Recognition of Substrates by Immobilized Bienzyme Membranes

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Glucose oxidase and/or ascorbate oxidase were immobilized (entrapped) in poly(L-leucine-co-N^{ε}-benzyloxycarbonyl-L-lysin) using a spin casting method. Current shifts and time lags for immobilized single-enzyme and bienzyme membranes were investigated as a function of the substrate concentration at 37°C. The current shifts of L-ascorbic acid in the bienzyme membrane were found to be 2—3.5 times higher than those of a single-enzyme membrane. Both the time lags of L-ascorbic acid and D-glucose were found to depend on the concentration of the substrates for both single-enzyme and bienzyme membranes. The method was presented in order to estimate the concentration of two substrates simultaneously. The concentration of D-glucose and L-ascorbic acid in a model solution was simultaneously estimated from an analysis of the current shifts and the time lags using a nonlinear least-squares method as an example.

An immobilized enzyme electrode (a biosensor) comprises a physical transducer and chemically selective layers. The advantage of the sensor is high selectivity to biological molecules due to a specific enzyme-substrate interaction in the selective layers, although the same number of sensors is required when analyzing multicomponent substrates in the sample solution.

Several immobilized enzyme electrodes were developed and investigated by many researchers; there have been several reviews concerning the immobilized enzyme electrodes.[1-6] These studies were probably stimulated by Updike and Hicks,[7] who immobilized glucose oxidase in a gel and developed a glucose sensor. Recently, an enzyme-FET sensor was constructed by using ISFET[8] that was a micro-pH device and was reported as being useful for the recognition of several substrates.[9-11]

There have been only a few studies[12-15] on two immobilized enzymes in a membrane for substrate recognition. Jensen and Rechnitz[12] studied a D-gluconase sensor by using a two-step enzyme sequence (gluconate kinase and 6-phosphogluconate dehydrogenase). F. -Filho et al.[13] constructed a bienzymatic electrode for the determination of aspartame by the coimmobilization of carboxypeptidase A and L-aspartase on an ammonia gas sensing electrode.

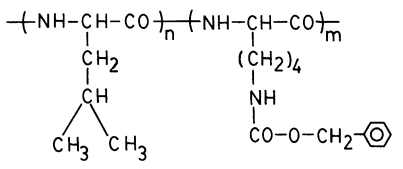
In our previous study, recognition of multicomponent ions was studied by analyzing the permeation of ions through a membrane at non-steady state.[16] The calculated concentration and the true concentration of multicomponent ions (LiCl, NaCl, and CsCl) agreed within a 10% error for a model solution. The previous study prompted us to develop an immobilized bienzyme electrode for the recognition of multicomponent substrates. Glucose oxidase and ascorbate oxidase were immobilized in a poly(α-amino acid) network as a model bienzymatic membrane in this study. The time lags and current shifts due to the response of D-glucose and/or L-ascorbic acid were studied using a bienzymatic electrode with an oxygen electrode at 37°C. The interaction of the response between the two enzymes is suggested in the bienzyme membrane, and the concentration of two substrates in a model solution is simultaneously estimated in this study.

Experimental

Reagents. Glucose oxidase (from Aspergillus niger, Grade II, 100 units per mg) and ascorbate oxidase (from Cucumis, Grade III, 200 units per mg) were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). Poly(4-methyl-1-pentene) was obtained as gift from Mitsui Oil Chemical Co. and purified by precipitation from a cyclohexene solution into methanol. Trichlorometyl chloroformate was also obtained as a gift from Hodogaya Chemical Co. Ultra-pure water by Toraypure LV-10T system was used throughout the experiments. All other chemicals were of reagent grade and were used without further purification.

Poly(α-Amino Acid). Poly(L-leucine-co-N^{ε}-benzyloxycarbonyl-L-lysine), LEU-ZLYS was synthesized by the polymerization of *N*-carboxyamino acid anhydride obtained by a reaction of the amino acid and phosgene in a benzene–dioxane mixture as a solvent for polymerization (Scheme 1).[17,18] The reaction was carried out at room temperature for >10 days, as described elsewhere in detail.[17,18] Triethylamine was used as an initiator.

