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# Recycling of $NAD^+$ Using Coimmobilized Alcohol Dehydrogenase and *E. coli*

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## Abstract

The use of immobilized enzymes has opened the possibility of large scale utilization of $NAD^+$-linked dehydrogenases, but the applications of this technique were limited by the necessity of providing the large amounts of $NAD^+$ required by its stoichiometric consumption in the reaction. After immobilization of alcohol dehydrogenase and intact *E. coli* by glutaraldehyde in the presence of serum albumin, the respiratory chain was found to be capable of regenerating $NAD^+$ from NADH. This $NAD^+$ can be recycled at least 100 times, and thus the method is far more effective than any other, and, moreover, does not require NADH oxydase purification. The total NADH oxidase activity recovered was 10–30% of the initial activity.

Although, NADH is unable to cross the cytoplasmic membrane, it was able to reach the active site of NADH dehydrogenase after immobilization. The best yield of NADH oxidase activity with immobilized bacteria was obtained without prior treatment of the bacteria to render them more permeable. The denaturation by heat of NADH oxidase in cells that are permeabilized was similar before and after immobilization. In contrast, the heat denaturation of soluble β-galactosidase required either a higher temperature or a longer exposure after immobilization. The sensitivity of immobilized NADH oxidase to denaturation by methanol was decreased compared to


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permeabilized cells. As a result, it is clear that the system can function in the presence of methanol, which is necessary as a solvent for certain water insoluble substrates.

**Index Entries:** Recycling of $NAD^+$; $NAD^+$, recycling of; coimmobilization, of alcohol dehydrogenase and *E. coli;* alcohol dehydrogenase, coimmobilization with *E. coli;* dehydrogenase, alcohol, coimmobilization with *E. coli; E. coli,* coimmobilization with alcohol dehydrogenase.

## Introduction

Immobilization and stabilization of enzyme systems by various methods (*1*) have increased the applications of enzyme technology. One of the limiting steps in the further development of enzyme utilization is the recycling of the cofactors that are involved in a large number of reactions.

During dehydrogenase reactions one molecule of $NAD^+$ is reduced for each molecule of substrate transformed according to the following stoichiometric equation:

Reduced substrate + Oxidized cofactor $\rightleftarrows$ Oxidized product + Reduced cofactor

Several methods for the regeneration of $NAD^+$ have already been proposed (*2, 3*). A soluble high molecular weight derivative of $NAD^+$ that can be returned in a continuous flow reactor by an ultrafiltration membrane has been used (*4*). Electrochemical regeneration of $NAD^+$ from its reduced form (*5*), cofactor regeneration in a liquid membrane–enzyme system (*6*), regeneration of $NAD^+$ within semipermeable aqueous microcapsules containing a multienzyme system (*7*), the oxidation of NADH with phenazine-methosulfate and derivatives (*8*), and the use of a second enzyme system (*9*) have also been described.

These methods present several drawbacks: instability of the system used, low yield, cost of the extra enzymes, and difficulties in eliminating contaminants.

In the present paper we describe the recycling of $NAD^+$ with the nonpurified respiratory chain of *E. coli*. $NAD^+$ was found to be regenerated while consuming only oxygen. After coimmobilization of the bacteria with the dehydrogenase, there was no need to eliminate contaminants. The alcohol dehydrogenase, *E. coli* cells, and serum albumin were copolymerized with glutaraldehyde. Stability to heat and methanol were compared before and after immobilization. β-Galactosidase, a soluble enzyme of *E. coli,* was also used for comparison.

## Material and Methods

### *Growth and Treatment of Bacteria*

*E. coli* K 12 strain 3300 constitutive for the lactose operon was grown in aerated culture at 37°C in a minimal medium of the following composition for 1 L:

6.0 g $H_2KPO_4$, 18 g $HK_2PO_4$, 4 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$, 1 mg $FeSO_4$

The medium was supplemented with 8 g glycerol/L and 1 mg thiamine/L. Bacteria were collected by centrifugation during exponential growth in 80-L fermentors ($A_{600}$ around 7.0) and washed in a 0.1*M* K-phosphate buffer at pH 7.5. After washing, batches of 10 g of wet bacteria were frozen and stored in liquid nitrogen. After rapid thawing at 20°C, 1 μg DNase/g wet bacteria was added to improve the resuspension of bacteria (some DNA is always released and tends to aggregate the bacteria).

