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The Electron-Transfer Effect of Coenzymes NAD(P)$^+$/NAD(P)H in Conjugated Transmembrane Oxidoreductase Reactions

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Conjugated oxidoreductase reactions across membranes prepared from PVA or polyurethane containing amino components were driven between systems of glucose 6-phosphate dehydrogenase (G6PD)/glucose 6-phosphate (G6P)/NADP$^+$ and glutathione reductase (GR)/glutathione oxidized form (GSSG)/NADPH. In order to obtain the optimum reaction system, membranes were prepared, while considering the membrane composition, membrane thickness, and membrane density. The kinetics and the mechanism of the conjugated transmembrane reaction systems have been discussed.

Consequently, it has been found that the coenzyme molecules adsorbed in the membrane can act as electron carriers, and that the used hydrogel membrane should be as dense as possible and positively charged.

Coupled two-oxidoreductase-reactions, in which, for example, amino[1—3] and carboxylic acids[4] can be produced from α-keto acids, are meaningful from the view point of the "fixation" of ammonia and carbon dioxide; many other coupled enzyme reactions[5—7] have also been investigated.

In these coupled reactions, coenzymes NAD(P)$^+$ and/or NAD(P)H are normally used mixing two different kinds of oxidoreductases. In these cases, one of the reactions is incorporated to reproduce the coenzyme in the reduced or oxidized form. One of the serious disadvantages of such mixed-reaction systems is that the purposive material is contaminated by the materials used for coenzyme reproducing.

In order to avoid contamination, the so-called transmembrane oxido-reductase reaction systems have been investigated,[8] in which the coenzyme-reproducing and product-producing systems are divided by a polyurethane membrane[9] incorporating an intercellular cement material, an excellent natural electron carrier extracted from wool.[10] Consequently, conjugated transmembrane reactions have been successfully driven. In such systems it is also possible that the coenzyme molecules adsorbed in the membrane could present an appropriate passage for electron transfer, due to their alternative arrangement in the oxidized and reduced forms. However, there have been no reports concerning the electron-transfer function of the coemzyme molecules adsorbed in the membrane.

In this study our attention was focused on two subjects associated with the construction of an efficient transmembrane oxidoreductase reaction system: One of them was to confirm whether the NAD(P)$^+$/NAD(P)H molecules adsorbed in the membrane can really act as electron carriers; the other was correlated to the membrane preparation to obtain an optimum reaction system. Thus, the effects of the membrane composition, membrane thickness, membrane density, and the other physicochemical properties of the membrane on the conjugated transmembrane reaction were investigated. In addition, the kinetics and the reaction mechanism of the total conjugated transmembrane reaction system are discussed.

Experimental

Materials: As two kinds of enzyme redox reactions to be conjugated, glucose 6-phosphate dehydrogenase (G6PD)/glucose 6-phosphate (G6P)/NADP$^+$ and glutathione reductase (GRD)/glutathione oxidized form (GSSG)/NADPH were selected, as described in a previous paper.[8] G6PD (from yeast), GRD (from yeast), NADP$^+$, and NADPH were purchased from Oriental Yeast Co., Ltd. GSSG, its reduced form (GSH), and G6P were all obtained commercially (Sigma) and were not purified further. The other reagents were of analytical grade and were used without further purification.

Electron Carrier: As possible electron carriers, coenzyme NADP(H) and nonkeratinous hydrophobic intercellular materials (δ_L) and hydrophilic one (δ_H) extracted from wool[8,10] were used.

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Membrane Preparation: For the purpose of this investigation, the membranes had to be hydrophilic and charged positively, because enzymes and coenzymes were used in aqueous systems; in addition, the negatively charged coenzyme molecules had to be adsorbed in and on them, as well as the enzyme molecules located on the membrane surfaces. Furthermore, the pore size of the membrane in swollen state had to be adequate so as to permit thermal motions of the δ_L and δ_H components in the membrane, and also to diminish the leakage of enzymes, substrates, and products through the membrane. In this investigation, poly(urethane) (Ure) and Poly(vinylalcohol) (PVA) were selected as membrane materials. In both cases, it was easy to change the membrane density, the membrane thickness and the pore size. Introducing positive charges on the membranes could be achieved by mixing a cross-linking agent, diethylenetriamine (DETA), or a positively charged second component, poly(ethylene imine) (PEI), in the preparation of Ure and PVA membrane, respectively.

