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Recycling of $NADP^+$ Using Immobilized *E. coli* and Glucose-6-phosphate Dehydrogenase

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Abstract

Recycling of $NADP^+$ using immobilized whole *Escherichia coli* cells as source of respiratory chain, glucose-6-phosphate, and soluble yeast glucose-6-phosphate dehydrogenase (1.1.1.49) is described. $NADP^+$ was recycled more than 10-fold.

We demonstrated NADPH respiration at pH 5.8 in *E. coli* membrane vesicles. The respiratory chain was involved most probably in NADPH oxidation.

1. The respiratory activity is localized at the level of the inner bacterial membrane. The active site for NADPH facing the cytoplasm.

2. NADPH respiration is inhibited by 10 m*M* cyanide, similar to the conditions of inhibition of NADH respiration.

3. NADPH dehydrogenase activity seems to be the limiting step of the respiratory chain: K_M for NADPH respiration and NADPH dehydrogenase activity are similar. The pH optima for these two activities are also comparable (around pH 5.8). Furthermore, the following properties are rather in favor of a common NADH dehydrogenase and NADPH dehydrogenase activity (1.6.99.2).

(1) At saturating concentrations of NADH and NADPH, neither respiration nor dehydrogenase activities were additive.

(2) Similar heat inactivation kinetics were observed for NADH and NADPH dehydrogenase-activity.

Protection against heat inactivation was obtained for the two activities with NAD^+ $NADP^+$, NADH, and NADPH.

All these results suggested the possibility of recycling of $NADP^+$ under similar conditions to those previously described for NAD^+ (Burstein et al., 1981). It becomes thus possible to use various NAD^+ and $NADP^+$-dependent dehydrogenases in enzyme technology.

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Index Entries: NAD^+ and $NADP^+$ recycling; NAD^+ and $NADP^+$ dependent dehydrogenases; immobilized *E. coli* cells as source of respiratory chain; recycling, of $NADP^+$; *E. coli* immobilized; glucose-6-phosphate dehydrogenase, immobilized.

Introduction

Immobilization of enzymes increases the possibilities for applications in enzyme technology [Mosbach (*1*)].

NAD^+ and $NADP^+$-dependent dehydrogenases represent at least 300 enzymes (*2*). One of the critical problems in the utilization of these enzymes is the necessity of recycling these cofactors. Effectively, NAD^+ and $NADP^+$ are utilized at substrate level concentrations and transformed into NADH and NADPH, respectively, in stoichiometric amounts with the second substrate of the dehydrogenase. These cofactors are extracted from living material, where they are found at millimolar concentrations. Therefore they are too expensive to be used in enzyme technology. Various methods have already been proposed for the recycling of NAD^+ (*1*, *1a*, *3–5*):

1. Electrochemical regeneration (*6*).
2. The use of a second enzyme system, for instance, pyruvate dehydrogenase (*7*, *8*).
3. The non-enzymatic oxidation of NADH with phenazine methosulfate (*9*).

All these methods present advantages and drawbacks. The main problems are low yield of recycling, instability of the system used, cost of extra enzymes and substrates, and difficulties in eliminating the contaminants.

4. The use of immobilized cells, mostly microorganisms, has often been proposed (*10*) because enzymes from microbial sources are most suitable for industrial purposes.

Immobilization of whole bacteria often permits to eliminate the costly procedure of extracting enzymes. We have proposed (*11*) utilization of immobilized *E. coli* cells that present an active respiratory chain capable of recycling NAD^+ from NADH with atmospheric oxygen.

Until 1973, it was generally agreed that the mitochondrial respiratory chain is incapable of oxidizing NADPH directly. Hatefi (*12*) demonstrated that NADPH is oxidized directly by the respiratory chain. This respiration proceeded five times more slowly with NADPH than with NADH at pH values between 5 and 6.

We demonstrated that NADPH was also respired by *E. coli* vesicles at pH 5.8. NADPH dehydrogenase activity was described in mitochondria (*13*), the activity at pH 6.5 was 700 times less than NADH dehydrogenase activity. NADPH dehydrogenase activity found in *E. coli* was comparable to NADH dehydrogenase at their respective pH optima.