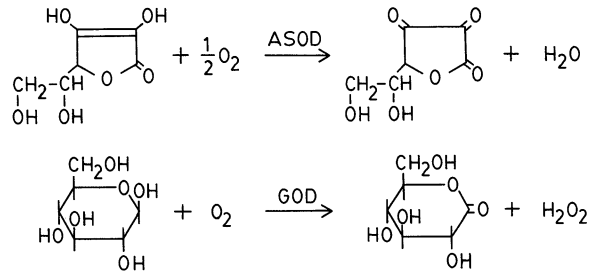
Immobilization Procedure. Glucose oxidase (GOD) and/or ascorbate oxidase (ASOD) were dissolved in 1 wt% of a benzene–dioxane solution of poly(α-amino acid)(4 mg ml^{-1} for GOD, 2.5 mg ml^{-1} for ASOD). Flat sheets of single-enzyme and bienzyme membranes (12 cm diameter) were prepared by a spin-casting method from enzyme solution



Scheme 1.

Table 1. Combination of Immobilized Enzyme Membranes and Substrates in Sample Solution

Membrane	Immobilized enzyme	Substrate in sample solution
SS-1	GOD	D-Glucose
SS-2	ASOD	L-Ascorbic acid
DS-1	GOD+ASOD	D-Glucose
DS-2	GOD+ASOD	L-Ascorbic acid
DD	GOD+ASOD	D-Glucose+L-ascorbic acid



Scheme 2.

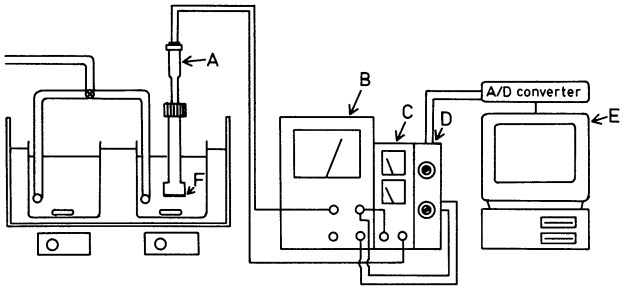


Fig. 1. Schematic diagram of apparatus: (A) oxygen electrode, (B) micro ammeter, (C) power supply, (D) DC amplifier, (E) personal computer, (F) immobilized membrane.

(400 RPM for 30 sec), followed by spin casting from a 3 wt% cyclohexene solution of poly(4-methyl-1-pentene)(500 RPM for 30 sec); they were prepared to be composite membranes, as described previously.[18] The concentration of GOD or ASOD was considered to be the same amount as that entrapped in the single-enzyme and bienzyme membranes, since the amount of enzyme in the casting solution was fixed to be the same amount in both membranes. The thicknesses of the immobilized enzyme layer and the supported layer were estimated be about 1 and 38 μm from SEM observations in this study.

Enzyme Electrode. An immobilized enzyme (single enzyme or bienzyme) electrode was prepared by placing an immobilized enzyme membrane on the platinum anode of an oxygen electrode (K-316 IPI type, Rikaseiki Kogyo Co., Ltd.), which was designed by one of the authors (T. N.). The oxygen consumption due to the enzyme-substrate reaction was measured by the oxygen electrode (Scheme 2).

Procedure of Measurement. A schematic diagram of the apparatus is shown in Fig. 1. The current under steady state conditions, $i(0)$, was measured after the enzyme electrode was introduced into cell A containing pure water in which oxygen gas was bubbled. The electrode was then shifted in

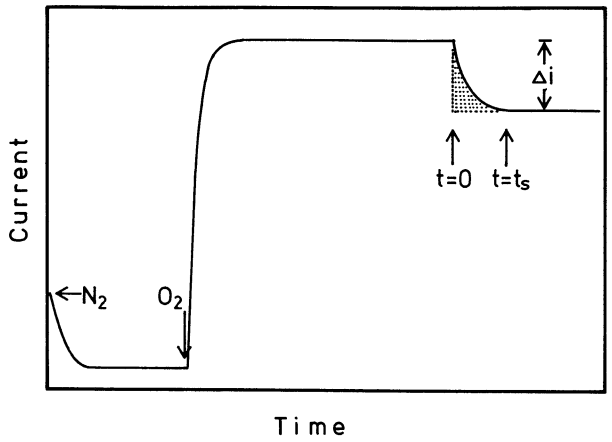


Fig. 2. Typical current curve for immobilized enzyme electrode.

the substrate solution (Cell B) at time (t)=0, and the current at t, $i(t)$, was measured. The solution in the cells was stirred at constant speed (i. e., 430 RPM in this study) by a magnetic stirrer having a revolution meter (HS-3D, Iuchi Seiseido Co., Ltd.). The current was transferred to a 16-bit personal computer (PC-9801VX, NEC Corp.) through an analogue-to-digital converter (EP-98ADC N, Elm Data Corp.).