The Ure membranes were mainly prepared by casting a mixture of urethane-prepolymer,[9] whose isocyanate groups were blocked with imidazol, and an aqueous solution of DETA on a glass plate framed by a silicone rubber ring at room temperature.[8] PVA membranes were prepared by a casting method and cross-linked with glutaraldehyde (Gla) or 2-ethyl-2-(hydroxymethyl)-1,3-propanediol (trimethylolpropane, TMP). In the case of cross-linking by Gla, an adequate amount of water or an aqueous solution of PVA (10 wt%, 1 g) was mixed with an appropriate amount of water or an aqueous solution of PEI (10 wt%, 0.5 g); the mixture was then cast on a glass plate and dried at 50 °C for 24 h to form a membrane. After that, the membrane was treated with a 0.1 wt% aqueous solution of Gla, containing 20 wt% Na_2SO_4 and 1 wt% H_2SO_4 at room temperature. The degree of cross-linking was varied by changing the Gla treating time. The membranes obtained in such way were coded by a Gla membrane. In the case of TMP cross-linking, the mixture of a PVA solution, a PEI solution and TMP was cast on a glass plate, dried at ca. 50 °C and then baked at 150 °C for 1 h. To change the degree of cross-linking the amount of TMP was varied. All of the membranes prepared were kept in water for 1 to 2 weeks in order to remove any excess of cross-linking agent.

The incorporation of δ components into the membrane was carried out by mixing the δ component in casting solution in the ratio of δ : DETA : urethane-prepolymer = 40 mg : 4 ml (0.5 mol dm^{-3}) : 3 g. NADP(H) could be easely introduced into the membrane by immersing the membrane in an aqueous NADP(H) solution.

Physical Properties of Membranes: The membrane thickness in both dried and water-swollen states was measured using a membrane thickness meter (Kobunshi Keiki Co., Ltd.). The errors were within ±4%. The porosity was obtained as the volume ratio of a cut membrane (ca. 5×5 cm) in the dried state against that in a completely swollen state. The average pore radius of the membrane was obtained by the water-permeation method;[12] the permeation rate of water through the membrane under pressure (2—5 kg cm^{-2}) was measured; from this value the average pore radius was calculated by Hagen–Poiseulle equation.

The membrane potential was determined using a two-compartment cell (described previously[13]). The potential was measured with a Digital Multimeter TR6843 (Takeda Riken Co., Ltd.) equipped with pin-hole-type of Ag/AgCl/sat.KCl electrodes (Horiba Co., Ltd., #2010). The mean value of two measurements obtained by exchanging the electrodes in both compartments was taken as a reliable membrane potential. The KCl solutions in the compartments were kept constant in one compartment at 10^{-3} M and in the other compartment at 10^{-4} M, respectively (1 M=1 mol dm^{-3}). The temperature was controlled at 25±0.2 °C.

Driving of Conjugated Transmembrane Oxydoreductase Reactions: A conjugated redox reaction was performed by using a two-compartment cell, as shown in a previous paper.[8] Both compartments (A and B) were filled with a 35 ml phosphate buffer solution of pH 7.8, an optimum pH for activating both enzymes. After that the diffusion flux of NADPH (J_{NADPH}) through the membrane was measured in the same buffer solutions by adding an NADPH solution into compartment A and monitoring the concentration of NADPH diffused to compartment B spectrophotometrically at 340 nm. The solutions were then taken away and a 0.25 mM $NADP^+$ aqueous buffer solution (pH 7.8) was again freshly filled in both compartments and 1 mmol G6P and 0.5 mmol GSSG in compartments A and B, respectively. The reaction was started by adding 0.3 mg G6PD to compartment A and 0.35 mg GRD to compartment B at the same time, respectively. The concentration change of GSSG and GSH in both compartments with time was followed either by the polarographic method[8] or the coloration method after Ellman.[11]

An additional experiment was carried out, in order to confirm whether protons are also transported through the membrane, accompanied by an electron transfer in driving the transmembrane redox reaction. For this purpose, a similar transmembrane redox reaction was drived in an unbufferized system; a 14 ml aqueous solution containing $MgCl_2$ (3.1 mM, an activating agent of G6PD), G6P (10 mM) and G6PD (5 units), and a 25 ml aqueous solution containing GSSG (0.53 mM), GR (5 units) and NAD^+ (0.1 mM) were prepared. The former and later solutions were added to compartments A and B, respectively. The reaction was started by adding 11.3 ml NAD^+ (0.53 mM) and 0.3 ml of 0.1 M NaOH aq solution in compartment A and B, respectively. Just after adding NaOH, the pH value of the solution in compartment B was 6.77. The change in the pH value in both compartments, and the GSH production rate in compartment B, were monitored at regular time intervals.

