# I. RATES OF THIOL-DISULFIDE INTERCHANGE REACTIONS INVOLVING PROTEINS

# II. REGENERATION OF THE NICOTINAMIDE COFACTOR NADH

by

Ze'ev Shaked

B.S., M.S., The Hebrew University, Jerusalem
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Signature of Author Signature redacted
Department of Chemistry

Certified by Signature redacted
George M. Whitesides

Accepted by Signature redacted
Chairman, Departmental Committee

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Submitted to the Department of Chemistry
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of the requirements for the
degree of Doctor of Philosophy

## ABSTRACT

I. Rates of Thiol-Disulfide Interchange Reactions Involving Proteins.

Brønsted coefficients were determined for the rates of thiol-disulfide interchange between low molecular weight thiols and the disulfide groups of four native or modified proteins: DNase ($\beta_{nuc} \simeq 0.36$), lysozyme ($\beta_{nuc} \simeq 0.55$), adenylate kinase $(SSCH_3)_2$ ($\beta_{nuc} \simeq 0.65$) and papain $(SSCH_3)$ ($\beta_{nuc} \simeq 0.45$). These values are similar to those observed for reductions of oxidized glutathione and Ellman's reagent by a similar set of thiols ($\beta_{nuc} \simeq 0.5$). Glutathione was anomalously slow in reduction of certain protein disulfide groups: although this effect may in part reflect steric hinderance to attack by the relatively large glutathione molecule at disulfides shielded by protein tertiary structure, other (presently undefined) factors appear also to be important, at least in the case of DNase. The rates of reduction of several disulfide derivatives of papain(SSR) by DTT were determined. These data provided estimates of the Brønsted coefficient for the "central" thiol in thiol-disulfide interchange: these estimates fall in the range $\beta_c \simeq -0.25$ to $-0.43$. Rates of reduction of protein disulfide moieties were analyzed by using a Brønsted equation developed previously to yield pKa values for the participating thiol moieties: in particular, for papain, pKa(Cys-25) = 8.4 at pH 9 and pKa(Cys-25) = 4.1 at pH 6. The thiols of the structurally essential cysteine group of lysozyme seem to have pKa $\simeq$ 11 which may suggest an important contribution to unexpectedly slow rates of reduction.

II. Regeneration of the Nicotinamide Cofactor NADH

Recycling schemes were developed for the employment of nicotinamide cofactors in synthetic application. The first is an enzymatic regeneration method based on gel-immobilized formate dehydrogenase (E.C. 1.2.1.2.) and formate. An enatioselective synthesis of D-lactate (94-96% enantiomeric excess, ee) from pyruvate demonstrated the operation of this regeneration system. The turnover numbers (TN = mol product/mol cofactor) achieved in this system were 1200 - 1750. The second system which is a combined electrochemical/enzymatic method regenerated NADH in situ from NAD by a process based on a novel cathodic reduction of lipoamide and oxidized dithiothreitol to their corresponding dithiols at tungsten or molybdenum cathodes. The dihydrolipoamide in the reaction mixture reduced NAD to NADH by using gel-immobilized lipoamide dehydrogenase. L-lactate was synthesized from pyruvate in 96% ee and the TN for NAD obtained in this electrochemical regeneration were 600 - 950. The third and last system developed was a liquid redox membrane in which the enzymatic reduction of NAD to NADH (by lipoamide dehydrogenase) and a co-substrate (lipoic acid, 1,3-dithio-2-propanol) was coupled with the oxidation of dithionite. L- and D-lactate were synthesized in 96% ee and the TN for NAD in this system were 20 - 40.

Thesis Supervisor: George M. Whitesides

Title: Professor of Chemistry

To Nitzi and Dan with Love

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# Chapter I. Rates of Thiol-Disulfide Interchange Reaction Involving Proteins and Kinetic Measurements of Thiol pKa Values.

## Introduction

Thiol-disulfide interchange reactions play an important role in biochemical processes.[1-5] The disulfide bond can be cleaved and formed rapidly and reversibly by thiol-disulfide interchange at neutral pH and room temperature, a property reflected in the formation and cleavage of structural cystines.[6-10] Covalent disulfide bonds provide bridges that are much stronger (55 kcal/mol) than the hydrophobic and hydrogen bonded interactions believed responsible for initial protein folding. The protein disulfide bonds can be formed, rearranged and broken up *in vitro* by systems involving low molecular weight thiol-disulfide couples.

The formation of disulfides from thiols occurs either by two-electron oxidation processes (thiol-disulfide interchange, oxidation by $H_2O_2$) or by one-electron processes (oxidation by certain transition metal ions). Indeed, by some of these processes, glutathione maintains the intracellular redox potential, and can be oxidized to its disulfide by oxygen, oxidized electron transport carriers, free radicals and a variety of disulfides.[11]

The thiolate anion in contrast to the thiol and the disulfide groups is a strong nucleophile, a property that is particularly important since thiols have been identified as essential components of the active site of many different types of enzymes.[12-17] Furthermore, in some proteins cysteine thiol groups participate in substrate binding and are often important

in hydrophobic interactions that contribute to the tertiary structure of proteins.[18-20]

A major mechanism by which enzyme deactivate is the autoxidation of essential cysteine thiol groups.[21] Protein autoxidation can be inhibited by the addition of thiol reducing agents such as dithiothreitol or 2-mercaptoethanol to the protein solutions.[22-24] These added thiols inhibit the irreversible oxidative deactivation of proteins by several mechanisms. One of these mechanisms is the thiol-disulfide interchange reactions. Whitesides et al., Hupe et al. and others have studied thiol-disulfide interchange reactions involving low molecular weight reactants.[25-29] The major conclusion of these studies is that the thiolate anion attacks the disulfide bond along the sulfur-sulfur axis by a $S_N2$ process which places appreciable charge on all three sulfur atoms in the transition state with little d-orbital participation. (eq 1)

$$R_{nuc}S^- + R_cS\text{-}SR_{lg} \rightarrow R_{nuc}S\text{-}SR_c + {}^-SR_{lg} \qquad (1)$$

Model studies clarified the basic physical-organic chemistry of thiol-disulfide interchange. Studies of the kinetics of these reactions demonstrated that the Brønsted coefficient characterizing the attack of thiolate anions on oxidized glutathione and 5,5'-dithobis-(2-nitrobenzoic acid) (Ellman's reagent), is approximately $\beta_{nuc} \simeq 0.5$. Brønsted coefficient for the "central" sulfur atom is approximately $\beta \simeq -0.3$ while the coefficient of the leaving-group sulfur has been estimated

as $\beta_{lg}$ -0.5 to -0.7 (not verified experimentally).[26]

The major purpose of this research was to determine the rates of thiol-disulfide interchange between low molecular weight mono- and dithiols and disulfide groups in enzymes and enzyme derivatives. The examination of these rates was important since they could establish the influence of steric and hydrophobic interactions on the reactivity of thiolate anions with the disulfide bonds in proteins. The other two objectives in this work were to explore the utility of thiol-disulfide interchange rates in measuring the pKa values and reactivities of thiol groups in proteins and to identify thiols of potential use as antioxidants for use in organic synthetic procedures requiring enzymatic catalysis. Full details of this work are provided in reference 30.

Results and Discussion

The proteins examined were deoxyribonuclease I (DNase), lysozyme, adenylate kinase (AdK) and papain. DNase[31] (two cystine groups)[32] and lysozyme[32] (four cystine groups) were used in their native configuration. The active-site cysteine moiety of papain[33] and the two cysteine groups of AdK[18] were modified to S-thioalkyl derivatives[20] [papain(SSR) and AdK$(SSCH_3)_2$]. Papain(SSR) was enzymatically inactive while AdK$(SSCH_3)_2$ showed $\sim$ 70% of the activity of the native enzyme.[34]

Reactions were followed by measuring enzymatic activity as a function of time in solutions containing a large excess of thiol.

The rates of reduction of the protein disulfide groups examined in this work all give a satisfactory Brønsted correlation. This suggests that steric and electronic effects peculiar to the protein tertiary structures are less important for these proteins and thiols than the electronic effects responsible for the reactivity-basicity relation which underlie Brønsted correlations. The Brønsted coefficients derived in this study are not very accurate, because the pKa range spanned and the number of thiols included are limited, and because the set of thiols studied would not necessarily provide a good set for Brønsted studies even in mechanistically uncomplicated cases. In addition, the precision of an individual rate constant is only modest ($\sim$ 15% or less).

Nonetheless, the values of these coefficients obtained by least-squares analysis (omitting the points for glutathione which fall appreciably off the lines) are similar to the value of $\beta \sim 0.5$ observed for thiol-disulfide interchange with Ellman's reagent[25] and glutathione:[26]

$$\beta_{nuc}^{papain(SSCH_3)} = 0.45 \ (r^2=0.77);$$

$$\beta_{nuc}^{AdK(SSCH_3)} = 0.65 \ (r^2=0.85);$$

$$\beta_{nuc}^{DNase} = 0.36 \ (r^2=0.80);$$

$$\beta_{nuc}^{lysozyme} = 0.55 \ (r^2=0.76).$$

The Brønsted lines although roughly parallel are significantly displaced from one another; thus, for example, the PS-$SCH_3$ disulfide bond of papain($SSCH_3$) is cleaved at pH 7 about $10^5$ times more rapidly than the cystine disulfide bond of lysozyme whose reduction is responsible for loss of its enzymatic activity. The rapid rate of reduction of the former is satisfactory rationalized by the high acidity of the active site thiol. The low rate of reduction of the structurally essential cystine of lysozyme apparently also reflects an anomalous but in this instance unexpectedly high thiol pKa (Table I). The high $pK_a$ value ($pK_a \sim 11$) suggests that there may be an important electrostatic contribution to unexpectedly slow rates of reduction. It is believed that proximate positively charged groups increase the acidity of the active site

TABLE I: $pK_a$ Values Inferred from Rate Constants [$k_1$($M^{-1}$ $min^{-1}$)] for Thiolate-Disulfide Interchange[a]

| compd | Group | pH | $10^{-2}k_1$ | $pK_a$ | lit. $pK_a$ |
|---|---|---|---|---|---|
| $CH_3OCOCH_2SH$ | SH | 4-9 | 52 | 7.9 | 7.91[b] |
| GHS ($NH_3^+$, $CO_2H$) | SH | 4 | 13 | 8.5 | 8.72[c] |
| GHS ($NH_3^+$, $CO_2^2$) | SH | 8 | 16 | 8.4 | 8.72[c] |
| Cys-Et ($NH_3^+$) | SH | 4-6 | 190 | 7.4 | 7.3[c] |
| Cys-Et ($NH_2O$ | SH | 9 | 11 | 8.6 | 8.87[c] |
| Cys-Et[d] | $NH_3^+$ | d | | 8.2 | 8.33[c] |
| papain | SH (Cys-25) | 6 | 6200 | 4.1 | |
| papain | SH(Cys-25) | 9 | 13 | 8.4 | |
| papain | $H^+$ (His-159) | d | | 7.5 | |
| DNase | e | 7 | 6.4 | 8.8 | |
| AdK | SH9Cys-25) | 7 | 22 | 7.5 | |
| Lysozyme | e | 7 | 0.042 | 11.0 | |

[a]DTT is the reducing dithiol in all experiments. [b]Wilson et al. (1980) [c]Reuben & Bruice (1976). [d]From inflections in pH-rate profiles.

[e]The location of the thiol group(s) determining these $pK_a$ values has not been established. The two protein cysteine thiol groups are assumed to have the same $pK_a$.

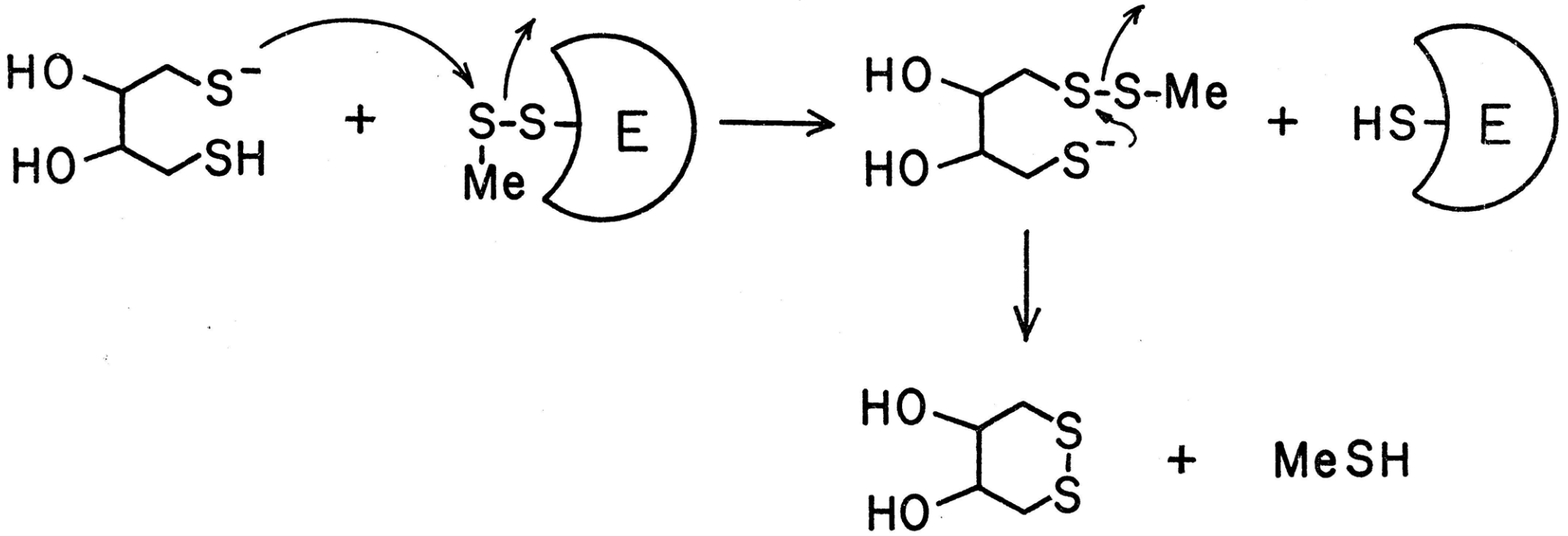
thiol of papain to a value appropriate for effective catalysis at pH 6. By analogous arguments it can be claimed that negatively charged or possibly hydrophobic groups around the structurally important disulfide of lysozyme might increase the $pK_a$ values of its constituent thiols to values which render reduction slow. The rates of thiol-disulfide interchange with DNase and $AdK(SSCH_3)_2$ are approximately those expected by analogy with the reduction rates of oxidized glutathione by the same set of thiols. The disulfide group of DNase responsible for maintaining the enzyme in its active conformation (i.e., the disulfide group whose reduction is rate limiting in loss of activity) has been suggested to lie close to the protein surface.[31] The $pK_a$ estimated in this work for the leaving thiol of this disulfide ($pK_a$ = 8.8) is close to that of glutathione and is compatible with this suggestion. The Cys-25-SH group of adenylate kinase which is closer to the active site and probably the one that upon blocking gives $\sim$ 70% of the activity of the native enzyme is abnormally acidic ($pK_a$ = 7.5). This thiol has been reported previously to react $\sim$ 40 times faster with NBD-chloride (7-chloro-4-nitrobenzo-2-oxa-1,3 diazole) than the more distant Cys-187.[18]

The rates of thiol-disulfide interchange involving proteins should provide a useful method for determining kinetic acidities of thiols. This method was used to characterize the active-site of papain. The $pK_a$ values obtained are summarized in Table I. When the thiol-disulfide interchange

reaction involving papain($SSCH_3$) is carried on at low pH, the active-site thiol of papain behaves as a leaving group with a pKa of $\sim 4$ (Scheme 1). At high pH it behaves as if it had a normal $pK_a$ of $\sim$ 8.5. The transition between these two regimes occurs at pH 7-8 and is attributable to protonation of a base with this $pK_a$ in the active site. These estimates of the $pK_a$ values of the active-site functional groups of papain are in general agreement with previous proposals by others.[39-42] A useful feature of the thiol-disulfide interchange reaction as a method for estimating thiol $pK_a$ values is that, unlike conventional methods, it can be carried out as a function of pH. It is therefore possible to observe changes in the thiol $pK_a$ with changes in the state of ionization of proximate acids and bases.

Scheme I. The Thiol-Disulfide Interchange Reaction Involving Papain($SSCH_3$) and Dithiothreitol.

Scheme I



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# Chapter II. Regeneration of the Nicotinamide Cofactor NADH

## Part A. Introduction

The practical application of enzymes in pharmaceutical,[1] food processing[2] and biomedical fields[3] is well established today in biotechnology (Scheme 2). During the recent decade, enzyme technology has been a synthetic technique in waiting never put through its full paces in synthetic organic chemistry.

Many enzymes are exceptional in their catalytic activity. Rates of enzyme-catalysed reactions can be faster than those of the corresponding non-enzymatic processes by factor of $10^9$ to $10^{12}$. Furthermore, enzymes may be very selective with respect to the type of reaction they catalyze. The stereospecificity of most enzymatic reactions is perhaps their most striking feature. Certainly it is their ability to discriminate between enantiomers, and between stereoheterotopic groups and faces, which is of greatest importance to the chemist engaged in asymmetric synthesis.

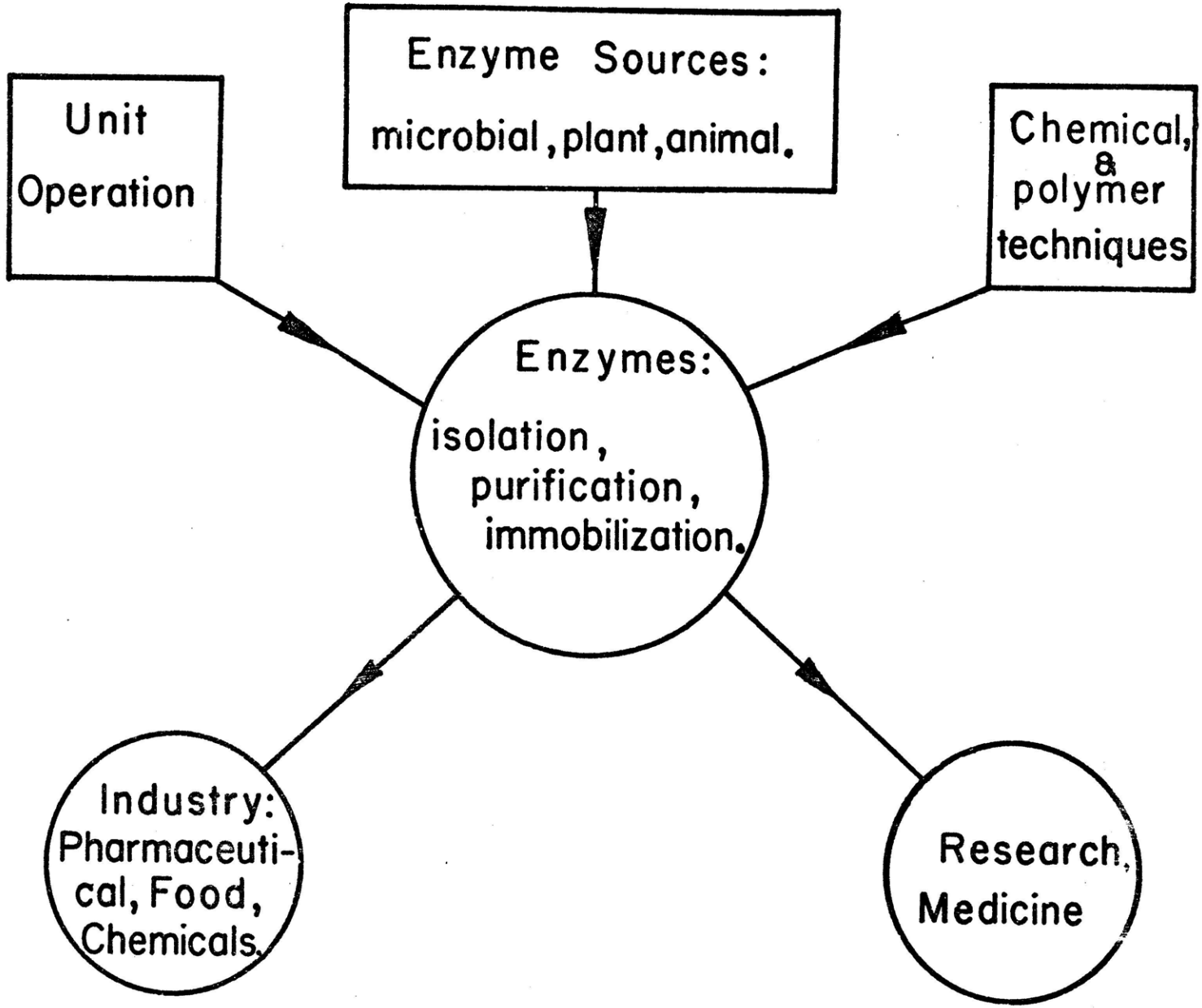
In the past most of the useful enzyme-mediated synthetic transformations were effected by growing micro-organisms. More recently, however, methods employing wholly or partly purified and immobilized enzyme preparation have become more prominent.[4-6]

Enzymes utilized to date on practical scale all belong to what might be termed the first generation of immobilized enzymes, that is simple enzymes consisting entirely of polypeptide chains with no coenzyme requirement.

Of the 2000 or so different enzymes to which a specific

Scheme 2. Applications of Immobilized Enzymes

# Scheme 2


Enzyme Sources:
microbial, plant, animal.
Unit
Operation
Chemical, & polymer techniques
Enzymes:
isolation, purification, immobilization.
Industry: Pharmaceutical, Food, Chemicals.
Research, Medicine

E.C. number has been assigned, over one-third require for catalytic activity one of the following five coenzymes: NAD(H), NADP(H), ATP, FAD and coenzyme A.[4,11] These enzymes that include the dehydrogenases, oxygenases, transferases and ligases are the most attractive ones for the organic chemist.[7-9]

Coenzymes are in reality cosubstrates because they undergo chemical transformation during the reaction and many are required in stoichiometric quantities. Some coenzymes do not present a problem in this regard, because they either are self-regenerating (biotin, pyridoxal phosphate) or they reform by catalytic oxidation with $O_2$ as the electron acceptor (FAD, NAD(P)). On the other hand, some coenzymes require an appropriate substrate other than oxygen for regeneration (ATP, $FADH_2$, SAM, NAD(P)H, coenzyme A).

The regeneration is not a problem when using growing microorganisms because all the cofactors required are present in the medium or are continuously produced during the fermentation process. Many of these fermentation processes are inefficient for secondary metabolites, and are slow. Moreover, the isolation of product(s) is tedious and expensive.

Cell-free enzymatic synthesis has the advantage of utilizing the exceptional properties of enzymes without the limitations of a complete fermentation. The cell-free enzymatic reactor has a higher productivity and selectivity for conversion of starting materials to products. The isolation

procedures for the products are relatively simple. Enzymes might also be used to produce quantities of intermediates present in vivo only in low steady state concentration (for example, 5'-phosphoribosyl-1-pyrophosphate).

