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ELECTROLYTIC PROCESS FOR REGENERATING NADP FROM NADPH IN A POLYMER MATRIX-BOUND FORM

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Regeneration of nicotinamide adenine dinucleotide phosphate (NADP) from its reduced form (NADPH) was performed in a matrix-bound form by an electrolytic method. NADP was immobilized to alginic acid. No significant loss of coenzymic function was induced by the immobilization of NADP on the matrix. Bound NADP was soluble in water. Glucose-6-phosphate dehydrogenase (G-6-PDH) was taken as a model system of coenzyme requiring enzyme. G-6-PDH immobilized on alumina particles was coupled with the soluble form of bound NADP in a fluidized bed type of reactor. The enzymatically reduced coenzyme was electrolytically oxidized in the coenzyme regenerator of NADP from NADPH, which was found to cause no harmful loss of coenzymic function.

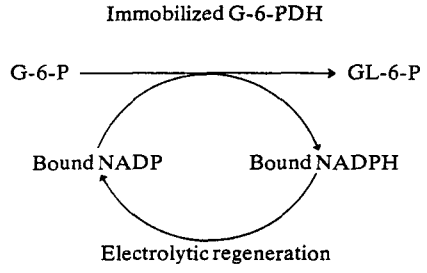
INTRODUCTION

Regeneration of cofactors has received considerable attention in connection with large-scale use of immobilized enzymes that involve such cofactors as nicotinamide adenine dinucleotide (phosphate) [NAD(P)] and adenosine triphosphate (ATP). In the case of enzymatic oxidation coupled with NAD(P), NAD(P) is enzymatically reduced to NAD(P)H. If the enzyme were immobilized on an insoluble matrix, it would be possible to use the immobilized enzyme over and over again in a continuous-flow reactor. For further use of the coenzyme, however, NAD(P)H would have to be oxidized to NAD(P). Unless regenerated to NAD(P), the NAD(P)H would have to be taken out of the reaction system, which would have to be charged with fresh NAD(P).

Several processes for regenerating NAD(P) from NAD(P)H and vice versa have been proposed. Both chemical and enzymatic regeneration methods have been applied (1–4). As additional chemicals and catalysts (or enzymes) are required, however, these methods are troublesome. In con-

trast, electrolytic regeneration could offer significant advantages in regeneration of such redox coenzymes as NAD(P) and NAD(P)H, since it works as a continuous-flow process without consuming any other chemicals.

Although fairly extensive investigations have been conducted on the electrochemical reduction of pyridine nucleotides (5, 6), there have been only a few systematic studies of the electrochemical oxidation of relevant dihydropyridine derivatives (7–9). From the point of view of enzyme engineering, electrolytic regeneration of NAD from NADH and of NADH from NAD has been found to be feasible in both free (10, 11) and immobilized forms (12,13). In the course of subsequent work it will be necessary to demonstrate the general applicability of this method. NADP was taken as an example because of its great similarity to NAD in structure but significant difference in enzymatic specificity. In addition, it is of great interest in enzyme technology to couple both NADP and its apoenzyme in immobilized forms (4). In the present work, oxidation of glucose-6-phosphate (G-6-P) into 6-phospho-gluconate (GL-6-P) by glucose-6-phosphate dehydrogenase (G-6-PDH) coupled with NADP was investigated as a model system.



MATERIALS AND METHODS

Materials. NADP, G-6-P and G-6-PDH were purchased from the Oriental Yeast Co. (Tokyo). Diepoxybutane, γ-aminopropyltriethoxy silane, 1-cyclohexyl-3-(2-morpholinomethyl)carbodiimide metho-*p*-toluene sulfonate (morpho CDI), and alginic acid were obtained from Tokyo Kasei Co. (Tokyo). Alumina particles used as matrix for enzyme immobilization had a surface area of 150–180 m^2/g, a porosity of 0.5–0.6 ml/g, and a particle size of 100 mesh.

