An Affinity Chromatographic Reactor for Highly Efficient Turnover of Dissociable Cofactors[†]

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A conjugated enzyme system of alcohol dehydrogenase and lactate dehydrogenase was immobilized in an ultrafiltration hollow fiber tube, which was inserted in a fine nylon tube to form a hollow-fiber-capillary reactor. To this reactor, the substrates, pyruvate and ethanol, were supplied continuously. The necessary cofactor, NAD, was supplied as a pulse for a short time. The retention time of NAD in the reactor, estimated from the response curve of lactate produced, was much longer than those of the other substrates and products because of the strong adsorption of NAD to the immobilized enzymes through affinity. Therefore, the reactor could produce lactate from pyruvate for a long time without any more NAD. As a typical case, when the enzyme concentration is sufficiently high, the estimated retention time of NAD was 50 times as long as those of other materials so that the NAD turnover obtained was 412,000. The effects of NAD pulse concentration and the immobilized enzyme concentration on the retention time of NAD and NAD turnover were investigated experimentally and theoretically.

To construct a bioreactor for the enzymatic synthesis of useful materials, the efficient regeneration of expensive dissociable cofactors such as NAD, NADP and ATP is necessary. Extensive work has been done on the regeneration of NAD because this cofactor is ubiquitous and involved in many reactions. To regenerate NAD, three methods were attempted; chemical,¹⁾ electrochemical^{2,3)} and enzymatic.⁴⁾ Among these three, the enzymatic method seems most promising because of its high reaction rate and excellent specificity. In this method, NAD is recycled by a conjugated enzyme system.

For a continuous reactor with continuous cofactor recycling, Chambers *et al.*⁵⁾ immobilized both NAD and the enzymes in a hollow fiber membrane, the cut-off molecular weight of which was smaller than that of NAD. However, since the molecular weight of NAD is 663, compatibility of good permeability of the membrane for substrates and products

with a sharp rejection of NAD seems difficult.

Gestrelus *et al.*⁶⁾ immobilized NAD on alcohol dehydrogenase itself to make the cofactor a prosthetic group of the enzyme. The immobilized NAD, however, worked only with the conjugated oxidoreduction of two substrates, both of which were catalyzed only by the alcohol dehydrogenase and did not work in a conjugated enzyme system combined with lactate dehydrogenase because the steric hindrance obstructed the interaction between the NAD immobilized on alcohol dehydrogenase and the free lactate dehydrogenase.

Extensive work has also been done on chemically modified NAD bound to a water soluble macromolecule such as dextran.⁷⁾ Various methods were used for the chemical modification of NAD^{8~13)} and the macromolecule-bound-NAD was coimmobilized with the conjugated enzymes in a membrane reactor,^{14,15)} in a microcapsule¹⁶⁾ or in a gel.¹⁷⁾ Wichmann *et al.*¹⁸⁾ applied this meth-

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od for the continuous production of optically active 2-hydroxycarbonic acid, obtaining a good cofactor turnover of 69,400. Although this cofactor turnover is fairly high, the co-enzymatic activity, the stability and the cost of the macromolecule-bound-NAD still seem to be problems in this method.

Recently, we found that dissociable cofactors without any chemical modification could be continuously recycled very efficiently in a hollow fiber reactor in which the conjugated enzymes were immobilized at a high concentration. The cofactor was not bound to any macromolecule but fed continuously at a concentration lower than those of the enzymes. Through the affinity between the enzymes and the cofactor, the cofactor was concentrated locally around the immobilized enzymes. A very high turnover of the cofactor was expected theoretically,¹⁹⁾ and proved experimentally for a conjugated system of alcohol dehydrogenase and lactate dehydrogenase which needed NAD as a dissociable cofactor.²⁰⁾ Similarly, NADP was produced from NAD with ATP recycling.²¹⁾ In these cases, the reactor was a complete mixing type and the enzymes were immobilized physically in hollow fibers.

To use the affinity between the enzymes and the cofactor more efficiently, an affinity chromatographic reactor can be considered where the highly concentrated conjugated enzyme system is immobilized in a column type reactor. To this reactor the substrates are fed continuously and the cofactor to be recycled is supplied as a pulse. Because of the affinity with the enzymes, the retention time of the cofactor must be much longer than those of substrates and products, so that very high turnover of the cofactor can be expected. In addition, the cofactor is easily recovered because it exists as a pulse in the column. Thus, one may call this an affinity chromatographic reactor. In this paper, we applied this principle for the recycling of NAD by an immobilized conjugated enzyme system of alcohol dehydrogenase and lactate dehydrogenase.