Unfortunately the nomenclature of the pyridine nucleotide dehydrogenases is somewhat confusing (*14*). Names can depend on the acceptors: quinones,

cytochrome C, ferricyanide, and so on. These pyridine nucleotide dehydrogenases are completely different from the about 300 NAD^+ or $NADP^+$-dependent dehydrogenases.

Although many results presented in this paper are in favor of single enzyme catalysis of NADH and NADPH dehydrogenation (1.6.99.2), we propose in the following to name the enzymatic activity according to the pyridine nucleotide substrate used: NADH or NADPH dehydrogenase.

Our results encouraged an attempt to recycle $NADP^+$ similarly to the recycling of NAD^+ (*11*). Immobilized *E. coli* with an active respiratory chain was used to recycle $NADP^+$ from NADPH.

Materials and Methods

Bacteria

E. coli K-12 was grown in mineral medium 63 supplemented with 8 g/L of glycerol. The bacteria were collected in exponential growth and washed in 0.05*M* K-phosphate buffer at pH 7.6. The bacteria were stored in the same buffer at 1 g/mL (wet weight) at −80°C.

Crude extract was obtained by rupturing the cells by extrusion at 20,000 psi (Ribi cell fractionator, Sorvall) followed by differential centrifugation. Unbroken bacteria and cell wall fragments were eliminated by 20 min centrifugation at 30,000*g*.

Immobilization (*15*) was achieved in 0.05*M* K-phosphate buffer, pH 7.5, with 4.6% bovine serum albumin, 0.4% glutaraldehyde and 0.3 g/mL of wet weight bacteria. Samples of 0.8 mL were spread on 10 cm^2 plate of glass and air dried at room temperature for approximately 3 h. The polymerized material was detached from the glass and stored at 4°C in distilled water.

NADH or NADPH Oxidation

Respirometry. Crude extract (50–500 μg/mL) or solid fragmented polymer (including immobilized cells) resulting from the above process was incubated at 25°C in 0.1*M* K-phosphate, pH 8.0, for respiration of NADH or pH 5.8 for NADPH. Oxygen consumption was recorded with a Clark electrode (Gilson oxygraph). Cofactor concentrations were 1 m*M*. Results were expressed either as oxygen consumption or NADH or NADPH oxidation (in nmol min^{-1} mg^{-1}).

Recycling System. $NADP^+$ recycling was measured by oxygen consumption (at 25°C) obtained in an incubation mixture containing immobilized *E coli* (10 units of NADPH respiration), 1 m*M* $NADP^+$, 3 m*M* glucose-6-phosphate and 5 units of soluble glucose-6-phosphate dehydrogenase from yeast (lyophilized powder from Boehringer).

Spectrophotometric Assay of NADH and NADPH Dehydrogenase. NADH or NADPH dehydrogenase activities were assayed at 25°C in crude extracts (1–50 μg/mL protein) in the presence of 0.5 m*M* ferricyanide in 0.1*M* K-phosphate, pH

6.8, and at pH 5.5 with 1 m*M* NADH or NADPH. Decrease of absorbance was recorded at 420 nm. Initial velocities were determined for 30 s to 5 min. Results were expressed as NADH or NADPH oxidized in nmol min^{-1} mg^{-1}.

Results and Discussion

Respiratory Chain Activities

Respiration of NADPH. In the presence of a crude extract of *E. coli*, oxygen consumption was observed after addition of NADPH at pH 5.8.

NADPH Respiration was Localized at the Level of the Inner Membrane From the crude extract, membrane vesicles were obtained by sedimentation at high speed (165,000*g* for 3 h). These vesicles are mainly inverted (*16*). They are able to respire NADPH. NADPH dehydrogenase seemed to be located similarly to NADH dehydrogenase on the inside surface of the inner membrane. Intact bacteria are unable to respire either NADH nor NADPH; these cofactors are unable to cross the inner membrane.

NADPH/Oxygen Stoichiometry. NADPH was completely transformed into $NADP^+$, as measured by complete disappearance of absorbance at 340 nm. NADPH was completely restored by addition of an excess of glucose-6-phosphate dehydrogenase and glucose-6-phosphate. During respiration, two molecules of NADPH were consumed per molecule of oxygen.