Preparation of Alginic Acid-Bound NADP. Five hundred milligrams of NADP was dissolved in 5 ml of water. Into the solution 2.5 g of γ-aminopropyltriethoxy silane was added under vigorous stirring. The pH was adjusted to 4 with about 15 ml of 1 N HCl. The resulting solution was stirred

at 40°C for 1 h. In order to precipitate the silanized NADP, isopropanol was added to the solution. The supernatant was replaced with acetone. The silanized NADP was filtered, washed with acetone, and was then dried.

Alginic acid (1.4 g) was swollen in 50 ml of water and was titrated with 1 N NaOH up to pH 4. This silanized NADP and 2.8 g of morpho CDI were added to the alginic acid solution. Vigorous stirring was continued at room temperature for 24 h. Acetone was added to precipitate the NADP–alginic acid composite. The precipitate was washed with acetone and acetone–0.1 N HCl (1 : 1), and was dried.

Preparation of Immobilized G-6-PDH. Alumina particles were extensively washed with water. One gram of alumina was contacted with 10 ml of 0.26 ml M diepoxybutane solution at pH 12. The mixture was allowed to stand at 50°C for 1 h. After filtration the alumina particles were extensively washed with pH 9 carbonate buffer, and were immersed in 20 ml of 1 mg/ml G-6-PDH in pH 9 carbonate buffer. The reaction was continued at room temperature for 16 h. Enzyme–alumina composite was washed with pH 9 carbonate buffer and was immersed in 10 ml of 1 M glycine in order to block the residual epoxide. The resulting mixture was kept at 4°C for 24 h. The immobilized enzyme was washed with pH 8 tris-buffer until the wash solution exhibited no absorbance at 280 nm.

Activity Assay. The coenzymic function of bound NADP was determined at 30°C using G-6-P and G-6-PDH. As stock solutions 2 mM NADP (or bound NADP), 70 mM G-6-P, and 0.12 mg/ml G-6-PDH in pH 8.0 tris-buffer were made. Into a cuvette were added 0.3 ml of G-6-P, 0.1 ml of G-6-PDH, and a requisite amount of NADP solutions. The final volume was made up to 3.0 ml with pH 8.0 tris-buffer. The concentration of NADP ranged from 0.01 to 0.17 mM. The initial velocity was obtained from the increase of absorbance at 340 nm.

The enzymatic activity of immobilized G-6-PDH was measured according to the same procedures.

Electrolytic Oxidation of NADPH. A glass cell having two compartments separated with fritted glass was used for voltammetric measurement and macroscale electrolysis. In the voltammetric measurement a platinum wire (1 mm × 10 mm) and a platinum plate (1 cm × 4 cm) served as the working and counter electrodes, respectively. The anolyte contained 0.11 mM free NADPH or 0.084 mM bound NADPH and pH 8.0 tris-buffer, and the catholyte pH 8.0 tris-buffer. The electrolytes were preliminarily deoxygenated by N_2 bubbling. The electrode potential of the working electrode was referred to a saturated calomel electrode (S.C.E.) at 25°C and was scanned at a rate of 100 mV/sec with a function generator and potentiostat (Model 101 made by Hokuto Denko Co., Tokyo). An X-Y recorder was used for obtaining current-potential curves.

Macroscale electrolysis was conducted, using the same cell. Both anode and cathode were platinum plates (1 cm × 4 cm). The volume of the anolyte was 20 ml. The supporting electrolyte was pH 8.0 tris-buffer for both anolyte and catholyte. The anolyte contained 0.26 mM bound NADPH that was enzymatically reduced. The anode potential was controlled at 0.9 V vs. S.C.E.

Immobilized-Enzyme Continuous-Flow Reactor Incorporating Continuous Electrolytic NADP Regenerator. The system consisted mainly of an immobilized G-6-PDH reactor and an electrolytic NADP regenerator as shown in Fig. 1. The reactor is a type of fluidized bed. Immobilized G-6-PDH was packed into the reactor through a peristaltic pump at a constant flow rate. From the top of the reactor the feed solution, which contained GL-6-P, NADPH and unreacted G-6-P, and NADP, flowed out. The electrolytic NADP regenerator was operated under the same conditions as those of the macroscale electrolysis mentioned above.