*Permeabilization*

Cytoplasmic enzymes of bacteria can be made accessible to impenetrable solutes by sonication or by treatment with 10 μL/mL toluene (and exposure to 37°C for 15 min) or by passage through a French press. Permeabilization was followed by measuring β-galactosidase activity using hydrolysis of β-O-nitrophenyl-galactoside in the presence of $10^{-4}M$ *p*-hydroxy-mercuri-benzoate (to inhibit the lactose permease) or by following the appearance of the NADH oxidase activity.

*Membrane Preparation (10)*

After French press extrusion, differential centrifugation was employed to give a fraction of light vesicles containing the cytoplasmic membrane with little contamination by outer membrane and soluble enzymes. The supernatant was a source of soluble β-galactosidase.

*Immobilization of the Bacteria (11)*

After thawing, the bacterial pellet was washed with 0.02*M* Na-phosphate buffer at pH 7.5. The bacteria were resuspended in the same buffer at approximately 1 g of wet weight/mL (corresponding to $A_{600}$ = 200 or 125 mg/mL protein). This suspension was diluted four times (250mg/mL final) in a solution containing 50 mg/mL serum albumin and 0.4% glutaraldehyde (final concentration). For $NAD^+$ regeneration experiments, 1 mg/mL lyophilized alcohol dehydrogenase from yeast (Boehringer) was added before immobilization. After rapid stirring at 0°C, the mixture was placed at −20°C for at least 2 h. The immobilized material was thawed and fragmented with a polytron in 0.1*M* Na-phosphate buffer at pH 7.5. This treatment gave particles of about 0.1 mm that could be readily collected by filtration. During filtration, three washes with 300 mL of distilled water were performed. β-Galactosidase activity was not detectable in the filtrates nor was alcohol dehydrogenase activity.

*Respiration (12)*

Air was bubbled through 0.1*M* Na-phosphate at pH 7.5 until dissolved oxygen was in equilibrium with the atmosphere at 25°C. Solid fragmented polymer was dried with porous paper and 100 mg (dry weight) were added to 1.7 mL of this buffer in a thermostated cell at 25°C. Oxygen consumption was measured with a Clark electrode using a Gilson oxygraph. With the regenerating system, 10 m*M* ethanol, 5 m*M* $NAD^+$, and 5m*M* NADH were added successively. Oxygen consumption

was measured after each addition. NADH-oxidase was also measured with a Gilford spectrophotometer at 25°C by the decrease of absorbance at 340 nm. A circulating system was then designed so that pieces of insoluble material would not enter into the measuring cuvette.

*Alcohol Dehydrogenase*

Alcohol dehydrogenase was measured in 0.1*M* Tris-HCl, pH 8.7, containing $3 \times 10^{-2}M$ ethanol and $1 \times 10^{-2}M$ $NAD^+$. Lyophilized alcohol dehydrogenase from yeast (Boehringer) was added and the increase in absorbance was recorded with a Gilford spectrophotometer at 340 nm.

β-*Galactosidase*

Hydrolysis of β-*o*-nitrophenyl galactoside was followed by the appearance of *o*-nitrophenol at 420 nm. The reaction was stopped by addition of 0.5*M* Na-carbonate. When immobilized material was used as the enzyme source, this was removed by filtration before taking a reading.

*Protein (13)*

Protein was determined according to Lowry et al. using bovine serum albumin as standard.

## Results

*Permeabilization of Internal Enzymes*

After freezing and thawing, bacteria were unable to oxidize exogenous NADH. NADH dehydrogenase is located on the inner face of the inner membrane and NADH molecules were not able to cross the inner membrane. Accessibility can be obtained by different techniques (Table 1). After immobilization, the best rate of respiration was unexpectedly observed with otherwise untreated whole bacteria (Table 1). If cells were permeabilized before immobilization, a much lower yield of NADH oxidation was obtained (Table 1). The measurement of activity of a soluble enzyme, β-galactosidase, also showed permeabilization to its substrate β-ONPG by immobilization of whole bacteria, but with minor loss of activity (Table 1).

*Optimization of NADH-Oxidase Activity after Immobilization*

It was observed that fragmentation of the immobilized material with scissors did not give as good results as fragmentation into smaller pieces with a polytron homogenizer. The optimal activity depended on both the time of fragmentation and on the power output of the homogenizer.