With purified and immobilized enzymes, however, maintaining a sufficient concentration of coenzyme can prove to be a major problem. Coenzymes are costly. For example, the cost of ATP is ∿$800/mol while NAD costs ∿$1200/mol and NADPH, the most expensive cofactor costs ∿ $210,000/mol (p.33). It is economically unacceptable to use them in stoichiometric amounts. The problem of developing more economical ways of using cofactors in large scale applications is particularly important in biotechnology.

In recent years different methods and approaches have been directed toward the development of coenzyme dependent large scale enzymatic processes.[10-12] These efforts were directed mainly towards two classes of cofactors; the nucleotide triphosphates (especially ATP) and the nicotinamide derivatives, NAD(P)(H). Since both cofactors are expensive they must be recycled to permit large scale processes. Adenosine triphosphate (ATP) is a relatively stable substance. Its cost remains high since the fermentation-based synthesis of this substance is still inefficient and expensive due to isolation and purification procedures.

The large-scale enzymatic regeneration of ATP was developed at MIT by an interdisciplinary group headed by Professor

# ECONOMICS

| | $/Kg |
|---|---|
| NAD | $ 1,600 – 8,100 |
| NADH | $ 24,000 |
| NADP | $ 73,000 |
| NADPH | $ 237,500 |

Wang.[13] The system, one of the best current methods for regenerating ATP, is based on the enzyme-c talyzed production of ATP from AMP or ADP by reaction with acetyl phosphate. These reactions are catalyzed by adenylate kinase (AdK) and acetate kinase (AcK) (Scheme 3). The utility of this system has been demonstrated in large-scale ATP dependent enzymatic syntheses.[14-16] Acetyl phosphate is the ultimate phosphoryl donor which is synthesized economically as the diammonium salt by acylation of anhydrous phosphoric acid with ketene[17] or by the reaction of acetic anhydride and phosphoric acid.[18]

The development of efficient recycling methods for the pyridine nucleotide cofactors (Figure 1) presents a difficult challenge. These cofactors are intrinsically unstable in aqueous solution. Both reduced [NAD(P)H] and oxidized [NAD(P)] forms degrade in aqueous solution, and both non-enzymatic and enzymatic routes for this degradation have been identified.[19-23]

Their high cost and short lifetime are the main reasons for the many efforts that have been directed toward the development of less expensive sources of these cofactors.[24-26]

A different approach has been to utilize analogs of unstable cofactors, especially NADP(H). Many analogs of NAD(P)(H) are known to be enzymatically active.[27-30] There seems to be little doubt, however, that the relative coenzymatic activities of nicotinamide coenzyme analogs are lower than those of the parent coenzyme

Scheme 3. Cell-Free Enzymatic Regeneration of ATP

Scheme 3

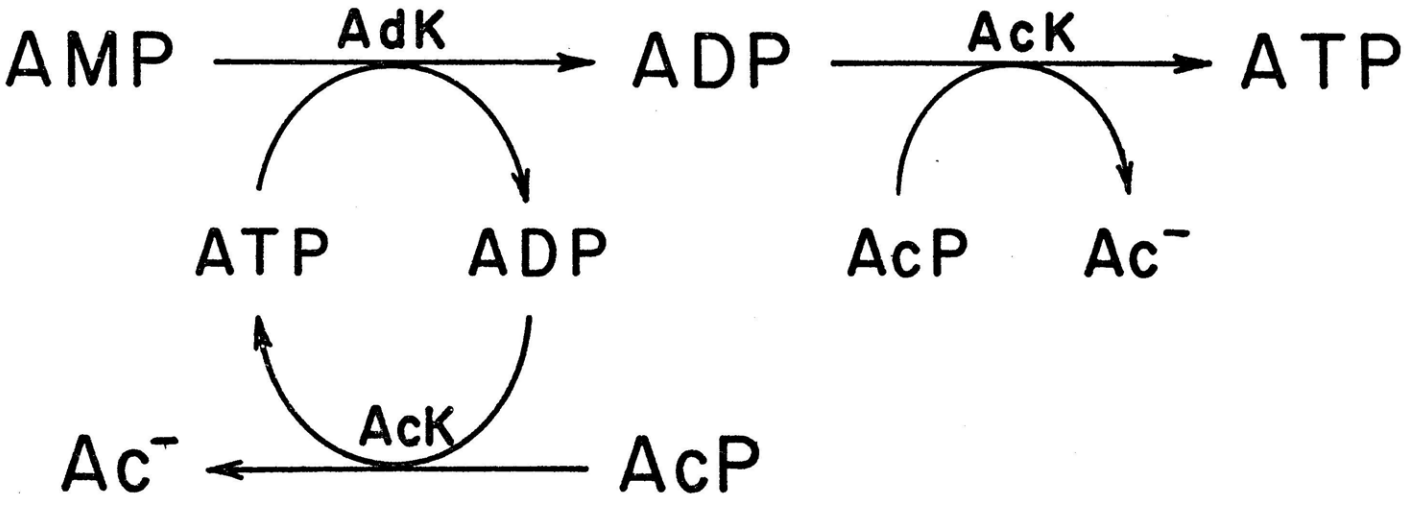
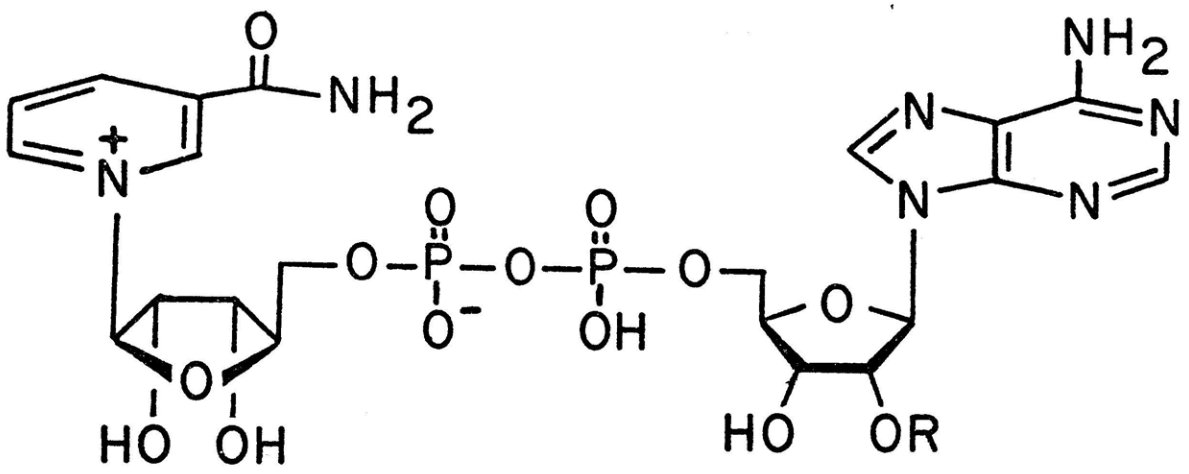


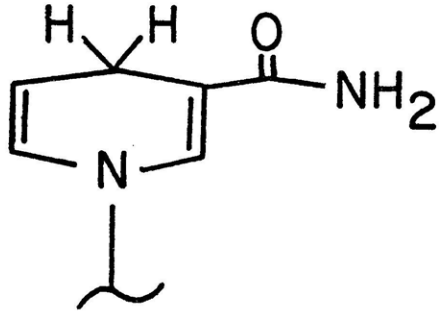
Figure 1. The Nicotinamide Cofactors

# Figure I



$NAD^+$, R=H

$NADP^+$, $R=PO_3^{=}$



NAD(P)H

under comparable conditions (lower $V_{max}$ and higher $K_m$ values). The activities decrease further on immobilization to support matrices. The sequence of activities: coenzyme ≥ coenzyme analogs > coenzymes bound to soluble support > coenzyme bound to insoluble support appears to be generally correct. A careful examination of these analogs might, however, yield inexpensive compounds which could substitute for the native cofactors.

Recently, immobilization of cofactors has been used in order to retain them in the reactor and to extend their lifetimes.[30-36] Water-soluble or insoluble support matrices have been tested by chemically modified coenzyme that has been covalently attached to the carrier. The much greater retention of coenzymatic activity of the analogs cofactors when compared to water-soluble polymers such as dextran, polyethyleneimine polylysine, and polyethyleneglycol is due to the greater steric accessibility of the ligands. That is not the case with particulate supports such as agarose. Another method is involves binding the coenzyme in the immediate vicinity of the active site of the enzyme and performing regeneration with an electron carrier (phenazine methosulfate).[37] Most immobilized cofactors are still expensive to produce and are insufficiently stable to be used in synthesis. Their application, however, seems to be a promising approach particularly to large-scale synthesis.

In any event, a large-scale nicotinamide dependent enzyme synthesis will require an efficient cofactor recycling

scheme. Many recycling methods that have been proposed are quite satisfactory for laboratory scale syntheses but are insufficiently efficient and not economical at this point for large-scale industrial applications.[10, 11, 12, 38, 39]

The regeneration of the nicotinamide cofactors can be done by either an enzymatic method (with free or immobilized coenzymes) or by a nonenzymatic method (chemical or electrochemical). This work presents three different recycling methods, an enzymatic method (formate/formate dehydrogenase), a combined electrochemical/enzymatic method, and a chemical method based on a liquid-membrane system.

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## Part B. NADH Regeneration by Using Formate and Forgmate Dehydrogenase

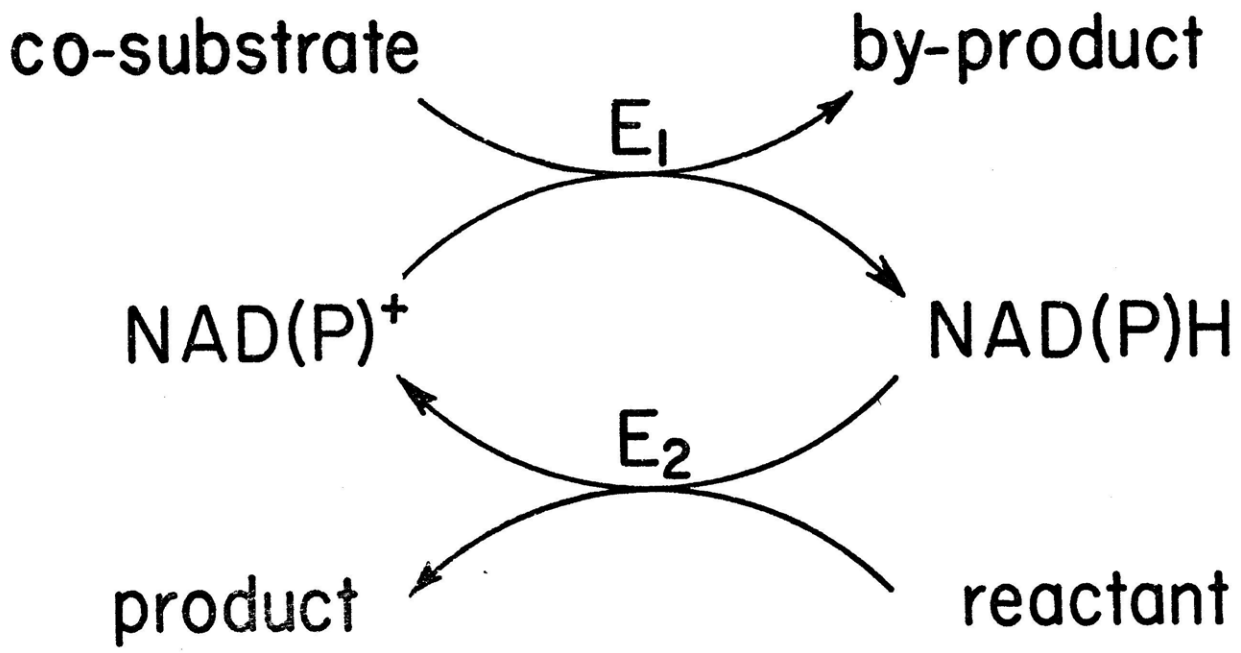
Background

Enzymatic regenerations of the nicotanimide cofactors received more attention than the non-enzymatic methods. Many enzymatic regeneration systems have been proposed, and some of these have been tested and utilized in enzyme requiring organic synthesis. In enzymatic recycling systems usually two substrates must be added to the reactor and two products are produced, only one of which is required. Thus, an additional step must be introduced to separate out the required product. The general scheme for this system is demonstrated (Scheme 4). The following recycling systems belong to this category: ethanol/alcohol dehydrogenase (ADH);[1-2] isopropanol/alcohol dehydrogenase;[3] glucose-6-phosphate/glucose-6-phosphate dehydrogenase.[4,5] The most common schemes for NAD(P)H regeneration are based on alcohol dehydrogenase (Scheme 5). These use relatively high concentrations of organic reactants and products in solution and generate aldehydes (acetaldehyde) or ketones which are reactive toward proteins. The reducing reagents (ethanol, isopropanol) are inexpensive. They are not, however, strong reducing agents and therefore have to be used in high concentration of alcohols.

The isopropanol/secondary alcohol dehydrogenase recycling system has some advantages over the ethanol/alcohol dehydrogenase. First, the equilibrium constant for reduction of NAD to NADH is quite favorable and second, isopropanol and acetone are less toxic towards proteins. An improvement to the

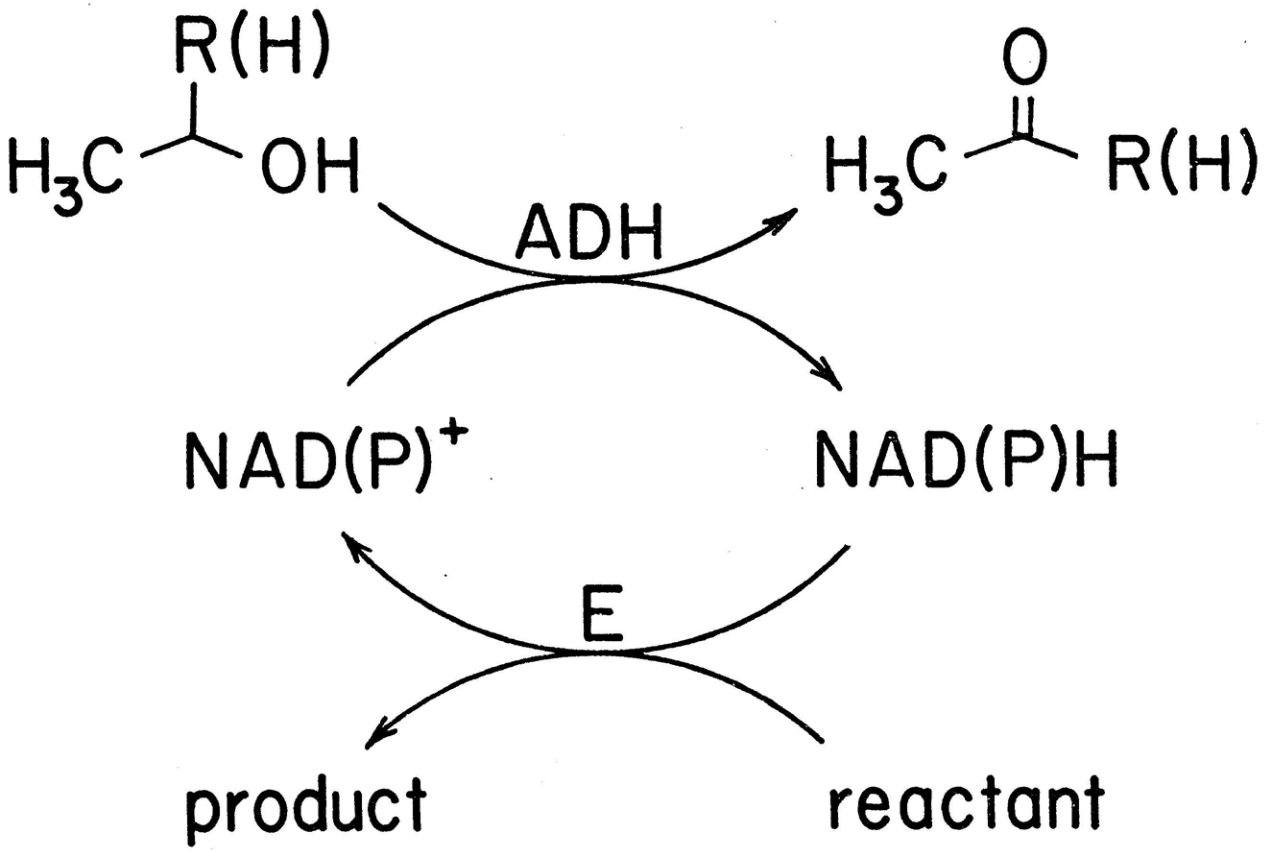
Scheme 4. Enzymatic Regeneration of the Nicotinamide Cofactors

Scheme 4



Scheme 5. The Enzymatic Regeneration of the Nicotinamide Cofactors Based on Alcohol Dehydrogenase.

Scheme 5

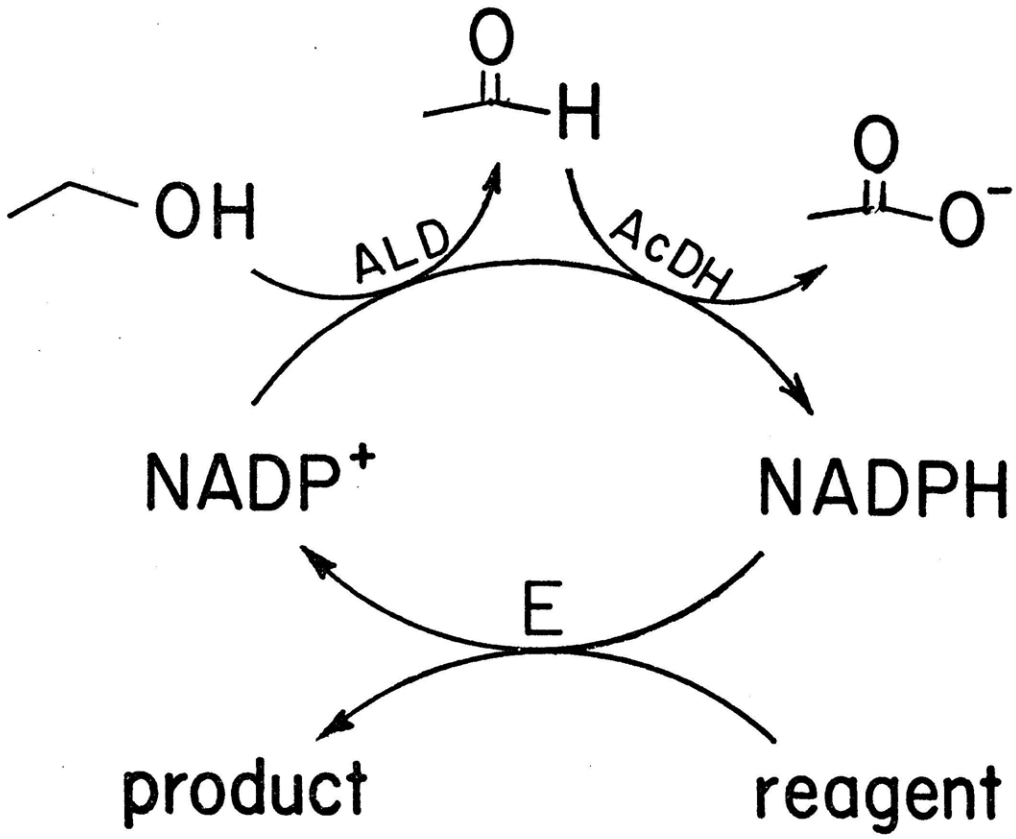


ethanol/alcohol dehydrogenase system is the addition of acetaldehyde dehydrogenase (Scheme 6).[6] This results in a high equilibrium constant for the reduction of NAD to NADH ($\Delta G_o' = -53$ kcal $mol^{-1}$). NADH is a substrate of acetaldehyde dehydrogenase from S. cerevisiae while NADPH is a substrate of this enzyme from L. mesenteroides. Both enzymes are stable and commercially available. The disadvantage is the need for two enzymes and the formation of acetate as the by-products.

The glucose-6-phosphate/glucose-6-phosphate dehydrogenase NAD(P)H regeneration system was developed recently[4] (Scheme 7). This system is useful in enzyme-catalyzed organic synthesis. Glucose-6-phosphate dehydrogenase from Leuconostic mesenteroides is commercially available, inexpensive, and stable. The enzyme has a high equilibrium constant for the reduction of both nicotinamide cofactors (NAD and NADP). This system possesses as well some disadvantages. First, glucose-6-phosphate is an expensive reducing reagent. Secondly, there are difficulties in separating the products from the by-product 6-phosphogluconate. Finally, glucose-6-phosphate is a catalyst for the decomposition of NAD(P)H. An improvement of this system is the substitution of glucose-6-sulfate for glucose-6-phosphate.[7] The reduced nicotinamide cofactors are more stable in solutions of glucose-6-sulfate than in solutions of glucose-6-phospate. Glucose-6-sulfate appears to be useful for the recycling of NADPH. In addition, glucose-6-sulfate is more easily prepared than

Scheme 6. The Enzymatic Regeneration of NAD(P)H Using Alcohol Dehyrogenase and Acetaldehyde Dehydrogenase.

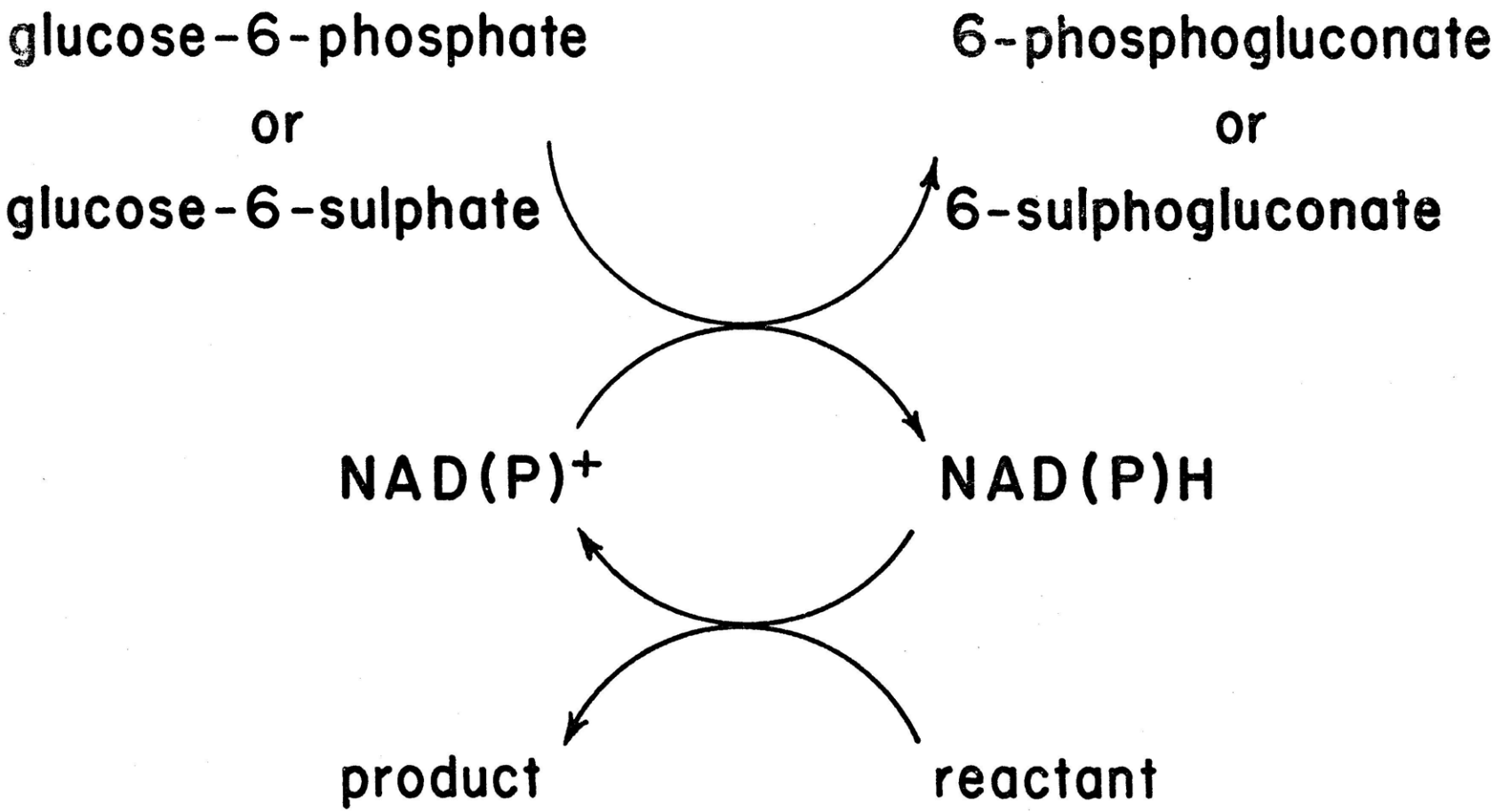
Scheme 6




AcDH — Acetaldehyde Dehydrogenase

Scheme 7. The Enzymatic Regeneration of NAD(P)H Using Glucose-6-Phosphate or Glucose-6-Sulfate and Glucose-6-Phosphate Dehydrogenase.