The current shift, Δi, was calculated from

$$\Delta i = i(0) - i(t_s), \tag{1}$$

where t_s is the minimum time that the current could be considered to be in the steady state. The time lag, θ, was calculated from[19]

$$\theta = [\int_0^{t_s} i(t)\mathrm{d}t - i(t_s)t_s]/i(t_s). \tag{2}$$

A typical current curve of the enzyme electrode is shown in Fig. 2. All measurements were performed at 37±0.1 °C.

Three types of enzyme membranes (GOD, ASOD and GOD+ASOD membranes) were used in this study. The experimental combination with the enzyme membrane and substrate solutions is summarized in Table 1. Each point in Figs. 4—7 is an average of 4—6 measurements. The standard deviations for the measurements of the current shifts and the time lags were found to be about 7 and 10%.

Results

SEM Picture. SEMs of LEU-ZLYS (a) an immobilized GOD+ASOD membrane (b) on the surfaces are shown in Fig. 3. The SEM observation shows that the

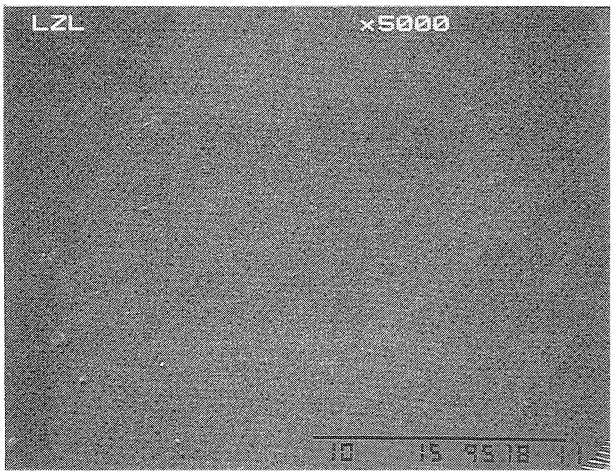


Fig. 3a. SEM picture of LEU-ZLYS surface (×5000, marker=100 μm).

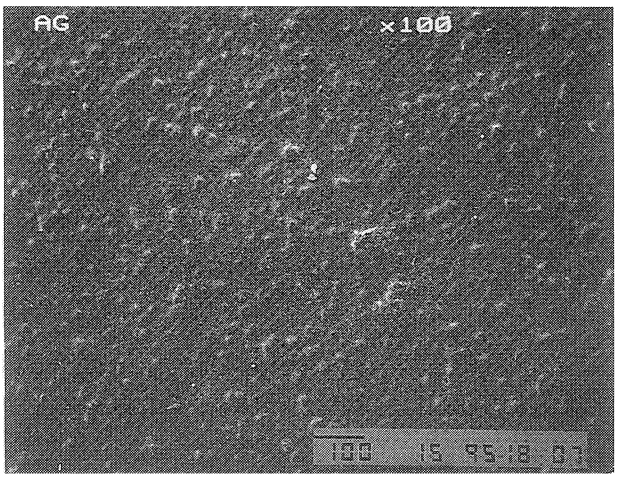


Fig. 3b. SEM picture of immobilized GOD and ASOD membrane on surface (×100, marker=100 μm).

surface of LEU-ZLYS is flat and has no pores. The surface of an immobilized enzyme membrane was found to be uneven and having dispersions of lumps comprising 2—10 μm spheres. The spheres are considered to be small lumps of enzymes that are caused by heterogeneous dispersions of enzymes in the casting solution, since enzymes are only slightly soluble in an organic solvent and most of the enzymes remain as insoluble in the casting solution.