*Storage of Immobilized Bacteria*

Immobilized cells were stable at −20°C for months. Lyophilized immobilized cells gave good activity and were stable at −60°C for 6 months. At 20°C addition

Table 1
Immobilization of NADH Oxidase from *Escherichia coli*[a,b]

| | Before immobilization | | After immobilization | |
|---|---|---|---|---|
| | β-Galacto-sidase activity | NADH oxidase activity | β-Galacto-sidase | NADH oxidase |
| Intact bacteria | 0 | 0 | 8.6 | 1.9 |
| Toluene treated bacteria | 9.8 | 10 | 8.4 | 0.3 |
| Bacteria disrupted by sonic oscillations | 10 | 9.5 | 8.5 | 0.2 |
| Membranes preparation | 0.07 | 40 | 0.05 | 0.1 |

[a]Cytoplasmic and membrane-bound enzymes according to treatments nmol/min/mg protein.
[b]Intact *E. coli* were opened and inner membrane vesicles were prepared. β-Galactosidase activity was measured by β-O-nitrophenyl-galactoside hydrolysis. With intact bacteria, $10^{-4}M$ PCMB was added in order to inhibit the β-galactoside permease. NADH oxidation was followed with an oxygraph. Immobilization was performed by polymerization with glutaraldehyde in the presence of serum albumin. The polymerized material was fragmented with a polytron.

of 10% methanol and 100 mg/mL streptomycin increased the stability of immobilized NADH oxidase to several days. Figure 1 shows the rate of recycling of $NAD^+$ versus time under conditions of continuous activity. Surprisingly the regeneration rate of $NAD^+$ initially increased by a factor of about 2 during the first few hours. Then a decrease with a half-life of about 1.5 days was observed. The activity remained greater than or equal to the initial activity for at least 2 days.

*Kinetic Parameters: "Apparent Values"*

The $K_M$ for $NAD^+$ and alcohol and the optimal pH of alcohol dehydrogenase were measured under the same experimental conditions in solution or after immobilization together with the recycling system. The $K_M$ for NADH and the optimal pH were measured for NADH oxidase. The results show that after immobilization, the kinetic parameters of NADH oxidase were unchanged, while those of alcohol dehydrogenase were modified (Table 2). They provide a sensitive assay for alcohol because the apparent $K_M$ for alcohol was decreased 60-fold.

*Methanol Denaturation*

The methanol denaturation kinetics of NADH oxidase activity were followed as a function of time (Fig. 2). Cells permeabilized by toluene were very unstable ($t_{1/2}$ = 1 min). In contrast, after immobilization NADH oxidase was more stable

