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ENZYME ELECTRODES USING ULTRAFILTRATION MEMBRANES AS ENZYME CARRIERS

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Two kinds of commercially available ultrafiltration membranes were used for binding enzymes. These enzyme-bound membranes were connected with oxygen electrodes to build up enzyme electrodes. Quantitative assays of glucose, sucrose, and hydrogen peroxide were carried out using these enzyme electrodes in concentration ranges for glucose from 1 to 20 mg/dl, sucrose from 0.5 to 5 mg/ml, and hydrogen peroxide from 5 to 200 ppm. Similarly, D-alanine was measured in the range 0.005 to 0.05 mg/ml, but the calibration curve was not a straight line.

INTRODUCTION

Enzyme electrodes have been attracting much interest in recent years, especially in clinical diagnosis, where Updike and Hicks in 1966 reported the determination of glucose (1). More recently, many kinds of enzyme electrodes have been introduced. Substances reported to be measurable are glucose (2), urea (3), D-amino acid (4), uric acid (5), alcohol (6), hydrogen peroxide (7), penicillin (8), and other substances as reviewed by Guilbault (9). However, the methods of immobilization of enzymes have not been sufficiently investigated. Immobilization methods applied so far to enzyme electrodes have been (*1*) acrylamide gel entrapment (1,4), (*2*) aqueous solution of free enzymes sealed with a semipermeable membrane on an electrode surface (10), (*3*) crosslinking with glutaraldehyde (5), and (*4*) collagen entrapment (7). Except for method (*4*) all immobilization methods required additional membranes to be connected to the electrodes. Thus, the construction of enzyme electrodes was very troublesome, and moreover they suffered from disadvantages of a long response time and a long recovery time. The collagen entrapment method gave membranes of good

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mechanical strength and enabled assay in a short response time, but the enzymes often lost activity during immobilization. However, ultrafiltration membranes have good permeability for relatively low-molecular-weight substances and good mechanical strength. Moreover, it is possible to immobilize enzymes within the matrix of these membranes, because the pore sizes are large enough to permit diffusion of the enzymes, even though their diffusion rate may be lower than that of the low-molecular-weight substances.

MATERIALS AND REAGENTS

Some physical properties of ultrafiltration membranes the authors used are shown in Table 1. Membrane I (trade name MRD 2370) was supplied by Mitsubishi Rayon Co., Ltd. (Tokyo). Membrane II (trade name Juragard 2400) was obtained from Celanese Co., Ltd. (U.S.A.). Glucose oxidase (from *Aspergillus niger*, 14 IU/mg powder), invertase (from yeast, 23100 IU/g powder), and catalase (from beef liver 9×10^3 IU/mg protein) were obtained from Kyowa Hakko Kogyo Co., Ltd. Mutarotase (from hog kidney, 6000 IU/mg protein) and D-amino acid oxidase (from hog kidney, 15 IU/mg protein) were obtained from Boehringer Mannheim. Hyland standard human plasma (Lot No. 0369 N 004AA) was obtained from Japan Travenol Corp., Ltd.

APPARATUS AND PROCEDURES FOR ASSAY

An oxygen electrode (Clark type) was provided by Tateishi Life Science Co., Ltd. (Kyoto). Enzyme-immobilized membranes were fitted on a teflon film, which covered the platinum surface of an oxygen electrode with the aid of an o-ring and a supporting cap. The enzyme electrode thus obtained was inserted into a cell (diameter of 2 cm and depth of 3.5 cm) which was covered with a water jacket. Water (35°C) was passed through this jacket. Eight

TABLE 1. **Properties of Ultrafiltration Membranes**

Membrane	Trade name	Functional group	Tensile strength (kg/cm^2)	Porosity	Thickness	Average pore size (Å)
I	MRD 2370	—C≡N,—COOR	100	0.53	80	90
II	Juragard 2400	none	>1400	0.35	25	1000×200

milliliters of 0.1-M phosphate buffer (pH 5.6 to 8.0) was poured into this cell and stirred with a teflon rod (length of 1.9 cm) at about 300 rpm. When the oxygen level reached a steady state, a certain quantity (1 to 5 μl) of the substrate solution was added.