Inhibition by Cyanide. NADPH respiration, like NADH respiration, was sensitive to 10 m*M* cyanide. Both reactions depend on electron transport through the cytochrome chain.

pH Dependence of NADPH Respiration. The respiration of NADH and NADPH were measured at different pH values (Fig. 1). Respiration of NADPH presented at pH optimum of 5.8. Respiration of NADH reached a plateau at pH 8.0 that remained constant at least until pH 9.4.

The differences in pH responses may be explained by the difference between the molecules NADH and NADPH.

Effectively, NADPH is more negatively charged because of the extra phosphate group. This may counteract the binding of NADPH to NADPH dehydrogenase. At a more acidic pH both negative charge of the enzyme and of the NADPH molecule are decreased, thus binding is facilitated.

Nonadditivity of NADH and NADPH Respiration. There appears to be at least one common limiting pathway for NADH and NADPH respiration. Respiration was successively measured in the presence of saturating concentrations of NADH, of NADPH, and of both substrates. No additive respiration was observed.

In order to circumvent the cytochrome chain as a rate-limiting factor, experiments were performed with NADH and NADPH dehydrogenase activities measured in the presence of ferricyanide.

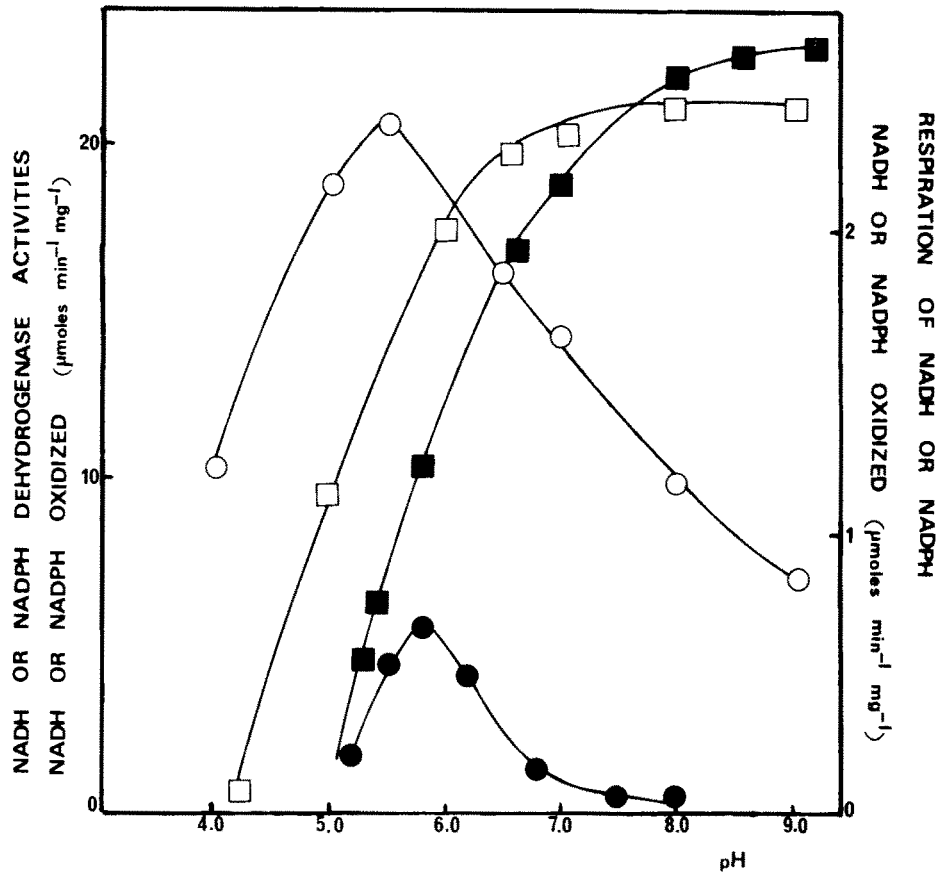

Fig. 1. pH dependence of NADH and NADPH respiration and of NADH and NADPH dehydrogenase activities. Disrupted bacteria (crude extract) were used. The respiration of NADH (■) and of NADPH (●) were measured by following oxygen consumption with a Clark electrode. NADH dehydrogenase activity (□) and NADPH dehydrogenase activity (○) were measured by following the decrease in absorbance at 420 nm in presence of ferricyanide. A 0.1*M* K-phosphate buffer was used between pH 5.8 and 7.8; for pH values below 5.8, a K-acetate buffer was used; for pH values above 7.8, Tris-HCl buffer was used. Initial velocities were measured at 1 m*M* concentration of substrates.