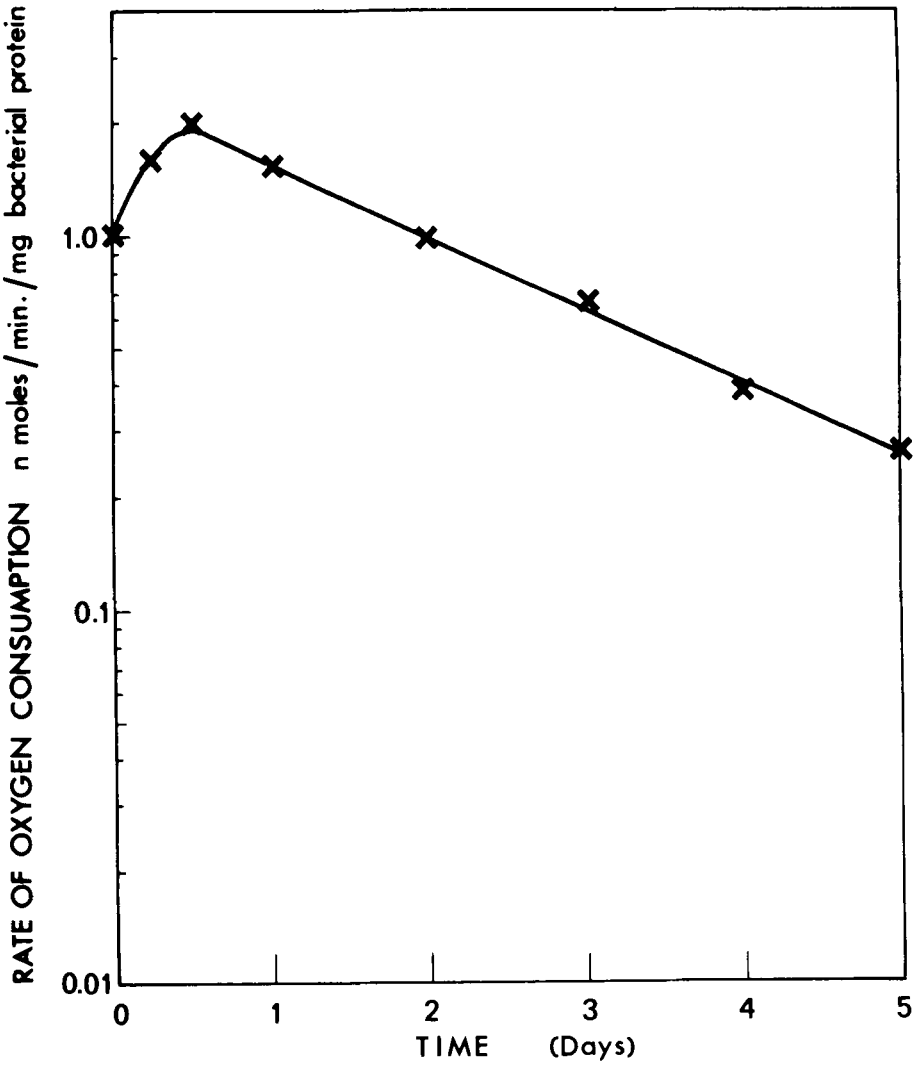


Fig. 1. Evolution of the rate of $O_2$ consumption with time during the activity of the regeneration system of $NAD^+$. *E. coli* and alcohol dehydrogenase were immobilized by glutaraldehyde in the presence of serum albumin. Fragmented polymer was kept at 20°C in a phosphate buffer under bubbling air in the presence of 10% ethanol and 5 m*M* $NAD^+$ Samples were collected at different times and tested in the oxygraph for $NAD^+$ regeneration capacity by measuring, in the polymer, NADH oxidase driven by alcohol dehydrogenase. ($NAD^+$ and ethanol were added and oxygen consumption was measured.)

($t_{1/2}$) = 20 min). These results permit water-insoluble substrates to be used if they are soluble in 30% methanol ($t_{1/2}$, 2 days).

*Heat Denaturation*

Heat denaturation kinetics were followed for NADH oxidase and β-galactosidase activities with or without immobilization. Denaturation kinetics were found to be first-order. Soluble β-galactosidase was stabilized by immobilization. In

Table 2
Kinetic Parameters of Alcohol Dehydrogenase, NADH Oxidase, and Recycling System [a]

| | NADH oxidase in toluene treated bacteria | Alcohol dehydrogenase in solution | Recycling system (immobilized) |
|---|---|---|---|
| Optimal pH | 7.5 | 9.2 | 7.5 |
| $K_M$ ethanol | — | $3 \times 10^{-3}M$ | $5 \times 10^{-5}M$ |
| $K_M$ $NAD^+$ | — | $1 \times 10^{-3}M$ | $3 \times 10^{-5}M$ |
| $K_M$ NADH | $3 \times 10^{-4}M$ | — | $3 \times 10^{-4}M$ |

[a]Alcohol dehydrogenase- and toluene-treated bacteria were either maintained in 0.1*M* Na-phosphate buffer at pH 7.5, or immobilized together (in a recycling system) by polymerization with glutaraldehyde in the presence of serum albumin. $K_M$ and pH optima were measured. Alcohol dehydrogenase was measured by the appearance of NADH at 340 nm. NADH oxidase by consumption of oxygen.

permeabilized cells, β-galactosidase and NADH oxidase were not stabilized by immobilization under the same experimental conditions (Fig. 3). These results allow the use of a reactor at 45°C, and this temperature will largely prevent bacterial contaminations (bacteria rarely grow above 42°C).

### *$NAD^+$ Recycling*

Intact *E. coli* cells were coimmobilized with alcohol dehydrogenase in the presence of an excess of serum albumin by polymerization at −20°C with glutaraldehyde. The fragmented polymer was introduced into a Gilson oxygraph cell. Limiting amounts of NADH were added and consumption of oxygen stopped rapidly. Excess ethanol was then added. Respiration resumed until complete exhaustion of oxygen (20-fold recycling of $NAD^+$). Oxygen was bubbled until saturation, and complete oxygen consumption was again observed (40-fold recycling of $NAD^+$). This was repeated several times; $NAD^+$ was recycled at least 100-fold.