RESULTS

Coenzymatic Function of Bound NADP. The bound NADP was essentially soluble above pH 3 at room temperature. Ultraviolet spectra were determined for a solution of bound NADP with reference to an alginic acid

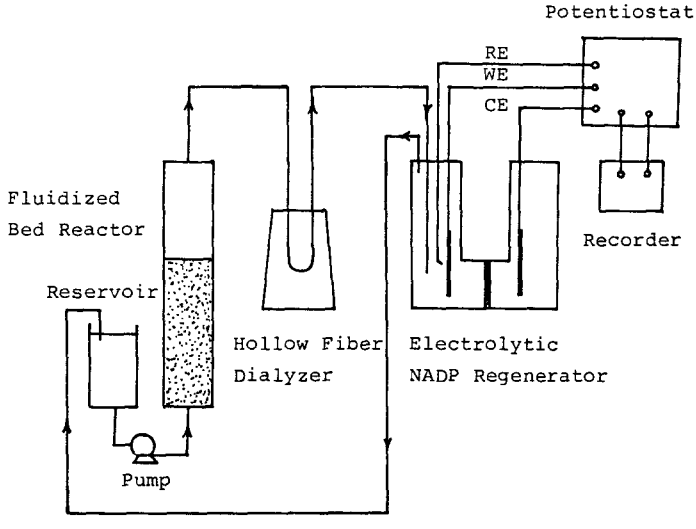


FIG. 1. Flow sheet of a continuous-flow system incorporating immobilized-enzyme reactor and electrolytic NADP regenerator.

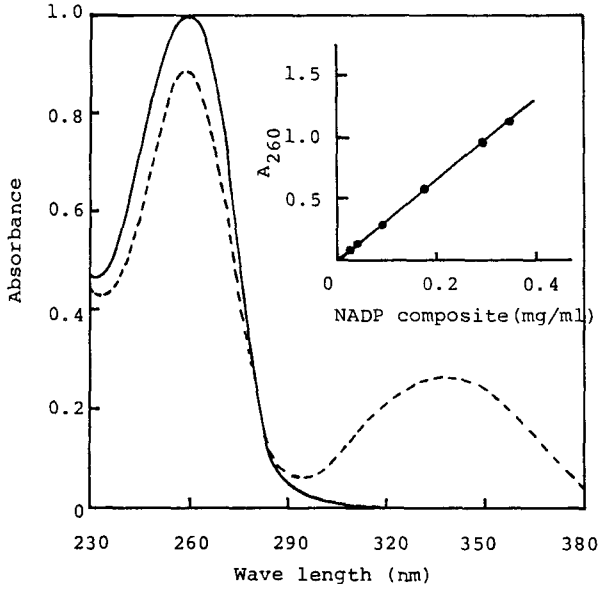
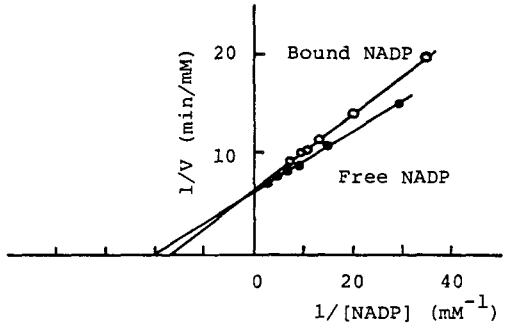


FIG. 2. Ultraviolet spectra of bound NADP in oxidized and reduced forms. Spectrum of the oxidized form (——) was obtained for a solution containing 0.29 mg/ml NADP–alginic acid composite. For determining the spectrum of the reduced form (- - -), the bound NADP was reduced by immobilized G-6-PDH. As a reference, a comparable concentration of alginic acid solution was employed.