Scheme 7


glucose-6-phosphate
or
glucose-6-sulphate
6-phosphogluconate
or
6-sulphogluconate
NAD(P)+
NAD(P)H
product
reactant

glucose-6-phosphate. Glucose-6-phosphate or glucose-6-sulfate and glucose-6-phosphate dehydrogenase can be regarded as one of the best systems available for laboratory scale preparation.

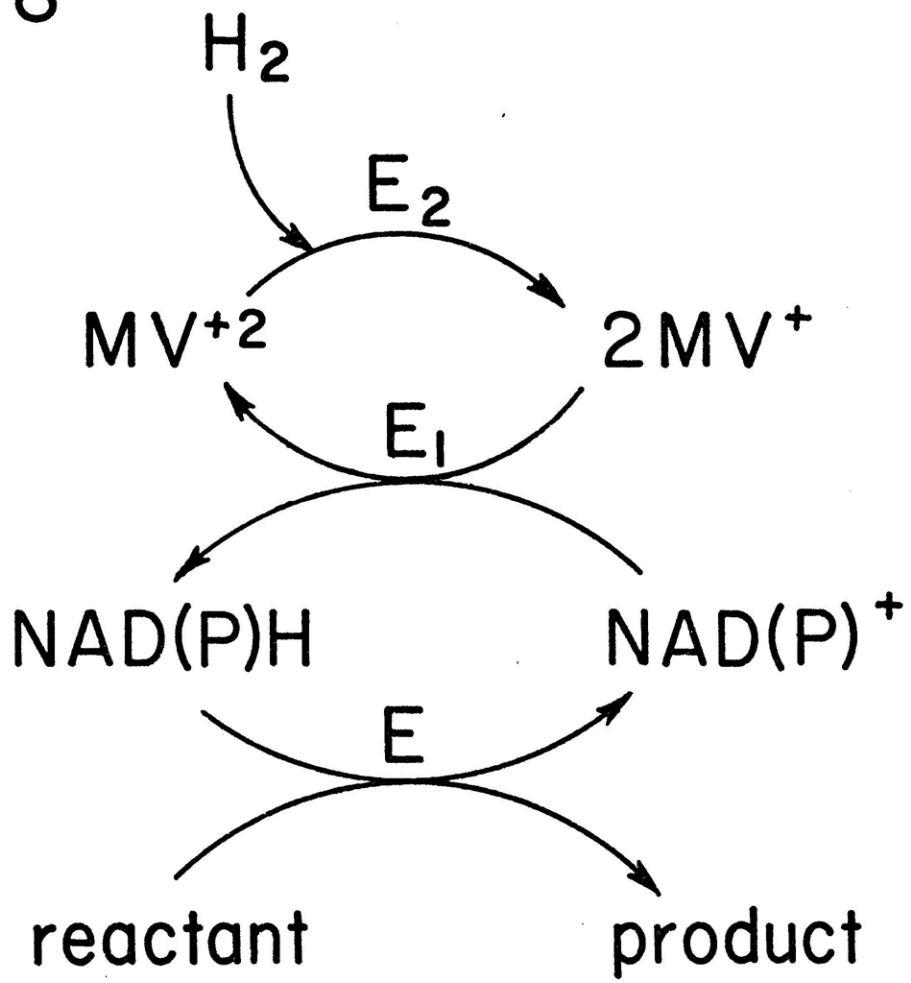
Many efforts have been directed toward the development of nicotinamide cofactor regeneration systems based on dihydrogen since it is the least expensive reducing reagent.[8-10] These systems are more complicated. A scheme has been proposed recently. It requires two enzymes: lipoamide dehydrogenase (NAD) or ferridoxin reductase (NADP) and hydrogenase and in addition, an electron transfer compound such as methyl viologen (Scheme 8).[11]

The hydrogenase based systems suffer from many disadvantages. They require hydrogenases that are unstable, expensive, and not available commercially. Furthermore, these systems contain additional enzymes that are not stable under the reaction condition. The electron transfer compounds (methyl viologen or diazaflavin) are toxic. An interesting system where hydrogenase has been replaced by a water-soluble rhodium(I) catalyst has been described recently.[12] This system, however, is presently not practical.

The purpose of this work was to develop a regeneration system based on formate and formate dehydrogenase. This system (Scheme 9) seemed to be attractive and advantageous over some of the other systems proposed. Formate is inexpensive (5¢/mol) and is a strong reducing agent. The equilibrium constant for the reaction:

Scheme 8. The Enzymatic Recycling of NAD(P)H Based on Hydrogenase and Lipoamide Dehydrogenase (NADH) or Hydrogenase and Ferridoxin Reductase (NADP).

Scheme 8



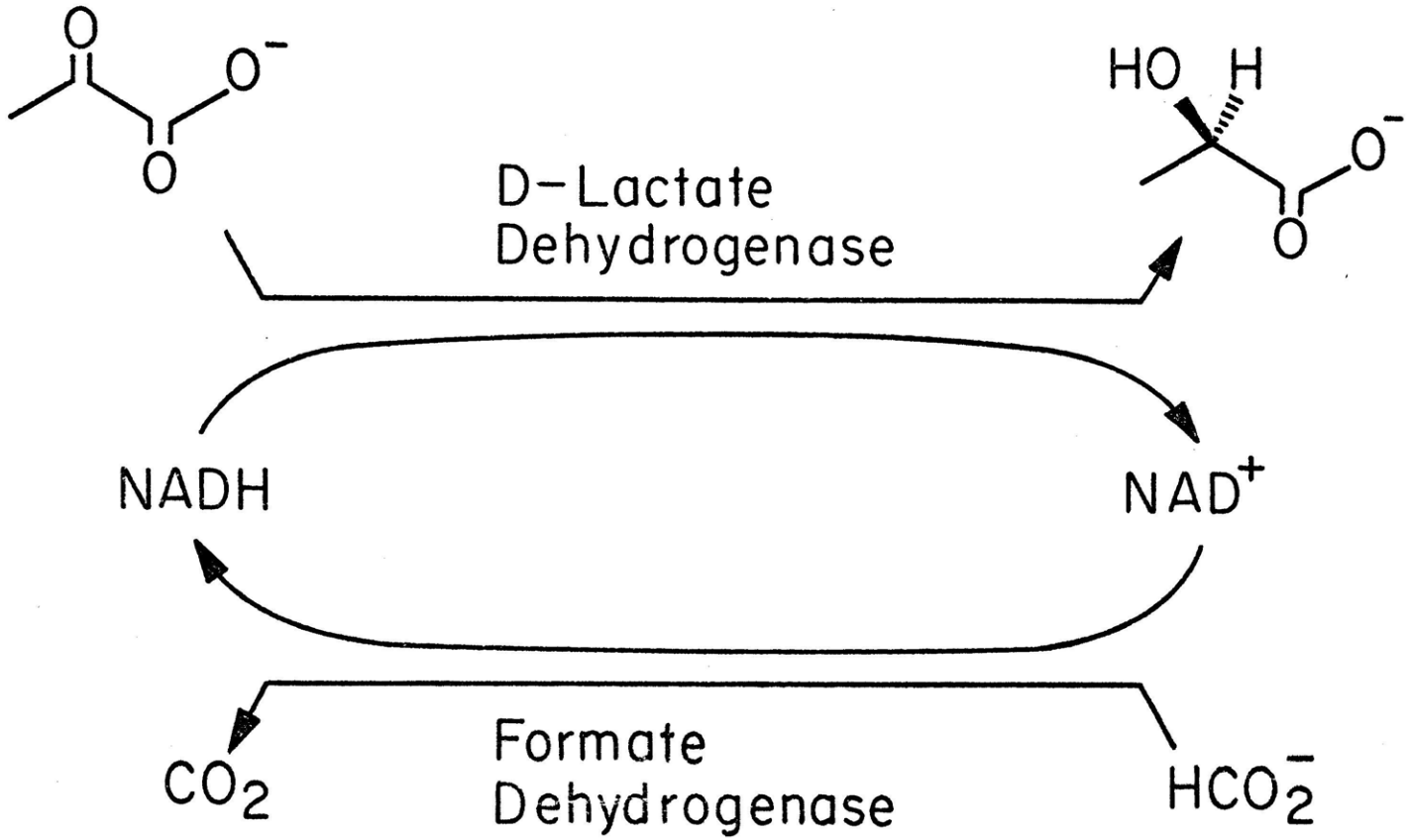

MV – Methyl Viologen

$E_2$ – Hydrogenase

$E_1$ – Lipoamide Dehydrogenase ($NAD^+$), Ferridoxin Reductase ($NADP^+$),

Scheme 9. The Enzymatic Recycling of NADH Using Formate and Formate Dehydrogenase.

# Scheme 9


D-Lactate
Dehydrogenase
NADH
NAD+
CO2
Formate
Dehydrogenase
HCO2-

$$HCO_2^- + NAD^+ \rightarrow CO_2 + NADH$$

$$\log K = 5.79 \text{ (based on } \Delta G = -7.95)^{14}$$

is very favorable in the forward direction. Moreover, $CO_2$ and formate are innocuous towards most enzymes. Carbon dioxide can be removed from the reactions as formed and does not complicate the workup of the reaction. Formate dehydrogenase (from Candida boidinii) is commercially available.[14] A large scale isolation and purification was described recently.[17] The enzyme is specific for NAD.[17] Other formate dehydrogenases specific to NAD and NADP are known, but are not commercially available.[18-20]

A recycling system based on formate/formate dehydrogenase seems to be especially attractive for large-scale application. This study examined some of the properties of formate dehydrogenase like stability, stability upon immobilization, stability under operating condition, and a partial characterization of the cysteine residue(s) in the enzyme.

A large scale synthesis (0.5 mol) of D-lactate using the formate/formate dehydrogenase NADH regeneration system was performed. A preliminary report of this work is described in reference 21.

## Results and Discussion

Properties of Yeast Formate Dehydrogenase from Candida boidinii. Yeast formate dehydrogenase (formate: NAD oxidoreductase (EC 1.2.1.2) catalyzes the oxidation of formate by

NAD (eq. 1). The properties of this enzyme were described recently.[16,17]

$$NAD^+ + HCO_2^- \rightarrow CO_2 + NADH \qquad (1)$$

The enzyme will utilize a number of NAD analogs but is specific for formate. The enzyme is a dimer, composed of two identical subunits, each with MW of 36000. All unchanged formate analogues, including formamide, formaldehyde, and methanol, neither undergo catalysis nor bind to the enzyme-nucleotide complex. No activity was detected at concentrations in excess of 1 mM with NADP, dichlorophenolindophenol, methyl viologen or methylene blue. The most effective inhibitors are linear triatomic anions, while the trigonal tetratomic anions are considerably poorer inhibitors. Azide is the most effective inhibitor tested. The Michaelis constants ($K_m$) are 0.03 mM for NAD and 13 mM for formate. The reaction rate increases quite steeply with temperature up to 55°C, and the enzyme has a broad pH optimum of activity between 7.5 and 8.5. The specific activity of the purified enzyme is 3 U/mg protein at 25°C.

Properties of Modified Formate Dehydrogenase from Candida Boidinii. Formate dehydrogenase (FDH) contains cysteine residue(s). p-Chloromercuribenzoate, $Cu^{+2}$, and $Hg^{+2}$ are strong inhibitors and it was suggested that there is at least one cysteine amino acid residue that is essential for the activity.[17] In this study the thiol chemistry of this enzyme was explored in some detail. The modified protein

FDH($SSCH_3$) was prepared by reaction of formate dehydrogenase with methyl methanthiosulfonate ($CH_3SSO_2CH_3$).[22,23] Prolonged treatment with $CH_3SSO_2CH_3$ produced a modified protein that retained 25%(± 3%) of the activity of the native enzyme. Assay of FDH($SSCH_3$) for residual thiol groups using Ellmans's reagent indicated that less than 0.001 of the thiols present in the native enzyme remained. It is reasonable to assume therefore, that all the cysteine residues of formate dehydrogenase were modified upon treatment with $CH_3SSO_2CH_3$. Full activity (>98%) was regenerated following incubation with dithiothreitol. Treatment of formate dehydrogenase with an excess of 5,5'-dithiobis-(2-nitrobenzoic acid), (Ellman's reagent), generated another modified protein FDH(SSEllman's) that had <0.1% of the activity of the native formate dehydrogenase. Incubation of FDH(SSEllman's) for 1 hr in the presence of dithiothreitol in excess restored the full activity of the enzyme (>98%). A detailed characterization of the modified formate dehydrogenase is difficult since the crystal structure of this enzyme is still unavailable. Nevertheless, these results indicate that at least one thiol group is close to the active center. It is not, however functionally involved in catalysis. Blocking this cysteine residue by an S-methyl group results in a substantial loss in activity (∿75%). One can assume therefore that this cysteine thiol participates in substrate binding or that its proximity to the active site results in severe steric interference upon modification. Studies with other enzymes (lipoamide dehydrogenase,

adenylate kinase) indicated that thiol(s) can be important in the interaction between $NAD^+$ and the enzymes.[24,25] Alkylation of the thiol(s) prevents charge transfer between $NAD^+$ and the enzyme.

Stability of Native and Modified Formate Dehydrogenase. The enzyme from yeast is relatively insensitive to oxygen and probably does not require a metal ion for enzymatic activity.[17] Thiols (dithiothreitol, mercaptoethanol) do not affect the activity of the native enzyme. This insensitivity to oxygen is unique among formate dehydrogenases since oxygen-induced inactivation is characteristic for most known formate dehydrogenases[18, 19, 20, 26]

The inactivation of formate dehydrogenase in the presence of various substances (Figure 2) showed that native formate dehydrogenase inactivates almost completely in 3-4 days at room temperature. The inactivation rate of the enzyme was significantly diminished in the presence of formate (0.3M) and NADH (3 mM). Formate was a better protecting agent than NADH. EDTA had a strong stabilizing effect and after two weeks the enzyme retained ~90% of the initial activity. The combination of EDTA (10 mM) and 1,3-dithiol-2-propanol[27] (DTP, 20 mM) was especially powerful in protecting the enzyme. Modified formate dehydrogenase ($FDH(SSCH_3)_n$ that retained 25% of the native enzyme upon modification was stable at room temperature for over three weeks, and at 4°C for one year when kept under air.

Figure 2. Stability of Native and Modified Formate Dehydrogenase in the Presence of Various Substances.

Enzyme activity was assayed as described for FDH. The enzyme (18U/mL) was stored in 50 m$\underline{M}$ Tris-$H_2SO_4$ buffer pH 7.6 at 25°C in the presence of air.

(●) native enzyme (no additives)

(Δ) 3 m$\underline{M}$ NADH

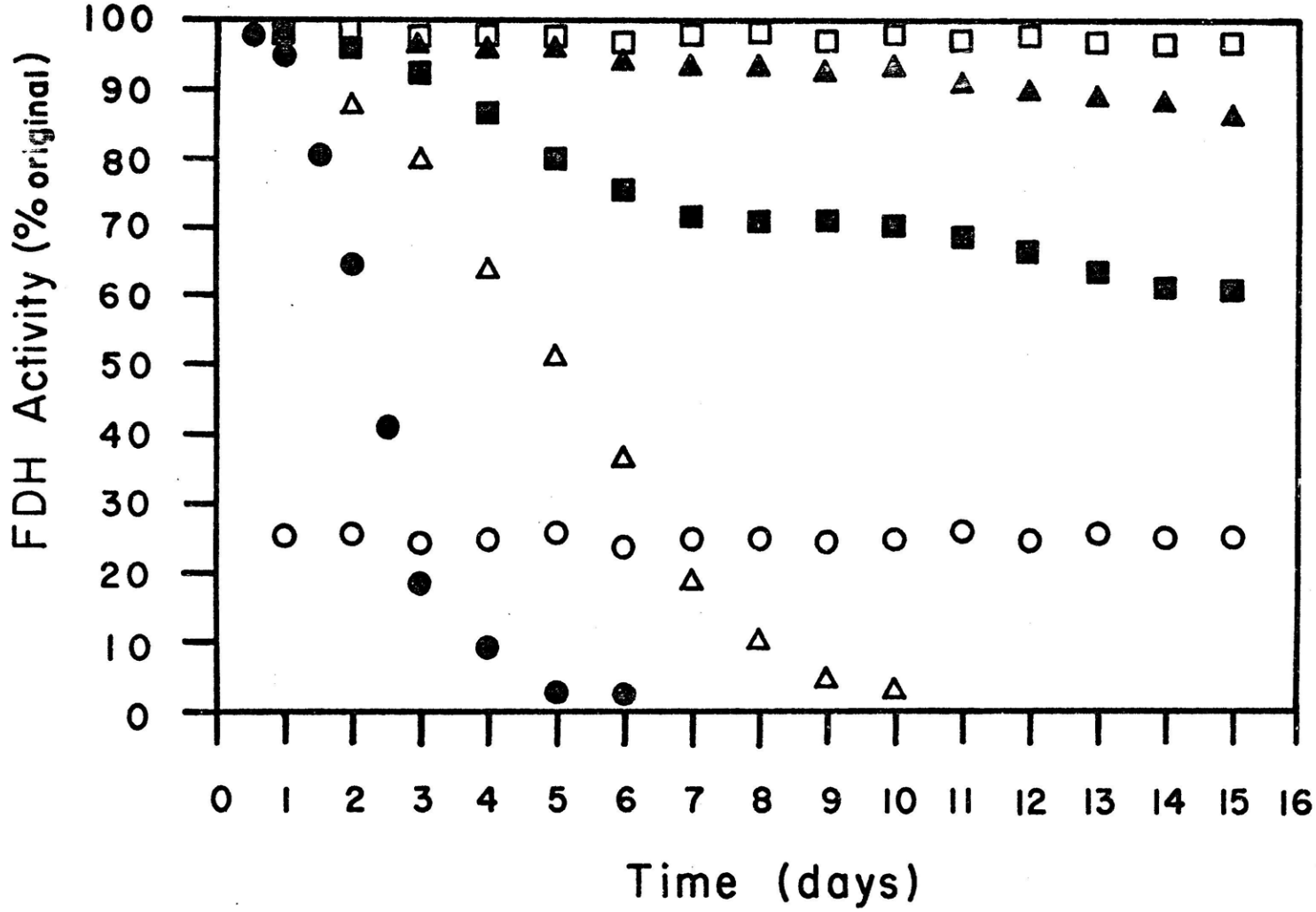
(■) 2 m$\underline{M}$ NADH + 0.3$\underline{M}$ formate

(▲) 10 m$\underline{M}$ EDTA

(□) 20 m$\underline{M}$ DTP + 10 m$\underline{M}$ EDTA

(○) modified enzyme [FDH$(SSCH_3)_n$] (no additives)

Figure 2

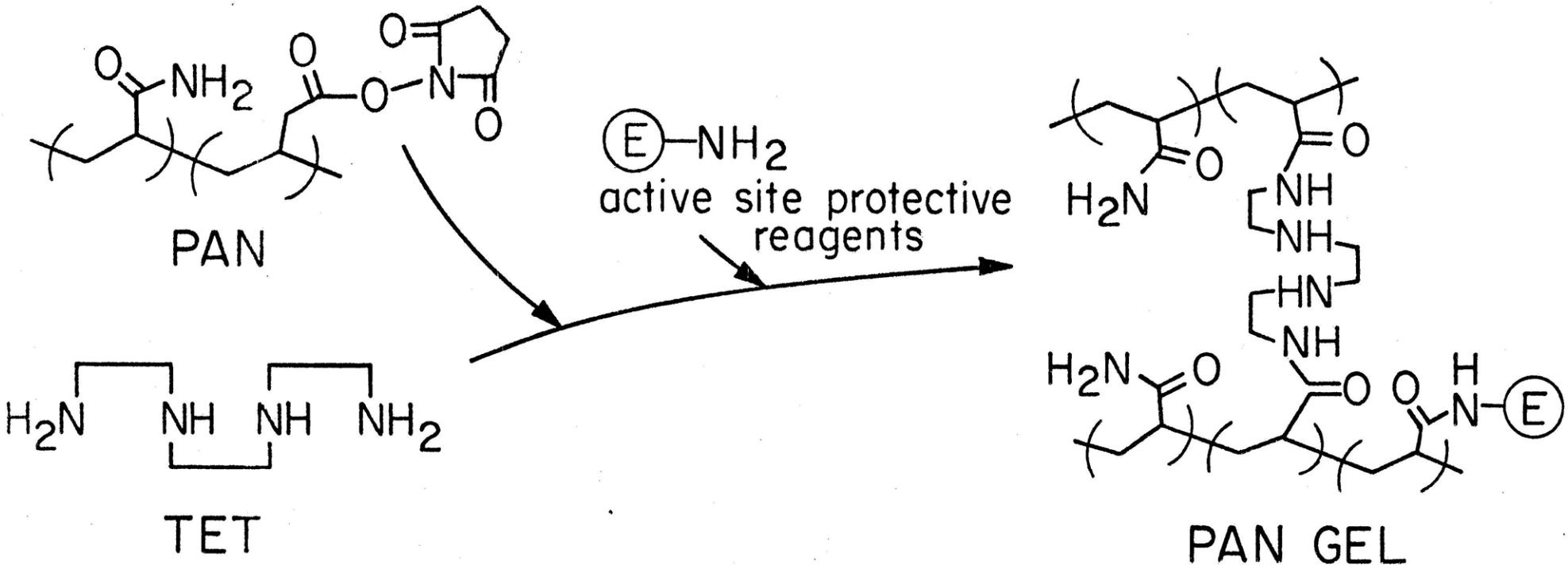

FDH Activity (% original)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)

Immobilization of Formate Dehydrogenase by Condensation Polymerization. The enzyme immobilization method is based on the condensation polymerization of poly (acrylamide-co-N-acryloxy-succinimide) (PAN) and the α, ω diamine triethyltetramine (TET) as crosslinking reagent in the presence of the enzyme(s) and additives (substrate and cofactors) at pH 7.6 (Scheme 10, 10a). This method that was developed by Whitesides et. al.[28] is especially useful in large-scale syntheses of fine chemicals. Yields for immobilization for formate dehydrogenase averaged 40-45%, In one case it went as high as 55%. The cofactors needed to obtain good immobilization yields were: formate (80 mM), NAD (3 mM), NADH (0.1 mM). The loading capacity of formate dehydrogenase for the polymer PAN-1000 was not optimized. Typically 5-10 mg of protein were dissolved in 3 ml of buffer for each gram of PAN-1000. The final activity of the gel was 0.2 - 0.25 U/mL of swollen gel.