METHOD OF IMMOBILIZATION

As shown in Table 1, membrane I has two chemically functional groups, a nitrile group and an ester group. We tried to immobilize enzymes covalently to the membrane through these functional groups using methods described by Zaborsky et al. (11). Polymers having a nitrile group can be activated to bind enzymes through transformation of the nitrile group to an imidate group. We applied this method to membrane I, but no enzyme was bound to the membrane. The ester group was easily activated by transforming the group into a hydrazide. The membrane was first immersed for 4 h at room temperature (about 20°C) in 20 vol.% hydrazine hydrate dissolved in methanol, then slightly washed with water and treated with 10 wt.% glutaraldehyde in buffer solution (0.1-M phosphate buffer, pH 8.0) for 20 min at room temperature, and then thoroughly washed with water. Finally, the activated membrane (1 cm^2) was mixed with the enzyme in buffer solution (5 ml) at 5°C for 16 h.

Enzyme solutions coupled using this method were glucose oxidase (20 mg powder/ml) in 0.1-M phosphate buffer of pH 6.0, and catalase (100 mg protein/ml) in 0.1-M phosphate buffer of pH 7.0.

Another immobilization method examined was as follows. First, the enzyme solution was prepared. Twenty milligrams of glucose oxidase was dissolved in 1 ml of buffer solution (0.1-M phosphate buffer, pH 6.0). One hundred milligrams of invertase, 12.5 mg of glucose oxidase, and 2.5 mg (as protein) of mutarotase were dissolved together in 1 ml of buffer solution (0.1-M phosphate buffer, pH 6.0). Five milligrams (as protein) of D-amino acid oxidase, being suspended in ammonium sulfate solution (3.2-M), was centrifuged at $10{,}000 \times g$ for 10 min, and the precipitate was dissolved in 0.2 ml of buffer solution (0.1-M phosphate buffer, pH 7.5). Then 3 pieces of membrane (1 cm^2) were immersed in these enzyme solutions at 5°C for 16 h, followed by slightly washing with water, then followed by immersion in 10% glutaraldehyde in buffer solution (0.1-M phosphate buffer, pH 8.0) for 20 min at room temperature.

Activities of the glucose oxidase immobilized by these two methods were almost comparable, as shown in Table 2. As for membrane II, having no chemically functional groups, only the adsorption-crosslinking method was applicable. The activity of immobilized glucose oxidase when attached to membranes I and II by the above-mentioned methods is summarized in

TABLE 2. **Immobilization of Glucose Oxidase**

Membrane	Method of immobilization	Activity of immobilized glucose oxidase (IU/cm^2)
I	Covalent Binding	
	(a) Imidate $[-C(OR)=NH_2^+$	0
	(b) Hydrazide $[-CONHNH_2]$	0.18
	Adsorption–crosslinking	0.18
II	Adsorption–crosslinking	0.11

Table 2. Patents for these methods are pending under Japan Open Patents 52-17889 and 53-19782.

RESULTS AND DISCUSSION

Properties of the Membrane-Bound Enzymes

The glucose oxidase bound to the membrane I was stable for 1 month storage (5°C, 30°C) in buffer solution (0.1-M phosphate buffer, pH 5.6) as shown in Fig. 1. But the activity of the enzyme immobilized to the membrane by simple adsorption and not crosslinked with glutaraldehyde decreased immediately. This result shows that the enzyme can be adsorbed only weakly to the membrane, and the leakage of the enzyme from the membrane can be prevented by crosslinking with glutaraldehyde. The membrane-bound glucose oxidase easily lost its activity when it was exposed to the air and