solution. Figure 2 demonstrates the spectra for a solution containing 0.29 mg of bound NADP per ml of pH 8.0 tris-buffer, and for that of an enzymatically reduced form of bound NADP. The enzymatically reduced form of bound NADP was prepared by reducing bound NADP with immobilized G-6-PDH, as will be described later. The oxidized form of bound NADP exhibited an absorption maximum at 260 nm. The concentration dependence of absorbance at 260 nm was quite linear, as shown in Fig. 2. For the reduced form of bound NADP, no significant shift at 260 and 340 nm was observed. No marked difference in UV spectra between free and bound NADP was found. On the supposition that bound NADP should exhibit the same molar absorption coefficient, the amount of bound NADP was determined as 190 μmol/g of dry alginic acid.

The coenzymatic function of bound NADP was assayed at pH 8.0 and 30°C by using G-6-PDH and G-6-P. From a Lineweaver–Burk plot of the initial rate against reciprocal concentration of NADP, K_m and V_{max} were



	K_m (M)	V_{max} (M/min)
Free NADP	5.0×10^{-5}	1.6×10^{-4}
Bound NADP	5.9×10^{-5}	1.5×10^{-4}

30.0 ± 0.5 °C, pH8.3

FIG. 3. Kinetic parameters of free and bound NADP coupled with native G-6-PDH.

determined, as shown in Fig. 3. The concentration of bound NADP was estimated from absorbance at 260 nm. The V_{max} of G-6-PDH coupled with bound NADP indicates that bound NADP fully retained the coenzymatic function of free NADP. No appreciable difference was observed between the K_m of free and of bound NADP.

The pH-activity relationship is profiled for G-6-PDH coupled with free and bound NADP in Fig. 4. Bound NADP induced no significant shift in the optimum pH, but caused a slight decrease in activity in the alkaline region.

Electrolytic Oxidation of Bound NADPH. Bound NADP was enzymatically reduced to NADPH using G-6-P and immobilized G-6-PDH. For the enzymatically reduced form of bound NADP a voltammetric characterization was performed. As was mentioned elsewhere, adsorption of NADP and NADPH at the electrode disturbs voltammetry (10). The working electrode was refreshed with 1 N H_2SO_4 before every measurement. The measurement of current-potential curve was carried out as soon as the working electrode was immersed in the sample solution. Both free and bound NADPH gave a peak potential of 0.64 V vs. S.C.E. at pH 8.0. No significant difference was detected between the peak potentials of these two substances. The oxidative current was attributed to the two-electron oxidation of NADPH to NADP.

The conditions of macroscale electrolysis were decided according to the voltammetric behavior of bound NADPH. Since it is necessary to control

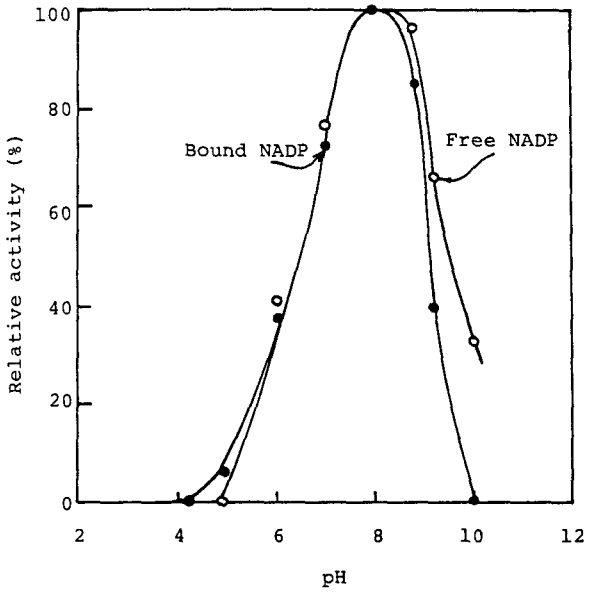


FIG. 4. pH-activity profiles for free and bound NADP couples with native G-6-PDH.