Stability of Immobilized Formate Dehydrogenase. The immobilization enzyme was stored in 50 mM Tris-$H_2SO_4$ buffer pH 7.6 at room temperature in the presence of EDTA(10 mM), DTP (10 mM), and formate (0.1 M) under nitrogen (Figure 3). In the presence of the additives the enzyme retains >98% of its original activity for at least two weeks at room temperature. Without the additives, under air and after two weeks the immobilized enzyme retains only ∿ 80% of the initial activity

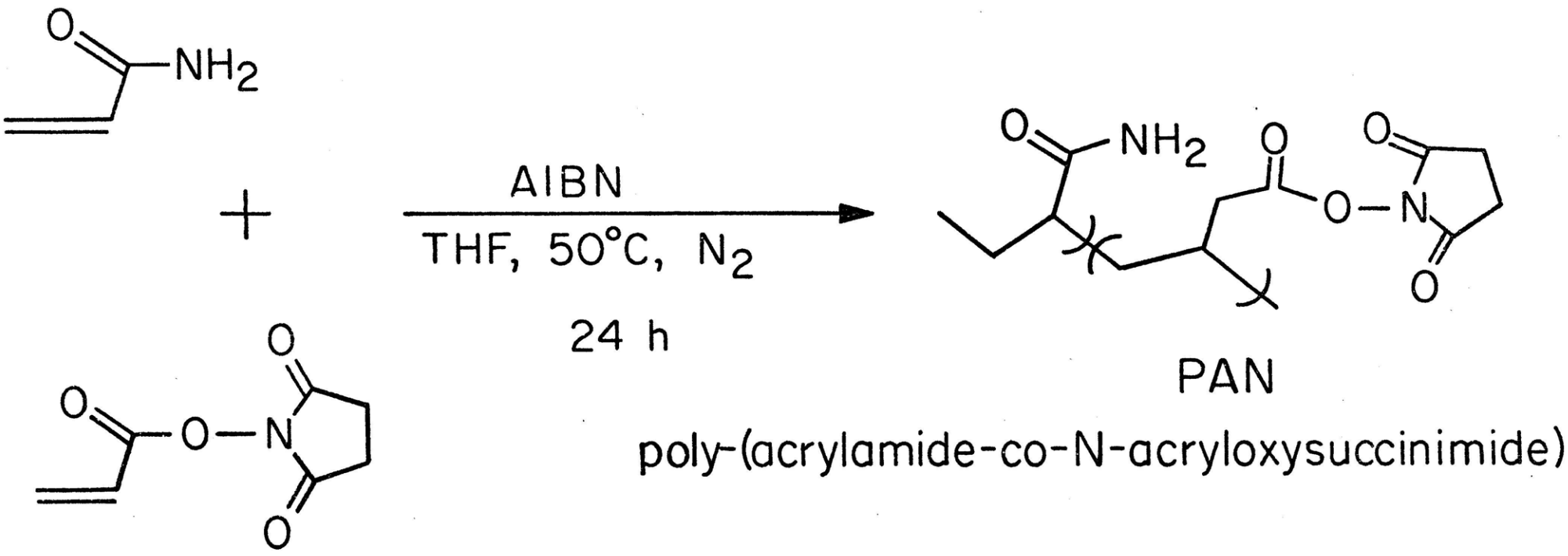
Scheme 10. The Enzyme Immobilization Method Based on the Condensation Polymerization of PAN and TET.

## Scheme 10



Scheme 10a. The Synthesis of PAN.

## Scheme 10a Synthesis Of PAN




PAN

poly-(acrylamide-co-N-acryloxysuccinimide)

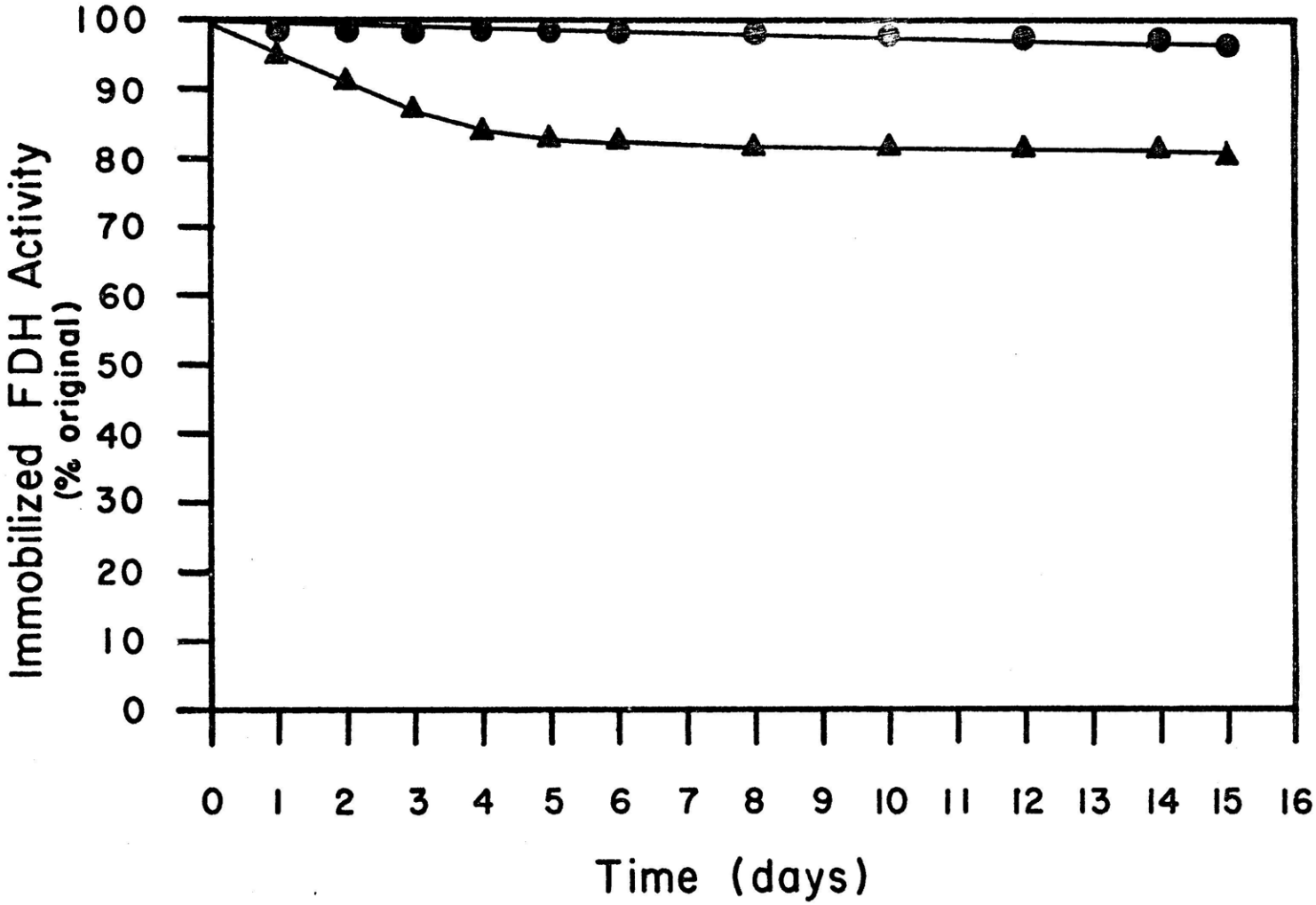
Figure 3. Stability of Immobilized Formate Dehydrogenase.

The enzyme was stored in 50 m$\underline{M}$ Tris-$H_2SO_4$ buffer pH 7.6 at 25°C.

(●) Immobilized FDH and EDTA (10 m$\underline{M}$ + DTP (10 m$\underline{M}$) and 0.1$\underline{M}$ formate under nitrogen

(▲) Immobilized FDH (no additives, under air)

Figure 3


Immobilized FDH Activity
(% original)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)

Coimmobilization of Formate Dehydrogenase and D-Lactate Dehydrogenase. Coimmobilization of the enzymes in the same polyacrylamide gel increases the efficiency of conversion. This coupling has been well established in enzymatic reactors where two enzymes are required for the conversion of reactants to products.[29] Formate dehydrogenase and D-lactate dehydrogenase were coimmobilized using PAN-1000. The yields of the coimmobilized formate dehydrogenase and L-lactate dehydrogenase were (5-10%) lower than that of the individually immobilized enzymes. Yields of immobilization for formate dehydrogenase averaged 40-50% and for L-lactate dehydrogenase 45-55%. The following substrates and cofactors were needed: pyruvate (30 mM), formate (80 mM), NAD (5 mM), and NADH (1 mM).

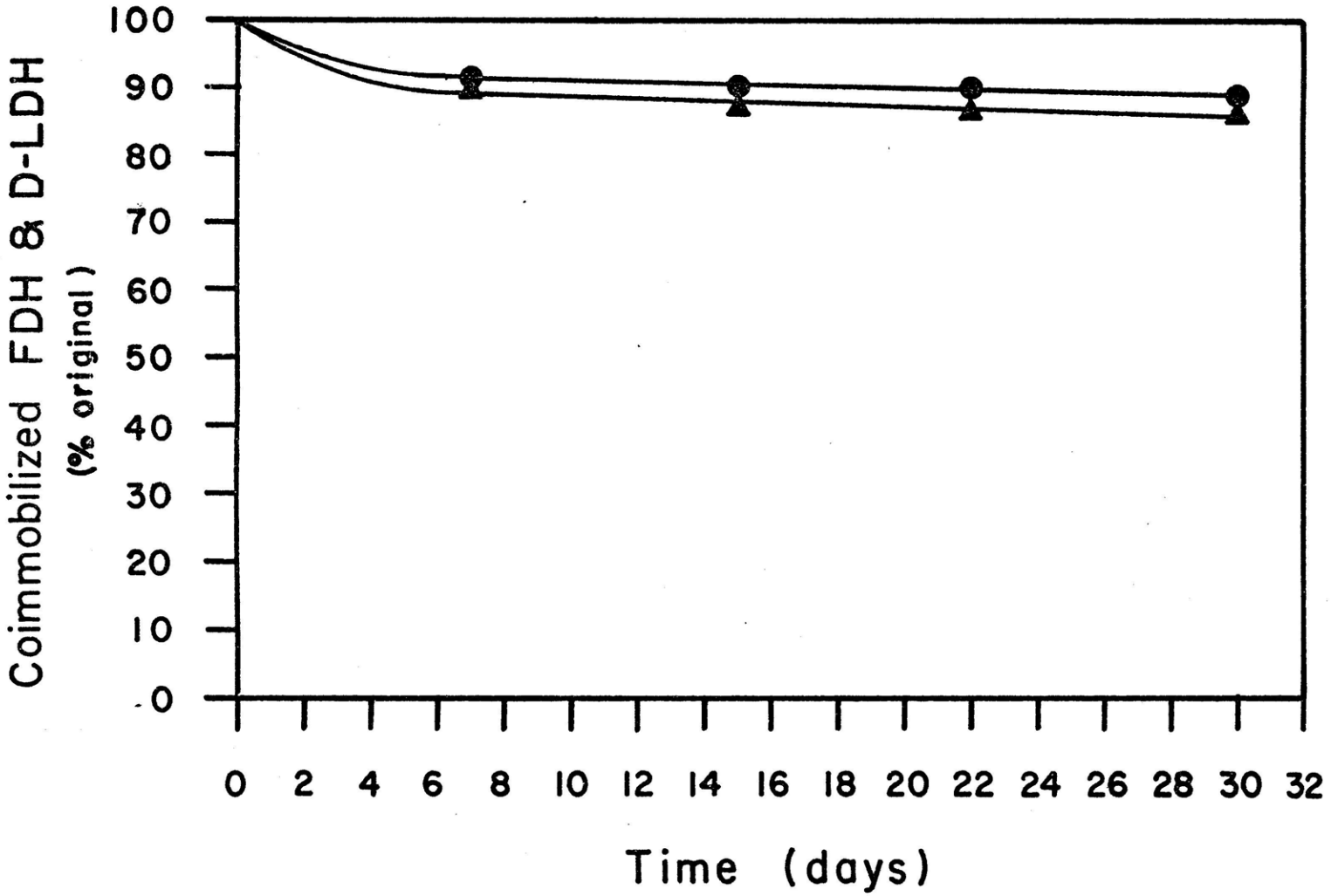
Stability of Coimmobilized Formate Dehydrogenase and D-Lactate Dehydrogenase. The stability of the coimmobilized enzymes were studied under operating conditions and upon long periods. The activities of formate dehydrogenase and D-lactate dehydrogenase after two consecutive runs (30 days) were 86% and 89% of the original immobilized activity (Figure 4). After storage for 3 months at 4°C under nitrogen and in the presence of formate (0.1 M), EDTA (10 mM), DTT (10 mM) the enzymes retained >98% of their activity. The activities of formate dehydrogenase and D-lactate dehydrogenase after storage at 4°C for 12 months in the presence of these additives and under $N_2$, were respectively 85% and 82% of the activities at the start of the storage.

Figure 4. Stability of Coimmobilized Formate Dehydrogenase and D-Lactate Dehydrogenase Under Operating Conditions

(●) D-Lactate Dehydrogenase

(▲) Formate Dehydrogenase

Figure 4


Coimmobilized FDH & D-LDH
(% original)
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (days)

Large-Scale Synthesis of D-Lactate. Coimmobilized formate dehydrogenase (52 units, 47% yield on immobilization) and D-lactate dehydrogenase (310 units, 51% yield on immobilization), were used for the large-scale synthesis of D-lactate. The reactor was loaded with 500 mmol of sodium pyruvate, 500 mmol of sodium formate and 0.39 mmol of NAD. Reaction was complete in 15 days (Figure 5). (See Figure 6 for the ractor set-up.). Enzymatic assay showed that 490 mmol of D-lactate had been produced in 1.1 L of solution. The activities of formate dehydrogenase and D-lactate dehydrogenase in the recovered gel after two consecutive runs (30 days) were respectively 86% and 89% of the original immobilized activities. The NAD turnover number[30] was 1250. The product was isolated as the zinc salt. A total of 47.9 g of white powder was recovered, 395 mmol of D-lactate, 70% yield based on pyruvate. The isolated product was 95% pure and it contained a 94% enantiomeric excess of D-lactate as determined by enzymatic assays for D- and L-lactate. The same reaction was carried out at 40°C. The conversion of pyruvate ($\sim$ 500 mmol) to lactate was completed in seven days. Both enzymes retained > 95% of their activity over the course of the reaction.

The cell-free enzymatic synthesis of D-lactate employing the formate/formate dehydrogenase NADH regeneration system was performed analogously on a 0.1-0.2 mol scale. The turnover numbers for NAD were between 1200 and 1500, and in one case as high as 1750.

Figure 5. Large Scale Synthesis of D-Lactate (mmol) at 25°C and 40°C as a Function of Time (Days)

(●) At 25°C

(■) At 40°C

Figure 5

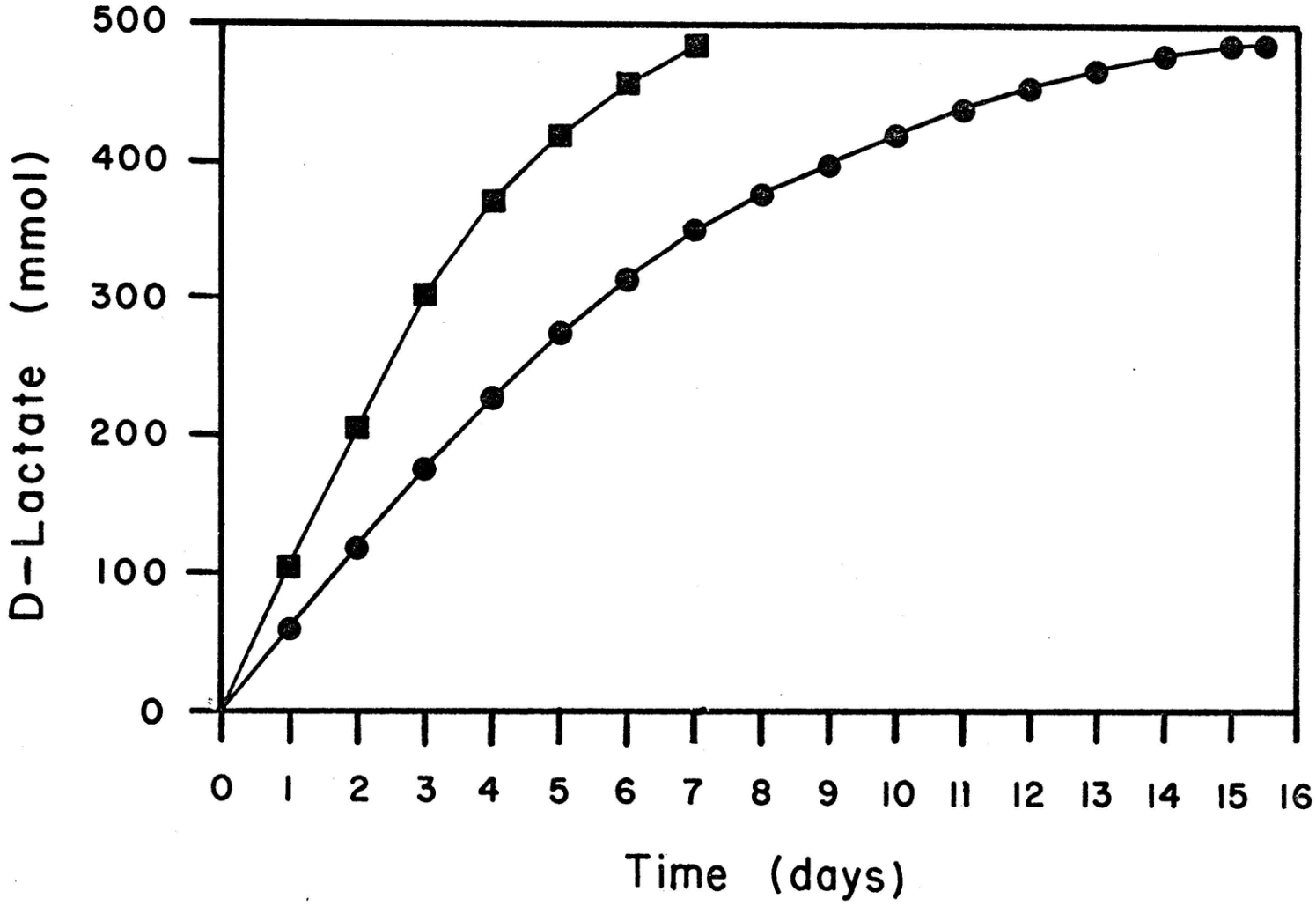
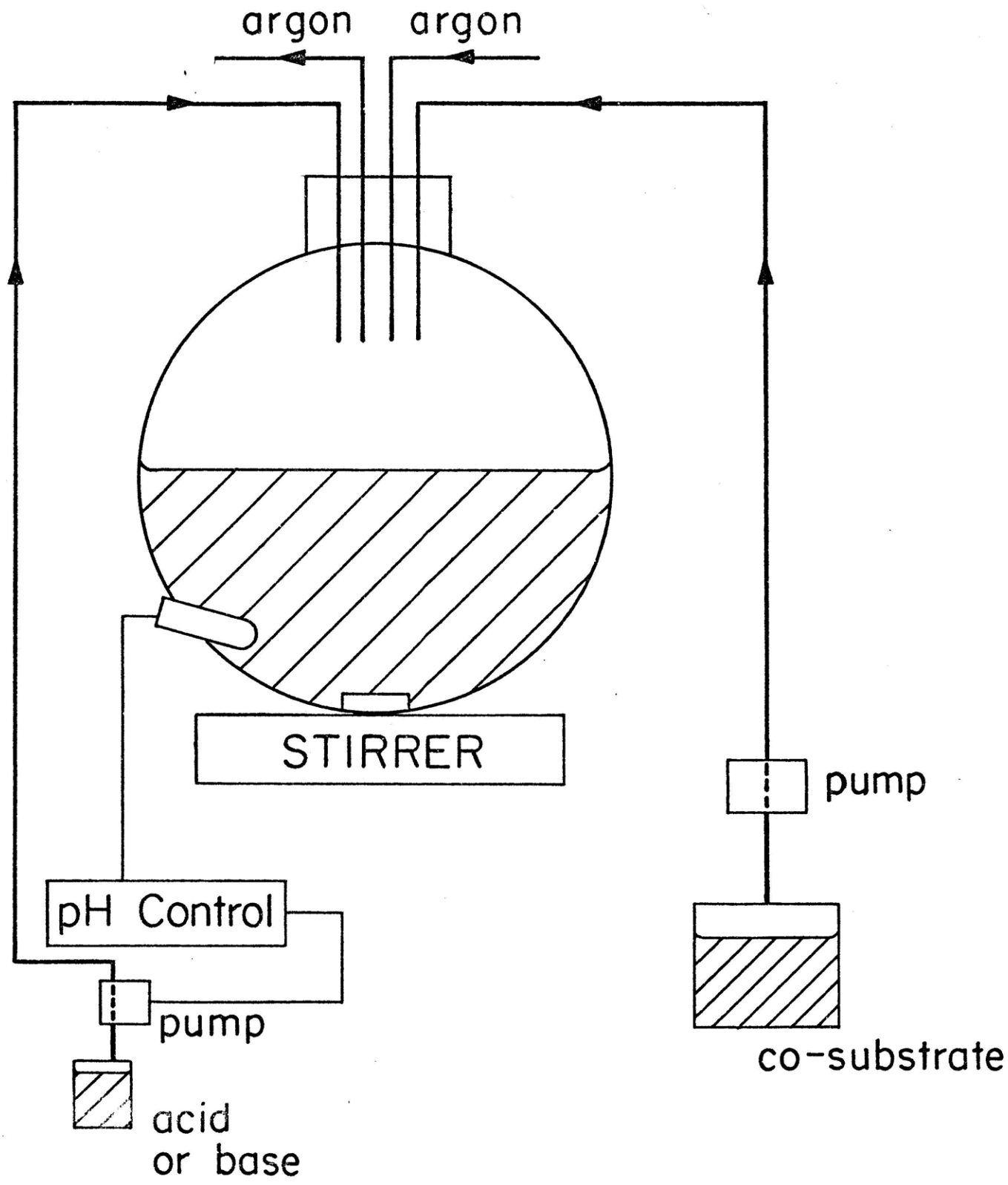

D-Lactate (mmol)
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)

Figure 6. Reactor Set-Up for the Synthesis of D-Lactate

# Figure 6: REACTOR



Recovery of the Coimmobilized Enzymes. The coimmobilized enzymes were recovered at the end of each reaction by allowing the gel to sediment and then were assayed for activity. The same coimmobilized enzymes were used 6 consecutive times for the synthesis of D-lactate.