Dehydrogenase Activities

Nonadditivity of NADH and NADPH Dehydrogenase Activities. When NADH and NADPH were added at saturating concentrations, similar dehydrogenase activity was measured. The simultaneous addition of both substrates did not increase the rate of ferrocyanide reduction, as it did not increase the oxygen consumption. Since here the rest of the respiratory chain is not at work, the common rate limiting step seems to be a single dehydrogenase.

Comparison of K_M Values of NADH and NADPH for Respiration and Dehydrogenase Activities. Comparable K_M values were found for NADH respiration and NADH dehydrogenase (K_M = 0.4 m*M*). Similar K_M values were ob-

tained for NADPH respiration and NADPH dehydrogenase (K_M = 1 mM). The dehydrogenase activities seem to be the limiting factor of the respiratory chain.

pH Dependence The pH dependence of NADH and NADPH dehydrogenase activities are given in Fig. 1. The pH curve obtained with NADH dehydrogenase is similar to that obtained with NADH respiration and the NADPH dehydrogenase pH curve is similar to that of NADPH respiration.

Heat Inactivation Kinetics of NADH and NADPH Dehydrogenase Activities. Upon heat inactivation of a crude extract from *E. coli* at 53°C, similar heat inactivation kinetics were observed for NADH and NADPH dehydrogenase in the same sample (Fig. 2). Furthermore, NAD^+, $NADP^+$, NADH, and NADPH were able to protect both activities to the same extent, as shown in Fig. 2 with NAD^+ and $NADP^+$.

All these results are compatible with the existence of a single dehydrogenase for NADH and NADPH with a common binding site for NAD^+, $NADP^+$, NADH, and NADPH.

These results permitted recycling of $NADP^+$ with the immobilized *E. coli* cells.

Immobilization of the Respiratory Chain for $NADP^+$ Recycling

Permeabilization of Bacteria by Immobilization. Intact bacteria were able to utilize exogenous NADPH only after permeabilization by toluene or after disruption of the bacteria resulting in the obtention of inverted vesicles. The observation that bacteria immobilized by glutaraldehyde in the presence of serum albumin were able to oxidize NADPH indicates that NADPH was able to reach the competent active sites accessible only from the inner side of the inner membrane. Similar results were obtained for the access of NADH dehydrogenase sites (*11*).

No obvious alterations in the membrane structure were observed on electron micrographs of immobilized bacteria. The bacteria and the membranes kept their native aspect (Barbotin, 1981 unpublished results).

Yield of NADPH Respiration after Immobilization of E. coli. About 30% of the activity found in crude extract (derived from the same amount of bacteria) was recovered.

This result suggested a stabilization of the respiratory chain because of the localization on a biological membrane.

Methanol Inactivation. Since methanol can be useful to solubilize water insoluble substrates (androsterone, for instance) of certain dehydrogenases (*9*), an experiment was designed to find out whether immobilized bacteria are able to oxidize NADPH in the presence of methanol.

Increased stability to methanol was obtained with the immobilized respiratory chain measured in the presence of NADPH. A half-life of about 20 h was observed during activity in presence of 30% methanol, while the non-immobilized membrane had a half life of less than 2 min in the same conditions.

