response was linear with the concentration.

Further experiments were carried out with these special membranes. The first experiment was the evaluation of the characteristics of the blocking membrane when assembled on the platinum electrode for hydrogen peroxide measurements. Calibration curves of hydrogen peroxide were performed using different lots of the same membrane to be sure the response of the electrode was not attributable to a possible malformation or microinfraction of the membrane. Surprisingly, calibration curves were linear in the range 5×10^{-6}–5×10^{-2} mol/L, with a detection limit of 2×10^{-6} mol/L, which is only one decade higher than that obtained with a bare platinum sensor. Figure 1 shows part of a calibration curve of the hydrogen peroxide electrode.

Reproducibility

The reproducibility of the sensor was tested by running calibration curves for several days, assembling different platinum electrodes. For each electrode, the membrane showed excellent response to hydrogen peroxide, and consecutive calibration curves did not show any decrease in the slope.

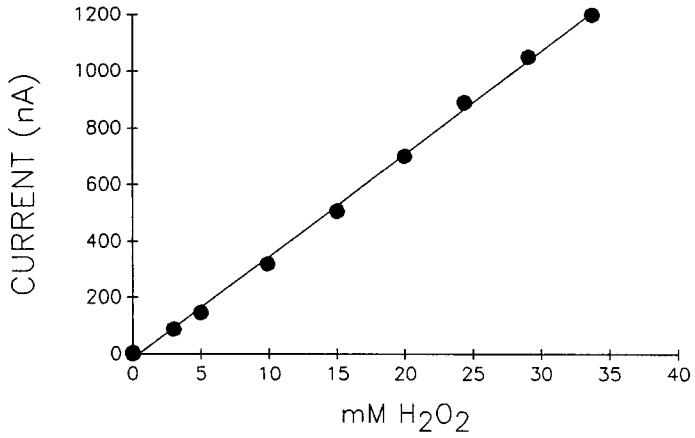

Fig. 1. Calibration curve of hydrogen peroxide electrode.

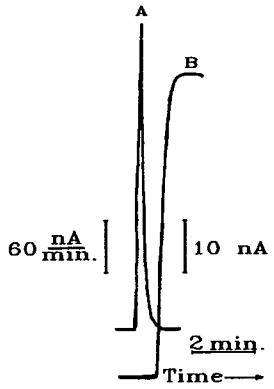

Fig. 2. Hydrogen peroxide electrode response, A = rate, B = steady-state.

Stability and Reusability

The stability of the electrode was tested by measuring hydrogen peroxide for 4 wks, using the same electrode and same membrane. The electrode was left in a solution of hydrogen peroxide for 6 h and the current output continuously recorded. No current drift was observed during this time-period. The electrode was continuously used for more than 20 d and it displayed the same current background and current/noise ratio at all times.

Response Time

The response time of the probe was less than 2 min by measuring the steady-state current and less than 10 s by measuring the rate of current change (Fig. 2).

Table 1
Response to Various Substances

Substance	Relative electrode response, nA/m*M*
D-Glucose	2.18
Ascorbic acid	none
D-Fructose	none
D-Galactose	none
Cholic acid	none
D-Mannose	0.05
Acetaminophen	none
Acetylsalicylic acid	none
Caffeine	none
EDTA	none
Uric acid	none
Cholesterol	none
Sodium citrate	none
Sodium fluoride	none
Hydrogen peroxide	20.30

None = current change was less than detectable (i.e., less than 0.01 nA).

Interferences

A platinum electrode needs a blocking membrane when used as a hydrogen peroxide sensor mainly because at the applied potential useful for hydrogen peroxide's electrochemical oxidation, several electroactive compounds are oxidized, giving severe interferences in many analytical applications 16–24. In order to use platinum electrodes as hydrogen peroxide sensors, electroactive compounds must be blocked. The ideal hydrogen peroxide selective membrane would block everything except hydrogen peroxide. Table 1 lists the relative response of several potential interferences tested at concentrations 10 or 100 times that present in blood. Owing to membrane hydrophobicity, none of the compounds responded, except D-Mannose, which is attributable to glucose impurity present.

pH Studies

The effect of pH on the hydrogen peroxide electrode was studied using different buffers ranging from pH 4 to 10. Results are displayed in Fig. 3. When increasing the pH from 4 to 10, no increase in current response was observed. This is in agreement with the hydrophobic character of the membrane, and demonstrates that hydrogen ion does not pass through the membrane and does not affect the electrolytic film over the platinum surface.