Stability of NAD Under the Operating Conditions. The intrinsic instability of NAD and NADH is a major obstacle to achieving efficient recycling systems with high turnover numbers ($10^3$-$10^5$). This problem is particularly severe in systems that employ enzymes with low specific activity (<30 U/mg protein). The formate/formate dehydrogenase system with a specific activity of 3 U/mg protein, certainly belongs to this category. The prolonged time of conversion of the reactant to product (especially on large-scale) contributes significantly to the inactivation of the cofactor. Half of the enzymatically active form of the cofactors (NAD + NADH) disappeared at 25°C in ∿ 15 days. When the reaction was performed at 40°C, 2/3 of the total initial concentration of the nicotinamide cofactors was inactivated after 7 days (Figure 7).

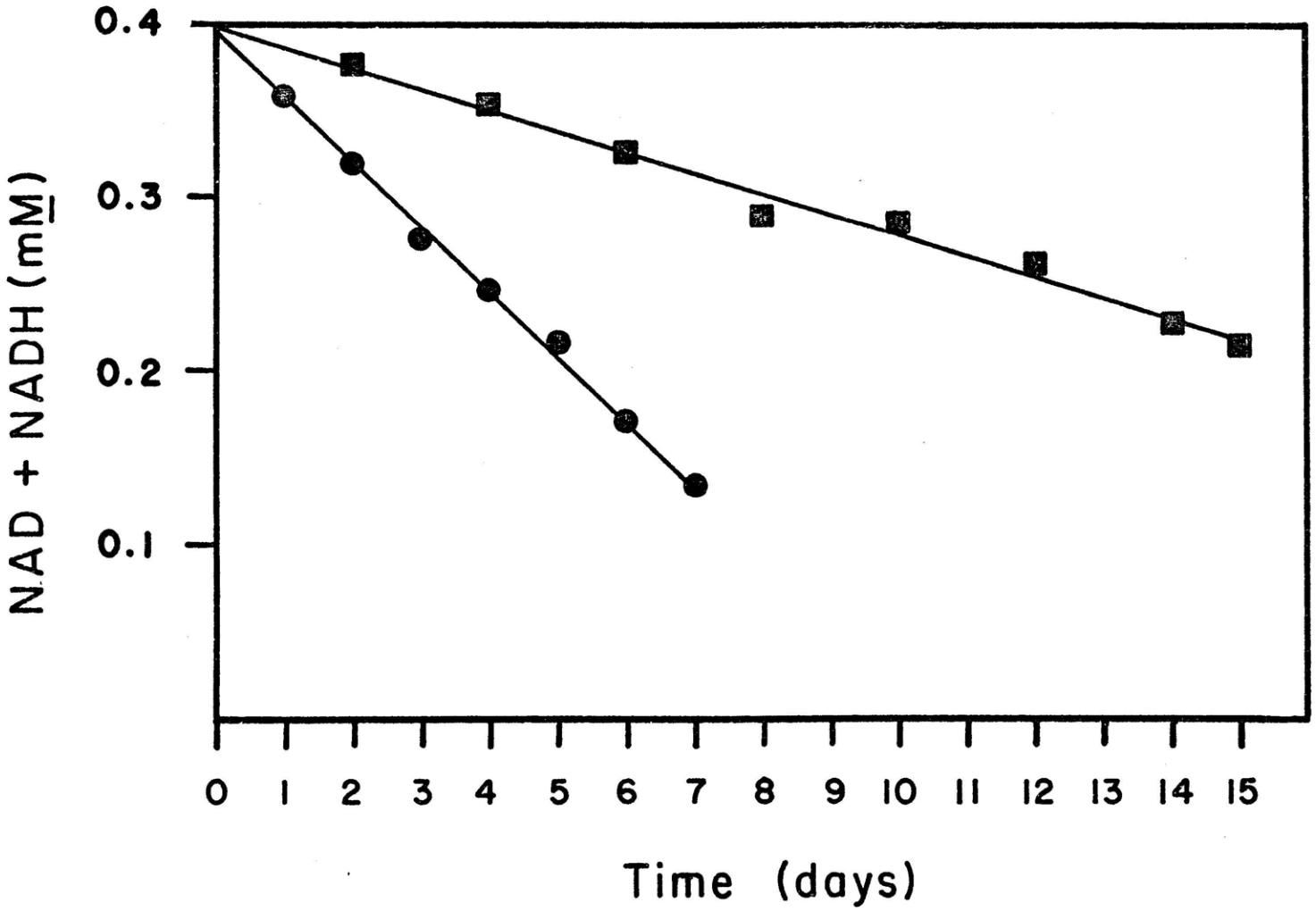
Figure 7. The Aggregate Concentration of NAD and NADH Over the Course of D-Lactate Synthesis at 25°C and at 40°C.

The cofactors were deterimed according to the enzymatic assays described for each of them in the experimental.

(■) At 25°C

(●) At 40°C

Figure 7



## Concluding Remarks

The NADH regeneration system based on formate and formate dehydrogenase is one of the most advantageous recycling schemes that has been proposed so far. Formate, a strong reducing agent, is relatively less expensive than other co-substrates such as ethanol, isopropanol or glucose-6-phosphate, and is innocuous toward most enzymes even at high concentrations. Furthermore, $CO_2$, the by-product, is removed from the reactor as it is formed and the workup of the reaction does not require tedious separation of the product from the by-product (aldehydes, ketones, acetic acid, glucose-6-phosphate) produced in other recycling systems.

Presently, the enzyme is expensive ($60/100 U) but a large scale (200000 U) isolation procedure has been described recently so that it is reasonable to assume that the price of this enzyme will come down. The most serious disadvantage of the formate/formate dehydrogenase system is the relatively low specific activity of the enzyme, 3U/mg protein (when compared to the S.A. of G-6-PDH of 400 U/mg). This disadvantage can in principle be overcome by finding sources for formate dehydrogenase having higher specific activities. Synthesis on a scale of several moles can be done by immobilizing large quantitites of the enzyme.

## Experimental

### General

Formate dehydrogenase (EC 1.2.1.2.) from Candida boidinii as a lyophilized powder from Boehringer Mannheim Biochemicals contained 3U/mg of protein. D-lactate dehydrogenase from Lactobacillus leichmanii (as a suspension in 3.2M ammonium sulfate,(320U/mg protein), NAD, NADH, DTT, sodium pyruvate and L-lactate dehydrogenase (from rabbit muscle lyophilized powder) were obtained from Sigma Chem. Co. Sodium formate, 5.5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), 1,3-dithiol-2-propanol were obtained from Aldrich Chem. Co. Dialysis was performed using an ultrafiltration cell (Amicon Diaflow, XM 10 membrane) or using cellophane tubing. The dialysis buffer was 100 mM Tris-HC , pH 7.6 containing 4 mM DTT and 5 mM pyruvate. pH measurements were done using a Radiometer PHM62 standard potentiometer. Water was doubly distilled using a Corning AG-1B still. Spectrophotometric measurements were carried out on either a Gilford Model 240 or Perkin Elmer Model 552 spectrometers, both equipped with a constant temperature cell compartment. Methanethiolsulfonate was synthesized according to a procedure described previously.[22]

Formate Dehydrogenase Assay.[31,17] The activity of formate dehydrogenase was determined by measuring the rate of NADH formation at 340 nm. The activity was calculated as enzyme units (U, i.e. μmol/min) by using $\varepsilon = 6220 \times mol^{-1} \times cm^{-1}$ for

NADH at 340 nm. The assay mixture for formate dehydrogenase was composed of the following standard solutions:

1. 1 mL of 50 mM Tris-HCl buffer solution (1.21 g/50 mL of water), pH 7.6
2. 1 mL of 75 mM NAD (0.49g/10 mL dd $H_2O$)
3. 1 mL of 80 mM formate solution (10.56 g of sodium formate in 200 mL of 50 mM Tris-HCl buffer pH 7.6).

After incubation of the 3 mL assay mixture for 4 min an aliquot of formate dehydrogenase (or gel suspension) was added (0.01-0.05 mL) to give a total assay volume of 3.01 - 3.05 mL units of activity were calculated according to equation 2.

$$U = \frac{O.D.}{min} \times \frac{\text{assay vol (mL)}}{6.22\ \text{mmol}^{-1}\text{cm}^{-1}} \qquad (2)$$

D-Lactate Dehydrogenase Assay.[32] Unlike L-lactate dehydrogenase, D-lactate dehydrogenase occurs only in some microorganisms and plants. It participates in the degradation of carbohydrates by Lactobacillus leichmannii and Escherichia coli and it regulates the pyruvate-lactate equilibrium. The activity of the enzyme is determined according to equation 3.

$$\text{Pyruvate} + \text{NADH} + H^+ \longrightarrow \text{D-Lactate} + NAD^+ \qquad (3)$$

The change in optical density of 340 nm per min time is a measure of the D-lactate dehydrogenase activity.

The following solutions were prepared:

1. Phosphate buffer, 0.1 M, pH 7.0 (530 mg $KH_2PO_4$ + 1.39 g $K_2HPO_4 \cdot 3H_2O$/100 mL $H_2O$)
2. Sodium pyruvate solution, 30 mM (326 mg sodium pyruvate/ 100 mL)
3. NADH solution, ∿ 24 mM (40 mg NADH, sodium salt/2 mL).

The assay mixture was made by mixing in a 3 mL cuvette; phosphate buffer (2.83 mL), sodium pyruvate solution (0.10 mL), NADH solution (0.05 mL). Then, after mixing and incubation for 4 min an aliquot of D-lactate dehydrogenase (0.01-0.02 mL) was added. After mixing again the optical activity is read after 1, 2 and 3 min against a cuvette that contained all the components except the enzyme sample. The calculated mean from the measured O.D./min values is substituted in eq. 2.

L-Lactate Dehydrogenase Assay.[33] L-lactate dehydrogenase was determined by a procedure analogous to that described for D-lactate dehydrogenase. The following solutions were prepared:

1. Phosphate buffer, 0.1 M (1.06 g $KH_2PO_4$ + 2.78 g $K_2HPO_4 \cdot 3H_2O$/1 L
2. Sodium pyruvate solution, 23 mM (250 mg sodium pyruvate/ 100 mL)
3. NADH solution, about (100 mg NADH, sodium salt/10 mL)
4. Commercially available L-lactate dehydrogenase (0.01 mL)

A 3 mL cuvette contained 2.85 mL phosphate buffer, sodium pyruvate solution (0.1 mL), NADH solution (0.05 mL). After mixing,

the sample (0.01 mL) was added and the optical density read at 1 min intervals. The blank contained all the components except instead of the sample, 0.01 mL of $H_2O$ was added. The activity of the enzyme (U/mL sample) was calculated according to eq. 2.

Enzymatic Determination of D(-)-Lactate.[34] D(-)-Lactate was determined quantitatively by a spectrophotometric assay using D-lactate dehydrogenase from Lactobacillus leichmannii. The NADH formed according to reaction (4) as measured by the change in optical density at 340 nm is proportionate to the amount of D-(-)-lactate in the sample.

$$\text{L-lactate} + NAD^+ \xleftrightarrow{\text{D-LDH}} \text{Pyruvate} + NADH + H^+ \qquad (4)$$

The equilibrium of the reaction lies well on the side of lactate and NAD. The equilibrium constant is $K = 2.9 \times 10^{-2}$ mol/L (25°C). For quantitative oxidation of D-lactate, the reaction product must be removed from the equilibrium. Protons are trapped by an alkaline reaction medium and pyruvate as the hydrazone. A large excess of NAD is used. The equation for the spectrophometric determination of L-lactate is:

$$\text{L-Lactate} + NAD^+ + \text{Hydrazine} \xrightarrow[\text{pH9.0}]{\text{LDH}} \text{Pyruvate Hydrazone} + NADH + H_3O^+$$

The equilibrium constant of this reaction is $K = 7 \times 10^2$ at 25°C and pH 9.5. A relatively high lactate dehydrogenase

activity is necessary for a sufficiently fast reaction. The following solutions were prepared.

1. Glycine buffer (500 mM 400 mM hydrazine; pH 9.0). Glycine (11.26 g) was dissolved in $H_2O$, hydrazine hydrate (25 mL was added followed by 300 mL of water. The pH was adjusted.
2. NAD (41 mM) obtained by dissolving 30 mg NAD in 1 mL water.
3. D-(-)-lactate dehydrogenase, (5 mg protein/mL) commercial preparation.

The assay mixture was prepared by pipetting successively into the cuvette: glycine buffer (3.0 mL), NAD solution (0.20 mL), sample (0.10 mL). After mixing the cuvette the optical density was zeroed against a blank that contained all the components and distilled water instead of sample. The D-LDH suspension (0.01 mL) (15 mg/mL or ∿ 5U/mL) was added, and the optical density change followed until constant (about 90 min). The obtained optical density was used to calculate the D-lactate concentration (eq. 6).

$$C = \frac{\text{volume in cuvette}}{\varepsilon \times \text{volume of sample}} \times \Delta O.D. \quad (\text{mmol/mL sample})$$

$$\varepsilon = 6.22 \text{ mmol}^{-1}/\text{cm}^{-2}$$

Enzymatic Determination of L-(+)-Lactate.[35] L-(+)-lactate is oxidized to pyruvate by NAD in the enzymatic reaction catalysed by L-lactate dehydrogenase according to the reaction.

$$\text{L-lactate} + NAD^+ \xrightarrow{\text{L-LDH}} \text{pyruvate} + NADH + H^+$$

The formation of NADH is measured by the increase in optical density at 340 nm. Because of the unfavorable equilibrium constant for the oxidation of lactate (see discussion in the enzymatic determination of D-lactate), hydrazine is added to the assay mixture. The 2.92 mL assay mixture contained the following solutions: 2.5 mL hydrazine/glycine buffer (0.4 M hydrazine; 0.5 M glycine pH 9.0), 0.2 mL NAD (40 mM), 0.2 mL sample. The optical density of this mixture was read against a blank that contained the buffer and NAD solution but instead of 0.2 mL of sample it contained 0.2 mL of 1 N perchloric acid. Then an L-lactate dehydrogenase suspension was added to the sample and the blank cuvettes. (0.02 mL, 20 U/mL). After mixing, the change in optical density is followed until a constant value (about 60 min). This value is used to calculate the L-lactate concentration (eq. 6).

Enzymatic Determination of $NAD^+$[36]. The determination of NAD is based on the reaction:

$$NAD^+ + \text{ethanol} \xrightarrow{\text{ADH}} NADH + H^+ + \text{acetaldehyde}$$

ADH - alcohol dehydrogenase.

The increase in optical density at 340 nm is due to the formation of NADH and is a measure of the NAD concentration.

The following solutions were prepared.

1. Sodium pyrophosphate semicarbazide buffer, 75 m$\underline{M}$, pH = 8.7 (33.3 g $Na_4P_2O_7 \cdot 10H_2O$, 8.4 g semicarbazide · HCl, 1.7 g glycine + 10 mL ethanol/1 L adjusted to pH = 8.7 with 2 $\underline{N}$ NaOH).
2. Alcohol dehydrogenase from yeast, commercially available (lyophilized 300 U/mg protein, 30 mg/1 mL). The buffer solution (2.89 mL) is transferred to a 3 mL cuvette followed by 0.10 mL sample. The ADH suspension is added (0.01 mL) and the optical density is read when the increase in absorbance due to the reaction stops. The concentration of NAD is calculated using eq. 7.

$$C[NAD] = \frac{\text{total volume in cuvette}}{6.22 \times \text{volume of sample}} \times \Delta O.C. \text{ (mmol/mL center)} \quad (7)$$

Enzymatic Determination of NADH.[37] The concentration of NADH is measured by using the enzyme L-lactate dehydrogenase (LDH) according to the equation:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+$$

The reaction is followed by the decrease in optical density at 340 nm.

The solutions required for the determination are:

1. Triethanolamine buffer, 0.1 $\underline{M}$; pH = 7.6 (18.6 g TEA. HCl in 1 L $H_2O$, adjusted to pH = 7.6 with 2 $\underline{N}$ NaOH.
2. Sodium pyruvate solution, 23 m$\underline{M}$ (250 mg sodium pyruvate in 100 mL $H_2O$)

3. L-lactate dehydrogenase (commercially available crystalline suspension from rabbit muscle 5 mg/mL about 500U/mg). The optical density is read against air at 340 nm. A 3 mL cuvette contains 2.49 mL of the buffer, 0.40 mL of the sodium pyruvate solution, 0.10 mL of the sample. After adjusting the absorbance to zero the enzyme suspension (0.01 mL) is added. The optical density obtained after the reaction stopped is read and used to calculate the NADH concentration according to eq. 7.

Modification of Formate Dehydrogenase by Methyl Methaethiolsulfonate and Ellman's Reagent. The preparation of formate dehydrogenase$(SSCH_3)_n$ is analogous to the procedure described described previously for papain and adenylate kinase.[22] A degassed solution (5 mL; 0.1 M Tris-HCl buffer, pH 7.5, $10^{-4}$ M in EDTA and 0.1 M in KCl) containing 40 U ($1.7 \times 10^{-4}$ mmol) of formate dehydrogenase was treated with 1 mg ($8.7 \times 10^{-4}$ mmol, 40 x excess) of $CH_3SSO_2CH_3$ under argon. The decrease in activity was monitored: after 5 h at 4°C under argon the residual enzymatic activity was 25% (± 3%). Excess $CH_3SSO_2CH_3$ was separated by using ultrafiltration tubing and dialyzing against 0.1 M Tris-HCl buffer pH 7.5. Formate dehydrogenase(SSEllman's) was prepared by an analogous procedure. The reaction time required to obtain < 0.1% of native formate dehydrogenase was 12 h at 4°C under argon.

Formate Dehydrogenase Immobilization. The enzyme was immobilized by the polymerization condensation. PAN-1000 (1000 micromole of active ester groups, 2g) was dissolved in 6mL of Hepes buffer (3 M, 71.5 mg/mL) pH 7.6 at room temperature, containing sodium formate (80 mM) NAD (3 mM) and NADH (0.1 mM) in a 50 mL glass vial equipped with a small magnetic stirring bar. The polymer dissolved completely after 1 min of vigorous stirring. DTT was added (0.02 mL, 0.3 M, 46 mg/mL) and after few seconds 1 mL of 0.1 M Hepes buffer solution containing formate dehydrogenase (60 U, 120 mg of lyophilized protein). After 10 sec., 1.5 mL of triethylenetetramine (0.5 M, 0.085 mmol of primary amino groups) was added. Within 2-3 min, the solution set to a gel, and was allowed to stand for 60 min at room temperature under argon. The gel was grinded in a mortar for 10-20 min adding each time small aliquots (1-2 mL) of washing solution (50 mM Hepes buffer, pH 7.6, 2 mM in DTT). The initial wash contained as well 0.5 mL lysine solution (0.1 M 14.6 mg/mL). The gel suspension was transferred to a centrifuge tube and agitated for a few minutes. The tube was sealed and after centrifugation (10-15 min) the washes (total of three) were recovered and assayed. The gel was assayed as described (page 85).

Large-Scale Coimmobilization of Formate Dehydrogenase and D-Lactate Dehydrogenase. An analogous procedure described for formate dehdrogenase was used for the coimmobilization.

PAN-1000 (10 g) was dissolved at room temperature in 40 mL of 0.3 M Hepes buffer (pH 7.6, containing pyruvate (30 mM), formate (80 mM), NAD (5 mM), NADH (1 mM) in a 50 mL beaker equipped with a magnetic stirring bar. After 2-3 min of vigorous stirring, the polymer dissolved. DTT (1 mL, 0.3 M) was added followed by the addition of 1 mL of the enzymes solution. (110 U, 220 mg of lyophlized formate dehydrogenase and 610 U of L-lactate dehydrogenase from rabbit muscle.) After 10-15 sec, TET (0.5M, 7.5 mL) was added. The solution turned to a gel within 2-3 min. The subsequent procedures were the same as those described for the formate dehydrogenase immobilization method. The gel was assayed for the activities of the coimmobilized enzymes.

Large-Scale Synthesis of D-Lactate. The reactor, a 3L three-necked round-bottomed flask was equipped with a pH electrode connected to a pH controller, an argon line, and a magnetic stirring bar. The flask was charged with 1 L of degassed distilled water. Sodium formate (99.9%) dithiothreitol (DTT, 10 mmol), and EDTA (10 mmol) were added. The solution was adjusted to pH 7.6. Formate dehydrogenase and D-lactate dehydrogenase were coimmobilized in PAN gel: 240 mL of swollen gel contained 52 U of formate dehydrogenase (47% yield on immobilization) and 310 U of D-lactate dehydrogenase (51% yield). The gel particles were suspended in the reaction mixture, and NAD (0.39 mmol) was added. The stirred reaction mixture was maintained at ambient temperature between

pH 7.4 and 7.8 by addition of 2 N HCl using an automatic pH controller. Argon was bubbled gently through the mixture. Reaction was complete in 15 days. The gel was allowed to settle, and the supernatant was decanted under positive argon pressure by using a stainless-steel cannula. The gel was washed, and the washings were combined with the original reaction solution. Zinc lactate (47.9 g, 395 mmol of lactate, 79% based on pyruvate was isolated in 95% purity, it contained a 94% enantiomeric excess of D-lactate.

Large-Scale Synthesis of D-lactate at 40°C. An analogous procedure was used for the synthesis of D-lactate except that a constant-temperature bath was used to keep the reaction at 40°C.

Isolation of D-Lactate. D-lactate was isolated as the zinc salt by using a modification of the procedure of Brin.[38] The supernatant (1100 mL) was concentrated under reduced pressure at 45°C to a volume of 500 mL. The pH of this solution was adjusted to 2.5 with concentrated HCl. Absolute ethanol (100 mL) was added and suspended solids removed by suction filtration. The solution was neutralized to pH 6.5 by addition of a slight excess of zinc carbonate and filtered. The solution was heated to 65°C and cold absolute ethanol was added to the solution until turbidity appeared. Crystallization was allowed to proceed for 24-48 hr. at 4°C. The crystals were collected by filtration and washed twice with

ethyl ether. Assays of chemical and enantiomeric purifies were based on enzymatic methods.

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## Part C. A Combined Electrochemical/Enzymatic Method for *in Situ* Regeneration of NADH Based on Cathodic Reduction of Cyclic Disulfides.

Background

Electrochemical methods of regeneration of $NAD^+$/NADH have received less attention than the enzyme-dependent processes. Recycling schemes that are based on a chemical catalyst or an electrode (as the source of reducing equivalents) have some distinct advantages. Chemical catalysts or electrodes are by far more stable than enzymes. Furthermore, these systems should be the least expensive, certainly less expensive than coupled enzyme systems.