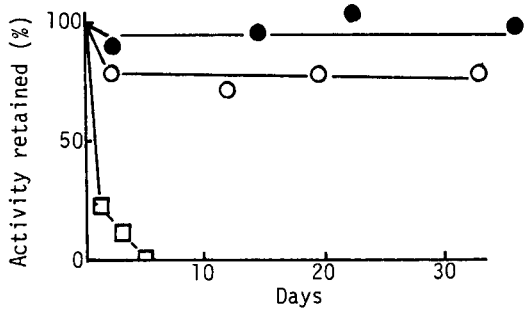

FIG. 1. Stability of immobilized enzyme. Glucose oxidase was adsorbed to membrane I and stored at 5°C (open squares), adsorbed and crosslinked with glutaraldehyde in membrane I and stored at 5°C (solid circles) and 30°C (open circles) in 0.1-M phosphate buffer of pH 5.6.

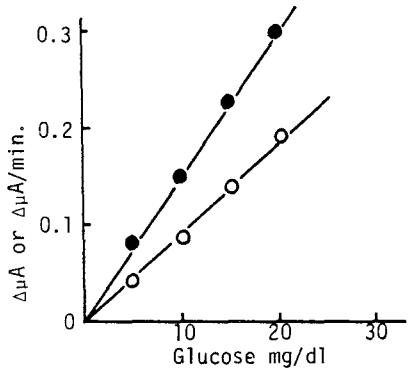

FIG. 2. Calibration curve for glucose in the steady-state method (open circles) and the kinetic method (solid circles) using membrane I at 35°C and pH 5.6.

dried. This enzyme-immobilized membrane must be stored in water of pH 5 to 7.

Glucose Sensor

Figure 2 shows the response curves for glucose with a steady-state method and a kinetic method. Using membrane I, both methods showed proportionality to the glucose at concentrations up to 20 mg/dl. Response times are shown in Fig. 3. Oxygen levels reached steady states within about 1 to 3 min, depending on the glucose concentration. With the kinetic method, it was possible to read out the glucose concentration in 10 to 20 sec, using a differentiator attachment. The enzyme electrode of membrane I has an additional advantage in repeated use, as it takes only 30 to 45 sec of washing for the electrode to reach the initial level of electric current. This excellent property is due to a high diffusion rate of glucose and oxygen through this

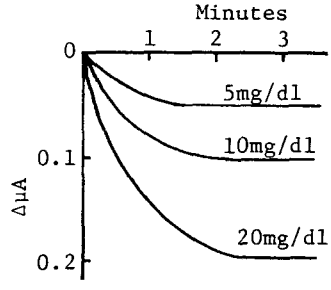

FIG. 3. Response time of the glucose sensor at glucose concentrations of 5 mg/dl, 10 mg/dl, and 20 mg/dl. The conditions were the same as in Fig. 2.

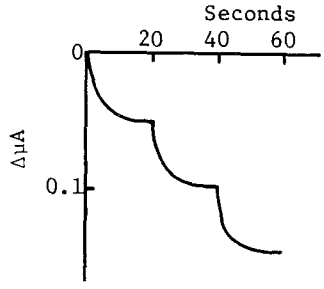

FIG. 4. Response time of the glucose sensor. Glucose was added stepwise to the concentration of 20 mg/dl at intervals of 20 sec. The conditions were the same as in Fig. 5.

membrane. With the membrane II, the initial rate of the change of oxygen levels was very high, as shown in Fig. 4, and the oxygen level reached a steady state very rapidly.

As shown in Table 1, the average pore size of membrane II (length of 1000 Å and width of 200 Å) is larger than that of membrane I, and membrane II is thinner than the membrane I. Thus, the substrates can diffuse through membrane II faster than through membrane I. This is why the response time of the enzyme electrode of membrane II is shorter than that of membrane I. A calibration curve is shown in Fig. 5, and good proportionality is observed up to 60 mg/dl. Calibration curves were influenced by temperature, as shown in Fig. 6. This is caused not only by high temperature dependency of the oxygen electrode, but also by diffusion rate dependency of glucose and oxygen through the micropores of this membrane to the electrode, and in addition by activity dependency of the immobilized glucose oxidase.