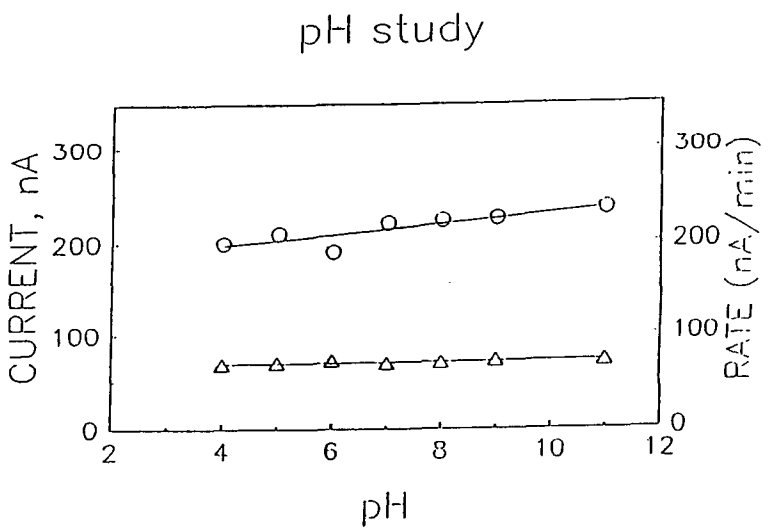

Fig. 3. Effect of the pH on the hydrogen peroxide electrode, △ = steady-state current response, ○ = rate response.

Temperature Studies

Temperature studies were carried out from 20 to 40°C to evaluate its effect on hydrogen peroxide diffusion through the membrane. Plots are given in Fig. 4, and as expected, an increase in current was observed with increase in temperature.

Lifetime Studies

As long as the hydrogen peroxide blocking membrane retains its properties, the peroxide electrode should continue to function suitably. After 4 mo of continuous use, the properties of these probes have not changed.

Glucose Studies

The novel hydrogen peroxide sensor demonstrates excellent characteristics for assembly as an enzyme sensor with oxidative enzymes producing hydrogen peroxide. We chose the oxidative enzyme glucose oxidase for this study for two reasons. First, this enzyme is stable, very active, inexpensive, and generally used as the "test enzyme" for biosensor assemblies. Second, and more important, was the need for reliable, selective glucose sensors with wide linear range, for blood analysis and industry process control, for use without sample dilution, and on-line continuous monitoring. Results with the preactivated membranes showed good response to glucose, but when they were assembled with the hydrogen peroxide and polycarbonate membranes, the response time and the sensitivity of the probes were not as good as the glutaraldehyde immobilization

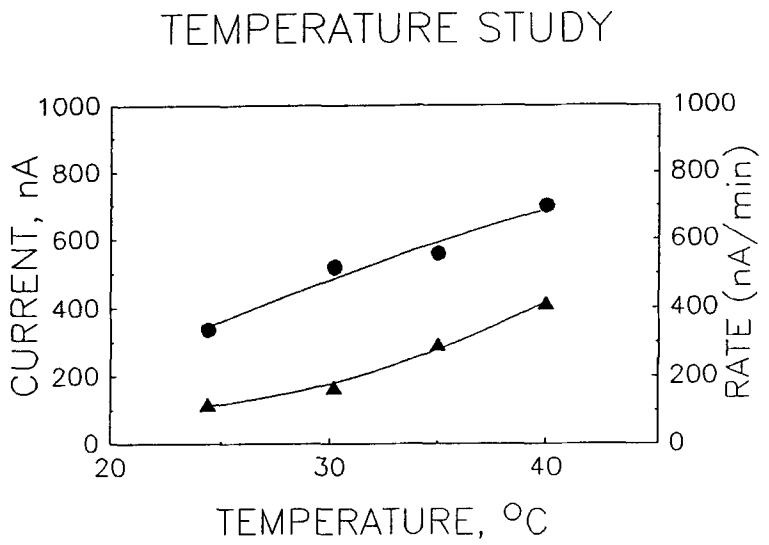

Fig. 4. Effect of temperature on the hydrogen peroxide electrode ▲ = steady-state current response, ● = rate response.

because of the thickness of the membranes. Here, further studies were carried out with the glucose oxidase immobilized directly on the polycarbonate membranes.