Results and Discussion

Properties of Prepared Membranes. Table 1 shows some properties of the prepared PVA and Ure membranes. In the case of PVA it is clear that by increasing the time of cross-linking, the porosity of the membrane in aqueous systems decreases. The increment of the membrane potential may be due to carboxylate groups introduced by the partial oxidation of glutaraldehyde. This means that the membranes cross-linked with glutaraldehyde are charged negatively.

In the case of the Ure membrane the mixing ratio of DETA and prepolymer (in molar ratio of the amino

Table 1. Properties of Prepared PVA and Ure Membranes

Membrane code	Preparation conditions	Thickness μm	Porosity	Pore size μm	Membrane potential mV
PVA-1	0.5 h[a)]	110	0.67	0.015	0.66
PVA-2	1.0 h[a)]	65	0.71	0.016	1.47
PVA-3	5.0 h[a)]	102	0.43	—	7.36
Ure-1	1 : 0.58[b)]	82	0.25	0.025	−22.9
Ure-2	1 : 0.75[b)]	119	0.58	0.027	−40.4
Ure-3	1 : 0.81[b)]	157	—	—	−41.3
Ure-4	1 : 0.87[b)]	167	0.52	0.028	−45.5
Ure-5	1 : 0.92[b)]	78	—	—	−51.5
Ure-6	1 : 1.01[b)]	63	—	—	−50.0
Ure-7	1 : 1.15[b)]	141	0.72	0.062	−41.9

a) Cross-linking time by glutaraldehyde (PEI was not incorporated). b) Molar ratio of [–NH_2] group of TEDA and [–NCO] group of urethane prepolymer.

group of DETA and the isocyanate group of the prepolymer) was varied, also to change the porosity of the membrane in the water-swollen state. By increasing the –NH_2 content in the mixture the porosity decreased, leading to a smaller pore size, which was measured by the water-permeation method.[12)] A negative membrane potential means that the membranes are charged positively because of the amino groups introduced by mixing DETA. Of interest is that the potential, which increases upon increasing the –NH_2 content, reaches the maximum at a ratio of –NH_2 : –NCO≈0.95, and then decreases after a higher –NH_2 content (Fig. 1). The decrement can be considered to be caused by an extremely higher porosity.

Confirmation of Parallel Transports of Electron and Proton. In Fig. 2 the rate of GSH production in compartment B and the pH change in compartments A and B are plotted against the reaction time for

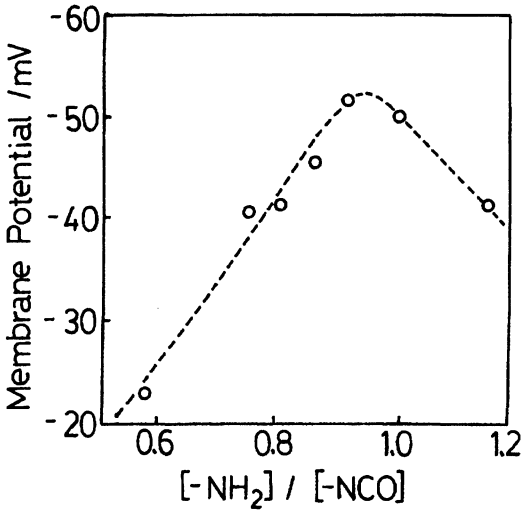


Fig. 1. Relationship between membrane potential and molar ratio of [–NH_2] of DETA and [–NCO] of urethane-prepolymer at preparation of polyurethan membranes.