Single Enzyme for Single Substrate. The effect of current shifts and time lags on the concentration of the substrate, [S], for an immobilized single-enzyme membrane (SS-1 for GOD-D-Glucose, SS-2 for ASOD-L-Ascorbic acid) is shown in Figs. 4 and 5. A linear in plots of Δi vs. log (C) was observed in the concentration range 10^{-5} to 10^{-1} M (1 M=1 mol dm^{-3}) with a slope of 0.94 μA/decade for SS/1, similar to results in a previous study,[18] and 0.65 μA/decade for SS-2. The

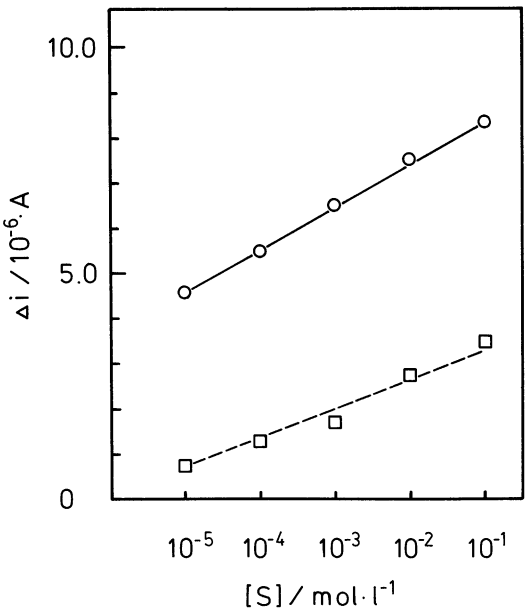


Fig. 4. Effect of current shifts on concentration of substrate for single enzyme membranes. (○) D-glucose; (□) L-ascorbic acid. Solid and broken lines are calculated lines from least squares methods.

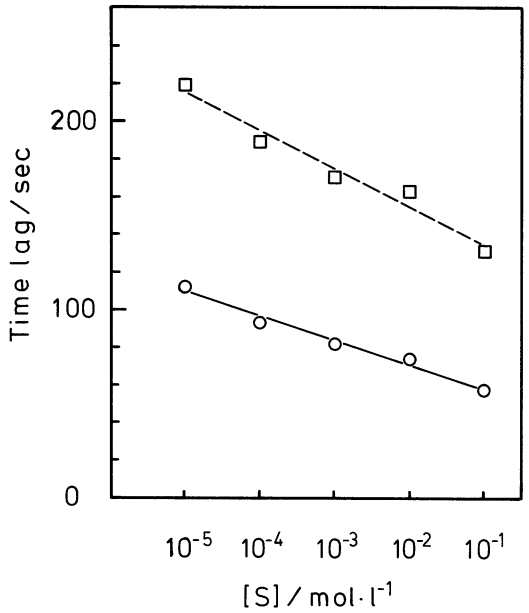


Fig. 5. Effect of time lags on concentration of substrate for single enzyme membranes. (○) D-glucose; (□) L-ascorbic acid. Solid and broken lines are calculated lines from least squares methods.

current shifts for SS-1 were found to be 2.5—4 times higher than those for SS—2. The time lags were found to be slightly dependent on the concentration and the linear response was observed in the concentration range 10^{-5} to 10^{-1} M with a slope of −13.2 sec/decade for SS-1 and −20.2 sec/decade for SS-2. The time lags for SS-2 were found to be approximately twice as large

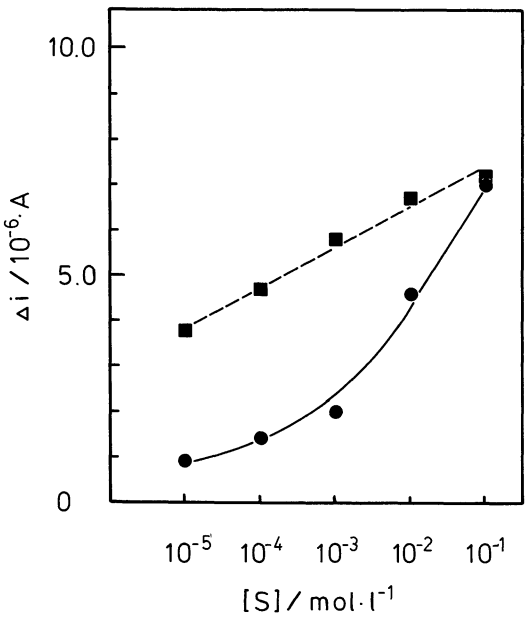


Fig. 6. Effect of current shifts on concentration of substrate for bienzyme membrane. (●) D-glucose; (■) L-ascorbic acid. A broken line is a calculated line from a least squares method.