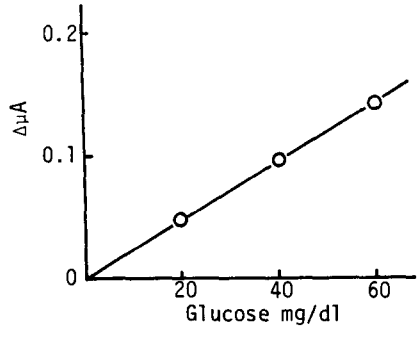

FIG. 5. Calibration curve for glucose in the steady-state method using membrane II at 35°C and pH 5.6.

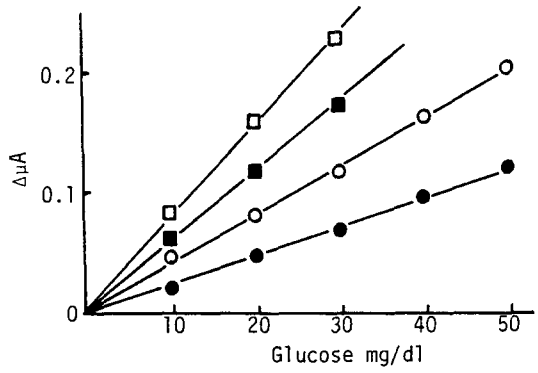

FIG. 6. Calibration curve for glucose using glucose-oxidase-immobilized membrane I at 1°C (solid circles), 10°C (open circles), 20°C (solid squares), and 30°C (open squares).

0.1-M phosphate buffer (pH 6.0) were poured into the cell and the enzyme electrode was inserted into it. Then 0.8 ml of the standard plasma incubated at 35°C beforehand and equilibrated to the oxygen level of the buffer solution in the cell was added to the cell. As shown in Table 3, the data obtained by this method show good agreement with the results obtained by other methods.

Sucrose Sensor

We immobilized invertase, mutarotase and glucose oxidase all together within a membrane I. Activities of the immobilized enzymes were 0.20 IU/cm^2 for invertase and 0.15 IU/cm^2 for glucose oxidase. Activity of the coimmobilized mutarotase was not measured. As reported by Suzuki et al. (12), addition of mutarotase enhanced the sensitivity of this sucrose

TABLE 3. **Assay of Plasmic Glucose**

Method of analysis	Concentration of glucose in human plasma[a] (mg/dl)
SMA 12/60[b]	92±6
o-Toluidine[c]	90±6
This method	90±6

[a] Hyland standard human plasma.
[b] Glucose oxidase method using Technicon autoanalyzer SMA 12/60 (14).
[c] Chemical colorization method (14).

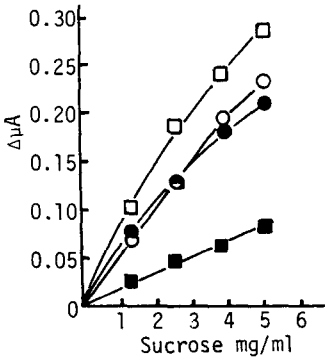

FIG. 7. Calibration curve for sucrose in the steady-state method using invertase, mutarotase, and glucose oxidase coimmobilized to membrane I at 35°C and pH 6.0 (open circles), pH 6.5 (open squares), and pH 7.1 (solid circles) using invertase and glucose oxidase coimmobilized to membrane I at 35°C, pH 5.6 (solid squares).

sensor to about 5 times that of an invertase and glucose oxidase system (Fig. 7). Maximum sensitivity was obtained at pH 6.5, an intermediate optimal pH for the three enzymes. Optima for each enzyme were as follows: invertase, pH 5.0; mutarotase, pH 7.1; and glucose oxidase, pH 5.6, as shown in Fig. 7.