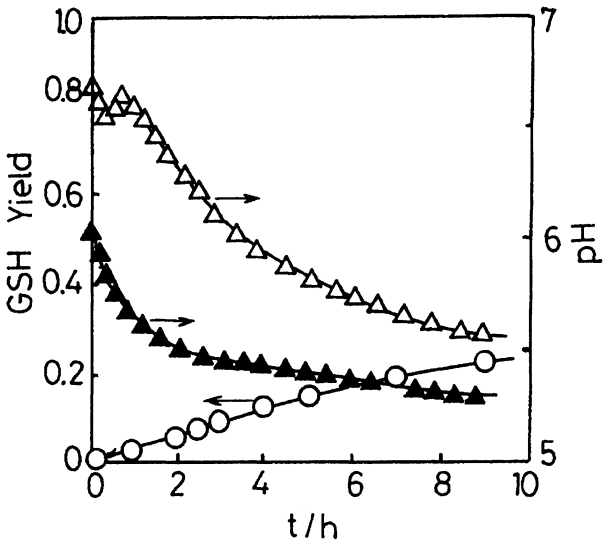


Fig. 2. GSH production and pH changes in the reaction bathes through the conjugated oxidoreductase reaction across the membrane Ure-1 in unbufferized system. ○: Production curve of GSH, ▲: pH in compartment A, △: pH in compartment B.

the conjugated reaction system of G6PD/G6P/NAD^+ and GR/GSSG/NADH without a buffer. The yield of GSH was lower than those in buffered systems (described later) due to a deviation from the optimum pH value for enzymic reactions. It is clear that a decrease of the pH values in both compartments was followed by GSH production. What is important is that the pH in compartment A decreased exponentially, very fast, but that in compartment B decreased after a certain time lag. This means, the reaction of GR/GSSG/NADH could occur, after the protons are transported from the compartment A to B, which was accompanied by the electrons produced by the reaction G6PD/G6P/NAD^+ in compartment A. The decrement in the pH of compartment A with the reaction time could be attributed to the formation of gluconic acid.

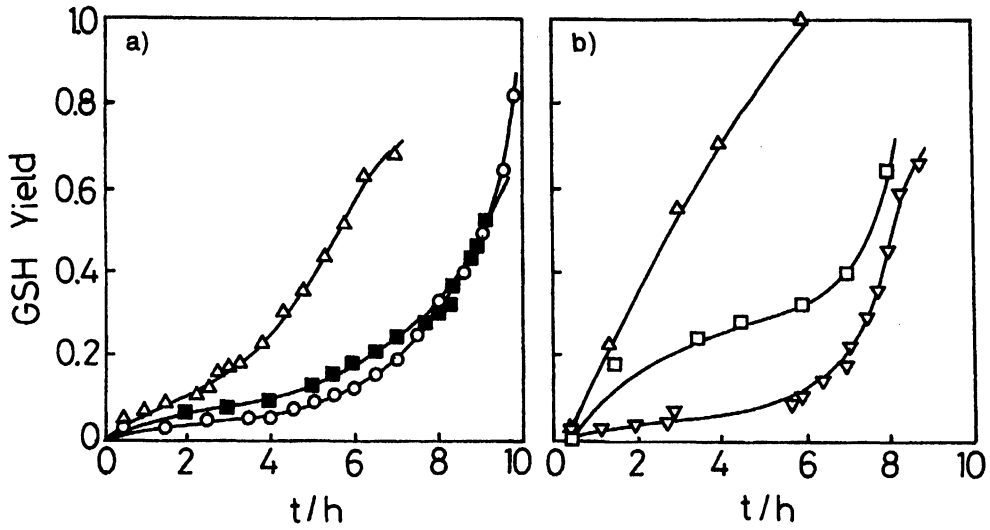


Fig. 3. Conjugated transmembrane oxidoreductase reactions using a) PVA membrane (■: PVA-1, △: PVA-2, ○: PVA-3). b) Polyurethane membrane (▽: Ure-1, □: Ure-2, △: Ure-7).

Table 2. Physical Properties and Kinetics Data on Conjugated Redox Reaction through Membrane

Exp. no.	Membrane code[a)]	$J_{NADPH} \times 10^{12}$ / mol cm^{-2} s^{-1}	M_{NADPH} / mol dm^{-3}	I.P. / h	$J_{NADPH \leftarrow GSH} \times 10^{10}$ / mol cm^{-2} s^{-1}	ETE
1	PVA-1	3.61	—	5.0	1.75	48.4
2	PVA-2	6.09	0.137	4.0	2.49	40.9
3	PVA-3	1.43	0.045	7.5	6.55	458.0
4	Ure-1	1.33	0.075	6.5	6.49	486.0
5	Ure-2	2.24	0.022	0.5	3.06	136.4
6	Ure-3	24.3	0.064	0.5	2.45	10.1

a) Preparation methods are shown in Table 1.