The electrochemical schemes that have been proposed for nicotinamide cofactor recycling are based on the direct electrochemical reduction of $NAD(P)^+$.[1-3] The proposed systems, however, have not been sufficiently selective for reduction at the 4-position of the nicotinamide ring to permit high turnover numbers for those cofactors. For example, the reduction must be > 99.8% selective for the 4-position to achieve turnover number of 1000 .

The electrochemical reduction of NAD and NADP has been studied by polarographic and voltammetry techniques.[4-8] It is generally believed that dimerization of the NAD radical produced as an intermediate during electrolysis prevents the further reduction of $NAD^+$ to NADH. The dimer $(NAD)_2$ that is formed during the direct electrochemical reduction of NAD is only one of the enzymatically inactive forms produced during electrolysis (Scheme 11). Immobilization of the cofactor to soluble or insoluble supports has been suggested

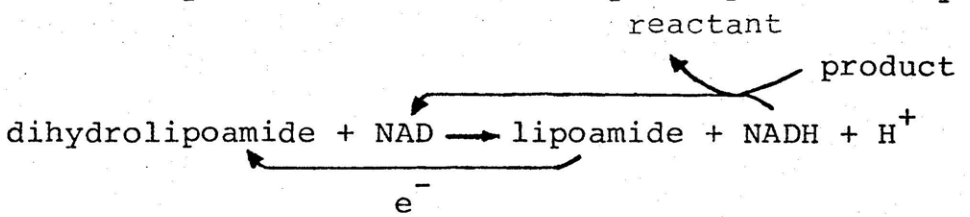
as a way to prevent the intermolecular coupling of the NAD radical. Immobilization of the nicotinamide cofactor, however, introduces problems such as low productivity and high cost, and none of these systems have been tested in large-scale synthesis.

A less complicated method to increase the selectivity is to use an indirect electrochemical step and to add an enzymatic step to reduce NAD(P) to NAD(P)H. An electrochemical regeneration of this kind using methyl viologen and flavoenzymes was described recently.[9] The proposed method (Scheme 12) gives a turnover number ∿ 1000.

The purpose of this work was to propose and demonstrate a new indirect electrochemical method for recycling NADH based on a new cathodic reduction of stable cyclic disulfides (lipoamide). A preliminary report of this work had been submitted for publication: Z. Shaked, J.J. Barber, G.M. Whitesides, J. Org. Chem., in press.

## Results and Discussion

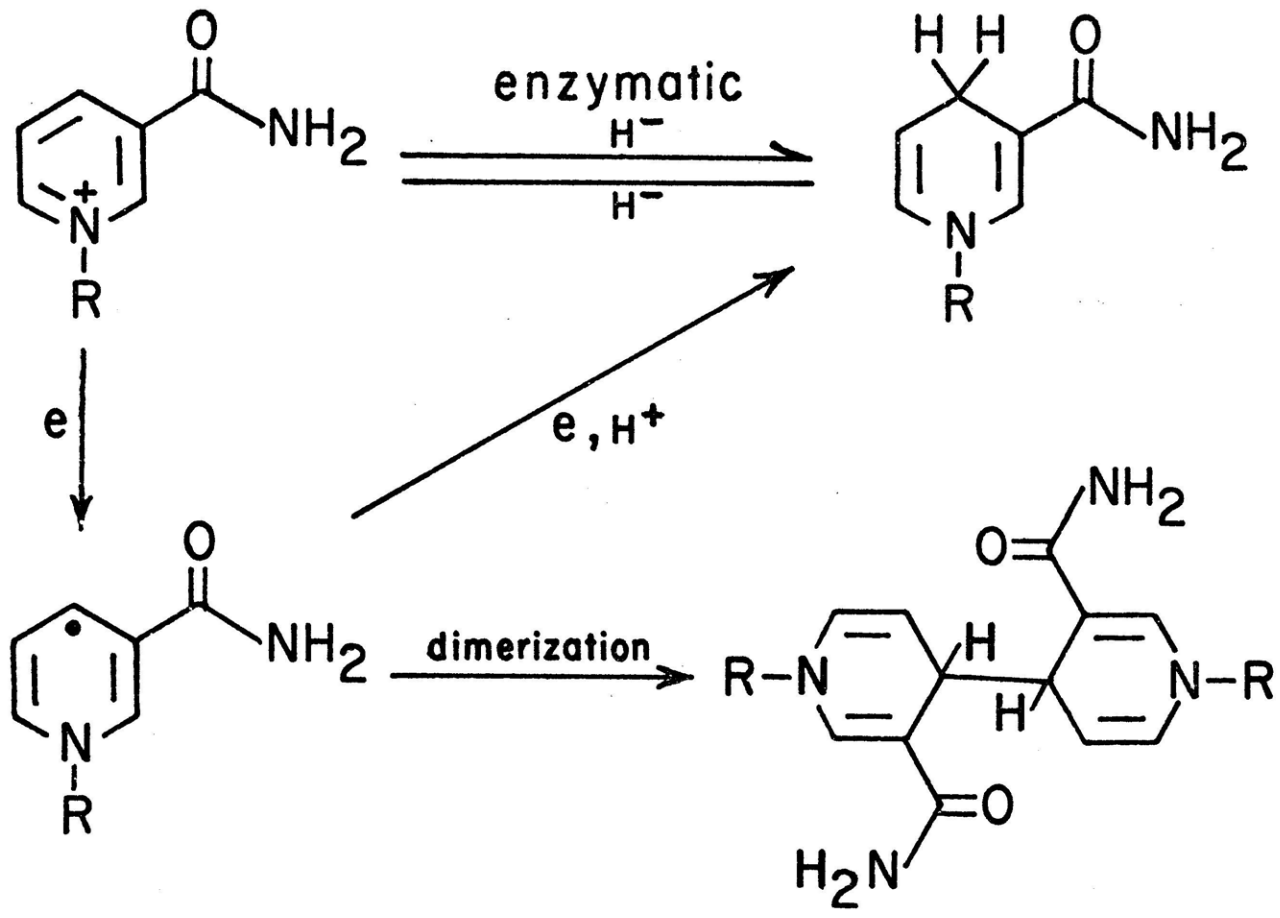
Cathodic Reduction of Cyclic Disulfides. Attempts to Reduce Cyclic Disulfides at the Gold, Platinum, Lead and Dropping Mercury Cathodes. The electrochemical reduction of lipoamide or lipoic acid was essential to the development of a regeneration system based on the enzyme lipoamide dehydrogenase(1)




(1)

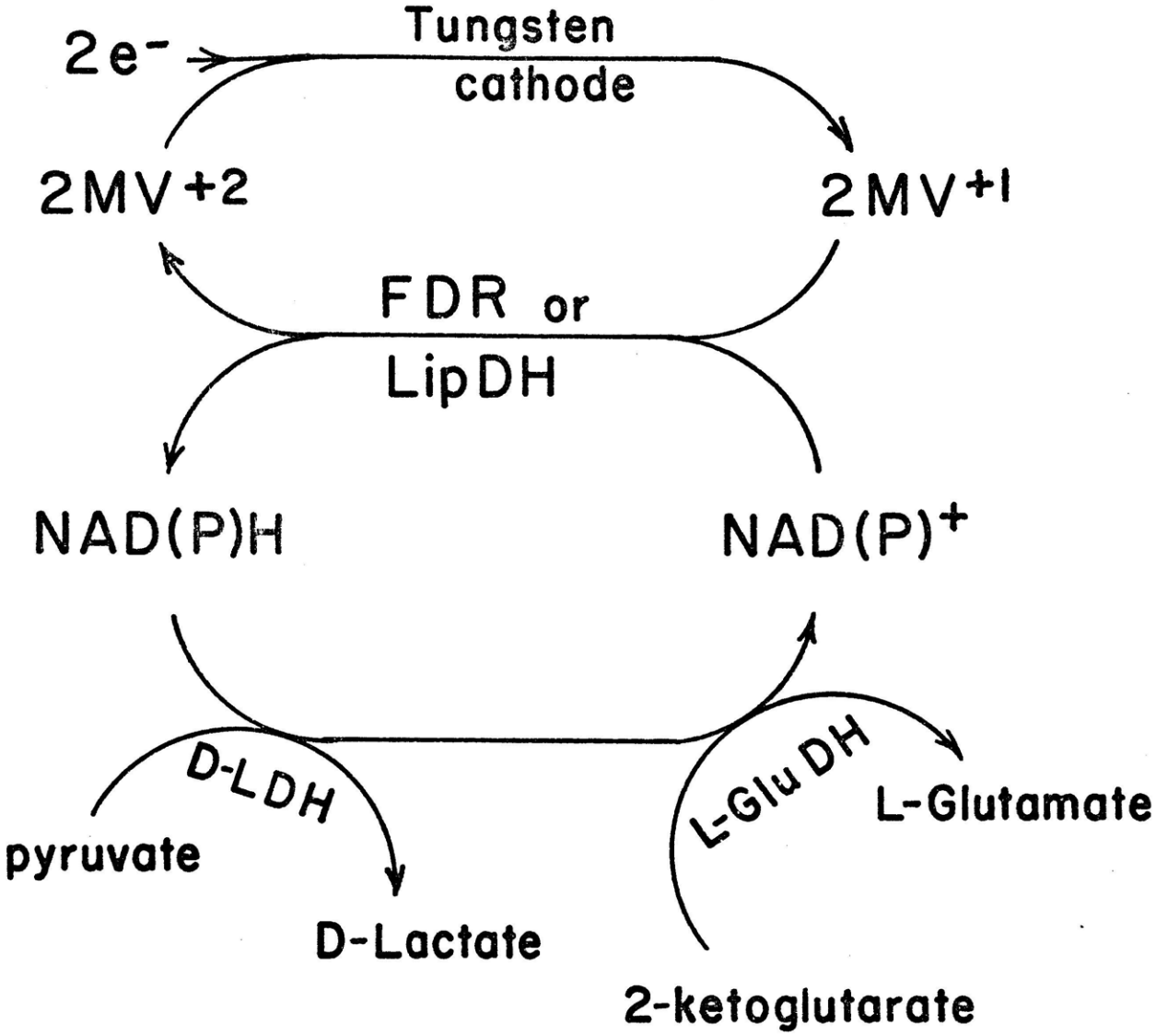
Scheme 11. Enzymatic and Electrolytic Reduction of $NAD(P)^+$

# Scheme II



Scheme 12. Electrochemical Regeneration of NAD(P)H Based on Methyl Viologen and Flavoenzymes

Scheme 12




MV— Methyl Viologen
FDR—ferridoxin-NADP reductase
D LDH—D-lactate dehydrogenase
LipDH—lipoamide dehydrogenase
GluDH—glutamate dehydrogenase

Therefore, many attempts were made to find an appropriate electrode that would quantitatively reduce cyclic disulfides in $H_2O$ and neutral pH. Cathodic reductions of linear disulfides (glutathione, cystine), in water at the dropping mercury electrode (DME) are known in the literature.[10-11] Several groups have reported the electrochemical reduction in $H_2O$ of cyclic disulfides (lipoic acid, 1,2-dithiolane, 1,2-dithiane and 1,2-dithiepane derivatives).[12-14] The product, however, has not been identified. On the other hand, one group reports no reduction of the disulfide bond of lipoic acid derivatives at the gold electrode at -2.0 V (SCE). The first attempts included the following metals as the cathode: gold, platinum, lead, and a mercury pool.

No reduction of the disulfide bond of $DTT^{ox}$ or lipoamide was observed at the gold and platinum electrodes even at very negative potentials (-3.0 V vs SCE). On the other hand, reduction of lipoamide at a mercury pool or lead cathode at -2.0 V (SCE) resulted in a product that reduced Ellman's reagent but was not a substrate for lipoamide dehydrogenase. This product did not match authentic dihydrolipoamide in hplc retention time. Treatment of this product with DTT released dihydrolipoamide. These complexes have high stability.[16]

Electrochemical Reductions of Cyclic Disulfides at the Tungsten and at the Molybdenum Electrodes. The reduction of stable cyclic disulfides was accomplished at the tungsten or

molybdenum electrodes in neutral, buffered aqueous solution. Lipoamide, oxidized dithiothreitol and lipoic acid were reduced quantitatively at the tungsten cathode at potentials of -1.0 to -1.5 V (vs. SCE) without corrosion or excessive dihydrogen production (Figure 8). Cyclic voltametry showed a broad, apparently irreversible wave for reductions of these disulfides. The cathodic reductions were performed with good current efficiencies (Figure 8). Oxidized dithiothreitol is reduced approximately twice as rapidly at -1.0 V (vs. SCE) (Figure 9). Molybdenum is also an effective cathode for the reduction of cyclic disulfides, but gives lower current efficiencies than tungsten. The conversion of the cyclic disulfides to their corresponding dithiols was quantitative as determined by assays based on Ellman's reagent, hplc, and enzymatic assays of lipoamide and lipoic acid using lipoamide dehydrogenase.

Cathodic Reductions of NAD at the Tungsten Electrode in the Presence of Disulfide and N-Heterocyclic Compounds. The $NAD^+$ - NADH cofactor redox couple is reversible under physiological conditions (eq. 2), with an apparent formal potential at pH 7 and 25°C of -0.56 V (vs SCE). Electrochemically, $NAD^+$ is reduced

$$NAD^+ + H^+ + 2e^- \rightleftharpoons (1,4)\ NADH \qquad (2)$$

in aqueous medium at the DME in a reversible $1e^-$ process ($\sim$ -0.9 V vs SCE) to a neutral free radical which rapidly dimerizes. Comparison of the behavior of $NAD^+$ and NMN indicates the major role of adenine in the adsorption process. NMN and its

Figure 8. Current Efficiency as a Function of the Applied Potential for the Electrochemical Reduction of Lipoamide at the Tungsten Cathode (10 ft, 0.030 in. in diameter). All the Reactions Were Performed at 25°C in Phosphate Buffer (0.05 M, pH 7.5) Under Argon.

# Figure 8

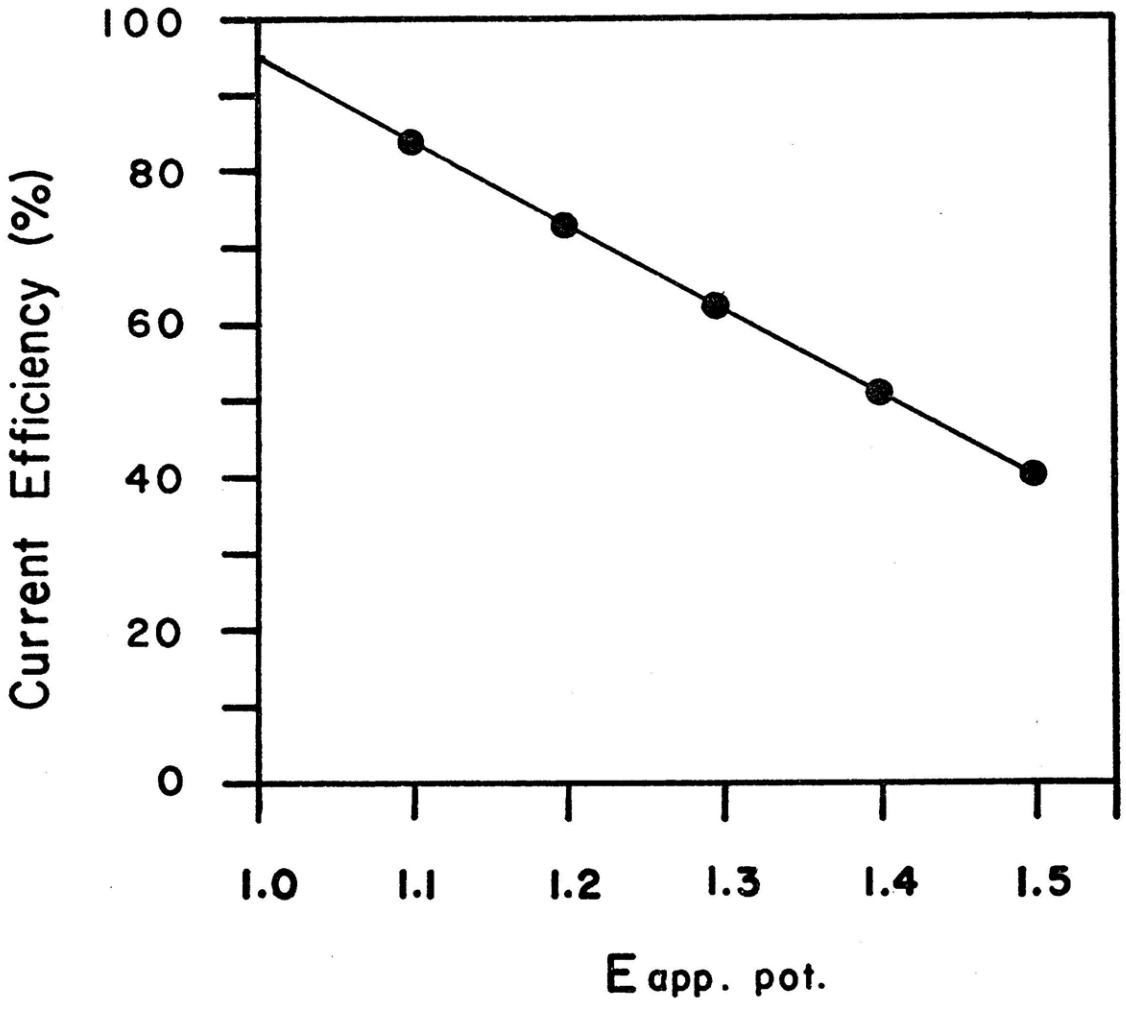


Figure 9. Electrochemical Reduction of Lipoic Acid and $DTT^{ox}$ at the Tungsten Electrode.

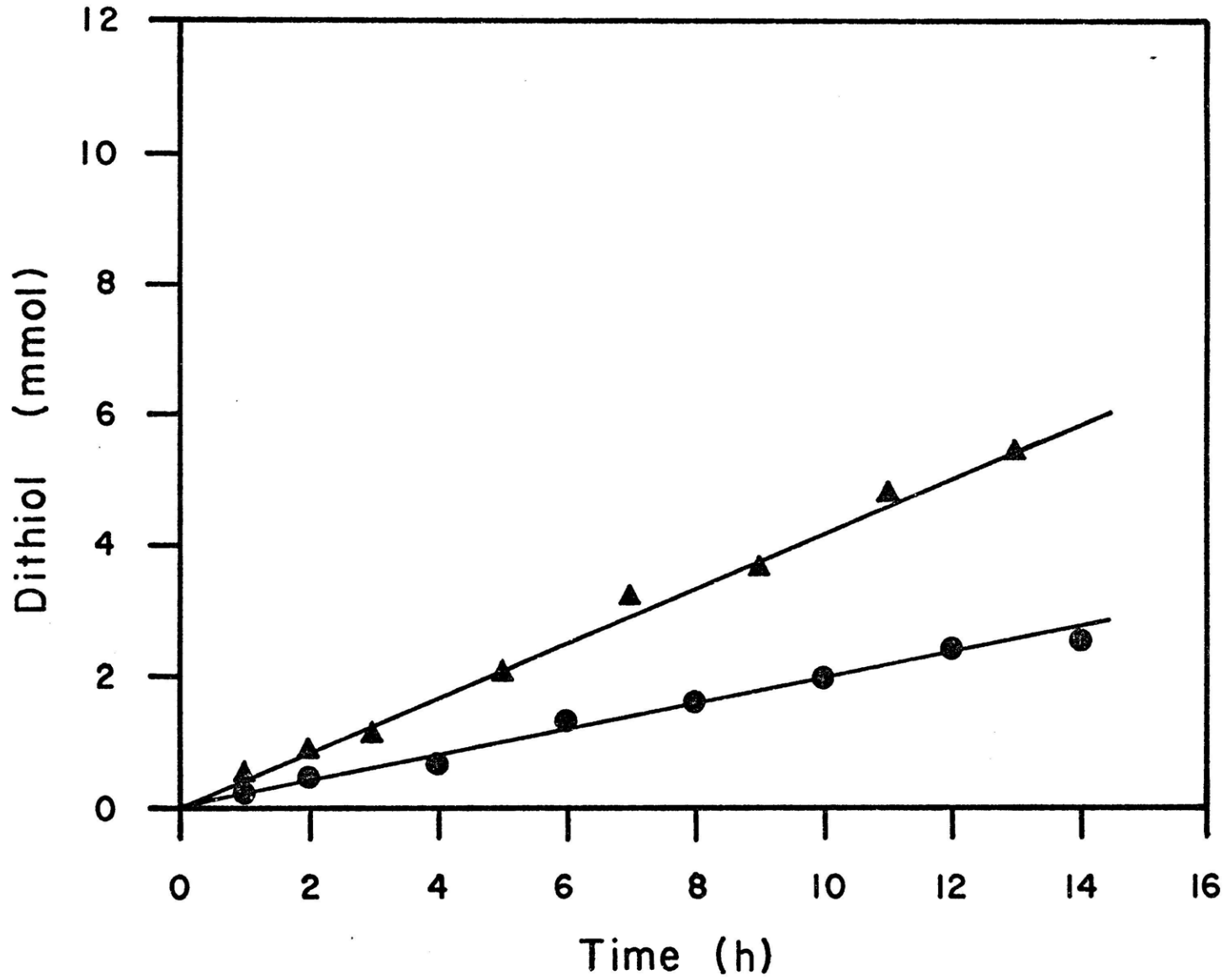
Cathodic reductions were performed in 500 mL, 0.1 M phosphate buffer, pH 7.5, at the tungsten electrode (10 ft, 0.030 in. in diameter, ∿ 75 $cm^2$ surface area). The potential of the tungsten working electrode was 1.0 V (vs. SCE). The reactions were stirred at 25°C.

(▲) $DTT^{ox}$ (30 mM)

(●) Lipoic acid (30 mM)

The reduced dithiols were determined by the Ellman's assay, and the current efficiencies during the reactions were over 85%.

Figure 9



reduction products are negligibly adsorbed. $NAD^+$ behaves similarly to other adenine nucleosides and nucleotides in being strongly adsorbed in the potential region prior to and in the vicinity of the electrocapillary maximum.[17-18]

Inclusion of nitrogen heterocycles (particularly those that contain the adenine moiety) in the reaction mixture substantially reduced the rate of cathodic reduction of NAD (Figure 10). The most effective additives are: ATP, AMP, xanthine 6-mercaptopurine, and adenine. Nicotinamide, nicotinic acid and thioimidazole are less effective (Figure 10), but their reduction to the corresponding dithiols is strongly inhibited by some of the additives, especially by ATP and 6-mercaptopurine (Figure 11).

The mechanism by which the additives change the selectivity of the tungsten electrode has not been established. NMR, Uv and fluorescence studies show considerable interaction in solution between the nicotinamide and adenine moieties of $NAD^+$ with a large fraction of the dinucleotide being in a folded conformation.[19-20] Obviously, it is important as to whether the reducible species at the interface is folded or unfolded, especially if adenine is the adsorption site in the molecule, and if it is the first species reduced. It has been postulated in pulse radiolysis studies of NAD that when an electron reacts with the adenine moiety, it is rapidly transferred to the nicotinamide.[21] It is reasonable to assume therefore that the additives compete with the adenine moiety of NAD for sites active for reduction on the electrode surface.