the anode potential at more positive potential than the peak potential for the sufficient oxidation of NADPH, the anode potential was controlled at 0.8 V vs. S.C.E. The electrolysis was conducted in a batch system. The decrease of bound NADPH in the electrolysis was followed by absorbance at 340 nm. Fractional conversion of NADPH into NADP increased during the electrolysis, as shown in Fig. 5. After 2 h of electrolysis, the electrolytically oxidized form of bound NADP was assayed for its coenzymatic function using G-6-PDH and G-6-P according to the same procedure as described in connection with Fig. 3. The value of K_m indicated that no noticeable decrease in coenzymatic function accompanied the electrolysis.

Continuous Operation of the Immobilized G-6-PDH Reactor Incorporating the NADP Regenerator. Figure 1 depicts a system composed of an enzyme reactor and a coenzyme regenerator. The reactor works for the oxidation of G-6-P to GL-6-P and the reduction of NADP to NADPH with the catalytic aid of immobilized G-6-PDH. The hollow fiber dialyzer (nominal surface area, 900 cm^2; nominal molecular weight cutoff, 5000) separates low molecular weight substances such as GL-6-P from the feed solution.

For the purpose of characterizing the reactor, K_m and V_{max} were determined for immobilized G-6-PDH in a batch system. The immobilized

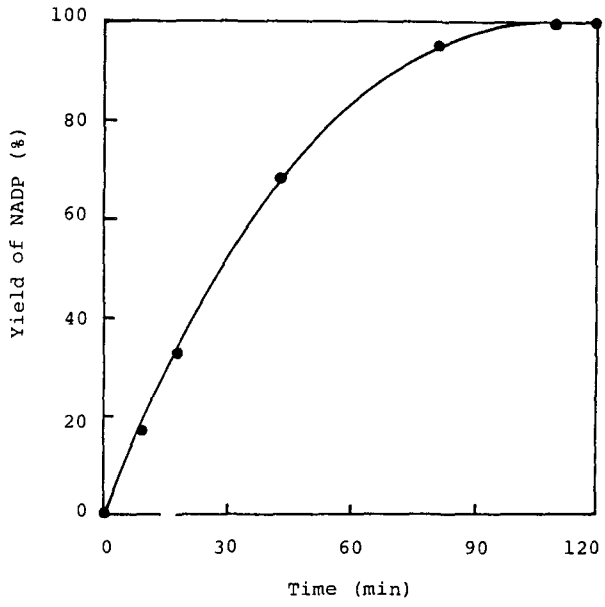


FIG. 5. Macroscale controlled-potential electrolysis for regenerating NADP from NADPH. Anolyte: 0.14 mg/ml NADPH-alginic acid composite (comparable to 2.6×10^{-4} M free NADPH); Catholyte: pH 8.0 tris-buffer. Anode potential was controlled at 0.8 V vs. S.C.E.

G-6-PDH was coupled with free and bound NADP. The initial rate was measured for various concentrations of NADP at a fixed amount of immobilized G-6-PDH. The concentrations of G-6-P was a hundred times greater than that of NADP. The pH was adjusted at 8.0 by tris-buffer. Both K_m and V_{max} for G-6-PDH and bound NADP were comparable to those for free NADP, which indicates that no marked decrease in enzyme–NADP affinity and enzyme activity was induced by attaching NADP to alginic acid.

Further characterization of the reactor was performed in a continuous-flow system. The reactor was packed with 840 mg of alumina G-6-PDH

TABLE 1. **K_m and V_{max} of Free and Bound NADP Coupled with Immobilized G-6-PDH and G-6-P**

	K_m (M)	V_{max} (M/min)
Free NADP	4.1×10^{-4}	8.5×10^{-6}
Bound NADP	2.0×10^{-4}	5.6×10^{-6}

composite. The feed solution contained 10 mM G-6-P and 0.26 mg/ml bound NADP (comparable to 0.48 mM NADP) in pH 8.0 tris-buffer. The immobilized G-6-PDH bed was fluidized at different flow rates. The conversion ratio of NADP to NADPH was followed by absorbance at 260 and 340 nm at the inlet and the outlet of the reactor. The fractional conversion ratio determined in a steady state changed, depending on the flow rate as shown in Fig. 6.