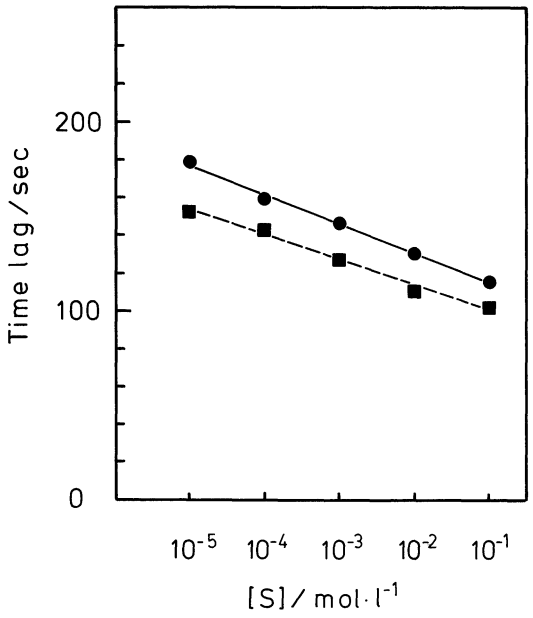


Fig. 7. Effect of time lags on concentration of substrate for bienzyme membrane. (●) D-glucose; (■) L-ascorbic acid. Solid and broken lines are calculated lines from least squares methods.

as those for SS-1.

The concentration decrease of oxygen, which is detected as the current shifts at the electrode, is mainly caused by two steps: (a) diffusion of the substrate to find an enzyme in the active layer and (b) reaction of the enzyme and the substrate followed by oxygen consumption.

The time lags are mainly influenced by the diffusion of substrates. Fig. 5 suggests that L-ascorbic acid diffuses faster than does D-glucose to find enzymes, since the diffusion coefficient, D, is known to be reversely proportional to the time lag,[20]

$$D = L^2/6\theta, \quad (3)$$

where L is the thickness of the membrane. We must, however, notice that D-calculated by Eq. 3 is only the apparent value in this system.

Bienzyme for Single Substrate. The effect of current shifts on the concentration of the substrate for immobilized bienzyme membranes (DS-1 for D-glucose and DS-2 for L-ascorbic acid) is shown in Fig. 6. A linear response of the current shifts was observed in the concentration range 10^{-5} to 10^{-1} M, with a slope of 0.88 μA/decade for L-ascorbic acid (DS-2); a linear response was only observed at concentrations of 10^{-3} to 10^{-1} M, with a slope of 2.5 μA/decade for D-glucose (DS-1).

The current shifts for L-ascorbic acid were found to be higher than those for D-glucose at [S]≤0.1 M in the bienzyme membrane, showing an opposite tendency to that found in the single-enzyme membrane. The current shifts of L-ascorbic acid in the bienzyme membrane was 2—3.5 times higher than those of the single-enzyme membrane. These results suggest that there exists an interaction between two enzymes, and that the activity of ASOD is, therefore, increased in the bienzyme membrane.

The time lags of D-glucose and L-ascorbic acid for the bienzyme membrane are shown as a function of the concentration in Fig. 7. It has been found that the time lags of both substrates in the bienzyme membrane show intermediate time lags between D-glucose and L-ascorbic acid in single-enzyme membranes. The time lags of D-glucose are also found to be close to those of L-ascorbic acid in the bienzyme membrane. This is probably due to the fact that both enzymes in the bienzyme membrane are entrapped at the same position in the membrane matrix, since these enzymes are finely aggregated in the casting solution, and that the diffusion path (tortuosity) of D-glucose to find GOD is probably the same that of L-ascorbic acid to find ASOD in the bienzyme membrane from the relation of Eq. 3.