Since the novel hydrogen peroxide probe is interference free, there is no reason to believe the glucose probe would suffer from electrochemical interferences. The only problem is to have an outer membrane that can protect the immobilized enzyme from biological materials, such as bacteria and proteins, and possibly slow down the glucose diffusion, thus increasing the linearity. We investigated several membranes for this purpose.

Polycarbonate Membrane Studies

It has already been demonstrated that polyurethane and silanized polycarbonate membranes slow down glucose diffusion and increase the linearity of the glucose response (*26*), so the first step in assembling the glucose sensor was to test the effect on glucose linearity of polycarbonate membranes with different porosity. To be sure we were controlling only glucose diffusion, the electrode was assembled without the hydrogen peroxide membrane, with the enzyme immobilized on the polycarbonate membrane, in contact with the electrode surface. In this case, the hydrogen peroxide produced by the enzymatic reaction of glucose can reach the platinum surface directly, giving a current signal function only of the glucose diffusion through the polycarbonate membrane. Figures 5 and 6 show the steady-state current and rate responses, respectively, of four membranes with porosities of 0.015–0.08 μm. Whereas the 0.05 and 0.08 μm membranes showed nonlinearities after the first injection of 90 mg/dL

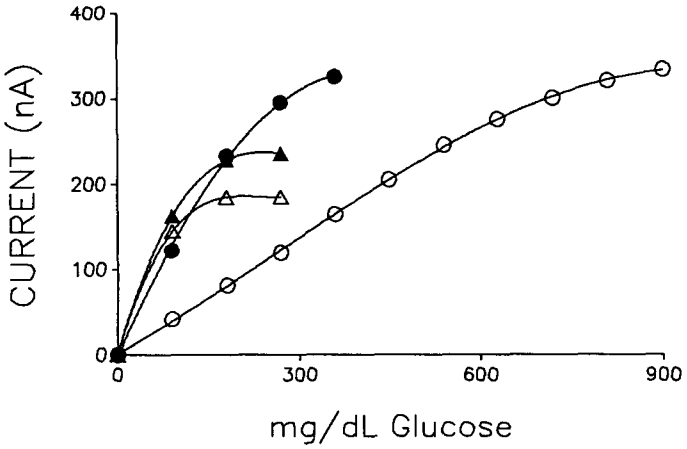

Fig. 5. Effect of porosity of polycarbonate membranes on the hydrogen peroxide electrode's steady-state current response, ○ = 0.015 μm, ● = 0.03 μm, △ = 0.05 μm, ▲ = 0.08 μm.

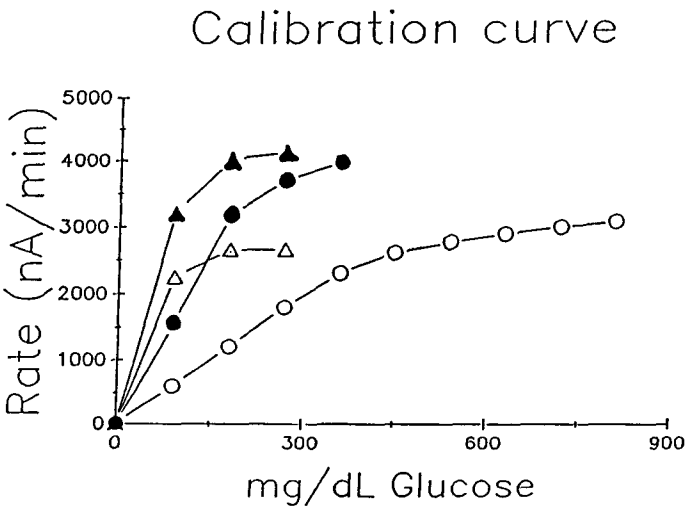

Fig. 6. Effect of porosity of polycarbonate membranes on the hydrogen peroxide electrode's current-rate response, ○ = 0.015 μm, ● = 0.03 μm, △ = 0.05 μm, ▲ = 0.08 μm.

glucose, the 0.03 μm membrane showed an extended linearity to 180 mg/dL and the 0.015 μm membrane, to 600 mg/dL. The maximum steady-state currents did not behave as expected. The membranes with higher porosity should always give a higher current than those with lower porosity before the saturation point, as long as they have the same thickness, the same enzyme activity, and are assembled on the same platinum electrode.