MATERIALS AND METHODS

Materials. Bovine heart L-lactate dehydrogenase (EC 1.1.1.27, abbreviated as LDH hereafter) was obtained from Sigma Chemical Co. Yeast alcohol dehydrogenase (EC 1.1.1.1, abbreviated as ADH) and β -NAD were obtained from Boehringer Mannheim Yamanouchi Co. All the other chemicals were of reagent grade.

Reactor. ADH and LDH were physically entrapped and immobilized in a hollow fiber tube (Asahi Kasei HC, nominal cut-off molecular weight = 13,000, 0.8 mm in inner diameter, 1.4 mm in outer diameter, 1440 mm in length). The hollow fiber tube with the immobilized enzymes was inserted in a silicon tube (same length as the hollow fiber tube, 2.0 or 2.5 mm in inner diameter) to form a hollow-fiber-capillary reactor, which can be considered as a column type reactor (Fig. 1). The immobilization of the enzymes and the change of enzyme loading were very easy with this reactor. The permeability of the hollow fiber membrane was high enough to allow the diffusion of NAD, substrates and products rapidly as compared with the retention time of these materials in the reactor²⁰⁾ (3.4~4.2 hr for the substrates and products). The reactor was immersed in a water bath controlled at 25°C. Substrates (0.05 M pyruvate and 0.835 M ethanol in 0.05 M Tris-buffer of pH 8) were fed continuously with a peristaltic pump and NAD was supplied as a pulse for 20 min. In the reactor, ADH catalyzed the reaction of ethanol with NAD^+ to produce acetaldehyde and NADH which reacted with pyruvate to produce lac-

FIG. 1. Hollow-fiber-capillary Reactor.

FIG. 2. Pulse Response of Affinity Chromatographic Reactor.

$\bar{e}_{ADH} = 2.99 \times 10^{-5}$ mol/liter; $\bar{e}_{ADH}/\bar{e}_{LDH} = 1.63$; $\tau = 3.63$ hr; $C_{NAD}^{pulse} = 5 \times 10^{-5}$ mol/liter; $\Delta t_{NAD}^{pulse} = 20$ min.

tate with the aid of LDH. The effluent from the reactor was collected with a fraction collector and the lactate produced was assayed enzymatically as described previously.²⁰ The enzyme concentrations were calculated based on a molecular weight of 141,000 with two active sites for ADH²² and a molecular weight of 150,000 with four active sites for LDH.²³

RESULTS

Figure 2 shows a response of the hollow-fiber-capillary reactor to the pulse injection of NAD at time zero. In the reactor, the mean enzyme concentration of ADH was 2.99×10^{-5} mol of active site per liter and the concentration ratio of the two enzymes, $\bar{e}_{ADH}/\bar{e}_{LDH}$, was 1.63. The production of lactate was observed shortly after the injection of an NAD pulse at 5×10^{-5} mol/liter. The peak in the lactate concentration produced, C_{Lac}^{peak} , was observed 4.1 hr after the pulse injection of NAD and was 0.0243 mol/liter. The half-value period, $\tau_{1/2}$, defined as the time between the NAD pulse injection and the time when the response curve of lactate produced attained one half of the peak value, was 57 hr. $\tau_{1/2}$ was considered to be the mean residence time of NAD in the reactor because at this time approximately half of the given NAD remained in the reactor. A direct measurement of the retention time of NAD was unsuccessful because of its low concentration. The retention

time for the substrates and products, τ , measured by a step response of pyruvate, was 3.4 hr. The comparison of $\tau_{1/2}$ with τ made it clear that NAD stayed in the reactor much longer than the other materials because of its affinity with the immobilized enzymes. NAD turnover in the reactor, R_N , was 79,000. R_N was calculated as following

$$R_N = C_{Lac}^{peak} \Delta t_{1/2} / C_{NAD}^{pulse} \Delta t_{NAD}^{pulse} \quad (1)$$

where $\Delta t_{1/2}$ is the half-width of the response curve of lactate produced and Δt_{NAD}^{pulse} is the pulse duration of NAD, which was 20 min in this case. In Fig. 2, the NAD pulse was injected again 56 hr after the first injection. The peak height in the second response curve was almost the same as that of the first response curve, showing the apparent good stability of the immobilized enzyme system.