The reactor was then connected with the NADP regenerator through a hollow fiber dialyzer as assembled in Fig. 1. The reactor, the dialyzer, the NADP regenerator, and all tubing were filled with pH 8.0 tris-buffer which amounted to 42 ml. Then, 48 ml of the feed solution was added to the reservoir. The feed solution was circulated at a constant flow rate. The bed in the reactor was homogeneously fluidized. NADP was continuously reduced at a conversion ratio, α, in the reactor. Simultaneously the anode potential was maintained at 0.8 V vs. S.C.E. for oxidizing NADPH into NADP in the regenerator. In a steady state a constant conversion ratio from NADPH to NADP, β, could be maintained in this regenerator. The conversion ratios were determined by following absorbance at 260 and 340 nm of the feed solution at the inlet and the outlet of the anodic compartment of the

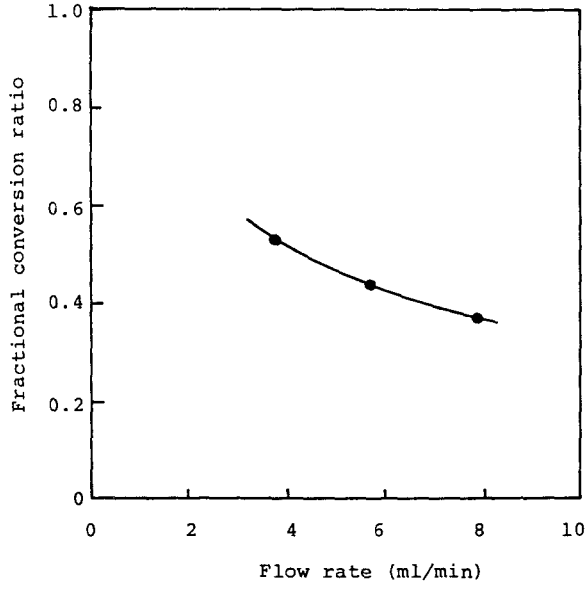


FIG. 6. Conversion of NADP to NADPH at various flow rates in the immobilized G-6-PDH reactor.

regenerator. Ratios α and β can be expressed by the following equations:

$$\alpha = (C_R - C_R^0)/C_O^0 \tag{1}$$

$$\beta = (C_O^0 - C_O)/C_R^0 \tag{2}$$

$$= (C_R - C_R^0)/C_R \tag{3}$$

$$C_O^0 + C_R^0 = C_O + C_R = C \tag{4}$$

where C_O^0 and C_R^0 are the concentrations of oxidized and reduced forms of bound NADP at the outlet of the regenerator, C_O and C_R are those at the inlet of the regenerator, and C is the total concentration of the oxidized and reduced form of bound NADP as presented in Fig. 7.

One of the typical results obtained for the system is shown in Fig. 7 for characterization of the actual operation. The feed solution contained 0.48 mM bound NADP, 10 mM G-6-P, and pH 8.0 tris-buffer. The experiment was carried out with a considerable excess of G-6-P, because regeneration of the coenzyme was expected to be performed effectively. The flow rate was maintained at 4.3 ml/min. In a steady state α was comparable to β. The operation was continued for several hours with α and β maintained constant.