Induction Period (IP) and Membrane Properties Influencing the Rate of GSH Production. Some results concerning the conjugated redox reaction through the membrane are shown in Fig. 3(a) for PVA membranes and (b) for Ure membranes. In these figures the concentration of the GSH produced in compartment B is plotted against time. The slow increment in the GSH yield at the beginning of the reaction means that electron transfer through the membrane could hardly proceed. After a certain time the reaction was steeply accelerated in every case. From this result it can be considered that an appropriate arrangement of adsorbed coenzyme molecules in $NADP^+$ and NADPH forms could be achieved in the membrane, so that the electrons could transfer smoothly between coenzyme molecules arranged alternatively in oxidized and reduced forms inside the membrane.

The maximum production rate of GSH and the permeation time-lag (induction period, IP) were obtained from the slope and the intercept on the time-axis of the asymptote of the GSH-production curve, respectively, as shown in a previous paper.[8)] The effect of the electron-transfer efficiency (ETE value[8)]) was estimated according to the following relation:

$$\mathrm{ETE} = J_{NAD(P)H \leftarrow GSH} / J_{NAD(P)H}, \quad (1)$$

where $J_{NAD(P)H \leftarrow GSH}$ is an imaginary diffusion flux of NAD(P)H reduced from the GSH production rate under the assumption that the production of GSH proceeds stoichiometrically according to the amount of NAD(P)H diffused out through the membrane ($GSSG + NAD(P)H + H^+ \rightarrow 2GSH + NAD(P)^+$).

The physical properties and kinetic data concerning the driving of the conjugated redox reaction across the membrane are summarized in Table 2. Here, experiment No. 1 was carried out without any additional charge of NADPH.

In Fig. 4 the IP values are plotted against the membrane potential. It is clear from this figure that the IP value decreases with increasing membrane potential to a more negative value for both the PVA- and Ure-membranes. It was, furthermore, found that the IP value is rarely affected by the membrane porosity. Since a negative membrane potential appeared on a positively charged membrane, this relation means that the adsorption of the apoenzyme on the membrane surfaces was important for starting the conjugated reaction. From this it can be explained that which type of coenzymes,

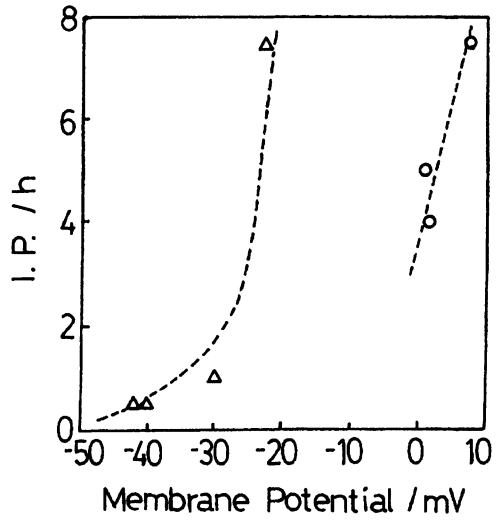


Fig. 4. Relationship between I.P. and membrane potential. △: Polyurethane membrane, ○: PVA membrane.

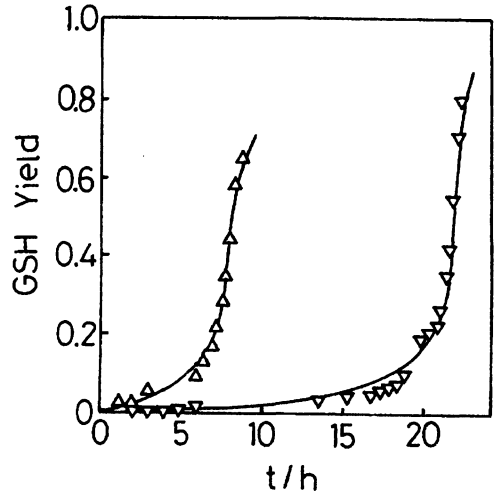


Fig. 5. Conjugated transmembrane oxidoreductase reactions using polyurethane membrane (Ure-1). △: Charging G6PD and G6P at 0 h. ▽: Charging G6PD and G6P after 13.8 h.

either $NADP^+$ or NADPH molecules, pre-adsorbed on the membrane does not affect the IP value and the GSH yield.