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Figure 10. Cathodic Reduction of $NAD^+$ at the Tungsten Electrode in the Presence of Disulfides and N-Heterocycle Additives.

The electrochemical reductions were done at 25°C at a constant potential (-1.0 V vs. SCE) in 500 mL phosphate buffer (0.1 M, pH 7.5). Tungsten wire (10 ft, 0.03 in. in diameter) was the working electrode. Platinum wire (1 ft, 0.04 in. in diameter) was the counter electrode. The $NAD^+$ concentration was determined by an enzymatic assay employing YALDH. The initial concentration of NAD in all experiments was 0.8 mM.

(●) ATP (10 mM)

(▲) Xanthine (2 mM)

(□) AMP (30 mM)

(o) Adenosine (5 mM)

(Δ) Nicotinic acid (60 mM)

(■) Nicotinamide (60 mM)

(◇) Thioimidazole (60 mM)

(◐) $DTT^{ox}$ (10 mM)

(◆) No additives

# Figure 10

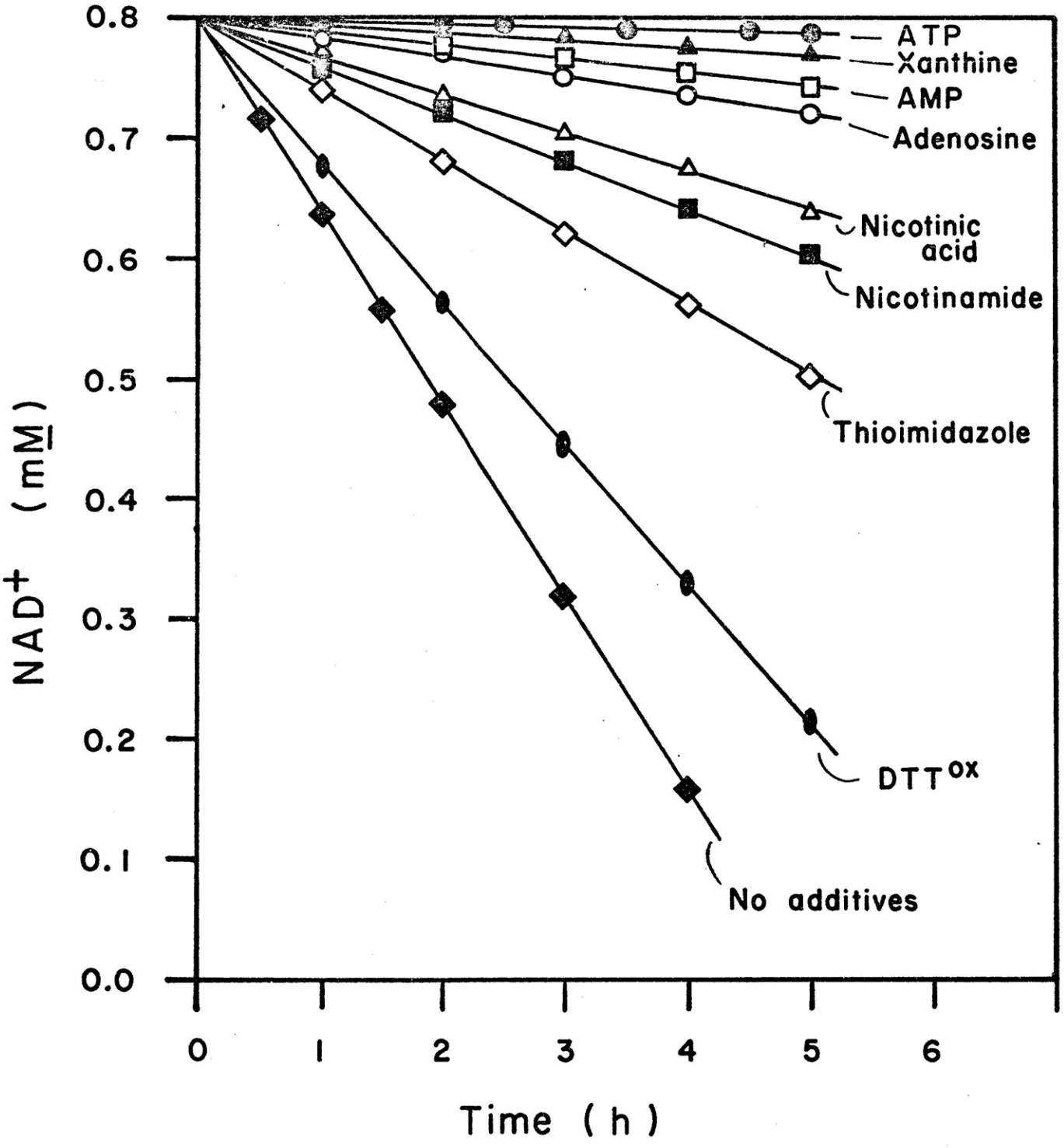

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NAD+ (mM)
0
1
2
3
4
5
6
Time (h)
ATP
Xanthine
AMP
Adenosine
Nicotinic acid
Nicotinamide
Thioimidazole
DTT ox
No additives

Figure 11. Electrochemical Reduction of $DTT^{ox}$ in the Presence of N-Heterocycle Additives.

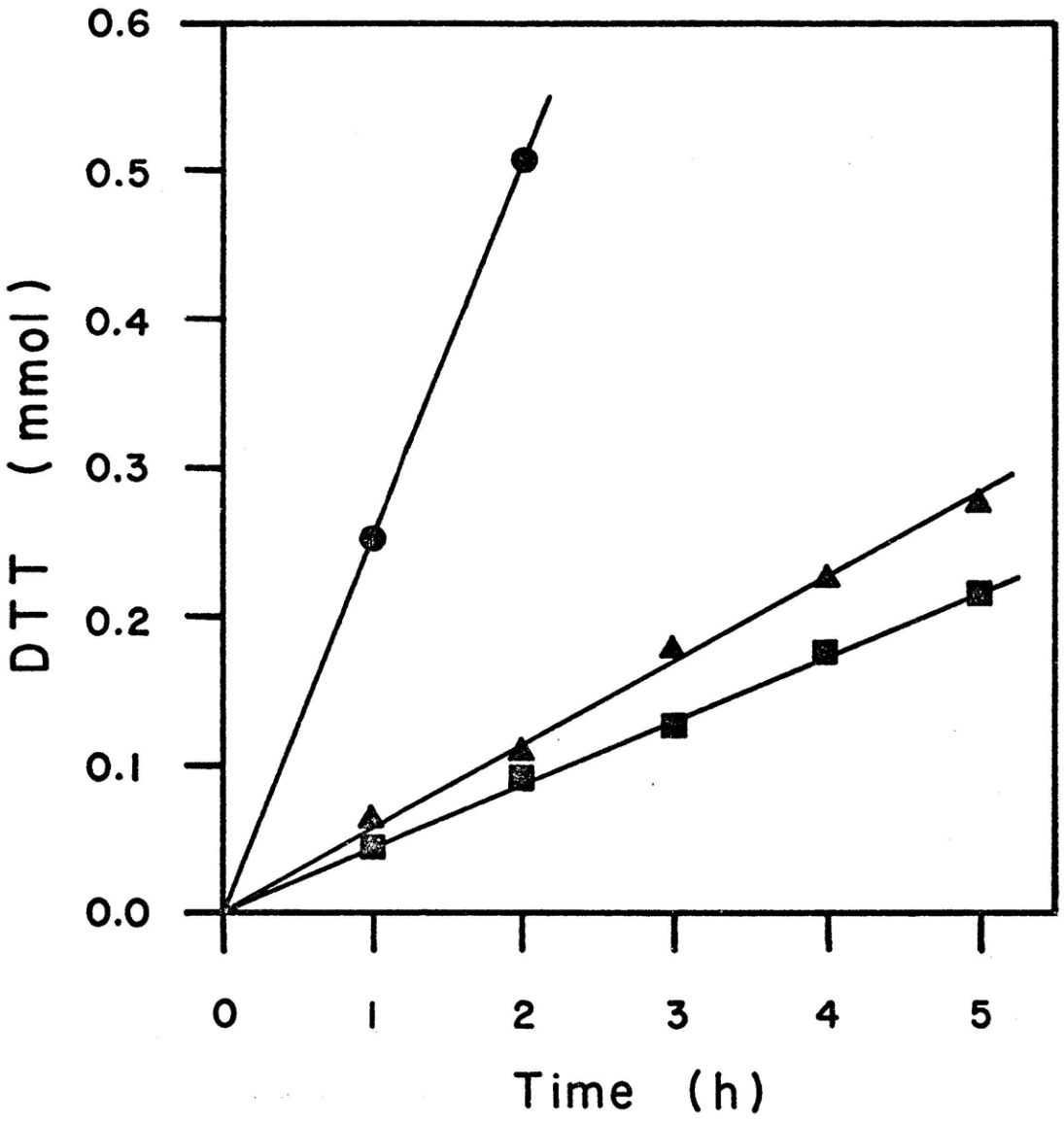
Cathodic reductions of $DTT^{ox}$ (15 mmol) in 500 mL of phosphase buffer (0.1 M, pH 7.5) at the tungsten electrode (10 ft, 0.03 in. in diameter). The reaction mixture stirred at 25°C.

(●) No additives

(■) ATP (10 mM)

(▲) 6-mercaptopurine (2 mM)

# Figure II



Coimmubilization of Lipoamide Dehydrogenase and L-Lactate Dehydrogenase.

Properties of Lipoamide Dehydrogenase. Lipoamide dehydrogenase functions in the oxidative decarboxylation of pyruvate and 2-ketoglutarate to reoxidize dihydrolipoic acid at the expense of $NAD^+$ as acceptor. The molecular weight of lipoamide dehydrogenase has been found to be 100,000 with 2 molecules of flavin adenine dinucleotide, and two active centers, per molecule of protein.[22-24] The $K_m$ for NAD is 0.2 mM and for dihydrolipoamide is 0.3 mM. The pH optimum of the pig heart lipoamide dehdrogenase in the direction of $NAD^+$ reduction by dihydrolipoamide is 7.9. In the direction of NADH oxidation by lipoamide the pH optimum is 6.5.

Coimmobilization of Lipoamide Dehydrogenase and L-Lactate Dehydrogenase by Condensation Polymerization. Lipoamide dehydrogenase and L-lactate dehydrogenase were coimmobilized to the same polyacrylamide gel using PAN-1000.[25] The substrates and cofactors used in the coimmobilization were lipoic acid (40 mM), pyruvate (20 mM), NAD (10 mM), and NADH (5 mM). Yields of immobilization of lipoamide dehdrogenase in the coimmobilized gel averaged 40-45% and those of L-lactate, 45-55%.

Stability of the Coimmobilized Enzymes in the Electrochemical System. The immobilization of the enzymes seems to be important to the success of the regeneration systems. Immobilization protects the enzymes against deactivation at the

electrode surface, and protects the electrode surface against poisoning by adsorbed proteins. The stability of the coimmobilized enzymes was studied under the experimental conditions. Stability of L-lactate dehydrogenase is slightly better than that of lipoamide dehydrogenase (Figure 12).

Large-Scale Synthesis of L-Lactate. The practicality and usefulness of the system is demonstrated by the large-scale synthesis of L-lactate (Scheme 13). Coimmobilized lipoamide dehydrogenase (340 U, 41% yield on immobilization) and L-lactate dehydrogenase (460 U, 47% yield on immobilization) were used for the synthesis. The working electrode was 40 ft of coiled tungsten wire (0.030-in. diameter) and the reference electrode was a standard calomel electrode. The counter electrode was a 3 ft coiled platinum wire (0.040-in. diameter). The reactor was charged with 700 mL of imidazole-$H_2SO_4$ buffer (50 mM, pH 7.8). A buffer was used and not a pH-controller since the high concentration of thiols in the reactor (30-40 mM) destroys the calomel electrode. Moreover, the pH measured by the electrode is 1-2 pH units lower than the actual pH of the solution.[27] Lipoamide (10 mmol), trans-4,5-dihydroxy-1,2-dithiane (dithiothreitol, oxidized, 10 mmol) sodium pyruvate (5 mmol), xanthine (2 mmol) were added. Lipoamide was added in large excess (5-10 times the solubility of lipoamide in the buffer employed) to increase the selectivitiy of the tungsten cathode against NAD and pyruvate reduction. Dithiothreitol

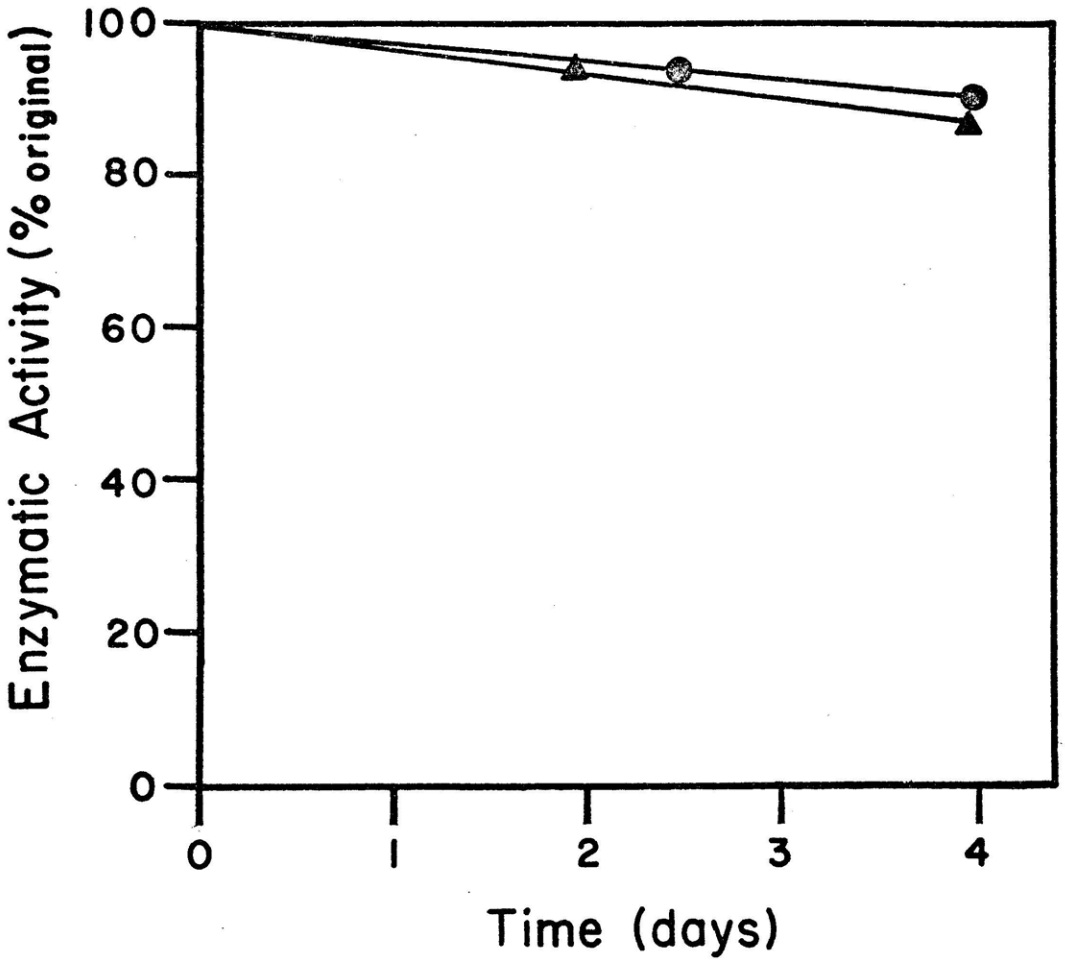
Figure 12. Stability of the Coimmobilized Enzymes in the Electrochemical System.

Stabilities were determined under operating conditions. The working electrode was 40 ft of coiled tungsten wire (0.030 in. in diameter). The counter electrode was a 3 ft coiled platinum wire (0.040 in. in diameter). The coimmobilized enzymes were maintained at 25°C in imidazole-$H_2SO_4$ buffer (50 mM), pH 7.8), and a constant potential (-1.0 V vs. SCE) was applied. The activities of the enzymes were determined according to the assays described.

(●) L-lactate dehydrogenase

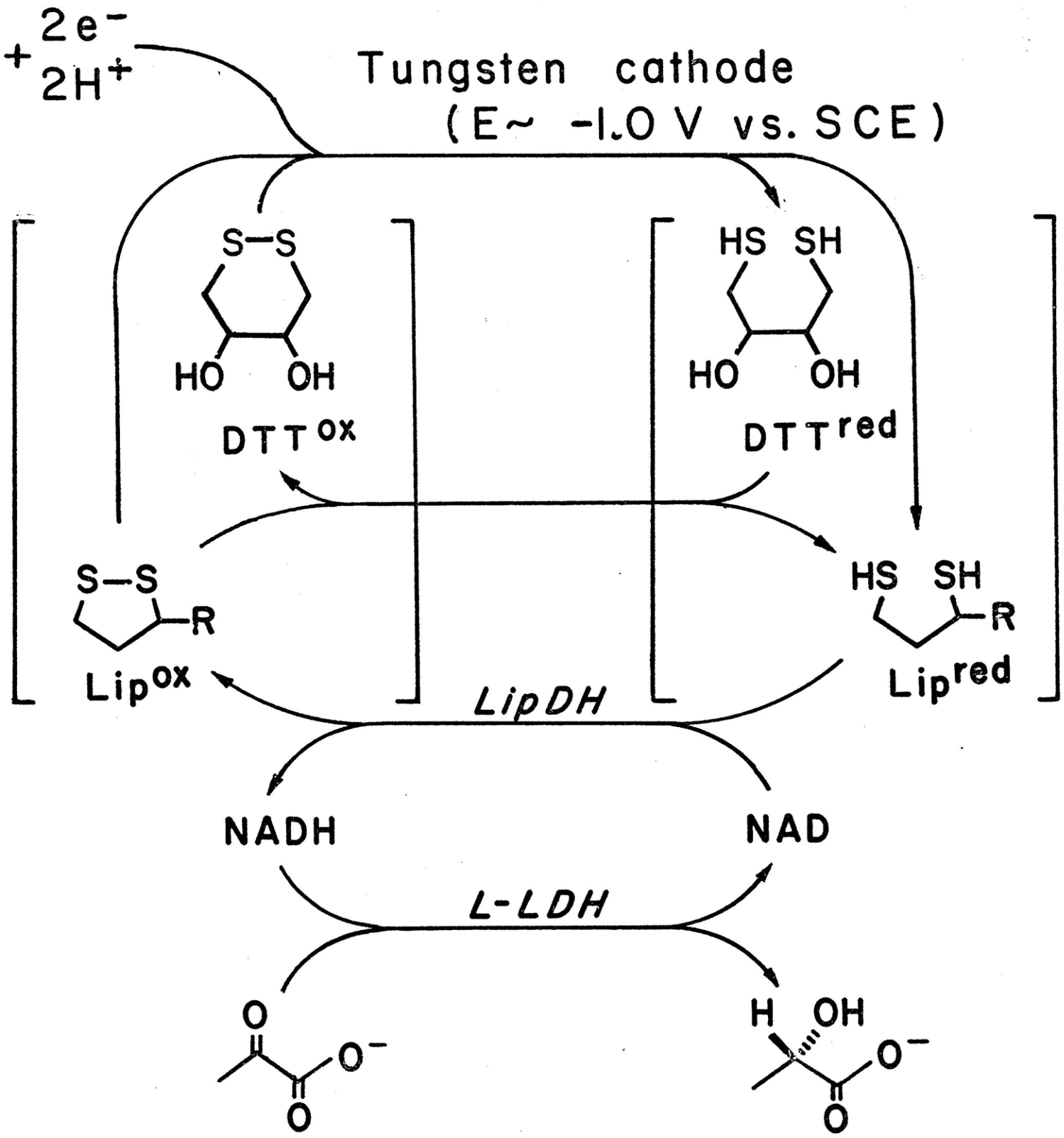
(▲) Lipoamide dehydrogenase

# Figure 12


Enzymatic Activity (% original)
100
80
60
40
20
0
0
1
2
3
4
Time (days)

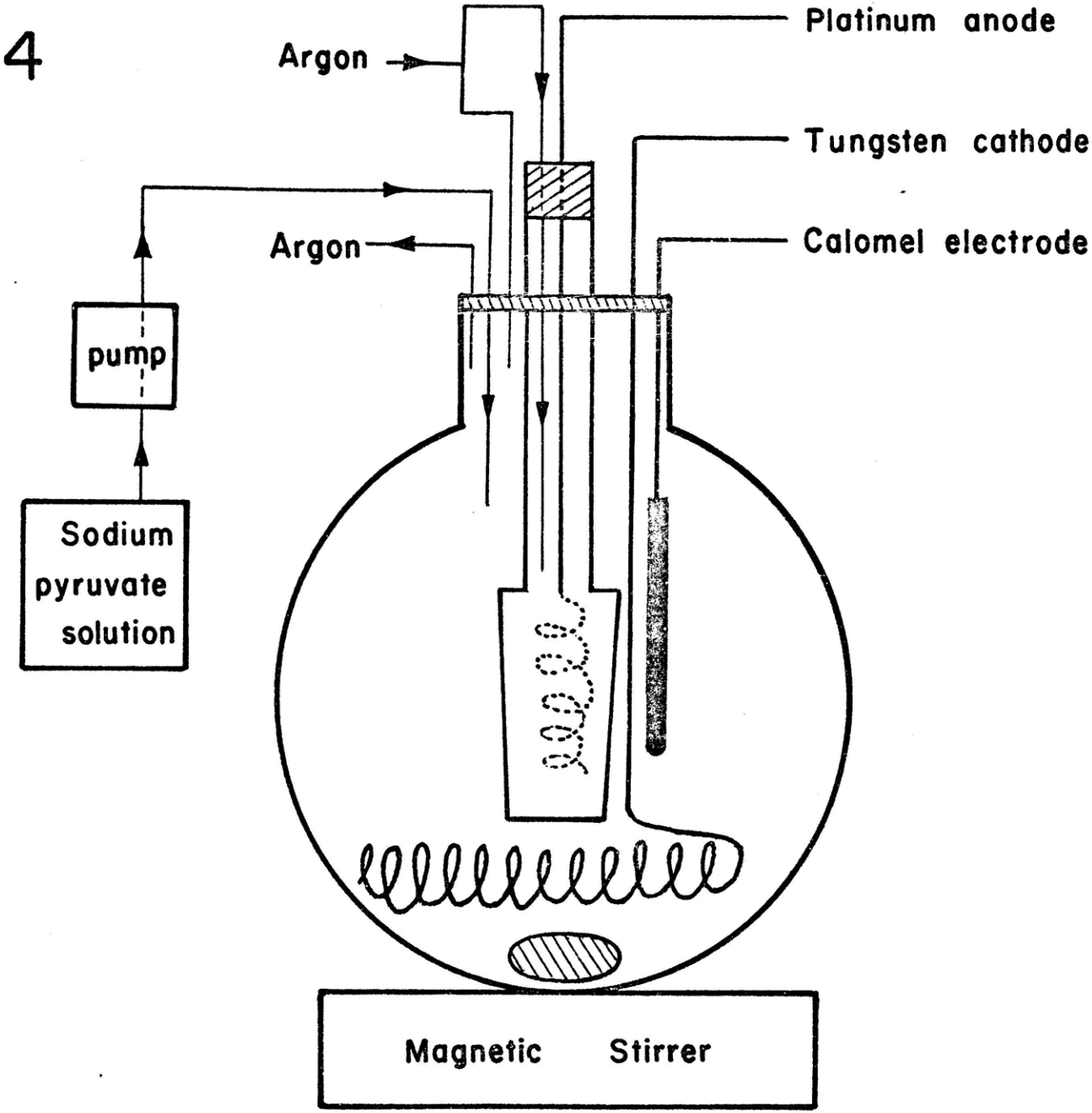
Scheme 13. The Recycling of NADH Based on the Electrochemical Reduction of Cyclic Disulfides.