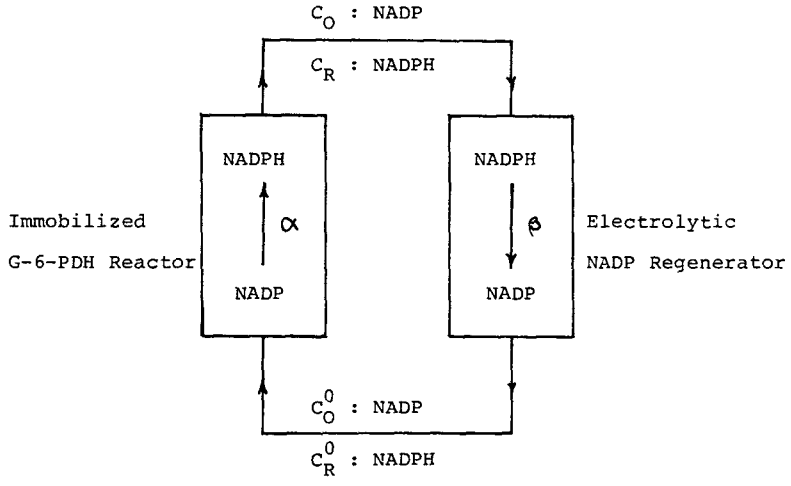


FIG. 7. Operational characteristics of the continuous-flow system incorporating an immobilized-enzyme reactor and an electrolytic NADP regenerator. At a flow rate of 4.3 ml/min, $C_R^0 = 0.21$ mM, $C_R = 0.26$ mM, $\alpha = 0.19$, and $\beta = 0.19$. $C = C_O + C_R = C_O^0 + C_R^0 = 0.48$ mM.

DISCUSSION

NADP was immobilized to alginic acid without noticeable loss of coenzymatic function. Bound NADP exhibited comparable V_{max} to free NADP when it was coupled with native G-6-PDH and G-6-P. The procedure employed for the immobilization apparently caused no harmful distortion of NADP, since bound NADP retained the coenzymatic function of free NADP. The amount of NADP bound to alginic acid was spectrophotometrically determined as 190 μmol/g dry alginic acid, which shows that every 25 units of mannuronic acid (a unit of alginic acid) immobilized one molecule of NADP. From K_m values of native G-6-PDH against free and bound NADP, it is to be noted that unreacted carboxyl groups of alginic acid had no marked effects on the affinity of the enzyme to NADP. Although several methods have been proposed for immobilizing NAD(P) to polymer matrices (1, 2, 4, 12–15), the immobilized NAD(P) has retained only a little coenzymatic activity. The present procedure offers the great advantages of simplicity and effectiveness in protecting coenzymatic function during the immobilization.

In the electrolytic regeneration of bound NADP from NADPH, the initial coenzymatic activity was fully recovered. Thus, the following mechanism for the electrochemical oxidation of NADPH in aqueous media is considered to best fit the results.

$$\text{NADPH} \rightarrow \text{NADP}^+ + \text{H}^+ + 2e \tag{5}$$

In an acidic solution the following decomposition process should occur, as pointed out by Elving and his co-workers (9).

$$\text{NADPH} + \text{H}^+ \rightarrow \text{H(NADPH)}^+ \tag{6}$$

$$\text{H(NADPH)}^+ \rightarrow \text{decomposition products absorbing at 280–290 nm} \tag{7}$$

where H(NADPH)^+ is a protonated form of NADPH. Since no noticeable excess absorbance at 280–290 nm was detected for the electrolytically oxidized form and the electrolysis was conducted at a slightly alkaline condition, little decomposition apparently occurs in the present electrolytic regeneration.

Desirable conversion ratios (α and β) in the system could be obtained by controlling the amount of immobilized G-6-PDH in the reactor and in the electrode area of the regenerator. Furthermore, the present NADP regenerator system could be applied to any NADP-requiring enzyme.

CONCLUSION

G-6-PDH immobilized on alumina particles was coupled with a soluble form of bound NADP in a fluidized bed type of reactor. The enzymatically

reduced coenzyme was electrolytically oxidized in the coenzyme regenerator attached to the reactor. No harmful effects on coenzymatic function accompanied the electrolytic regeneration of NADP from NADPH. The electrolytic regeneration could be feasible in a continuous-flow process without requiring any chemicals for oxidizing NADPH to NADP.

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