From Table 2 it is also clear that the ETE value decreases with increasing J_{NADPH} value, which is, of course, correlated with the membrane porosity. In the case of a membrane having $J_{NADPH} = 1 \times 10^{-12}$ mol cm^{-2} s^{-1} the ETE value reaches to a extremely high value (ca. 500). This means that for an adequate charge density the denser membranes are more effective for a conjugated redox reaction system, due to a smooth electron transfer between densely adsorbed coenzyme molecules.

Confirmation of the Electron-Transfer Function of Coenzymes. For a confirmation of the function of coenzymes as an electron carrier the following

Table 3. Comparison of Enzymatic Redox Reaction Rates

	Initial rate in free system		$(dGSH/dt)_{max}$
	G6P-DH	GRD	in conjugated system
	M min^{-1}	M min^{-1}	M min^{-1}
NADP(H)	6.17×10^{-4}	3.10×10^{-4}	4.70×10^{-6}
NAD(H)	5.90×10^{-4}	3.34×10^{-5}	1.07×10^{-6}

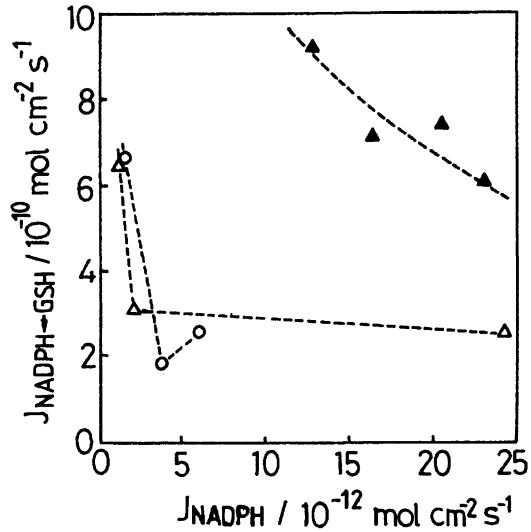


Fig. 6. Relationship between $J_{NADPH \leftarrow GSH}$ and J_{NADPH}. ○: PVA membrane, △: polyurethane membrane, ▲: polyurethane membrane containing δ-component.

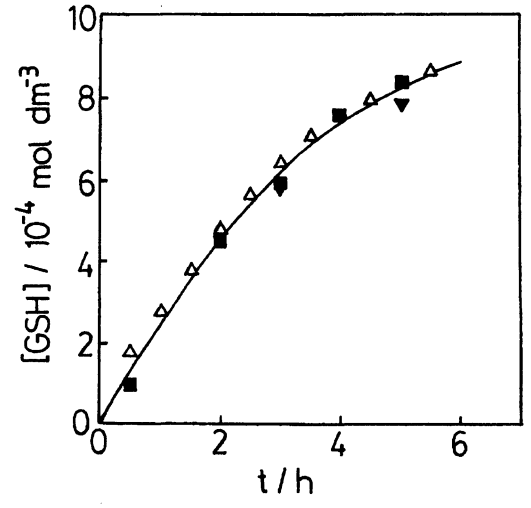
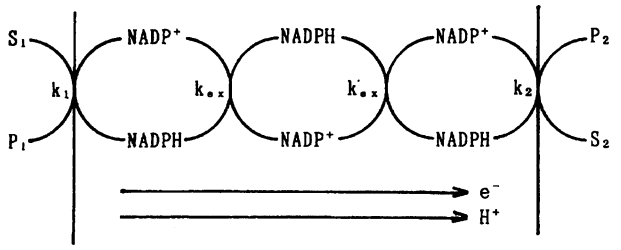


Fig. 7. Conjugated oxidoreductase reactions driven across the PVA–PEI membranes cross-linked by glutaraldehyde for 1 (▼), 3 (■), and 5 (△) h. Membrane thickness of ▼, ■, and △ was 149, 115, and 117 μm.

experiments were carried out: NADPH (2.5×10^{-4} M) only was placed in compartment A, and was allowed to diffuse into compartment B to produce the reaction for GSH production in it; after a certain time G6PD and G6P were put in compartment A. The results are in-



Scheme 1. Three kinds of electron transfer steps in the transmembrane redox reaction.