In Fig. 3, $\bar{e}_{ADH}$ was increased to 1.48×10^{-4} mol/liter with $\bar{e}_{ADH}/\bar{e}_{LDH}$ of 3.16. Then, $\tau_{1/2}$ was as long as 213 hr, 50 times that of τ (=4.2 hr), and R_N obtained was as high as 412,000.

Figure 4 shows the effect of C_{NAD}^{pulse} on the response curve with a fixed enzyme concentration. As C_{NAD}^{pulse} increased C_{Lac}^{peak} increased out of proportional to C_{NAD}^{pulse} . The dimensionless half-value period, $\tau_{1/2}/\tau$, decreased with an increase in C_{NAD}^{pulse} . As a result, R_N increased with a decrease in C_{NAD}^{pulse} .

FIG. 3. Pulse Response of Affinity Chromatographic Reactor When Enzyme Concentration Is High.

$\bar{e}_{\text{ADH}} = 1.48 \times 10^{-4}$ mol/liter; $\bar{e}_{\text{ADH}}/\bar{e}_{\text{LDH}} = 3.16$; $\tau = 4.24$ hr; $C_{\text{NAD}}^{\text{pulse}} = 5 \times 10^{-5}$ mol/liter; $\Delta t_{\text{NAD}}^{\text{pulse}} = 20$ min.

FIG. 4. Effect of NAD Pulse Intensity on Response Curve of Lactate Produced.

$\bar{e}_{\text{ADH}} = 6.66 \times 10^{-6}$ mol/liter; $\bar{e}_{\text{ADH}}/\bar{e}_{\text{LDH}} = 1.63$; $\tau = 4.24$ hr; $\Delta t_{\text{NAD}}^{\text{pulse}} = 20$ min. ---△---, $C_{\text{NAD}}^{\text{pulse}} = 5 \times 10^{-6}$ mol/liter; —○—, $C_{\text{NAD}}^{\text{pulse}} = 5 \times 10^{-5}$ mol/liter; —●—, $C_{\text{NAD}}^{\text{pulse}} = 5 \times 10^{-4}$ mol/liter.

Figure 5 shows the effects of $C_{\text{NAD}}^{\text{pulse}}$ on $\tau_{1/2}/\tau$ and R_N . The dimensionless half-value period of the response curve of the lactate produced

FIG. 5. Effects of NAD Pulse Intensity on Dimensionless Half-value Period and Turnover of NAD.

$\bar{e}_{\text{ADH}} = 6.66 \times 10^{-6}$ mol/liter; $\bar{e}_{\text{ADH}}/\bar{e}_{\text{LDH}} = 1.63$; $\Delta t_{\text{NAD}}^{\text{pulse}} = 20$ min.

was significantly bigger than 1, suggesting that the retention time of NAD supplied as a pulse was much longer than those of the other materials because of its affinity with the enzymes immobilized. The dimensionless half-

FIG. 6. Effect of Immobilized Enzyme Concentration on Response Curve of Lactate Produced.

$\bar{e}_{\text{ADH}}/\bar{e}_{\text{LDH}} = 1.63$; $C_{\text{NAD}}^{\text{pulse}} = 5 \times 10^{-5}$ mol/liter, $\Delta t_{\text{NAD}}^{\text{pulse}} = 20$ min. ---□---, $\bar{e}_{\text{ADH}} = 8.52 \times 10^{-7}$ mol/liter; —○—, $\bar{e}_{\text{ADH}} = 6.66 \times 10^{-6}$ mol/liter; —●—, $\bar{e}_{\text{ADH}} = 2.99 \times 10^{-5}$ mol/liter.

FIG. 7. Effect of Immobilized Enzyme Concentration on Dimensionless Half-value Period.

$\bar{e}_{\text{ADH}}/\bar{e}_{\text{LDH}} = 1.63$; $C_{\text{NAD}}^{\text{pulse}} = 5 \times 10^{-5}$ mol/liter; $\Delta t_{\text{NAD}}^{\text{pulse}} = 20$ min. —○—, $\tau_{1/2}/\tau$; ----, τ_R/τ .

value period and the NAD turnover increased with a decrease in $C_{\text{NAD}}^{\text{pulse}}$. However, the increase in R_N with a decrease in $C_{\text{NAD}}^{\text{pulse}}$ was inevitably accompanied by a decrease in the conversion from pyruvate to lactate. So, practically a lower limit of $C_{\text{NAD}}^{\text{pulse}}$ existed if a certain conversion was needed.