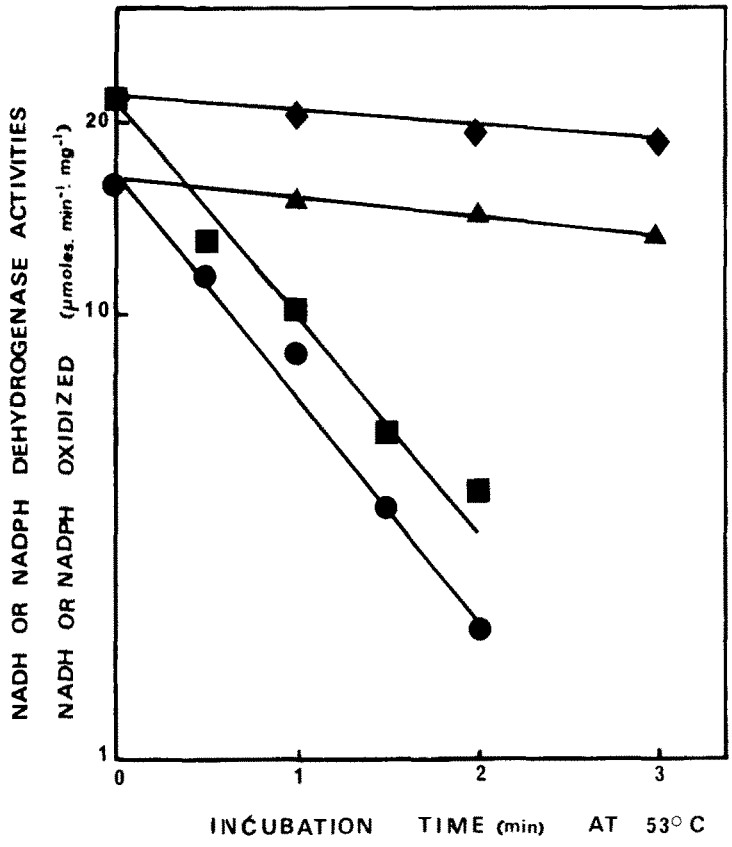

Fig. 2. Heat inactivation of NADH and NADPH dehydrogenase activities. Disrupted bacteria (crude extract) at 1 mg/mL of protein were incubated at 53°C in 0.1*M* K-phosphate buffer at pH 6.0 in the presence of 10 m*M* KCN. Samples were diluted 100-fold in the same buffer at 0°C in order to stop the heat denaturation at the indicated times. Dehydrogenase activities were measured directly in the presence of ferricyanide: NADH dehydrogenase (■) and NADPH dehydrogenase (●). Another portion of crude extract was incubated at 53°C in the presence of 2 m*M* NADH. Remaining NADH dehydrogenase activity was measured after dilution at 0°C (♦) as well as remaining NADPH dehydrogenase activity (▲). Similar results were obtained by protection with 2 m*M* NADPH, NAD^+, and $NADP^+$ (not shown).

Storage of NADPH Respiration after Immobilization. The activity of immobilized respiratory chain with NADPH as substrate had a half-life of approximately 30 days when stored at 4°C in distilled water (Fig. 3).

Recycling of $NADP^+$

Experiment Showing the Recycling of $NADP^+$. The recycling of $NADP^+$ was performed by utilization of the immobilized cells of *E. coli* and an $NADP^+$-dependent dehydrogenase. The $NADP^+$-dependent dehydrogenase used here, as