Discussion

Apparent Kinetic Constants. The current shifts observed in the enzyme electrodes are affected by two steps: a diffusion of the substrate and a reaction of the substrate on the enzyme. The enzyme kinetics is usually represented by Michaelis-Menten kinetics, although the reaction in the immobilized enzyme membrane is expected to be very complicated:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E. \quad (4)$$

The reaction rate, r, is given by

$$r = d[P]/dt = k_2[E][S]/([S] + K_M) \quad (5)$$

and

$$d[P]/dt = -Zd[O_2]/dt, \quad (6)$$

where [E] is the enzyme concentration and [P] is the product concentration (Z=1 for D-glucose and Z=2 for L-ascorbic acid from Scheme 1 and $K_M=(k_{-1}+k_2)/k_1$). The reaction of oxygen at the electrode is expressed by[19]

$$d[O_2]/dt = -\Delta i/(NFA), \quad (7)$$

where N=4 (number of electrons in the reaction), F is the Faraday constant, and A is the area of platinum anode (0.1983 cm^2 in this study).

By combining Eqs. 5—7 we obtain

$$\Delta i^{-1} = (Z/NFA[E])\cdot\{(K_M/k_2)\cdot[S]^{-1} + k_2^{-1}\}; \quad (8)$$

K_M and $[E]k_2$ can be obtained from the slope and the intercept in plots of Δi^{-1} vs. $[S]^{-1}$. Fig. 8 shows plots of Δi^{-1} vs. $[S]^{-1}$ for the single-enzyme and bienzyme membranes at $[S]\geq 0.001$ M. K_M and $[E]k_2$ were calculated using a least-squares method, and are summarized in Table 2.

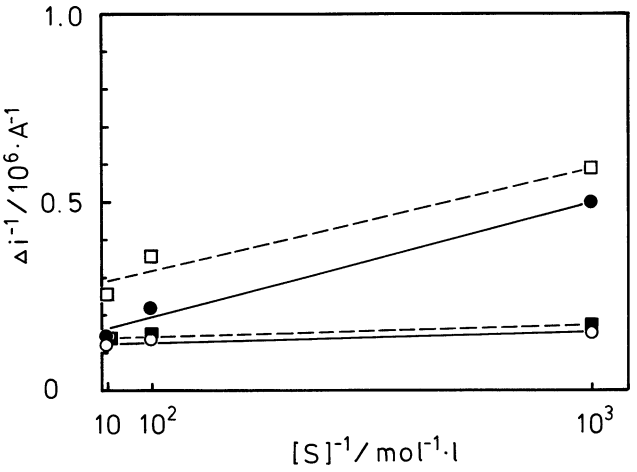


Fig. 8. Plots of Δi^{-1} vs. $[S]^{-1}$ for immobilized enzyme membranes. (○) SS-1; (□) SS-2; (●) DS-1; (■) DS-2.

The K_M of D-glucose in the single-enzyme and the bienzyme membrane were found to be approximately the same as K_M in bulk water (0.77 mM from Ref. 21), in spite of the fact that the enzymes in the membrane are entrapped in a polymer matrix, and the kinetic parameters should have the effect of substrate diffusion to find and meet enzymes in the membrane.

$[E]k_2$ of L-ascorbic acid in the bienzyme membrane was found to be two-times higher that that in the single-enzyme membrane (Table 2). This contributes to the high Δi of L-ascorbic acid in the bienzyme membrane compared to that in the single-enzyme membrane, since a high k_2 contributes to a high Δi (from Eq. 8) at the same value of [E] for the single-enzyme and the bienzyme membranes.

Estimation of [S] of Two Substrates. The concentration of two substrates in a model solution was simultaneously estimated from the current shift and the time lag in this study. Two independent equations are sufficient to estimate two parameters ([S] of D-glucose and L-ascorbic acid). When it is supposed that the current shift and the time lag for bienzyme-two substrate system (DD) can be estimated from the independent contributions of those for bienzyme-single substrate system (DS-1 and DS-2), the current shift, Δi_{DD}, and the time lag, θ_{DD}, for DD system can be expressed by

$$\Delta i_{DD} = \Delta i_G + \Delta i_A, \quad (9)$$

and

$$\theta_{DD}^{-1} = ([S]_G/\theta_G + [S]_A/\theta_A)/([S]_G + [S]_A). \quad (10)$$