Figure 6 shows the effect of the immobilized enzyme concentration on the response curve of the lactate produced with fixed $\bar{e}_{\text{ADH}}/\bar{e}_{\text{LDH}}$ ($=1.63$) and $C_{\text{NAD}}^{\text{pulse}}$ ($=5 \times 10^{-5}$ mol/liter). With an increase in the enzyme concentration, $\tau_{1/2}/\tau$ and R_N increased greatly. When $\bar{e}_{\text{ADH}}$

was 2.99×10^{-5} mol/liter, $\tau_{1/2}/\tau$ reached 16.9, which suggested that the retention time of NAD in the reactor was 16.9 times as long as those of other materials.

In Fig. 7, $\tau_{1/2}/\tau$ was plotted against $\bar{e}_{\text{ADH}}$ with fixed $\bar{e}_{\text{ADH}}/\bar{e}_{\text{LDH}}$ and $C_{\text{NAD}}^{\text{pulse}}$. $\tau_{1/2}/\tau$ increased acceleratively with an increase in the immobilized enzyme concentration, especially when the enzyme concentration was higher than $C_{\text{NAD}}^{\text{pulse}}$ ($=5 \times 10^{-5}$ mol/liter). As Fig. 8 shows, R_N also increased acceleratively with an increase in the enzyme concentration when the enzyme concentration was higher than $C_{\text{NAD}}^{\text{pulse}}$.

FIG. 8. Effect of Immobilized Enzyme Concentration on NAD Turnover.

$\bar{e}_{ADH}/\bar{e}_{LDH} = 1.63$; $\Delta t_{NAD}^{pulse} = 20$ min. —○—, $C_{NAD}^{pulse} = 5 \times 10^{-5}$ mol/liter; —●—, $C_{NAD}^{pulse} = 5 \times 10^{-4}$ mol/liter.

DISCUSSION

The effects of immobilized enzyme concentration and C_{NAD}^{pulse} on $\tau_{1/2}/\tau$ were investigated theoretically with the following assumptions. 1) The concentrations of substrates such as pyruvate and ethanol were so high that the effects of these concentrations on the enzyme kinetics were neglected. 2) The backward reactions were neglected for both enzymatic reactions of ADH and LDH. 3) As for the mass transfer, rapid radial mixing and no axial dispersion were assumed. 4) Cha-Cha's equation was used to describe the enzyme kinetics because the enzyme concentrations were relatively high and not negligible compared with the concentrations of substrates (NAD^+ and $NADH$).

Then the system equation to describe the part of the reactor where the NAD pulse exists is:

$$v = k_{ADH} \bar{e}_{ADH} C_{NAD} / (K_{ADH}^{NAD} + C_{NAD} + \bar{e}_{ADH})$$

$$= k_{LDH} \bar{e}_{LDH} C_{NADH} / (K_{LDH}^{NADH} + C_{NADH} + \bar{e}_{LDH}) \quad (2)$$

$$C_{NAD} + C_{NADH} = C_{NAD}^{pulse} \quad (3)$$

The rate parameters and the Michaelis constants in Eq. (2), obtained from the literature,^{23,24} are:

$$k_{ADH} = 556 \text{ sec}^{-1} \quad (4)$$

$$k_{LDH} = 217 \text{ sec}^{-1} \quad (5)$$

$$K_{ADH}^{NAD} = 1.19 \times 10^{-4} \text{ mol/liter} \quad (6)$$

$$K_{LDH}^{NADH} = 2.0 \times 10^{-5} \text{ mol/liter} \quad (7)$$

From Eqs. (2) and (3), C_{NAD} and C_{NADH} are calculated. Then the concentrations of the enzyme-cofactor complex are calculated:

$$[AO] = \bar{e}_{ADH} C_{NAD} / (K_{ADH}^{NAD} + C_{NAD} + \bar{e}_{ADH}) \quad (8)$$