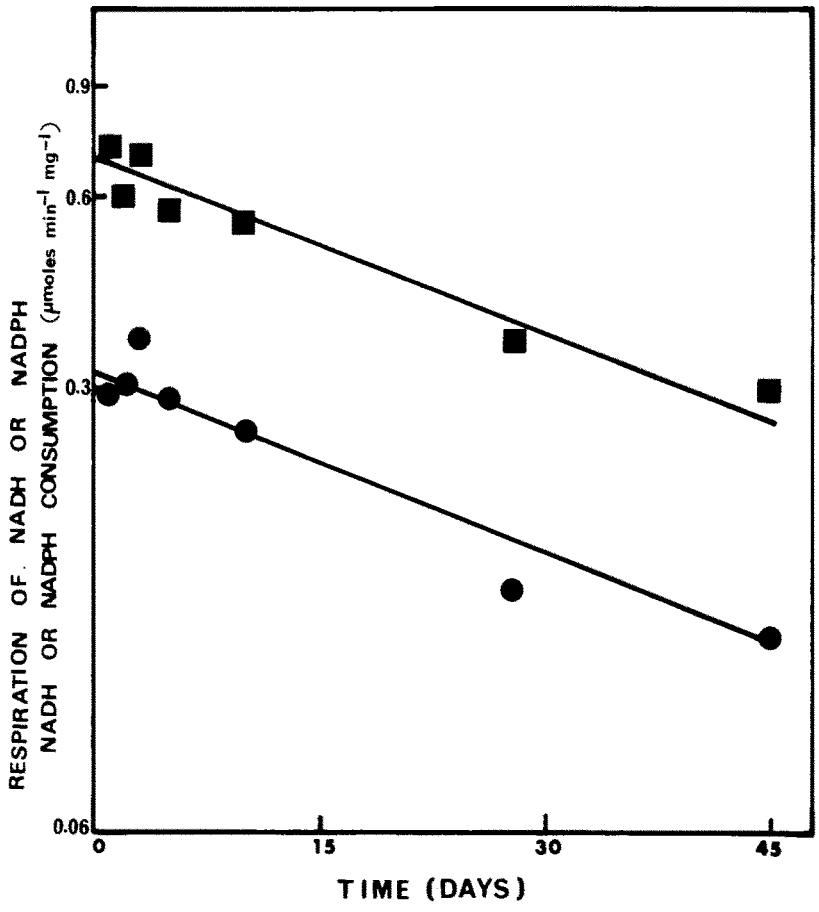

Fig. 3. NADH and NADPH respiration after storage of the polymer at 0°C. Respiration of NADH (■) and NADPH (●) was measured at different times of storage at 0°C in distilled water. Oxygen consumption was measured.

an example, was glucose-6-phosphate dehydrogenase (1.1.1.49) in the presence of glucose-6-phosphate.

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow[\text{Dehydrogenase}]{\text{Glucose-6-P}} \text{NADPH} + \text{H}^+ + \text{Gluconate-6-P}$$

$$\tfrac{1}{2}\text{O}_2 + \text{NADPH} + \text{H}^+ \xrightarrow[\text{Chain}]{\text{Respiratory}} \text{NADP}^+ + \text{H}_2\text{O}$$

$$\text{Glucose-6-P} + \tfrac{1}{2}\text{O}_2 \xrightarrow[\text{Respiratory Chain}]{\text{G-6-P Dehydrogenase}} \text{H}_2\text{O} + \text{Gluconate-6-P}$$

Oxygen consumption was followed (Fig. 4) with the oxygen electrode. Immobilized bacteria were added at (A); no oxygen uptake was seen in contrast to whole cells which always exhibit endogenous respiration. A limiting amount of NADPH (200 nmol) was added at (B). NADPH was completely oxidized into $NADP^+$ at