Here, Δi_i and θ_i (i=G or A) are the current shift and the time lag of i substrate at $[S]_i$ in DS system (DS-1 and DS-2), and subscripts G and A indicate D-glucose and L-ascorbic acid. Eq. 10 was obtained from Eq. 6 in Ref. 16. Δi_i and θ_i were observed to be a function of $[S]_i$ (see Figs. 6 and 7), and can be expressed by experimental equations in this study as follows:

$$\Delta i_G = 10^{\alpha}, \quad (11)$$

$$\Delta i_A = 8.28 + 0.88 \log [S]_A, \quad (12)$$

$$\theta_G = 99.1 - 15.5 \log [S]_G, \quad (13)$$

and

Table 2. Rate Constants Calculated from Michael-Menten Equation for Immobilized Enzyme Membranes

Membrane	Substrate	$[E]k_2/10^3$ M sec^{-1}	K_M/mM
SS-1	D-Glucose	0.61	0.21
DS-1	D-Glucose	0.48	2.13
GOD in Bulk water	D-Glucose		0.77
SS-2	L-Ascorbic acid	0.53	1.05
DS-2	L-Ascorbic acid	1.08	0.22

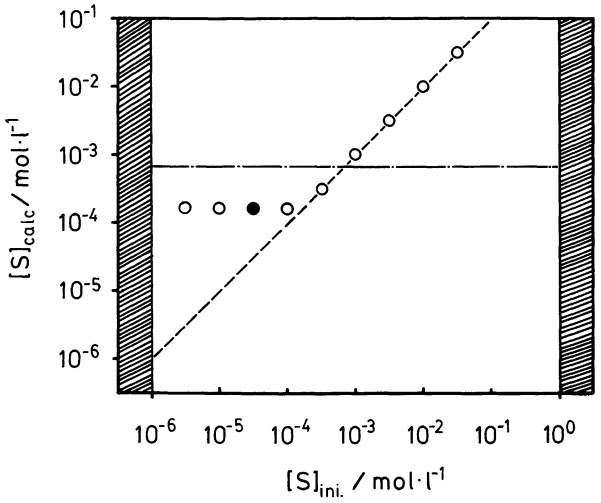


Fig. 9. Dependence of estimated concentration of D-glucose on initial concentration of calculation. -----: $[S]_{calcd}=[S]_{ini.}$: —·—: true concentration of D-glucose. (●) best fitting point; ■: [S] could not be estimated due to high errors in the calculation.

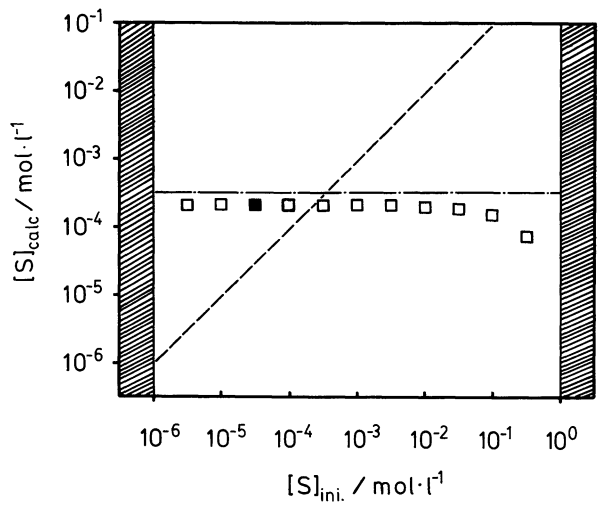


Fig. 10. Dependence of estimated concentration of L-ascorbic acid on initial concentration of calculation. -----: $[S]_{calcd}=[S]_{ini.}$: —·—: true concentration of L-ascorbic acid. (●) best fitting point; ■: [S] could not be estimated due to high errors in the calculation.

$$\theta_A = 86.8 - 13.2 \log [S]_A, \quad (14)$$

where $\alpha=1.07+0.23 \log [S]_G$.

The mixture solution ($[S]_G=0.667$ mM and $[S]_A=0.333$ mM) was simultaneously estimated from Eqs. 9—14 as a model example using a nonlinear least-squares method. The bienzyme membranes which were taken from the same sheet employed in Figs. 6 and 7 were used in this estimation.