$$[LR] = \bar{e}_{LDH} C_{NADH} / (K_{LDH}^{NADH} + C_{NADH} + \bar{e}_{LDH}) \quad (9)$$

where $[AO]$ and $[LR]$ are the concentrations of $ADH-NAD^+$ complex and $LDH-NADH$ complex, respectively. The total amount of cofactor adsorbed on the immobilized enzyme is $[AO] + [LR]$ and the concentration of the free cofactor is $C_{NAD}^{pulse} - [AO] - [LR]$. According to the theory in chromatography, the retention time of NAD, τ_R , will be:

$$\begin{aligned} \tau_R/\tau &= 1 + [\text{Adsorbed cofactor}]/[\text{Free cofactor}] \\ &= 1 + ([AO] + [LR]) / (C_{NAD}^{pulse} - [AO] - [LR]) \end{aligned} \quad (10)$$

In Table I, τ_R/τ calculated and $\tau_{1/2}/\tau$ observed are shown. τ_R/τ is also plotted in Fig. 7. In both cases, the theoretical model seems to explain the dependence of $\tau_{1/2}/\tau$ on C_{NAD}^{pulse} and the enzyme concentration to some extent. However, τ_R/τ differed greatly from $\tau_{1/2}/\tau$ quantitatively. Several reasons should be considered. First, $\tau_{1/2}$ is the half-value period measured from the product response. $\tau_{1/2}$ is expected to have a strong relationship with τ_R but still it is not the retention time as τ_R in its strict meaning. Second, Cha-Cha's model was used in the theoret-

TABLE I. EFFECTS OF IMMOBILIZED ENZYME CONCENTRATION AND NAD PULSE INTENSITY ON NAD RETENTION TIME; COMPARISON BETWEEN OBSERVED AND CALCULATED RESULTS

$\bar{e}_{ADH}$ [mol/liter]	$\bar{e}_{ADH}/\bar{e}_{LDH}$	C_{NAD}^{pulse} [mol/liter]	$(\tau_{1/2}/\tau)_{obsd}$	$(\tau_R/\tau)_{calcd}$
8.52×10^{-7}	1.63	5×10^{-5}	1.51	1.009
6.66×10^{-6}	1.63	5×10^{-5}	1.89	1.068
1.37×10^{-5}	1.63	5×10^{-5}	3.63	1.139
2.99×10^{-5}	1.63	5×10^{-5}	16.9	1.301
1.48×10^{-4}	3.16	5×10^{-5}	50.2	1.882
6.66×10^{-6}	1.63	5×10^{-6}	2.62	1.110
6.66×10^{-6}	1.63	5×10^{-4}	1.97	1.011

tical model. Cha-Cha's model cannot give the accurate concentration of enzyme-cofactor complex in Eqs. (8) and (9) especially when the Michaelis constant is low compared with the concentrations of enzymes and cofactor. Without Cha-Cha's equation, however, the concentration of enzyme-cofactor complex cannot be given explicitly as a function of the concentrations of enzymes and cofactor. Third, in the theoretical model, the Michaelis constants in Eqs. (2), (8), and (9) stand for both the kinetic parameters of the enzyme reactions and the dissociation constants of enzyme-cofactor complexes. As for the kinetic parameters, the values in the literature from Eqs. (4) to (7) were employed. The accuracy of these values were checked experimentally and the results were quite reasonable. However, the Michaelis constants as the kinetic parameters were measured at a very low enzyme concentration where the enzyme concentration was negligible as compared with that of NAD^+ and NADH. The present situation is much different. The enzyme concentration is comparable or even higher than that of NAD^+ or NADH. Therefore, the values of the Michaelis constants may be much different. Further studies are necessary considering these problems to improve the theoretical model.

The concept of the affinity chromatographic reactor is described here. The major difference between this and the conventional chromatographic reactor is that, in the former, the material to be adsorbed in the reactor is neither

substrate nor product but a cofactor which is a part of the catalyst. The cofactor turnover obtained was surprisingly high in this system. Thus the cost of the cofactor will be very low because of its high turnover and no need for chemical modification, but the cost for the enzymes will be high because of its high concentration. Therefore, high stability will be needed for the immobilized enzymes. To reduce the enzyme cost, displacement of a part of enzymes with an appropriate adsorbent may be considered. To raise the cofactor turnover more, a recovery of the cofactor pulse injected must be considered. The corresponding project is under investigation.

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