## Discussion

$NAD^+$ can be easily recycled using the respiratory chain of *E. coli*. The consumption of oxygen is inhibited by $10^{-2}M$ cyanide. The respiratory chain used is a multienzyme membraneous complex. It is probably because of this localization in vivo that the respiratory chain is already stabilized against heat denaturation. A soluble enzyme such as β-galactosidase, free in solution, was much more thermosensitive and was stabilized by immobilization. In contrast, the respiratory chain was very sensitive to 50% methanol ($t_{1/2}$ = 1 min). Immobilization dramatically decreased the effect of methanol ($t_{1/2}$ = 20 min), opening the possibility of

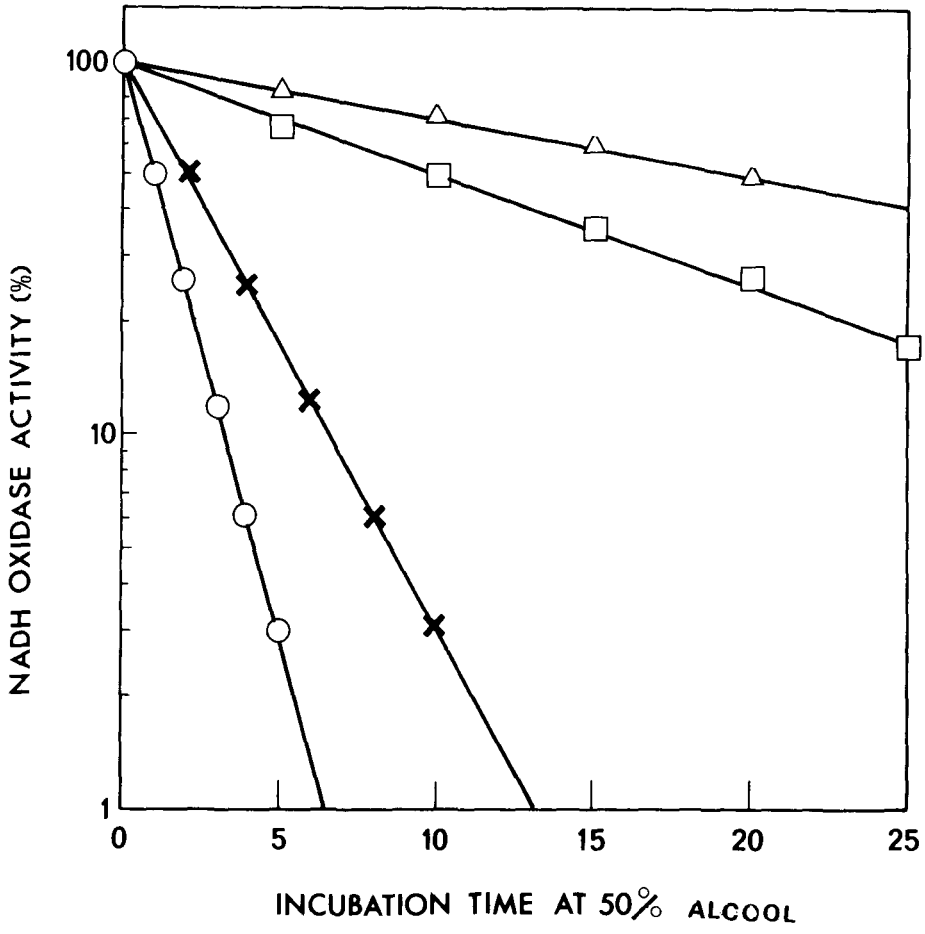


Fig. 2. Denaturation of NADH oxidase by alcohols. Biological samples were incubated at 37°C with the denaturing agent in 0.02*M* Na-phosphate buffer at pH 7.5. Aliquots were diluted 10-fold in 0.1*M* buffer at different times. Residual activities were measured as described in Material and Methods. Bacteria permeabilized with toluene were incubated with 50% methanol in the absence (○) or presence of 50 mg/mL serum albumin (**X**). Intact whole bacteria were first immobilized, fragmented with the polytron, and then incubated with 50% methanol (△) or 50% ethanol (□). NADH oxidase was measured with the oxygraph; 100% represents an oxygen consumption of 1 nmol/min/mg bacterial protein.

the utilization of water-insoluble substrates. Studies on steroid dehydrogenase are in progress (*14*). Other particularly unstable multienzyme systems were also stabilized by immobilization: chloroplasts (*15*) and chromatophores (*16*).