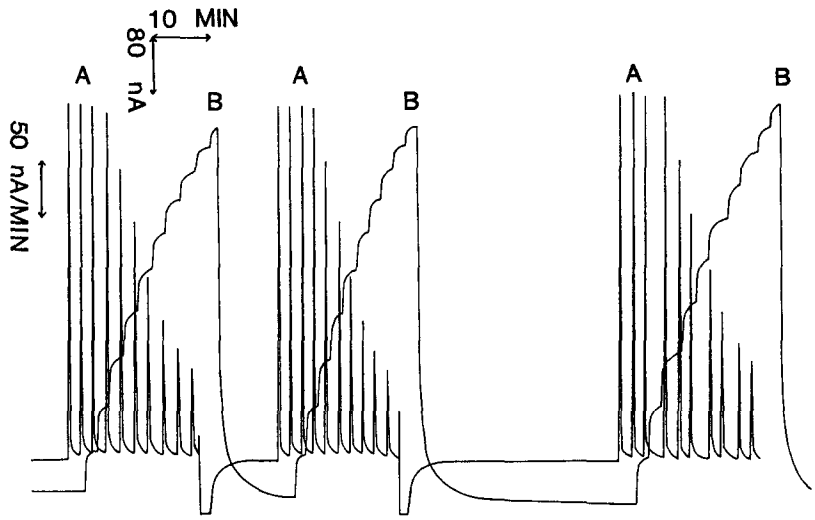

Fig. 7. Representative steady-state current and rate response curves of the glucose probe, each injection is an increment of $10^{-2}M$ (180 mg/dL glucose), **A** = rate response, **B** = steady-state current response.

Also, these currents should have the same magnitude. As shown in Figs. 5 and 6, this did not happen, because of the difficulty to control the thickness of enzymatic membranes and the impossibility to have on the tips of the platinum the immobilized enzyme with the same specific activity. However, what was important in this case was not the absolute current obtained for each sensor, but the extended linearity achieved for membranes with different porosity.

The 0.015 μm membrane was chosen for further studies. Polycarbonate membranes are characterized by a dull and a shiny side. Calibration curves using both faces as outer ones were carried out. No difference in current response was observed to glucose injections.

For the final glucose probe assembly, the polycarbonate membrane was secured onto the electrode jacket with the shiny side out. This was done for two reasons: One, the dull side could better retain the immobilized enzyme prepared directly on the membrane; two, the shiny side would absorb less "undesired" compounds, therefore allowing a better control of glucose diffusion.

Response Time and Reproducibility

Figure 7 shows the response time and the linearity of the glucose probe by measuring the steady-state current variation and the rate. The good reproducibility of the steady-state current and the rate during glucose injections is clearly evident in the figure and do not need further comments.

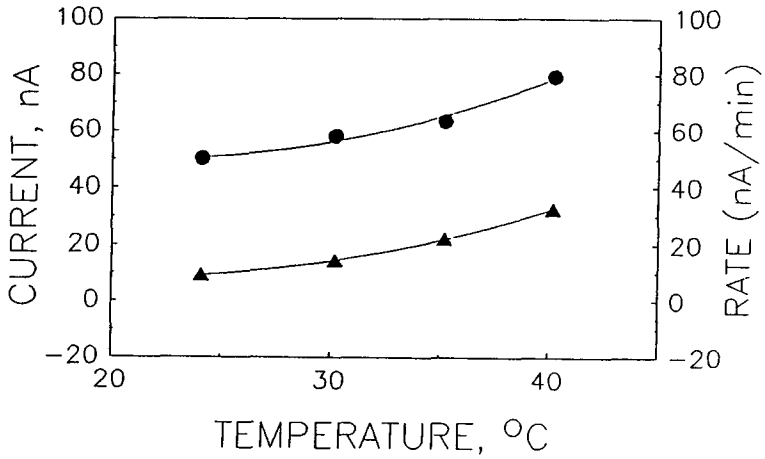

Fig. 8. Effect of temperature on the glucose probe ▲ = steady-state current response, ● = rate response.