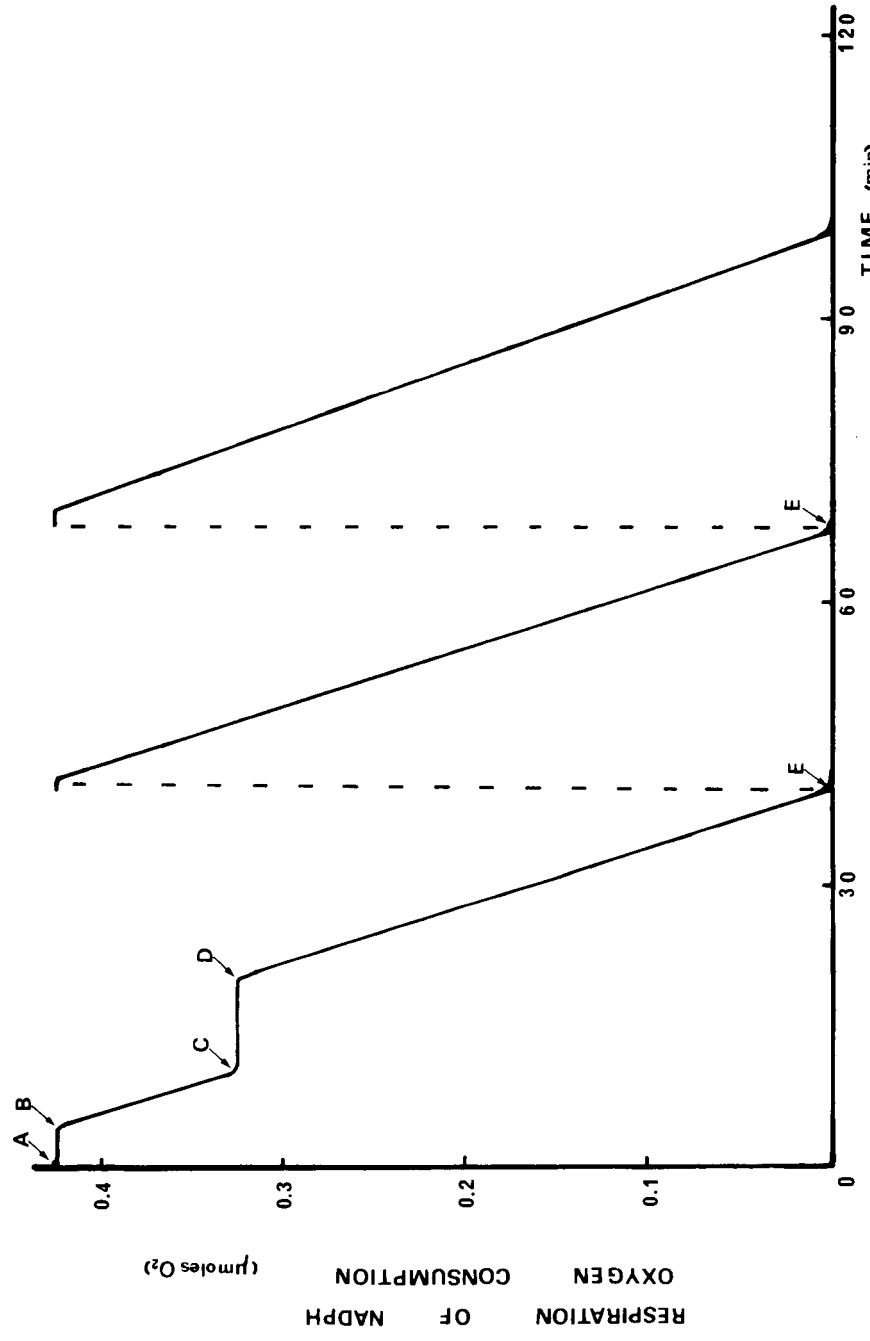

Fig. 4. Recycling of $NADP^+$ measured by oxygen consumption. The immobilized bacteria (10 units of NADPH respiration) were continuously stirred at 25°C in an oxygraph cell with 1.7 mL air saturated 0.1*M* phosphate buffer at pH 6.8 (A). Oxygen consumption was recorded. 0.2 m*M* NADPH was added at (B). NADPH was fully consumed at (C). The recycling reaction was started by the addition of 3 m*M* glucose-6-phosphate and 5 units of glucose-6-phosphate dehydrogenase at (D). Oxygenation was performed by whirling with a vortex during 1 min the liquid phase at (E).

(C). Corresponding to 100 nmol of oxygen consumption. The further addition of glucose-6-phosphate and soluble glucose-6-phosphate dehydrogenase from yeast at (D) reinitiated the oxygen consumption. This continued until all the available oxygen was consumed (425 nmol at 25°C). Further aeration at (E) allowed more recycling of $NADP^+$. Altogether, $NADP^+$ was recycled 13-fold during the 105 min recording of this experiment; much more recycling might still be possible since the rate of respiration decreased only 30% during the same time. In other experiments we were able to reach approximately 100 cycles without further optimization.

Some variants designed to improve the yield and the stability of the recycling system can be envisaged.

A thermophilic strain PS_3 (*17*) which is a gram positive microorganism growing at 65°C, was successfully used at 55°C. This high temperature increased the rate of recycling; the stability of glucose-6-phosphate dehydrogenase remained adequate for the duration of the experiment (2 h).

Many reports (D. Thomas, personal communication) suggest that coimmobilization increases the efficiency of bienzyme reactions. Because each NADPH dehydrogenase may need a special study for immobilization, no systematic study of coimmobilization of the bacteria and NAD^+ or $NADP^+$ dehydrogenase was done at the present time.

The immobilization of the cofactors might also be studied, although some difficulties because of diffusion limitation may be encountered.

To provide the best possible protection against bacterial contamination, we undertook to set up a small closed reactor that was able to function at least 5 h at 45°C in the presence of 10% methanol with less than 50% loss of activity. Most bacteria are unable to grow under these conditions.

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