Hydrogen Peroxide Sensor

High activity was found in membrane I, namely, 0.78 IU/cm^2 (at 2°C in 0.1-M phosphate buffer, pH 7.0) because of a very large turnover number of this enzyme. A hydrogen-peroxide sensor composed of a catalase-bound membrane (membrane I) and an oxygen electrode can indicate the increase or the increasing rate of oxygen level. As shown in Fig. 8, both methods, a steady-state method and a kinetic method, can be applied to the assay of hydrogen peroxide at concentrations up to 200 ppm.

As reported by Chance and Herbert (13), the Michaelis constant of catalase for H_2O_2 is of the order 10^{-9}–10^{-10} M. In spite of this fact, H_2O_2 can be measured by the present method up to 200 ppm (5×10^{-3} M). Consequently, it can be assumed that the rate-determining step is not the enzyme reaction but the diffusion of hydrogen peroxide through the membrane.

D-Amino Acid Sensor

Poor activity was found in membrane I, namely, 0.02 IU/cm^2 (at 35°C in 0.1-M phosphate buffer, pH 8.5). A calibration curve against D-alanine in

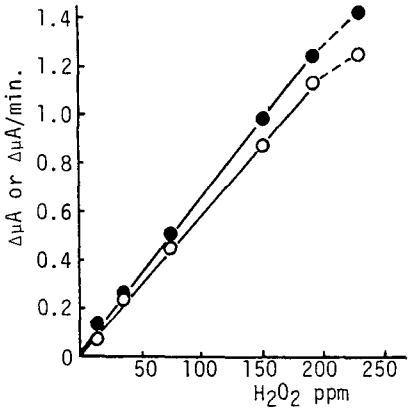

FIG. 8. Calibration curve for H_2O_2 in the steady-state method (open circles) and the kinetic method (solid circles) using catalase immobilized to membrane I at 2°C and pH 7.4.

a steady-state method is shown in Fig. 9. Good proportionality was not obtained in this case because of poor activity of the bound D-amino acid oxidase. When the sensor once used was stored at 5°C for 9 days, the sensitivity to D-alanine slightly decreased, as shown in Fig. 9. This may have been caused by inactivation of the enzyme by hydrogen peroxide produced by its own reaction.

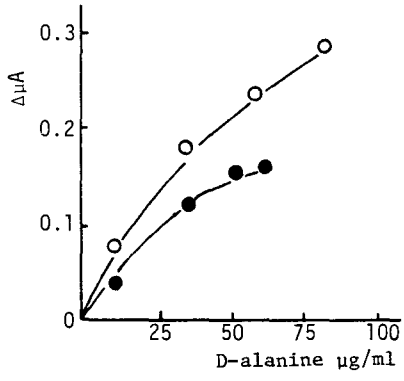

FIG. 9. Calibration curve for D-alanine in the steady-state method using D-amino acid oxidase immobilized to membrane I at 35°C and pH 8.0. Results are shown for freshly made enzyme membrane (open circles) and once-used enzyme membrane stored at 5°C and pH 8.0 for 9 days (solid circles).

CONCLUSIONS

Ultrafiltration membranes made of synthetic organic polymers can be used successfully as supports for enzymes in an enzyme electrode. Advantages of these membranes are as follows: (*1*) the ease with which one can immobilize enzymes; (*2*) good stability in the preservation of the enzyme-immobilized membranes unless exposed to air; (*3*) good mechanical strength; and (*4*) short response time in an assay of specific substrate and only a short time required for washing after each assay. Larger amounts of enzyme activity were preferably bound to the membranes for the preparation of enzyme electrodes. The minimum amount of enzyme activity that gave a good calibration curve was about 0.10 IU/cm^2 in the case of membrane I.

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