Table 4. Apparent Diffusion Coefficient of H^+ Followed by Electron Transfer in Comparison with That Followed by Chloride Ion

Diffusant	Membrane	$D_{app}/10^{-6}$ cm^2 s^{-1}
H^+, e^-	Ure-1	30.9
H^+, Cl^-	Ure-1	3.5[a)]
H^+, Cl^-	Cellophane	3.7[a)]

a) Concentration of HCl of up-stream was 1×10^{-3} M.

dicated in Fig. 5 by a symbol (▽). For a comparison, the normal conjugated redox reaction was also driven; the results are included by using the symbol (△) in the figure. From these results it can be emphasized that the GSH production became remarkable when the reaction for NADPH recycle was driven. Consequently, an important conclusion was introduced that substantial GSH production does not proceed due to the NADPH molecules diffused out from the membrane, but due to the electrons and protons transported toward the membrane surface facing the GSH production phase. The apparent diffusion coefficient of the protons accompanied by the electron transfer, which can be calculated from the reaction rate, is much larger than the apparent diffusion coefficient of protons accompanied by chloride ions, obtained by the membrane permeation method[12] (Table 3). This fact confirms that electron-transfer does not occur as the result of proton permeation based on the concentration gradient.

Effect of δ-Component on Electron Transfer. The relationships between the $J_{NADPH\leftarrow GSH}$ and J_{NADPH} values are shown in Fig. 6. In this figure the data for the membrane-immobilizing δ components are also included. It is clear that the $J_{NADPH\leftarrow GSH}$ values decreases with increasing J_{NADPH} value. Of interest is that the membranes immobilizing δ component are more efficient than the other membranes. This means that the δ component can act as a proton carrier, which is necessary for electron-transfer via the NADP(H) arrangement.

Effect of PEI-Mixed PVA Membrane. Figure 7 shows the GSH production rate in the conjugated redox reaction driven through the PVA-PEI membranes cross-linked by glutaraldehyde for several hours (1, 3, and 5 h). Because PEI has a high affinity to NADP(H), NADP(H) should be sufficiently adsorbed into these membranes. This is considered to be a reason why the IP value in these system is much shorter. Although the thicknesses of the membranes used were different, the rate of GSH production does not differ among the membranes. This means that the reaction rate does not depend on the membrane thickness, or on the condition of the cross-linking over their investigated regions.

Enzymic Redox Reaction Rates in Various Systems. The elementary steps of the conjugated redox reaction across the membrane are shown schematically in Scheme 1, where k_1, k_2, and k_{ex} are the rate constants of electron transfer on membrane surface 1 and 2, and in the membrane, respectively. Some data necessary to discuss the rate-determining step are summarized in Table 4. Here, the individual redox reactions correlating the conjugated redox reaction were measured using NADP(H) or NAD(H) in a free system (in mixed aqueous solution). The initial rates of such reactions are given in the second and third columns. In the fourth column the maximum rate $(dGSH/dt)_{max}$ is two orders of magnitude smaller than the rates of individual reactions. From this results it can be concluded that electron transfer in the membrane is mainly the rate-determining step in the transmembrane redox systems. Further details concerning the rate-determining step can be described as follows: It can be the step of electron exchange between the oxidized and reduced forms of the coenzyme in the membrane for the cases that the coenzyme concentration in the membrane is lower and the enzyme reactions occur at the membrane surfaces, while it can be the step of the electron exchange at membrane surfaces 1 and 2 for the cases that the coenzyme concentration in the membrane is higher and the enzyme reactions occur in the outer solutions.

Conclusion

A conjugated transmembrane reaction employing $NADP^+$/NADPH as a common coenzyme was investigated using PVA-based and polyurethan-based membranes. The effects of several chemical and physical properties of the membranes, such as the chemical composition, membrane potential and average pore size of the membrane in the swollen state, and immobilizing of the proton carrier, the intercellular component of wool fiber on the reaction rate and the induction period for the reaction, were checked. Consequently, important information, that the $NAD(P)^+$/NAD(P)H molecules adsorbed in the membrane act as a good electron carrier was obtained. Further, good relationships were found between the reaction rate in the transmembrane redox reactions and various physical properties of the systems, such as the membrane potential, structure density, induction period of the reaction. From these results it was proved that the hydrogel membranes used for the conjugated transmembrane oxidoreductase reactions should be as dense as possible and positively charged.

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