When these equations were solved numerically using a nonlinear least-squares method, it was sometimes observed that their solutions were dependent on the initial values of the calculations. The dependence of $[S]_G$ and $[S]_A$ on the initial concentration of the substrates, $[S]_{ini}$, in the calculations was investigated and is shown in Figs. 9 and 10. Although the estimated concentration, $[S]_{calcd}$, for L-ascorbic acid does not depend on the initial concentration (see Fig. 10), the estimated concentration for D-glucose depends on the initial concentration in Fig. 9. Since the time lags of D-glucose were found to be close to those of L-ascorbic acid in the bienzyme membrane at the same substrate concentrations (see Fig. 7), it is rather difficult to accurately estimate the concentration of two substrates simultaneously in this study. The concentration of L-ascorbic acid, however, shows almost the same concentration (0.22 mM) as the true value (0.333 mM) at the best fitting point, and the concentration of D-glucose is approximately within one order of difference (0.18 mM) from the true concentration (0.667 mM) in the simultaneous estimation (i. e., Figs. 9 and 10)

Conclusion

The current shifts and the time lags for the immobilized single-enzyme and bienzyme membranes were investigated as a function of substrate concentration at 37 °C. The current shifts of L-ascorbic acid in the bienzyme membrane were observed to be 2—3.5 times higher than those in the single-enzyme membrane. These results suggest that there exists an interaction between two enzymes; the activity of ASOD therefore increased in the bienzyme membrane. The simultaneous recognition of two substrates was theoretically and experimentally presented in this study, although the experimental example showed unsatisfactory results. Recognition will surely be possible with one sensor when we select a bienzyme membrane that shows different current shifts and time lags at the same concentration of each substrate.

References

1) J. Janata and A. Bezegh, *Anal. Chem.*, **60**, 62R (1988).
2) A. P. F. Turner, I. Karube, and G. S. Wilson, "Biosensors: Fundamentals and Applications," Oxford University Press, New York (1987).
3) J. S. Sidwell and G. A. Rechnitz, *Biosensors*, **2**, 221 (1986).
4) E. A. H. Hall, "The Developing Biosensor Arena," *Enzyme Microb. Technol.*, **8**, 651 (1986).
5) R. Renneberg, F. Schubert, and F. Scheller, *Trends Biochem. Sci. (Pers. Ed.)*, **11**, 216 (1986).
6) L. D. Bowers, *Anal. Chem.*, **58**, 513 (1986).
7) S. J. Updike and G. P. Hicks, *Nature*, **214**, 986 (1967).
8) P. Bergveld, *IEEE Trans. Biomed. Eng.*, **17**, 70 (1970).
9) M. Gotoh, E. Tamiya, and I. Karube, *J. Membr. Sci.*, **41**, 291 (1989).
10) S. Caras and J. Janata, *Anal. Chem.*, **52**, 1935 (1980).
11) Y. Ikariyama, S. Yamauchi, T. Yukiashi, H. Ushida, and M. Aizawa, *Bull. Chem. Soc. Jpn.*, **62**, 1869 (1989).

12) M. A. Jensen and G. A. Rechnitz, *J. Membr. Sci.*, **5**, 117 (1979).
13) O. Fatibello-Filho, A. A. Suleiman, G. G. Guilbault, and G. J. Lubrano, *Anal. Chem.*, **60**, 2397 (1988).
14) F. Scheller and D. Pfeiffer, *Anal. Chim. Acta*, **117**, 383 (1980).
15) J. Kulys, V. V. Sorochinskii, and R. Vidziunaite, *Biosensors*, **2**, 135 (1986).
16) A. Higuchi, T. Katoh, and T. Nakagawa, *J. Chem. Soc., Faraday Trans. 1*, **85**, 127 (1989).
17) E. R. Blout and R. H. Karlson, *J. Am. Chem. Soc.*, **78**, 941 (1956).
18) T. Nakagawa and T. Uchida, *Nippon Kagaku Kaishi*, **1987**, 502.
19) S. Aiba, M. Ohashi, and S. Y. Huang, *Ind. Eng. Chem. Fundam.*, **7**, 497 (1968).
20) J. Crank, "The Mathematics of Diffusion," Oxford University Press, London (1975), p. 51.
21) H. H. Weetall, *Res. Dev.*, **22**, 18 (1971).