Immobilized "NADH oxidase" from *Leuconostoc mesenteroides* has also been described (*17*). Partially purified enzyme was used. We propose instead to use directly immobilized bacteria, which are much more easily and much less time-consuming to prepare.

A better knowledge of activities found at the level of biological membranes may also arise from this kind of study on immobilized enzymes of multienzyme systems.

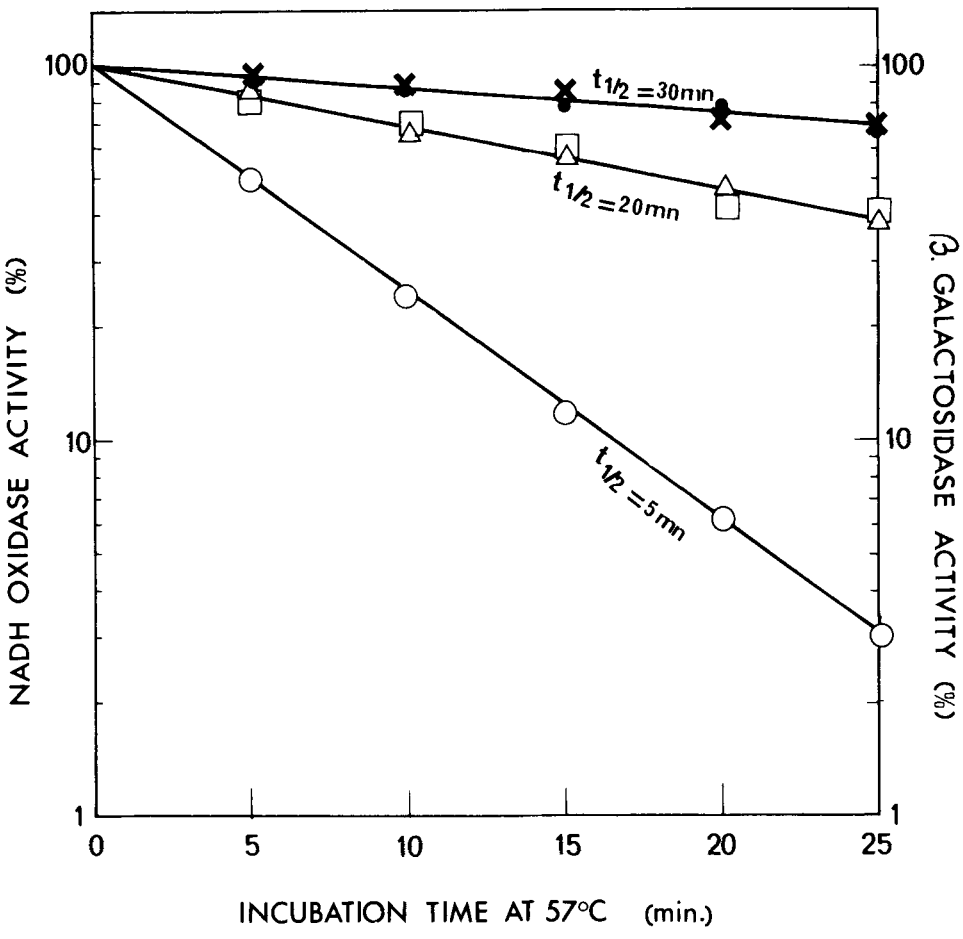


Fig. 3. Denaturation at 57°C of NADH oxidase or β-galactosidase. The experimental conditions of denaturation are described in Fig. 2, except that methanol was replaced by heating at 57°C. The supernatant from the ultracentrifugation during *E. coli* membrane preparation was the source of soluble β-galactosidase (○). This preparation was immobilized (**X**). Bacteria permeabilized with toluene (●) were used as another source of β-galactosidase. For NADH oxidase, a suspension of toluenized cells (△) and immobilized intact untreated bacteria (□) were used. All these preparations were incubated at 57°C for different periods of time.

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