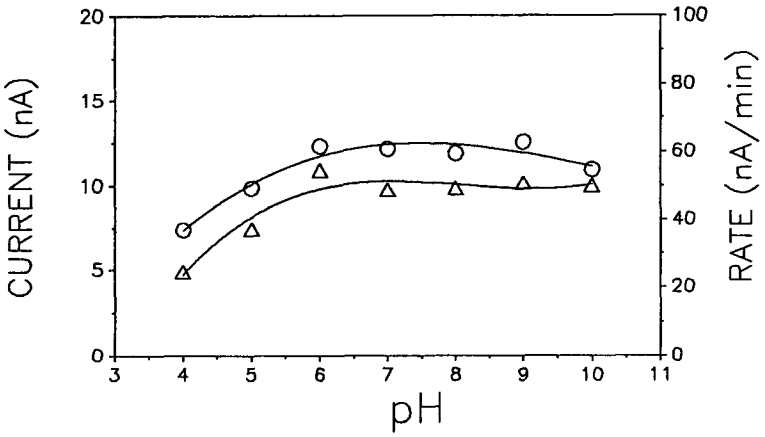

Fig. 9. Effect of the pH on the glucose probe, △ = steady-state current response, ○ = rate response.

Glucose Probe Assembled with Hydrogen Peroxide Membrane

pH and temperature studies were performed using the glucose electrode assembled in the following way: Pt/Peroxide memb./GOD/Polycarbonate membrane. As expected, glucose calibration curves showed an increase in current with increasing temperature from 20 to 40°C, Fig. 8, and calibration curves at different pHs did not show large variations, Fig. 9. The response at pH 6 was almost the same as that obtained at pH 9.6.

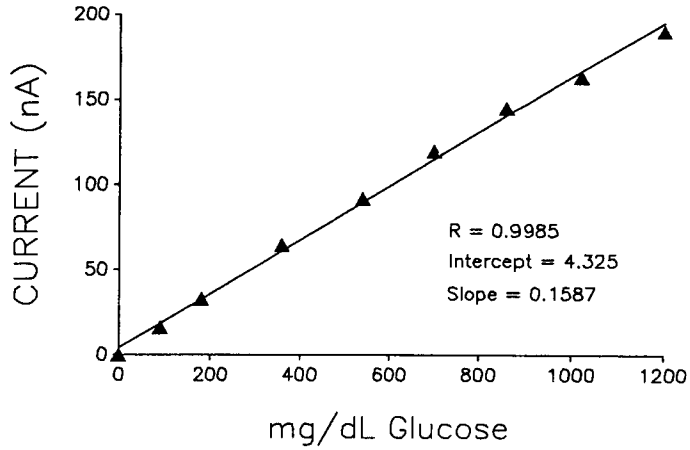

Fig. 10. Representative extended linearity of the glucose probe. R=0.9985, Intercept=4.325, Slope=0.1587.

Glucose calibration curves using this probe at pH 7.4 and room temperature (20–25°C) showed lower current changes than those assembled without the hydrogen peroxide membrane, but with a much higher extended linearity (to 1200 mg/dL, Fig. 10). Since the electrodes were assembled with the same polycarbonate and enzyme membranes, this effect can be attributed only to the hydrogen peroxide membrane.

Oxygen diffuses to the enzyme electrode membrane from both the electrode's filling solution and the sample solution. One possible explanation for the extended linearity could be that the hydrophobic membrane has a high capacity to retain molecular oxygen. This also agrees with the fact that when this membrane was assembled as an oxygen membrane in Clark-type electrode, the background current was more than 10 times higher than that obtained with electrode assembled with standard oxygen electrode membranes. The increase in linearity has been observed every time the hydrogen peroxide membrane was used in glucose probes with the polycarbonate membranes. This has been confirmed by using the 0.03 μm polycarbonate membrane, which gave a linearity to 300 mg/dL glucose.

In conclusion, the combination of the hydrogen peroxide and 0.015 μm polycarbonate membranes makes this probe a very useful glucose sensor for any practical application.

SUMMARY

A glucose probe based on a novel hydrogen peroxide electrode was developed and extensively studied. Both the hydrogen peroxide electrode and glucose probe showed great performance in terms of stability and

reproducibility, with no electrochemical interferences resulting from a new blocking membrane used to protect the platinum electrode surface. pH and temperature characteristics were excellent. The glucose probe incorporated an outer polycarbonate membrane. The linear range was from 1 to 1200 mg/dL. The steady-state response was measured in less than 2 min and the rate in less than 10 s, making this sensor a useful probe for hydrogen peroxide and glucose analyses in clinical, environmental, and industrial processes.

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