# Scheme 13


2e⁻
+ 2H⁺
Tungsten cathode
(E~ -1.0 V vs. SCE)
S—S
HO OH
DTT ox
HS SH
HO OH
DTT red
S—S
R
Lip ox
HS SH
R
Lip red
LipDH
NADH
NAD
L-LDH
O
O⁻
O
H OH
O⁻
O

Scheme 14. A Combined Electrochemical/Enzymatic Reactor

# Scheme 14


Platinum anode
Argon
Tungsten cathode
Argon
Calomel electrode
pump
Sodium pyruvate solution
Magnetic Stirrer

(oxidized form) was added to the reaction mixture since the corresponding five-membered ring disulfides are reduced less rapidly than six-membered ring disulfides at -1.0 V vs. SCE (lipoamide is reduced half as fast as oxidized DTT), and a major pathway in the reduction of a mixture of the two disulfides is one in which oxidized dithiothreitol is converted to DTT, and this reduced form in turn reduces lipoamide to dihydrolipoamide by thiol-disulfide interchange. The equilbrium constant for reduction of NAD to NADH by DTT is $\sim$ 1.3.[26]

Xanthine was added to the reaction mixture. Inclusion of nitrogen heterocycles in the reaction mixture substantially reduced the rate of cathodic reduction of NAD, probably by competition for sites active for the reduction of xanthine and other heterocycles on the electrode surface. NAD (0.14 mmol) was added and the potential of the tungsten electrode was adjusted to -1.0 V (vs. SCE). Sodium pyruvate (a total of 130 mmol) was added dropwise to prevent any electrochemical reduction of pyruvate by the tungsten electrode. (In one experiment, sodium pyruvate 120 mmol was added to the imidazole-$H_2SO_4$ buffer, pH 7.9 at the beginning of the reaction together with all the other components, and the electrochemical reduction of pyruvate was not observed.) The enzymes are coimmobilized in such a fashion that the reaction results in a high steady-state value for [NAD]/[NADH]. NAD is intrinsically more stable than NADH under these solution conditions, but more rapidly destroyed by cathodic reduction. Reaction was

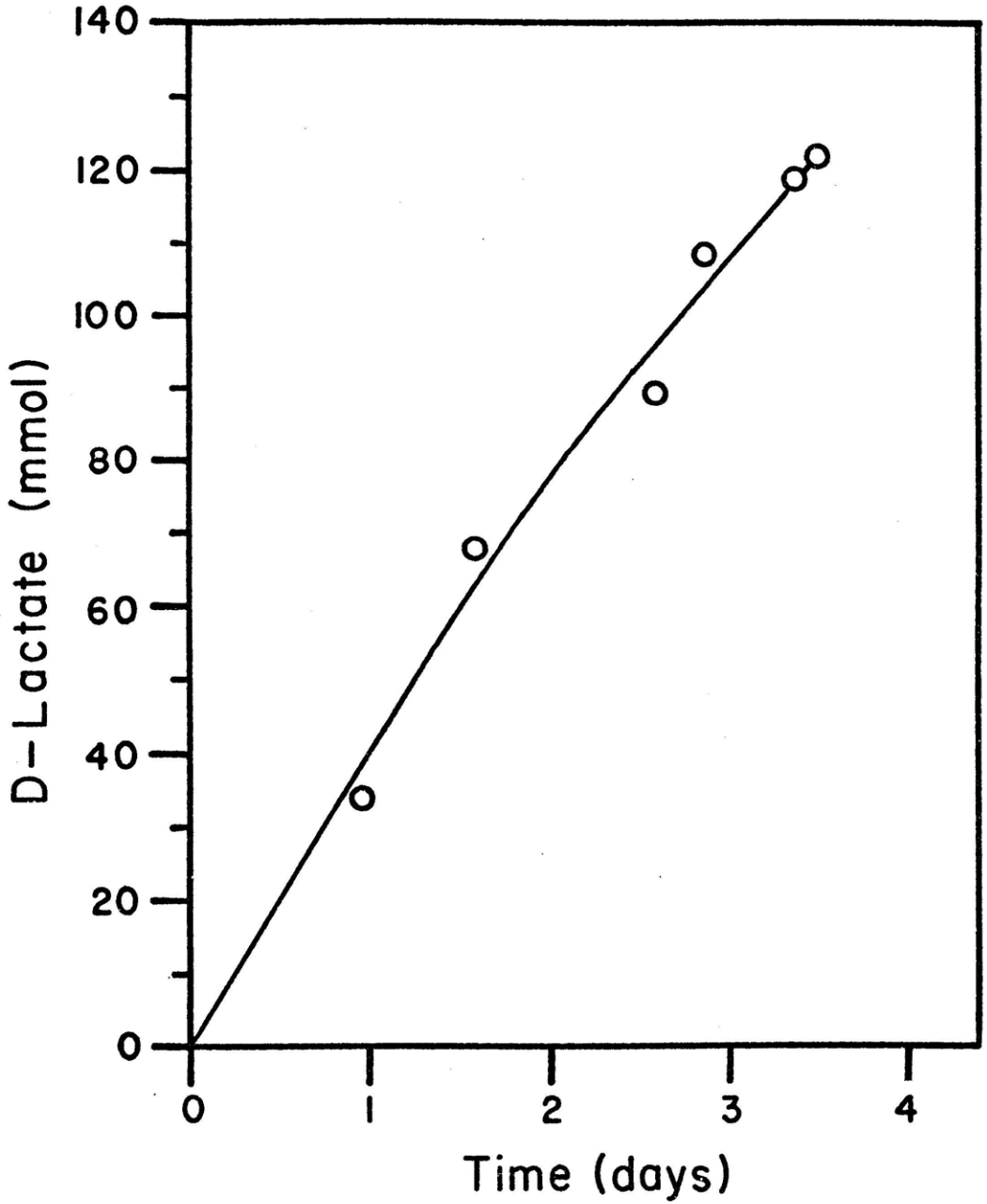
complete in 3.5 days (Figure 13). The current efficiency during the reaction was about 85%.

The lactic acid produced in the reactor was 135 mmol. The product (115 mmol) was isolated as the zinc salt (13.8 g, 96% pure, 85% yield based on pyruvate, 96% ee). The turnover numbers (and recovered activities) of the components were: lipoamide dehydrogenase, $2.6 \times 10^7$ (88%); L-lactate dehyrogenase, $3.6 \times 10^7$ (90%); NAD, 920; lipoamide, 13. Only approximately 5% of the NAD(H) originally added to the reaction mixture remained active at the conclusion of the reaction and most of the lipoamide was still present in active form. No effort was made to recover lipoamide or NAD since most of the lipoamide settled down with the gel that contained the coimmobilized enzymes. Turnover numbers (TN) for NAD (400-950) were typically obtained by using amalogous procedures. The lowest TN (420-600) were obtained when nicotinic acid or adenosine were used as an additive, while the highest TN were achieved with xanthine.

Figure 13. Large-Scale Synthesis of L-Lactate.

Coimmobilized LipDH (340 U) and L-LDH (460 U) were used for the synthesis. The cathode was a tungsten wire (40 ft, 0.030 in. in diameter), the anode was a platinum wire (3 ft, 0.040 in. in diameter) and the constant potential applied during the synthesis was -1.0 V (vs. SCE). The reaction was performed at 25°C and in imidazole-$H_2SO_4$ buffer (0.05 M pH 7.9).

Figure 13



## Concluding Remarks

The combined electrochemical/enzymatic NADH regeneration system described in this work presents a practical electrochemical method for recycling of the nicotinamide cofactor. The electrochemical reduction of stable cyclic disulfides at the tungsten electrode is a new synthetic method. The reaction is performed under mild conditions (25°C, pH 7) and can be useful in other applications such as reduction of protein disulfide bonds.

The regeneration system has disadvantages: first, the TN for NAD is 2-5 times lower than that in other methods, the TN for lipoamide is low (13) and finally, the reactor set-up is complicated.

## Experimental

General. Lipoamide dihydrogenase (EC 1.6.4.3) from porcine heart [suspension in 3.2 M$(NH_4)_2SO_4$] or from torula yeast (stable lyophilized powder), L-lactate dehydrogenase from rabbit muscle (lyophilized, salt free powder), trans-4,5-dihydroxyl-1,2-dithiane (oxidized DTT), DTT, DL-6,8-thioctic acid (DL-α-lipoic acid, oxidized form), DL-6,8-thioctic acid amide (DL-lipoamide), xanthine (2,6-dihyrodxypurine), NAD (sodium salt), NADH (disodium salt, ATP, ADP, AMP, nicotinic acid, nicotinic acid amide were obtained from Sigma Chem. Co. Imidazole, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and 1,3-dithiol-2-propanol were obtained from Aldrich Chem. Co. pH measurements were done using a Radiometer PH M62 standard potentiometer. Water was double distilled using Corning AG-1B still. Spectrophotometric measurements were carried out on either a Gilford Model 240 or Perkin Elmer Model 552 spectrometer, both equipped with constant temperature cell compartments. Cathodic reductions were performed by using a constant potential applied to the cathode with a Princeton Applied Research Model 371 Potentiastat-Galvanostat.

Cathodic Reduction of Cyclic Disulfides. A 1 L three-necked flask was equipped with a magnetic stirrer, a saturated calomel reference electrode, a working electrode (5-40 ft of tungsten or molybdenum wire), and a platinum wire (1-3 ft) counter electrode. The counter electrode was separated from

the solution containing the disulfide with a VWR Scientific medium or coarse porosity alundum thimble. Argon was passed separately through the counter electrode and working electrode compartments. The flask was charged with 500 mL of degassed phosphate (0.05 M, pH 7.5) or imidazole-$H_2SO_4$ (0.05 M, pH 7.8) buffers and each one or a mixture of the corresponding disulfide: $DTT^{ox}$, Lipoamide, Lipoic acid. The stirred reaction was maintained at ambient temperature (25°C) and a constant potential (-1.0 to -1.5 V vs. SCE) was applied to the working electrode. During the reduction the current was followed and the reaction was monitored spectrophotometrically by following the formation of dithiols using the Ellman's assay.

Spectrophotometric Determination (Ellman's Assay) of Dithiols. The cathodic reduction of cyclic disulfides to their corresponding dithiols was followed spectrophotometrically by using the method developed by Ellman.[28] The reagent 5-5'-dithiobis-2-nitrobenzoic acid (DTNB, commercially available) reacts with aliphatic (di)thiols by a thiol-disulfide interchange reaction. A stock solution of DTNB was prepared (0.396 g in 5 mL of acetone, then 95 mL of phosphate buffer 0.05 mM, pH 7.0, 2 mM, in EDTA added). The sample (0.01 - 0.5 mL) was added to a 3 mL cuvette that contained the DTNB solution (2.95 - 2.99 mL) and that was incubated in the spectrophotometer at 25°C. The optical density obtained after 15 min at 412 nm (the $\lambda_{max}$ for the anion of 2-nitro-5-thiobenzoic acid, Ellman's anion) was a measure of the dithiol concentration.

An extinction coefficient of 13700 $M^{-1}cm^{-1}$ was used for Ellman's anion.[29]

Cathodic Reduction of NAD in the Presence of Disulfides and/or N-Heterocycle Compounds. The electrochemical reduction of the nicotinamide adenine dinucleotide in buffered solutions (that contained disulfides and N-heterocycle compounds) was performed by an analogous procedure described previously for the cyclic disulfide. (NAD (0.2 - 0.4 mmol) was dissolved in 500 mL phosphate (0.05 M pH 7.5) or imidazole-$H_2SO_4$ buffer (0.05 M, pH 7.8) that contained the disulfides and/or the N-heterocycle additive. The actual concentration of NAD was measured enzymatically by employing the NAD assay. A constant potential (-1.0 to -1.1 V vs. SCE) was applied to the tungsten electrode. The electrochemical reduction was monitored by following enzymatically the concentrations of NAD and NADH, according to the procedure described (pages

Enzymatic Determination of Dihydrolipoamide and 6,8-Dihydrothiooctanoate. Dihydrolipoamide and 6,8-dihydrothio-octanoate (lipoic acid, reduced form) were determined by using the enzyme lipoamide dehydrogenase according to one of the reactions:

$$\text{dihydrolipoamide} + NAD^+ \xrightarrow{\text{LipDH}} \text{lipoamide} + NADH + H^+$$

$$\text{dihydrolipoic acid} + NAD^+ \xrightarrow{\text{LipDH}} \text{lipoic acid} + NADH + H^+$$

The following solutions were prepared:

1. Triethanolamine buffer, 0.2 M, pH 7.8 (18.6 g. triethanolamine hydrochloride/1L, adjusted to pH 7.8 with 2N KOH), $10^{-4}$M in EDTA
2. NAD solution, 30 mM (20 mg NAD/mL)
3. Lipoamide dehydrogenase suspension in 2.3 M ammonium sulphate solution about 150U/mg
4. Sample

The spectrophotometric measurement is done at 340 nm at 25°C against a blank that contains all the components except the sample. A 3 mL cuvette contains the buffer (2 mL), NAD solution (0.2 mL) the enzyme solution (0.03 mL) approximately 20U/mL. After mixing and incubation for 3-5 min the sample (0.05-0.1 mL) is added and the optical density was monitored until the reaction stopped. This optical density was used for the calculation (eq. 3).

$$c = \frac{\text{total volume in cuvette}}{\varepsilon x \text{ volume of the sample in cuvette}} \times \text{O.D.} \quad \varepsilon = 6.22 \text{ mmol}^{-1}\text{cm}^2 \qquad (3)$$

Lipoamide Dehydrogenase Assay. The enzyme's activity was determined by following the change in optical density at 340 nm per unit time according to the reaction:

$$\text{dihydrolipoamide} + \text{NAD}^+ \xrightarrow{\text{LipDH}} + \text{NADH} + \text{H}^+$$

The following solutions were prepared:

1. Triethanolamine buffer, 0.2 M pH 7.8 (18.6 g. triethanolamine hydrochloride/1 L adjusted to pH 7.8 with 2 N KOH), $10^{-4}$ M in EDTA

2. NAD solution, 30 m$\underline{M}$ (20 mg NAD/mL)
3. Dihydrolipoamide solution 6 m$\underline{M}$, (obtained by a sodium borohydride reduction of a lipoamide solution (10 m$\underline{M}$) that was prepared by dissolving 20.5 mg of lipoamide in 0.5-1 mL of DMSO and then adding $H_2O$ to get a total volume of 10 mL. $NaBH_4$ (3% solution) was used for the reduction. The dihyrolipoamide solution was acidified to pH3 with 6 $\underline{N}$ HCl to destroy any $NaBH_4$ left in the solution. This dihydrolipoamide solution has to be prepared a short time (5 min) before the assay due to the tendency of the compound to reoxidize back to the disulfide form.
4. Sample (commercially available lipoamide dehydrogenase).

The cuvette contained the TEA buffer (1.5 mL), NAD solution (0.4 mL) and dihydrolipoamide solution (1 mL). A blank contained the same components. The reaction was started by adding the sample (0.01 mL of commerciall available enzyme diluted 1:100 with the TEA buffer). The optical density was read after each minute and the average O.D./min calculated and used to obtain the activity of the enzyme (U).

Enzymatic Determination of NAD. NAD was assayed as described on page 90.

Enzymatic Determination of NADH. NADH was assayed as described on page 91.

L-Lactate Dehydrogenase Assay. L-LDH was assayed as described on page 87.

Enzymatic Determination of D- and L-Lactate. D- and L-lactates were determined as described on pages 88 and 89

Large-Scale Coimmobilization of Lipoamide Dehydrogenase and L-Lactate Dehydrogenase. PAN-1000 (5g) was dissolved at room temperature in 20 mL of 0.3 M Hepes buffer (pH 7.6). The buffer contained 40 mM lipoic acid, 10 mM NAD, 5 mM NADH and 20 mM pyruvate in a 25 mL beaker equipped with a magnetic stirring bar. After 2 min of vigorous stirring all the polymer dissolved. DTT, 0.1 mL (0.3 M solution) was added immediately followed by the addition of 1 mL of the enzyme's solution. The enzyme solution contained 870 U of lipoamide dehydrogenase and 505 U of L-lactate dehydrogenase. Finally, 3.5 mL (0.5 M) of triethylenetetramine was added. Within 4 min, the solution became a transparent gel, which was allowed to stand for 60 min at room temperature under argon atmosphere. The gel was ground in a mortar for about 10-15 min with periodic additions of aliquots (1-2 mL) of washing solution (50 mM Hepes buffer, pH 7.6, degassed). The initial wash contained, in addition, 0.3 mL lysine solution (0.1 M; 14.62 mg/mL) in 50 mM Hepes buffer pH 7.6. The gel suspension was transferred to a centrifuge tube (sealed with serum stopper) and separated from the washing solution by centrifugation (5 min at 3000 rpm). The washes (total of three) were recovered and together

with the gel suspension assayed for activity.

Large-Scale Synthesis of L-Lactate. A two L, three-necked, round-bottomed flask containing a stirring bar and attached to an argon line was used as the reaction vessel. The working electrode was 40 ft of coiled tungsten wire, 0.030- in diameter and a surface area of approximately 300 $cm^2$ and the reference electrode was a standard calomel electrode. The counter electrode was a 3 ft coiled platinum wire 0.040 in diameter that was separated from the working solution by a porcine ceramic Soxhlet extraction thimble (VWR) inserted in the center neck of the flask. During electrolysis, the solution in the anode compartment was purged with a slow stream of argon to remove any $O_2$ formed.

The flask was charged with 700 mL of imidazole-$H_2SO_4$ buffer (50 mM, pH 7.8). Lipoamide (2.05 g, 10 mmol), $DDT^{ox}$ (1.52 g, 10 mmol), sodium pyruvate (0.55 g, 5 mmol), and xanthine (0.3 g, 2 mmol) were added, and the solution readjusted to pH 7.8. Lipoamide dehydrogenase (340 U) and L-lactate dehydrogenase (460 U). NAD (0.14 mmol) was added and the concentration of the cofactor enzymatically measured. The potential of the tungsten electrode was adjusted to -1.0 V (vs. SCE), and sodium pyruvate (14.3 g, 130 mmol, in 100 mL of imidazole-$H_2SO_4$ buffer) was added at 1.5 mmol/h. Reaction was complete in 3-5 days. The L-lactate produced in the reactor as determined enzymatically was 135 mmol.

Isolation of L-Lactate. The gel was allowed to settle for about 12 h. and the supernatant decanted. The L-lactic acid produced was isolated as its zinc salt. The isolation was performed according to the procedure described for D-lactate (page 95).

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## Part D. The Regeneration of NADH Using a Liquid Thiol-Disulfide Redox Membrane System.

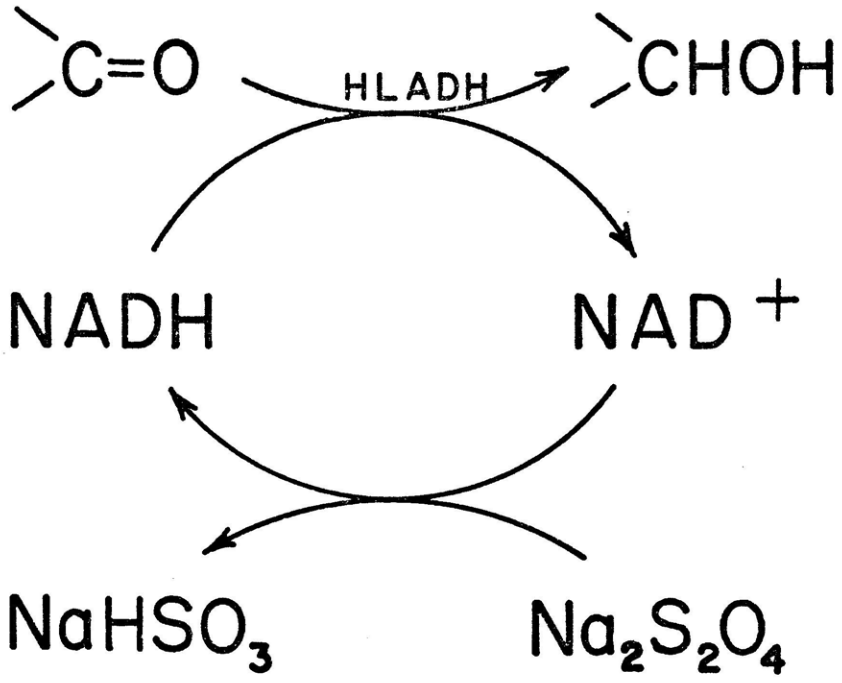
Background

Purely chemical methods for regenerating the nicotinamide cofactors have received less attention than enzymatic methods.[1] The chemical systems which have been proposed are all less efficient than the enzyme-dependent processes. The schemes that have been seriously proposed regenerate NAD(P)$^+$ from NAD(P)H oxidatively.[2-4] The exception to this generalization is the recycling of NAD(P)H by direct reduction of NAD(P)$^+$ using dithionite (Scheme I5).[5] Sodium dithionite has some advantages: it is inexpensive and highly selective for reduction at the 4-position of the nicotinamide ring to give (1,4)NADH. Turnover numbers (mol of product/mol of coenzyme) for regeneration of NAD as high as 105 have been obtained.[5] Dithionite is, however, also a reducing reagent for some of the substrates used (especially cyclic ketones). Furthermore, many enzymes are inactivated in the presence of dithionite. An indirect chemical method for regeneration of NADH using dihydrogen and a water-soluble diphosphinorhodium complex has been proposed recently.[6] The catalyst and two enzymes (L- and D-lactate dehyrogenase) catalyze the regiospecific reduction of NAD$^+$ to NADH by $H_2$. This coupled organometallic-enzymatic system is, however, not very practical.

The low efficiencies of chemical recycling methods and the low turnover numbers observed based on NAD$^+$ usually reflect low selectivity for reduction at the 4-position of NAD$^+$.

Scheme 15. HLADH-Catalyzed Aldehyde and Ketone Reduction Using Sodium Dithionite Recycling of NADH.

# Scheme 15



This work describes a study whose purpose was to develop liquid redox membranes, to allow redox coupling of separate aqueous phases without mixing ionic species present in these phases. The system (Scheme 16) provides a method of injecting reducing equivalents into biological systems using non-biological reducing agents while protecting the enzymes from direct contact with these reducing agents. The general features of the system are the following: (a) the two aqueous regions 1 and 2 are separated by an organic phase; (b) the organic phase (chlorobenzene, o-dichlorobenzene or a mixture of the two), permits the thiols or disulfides to move from one aqueous phase to the other and at the same time separates the two aqueous media; (c) all three regions are stirred during the reaction and the two aqueous phases are kept under argon.

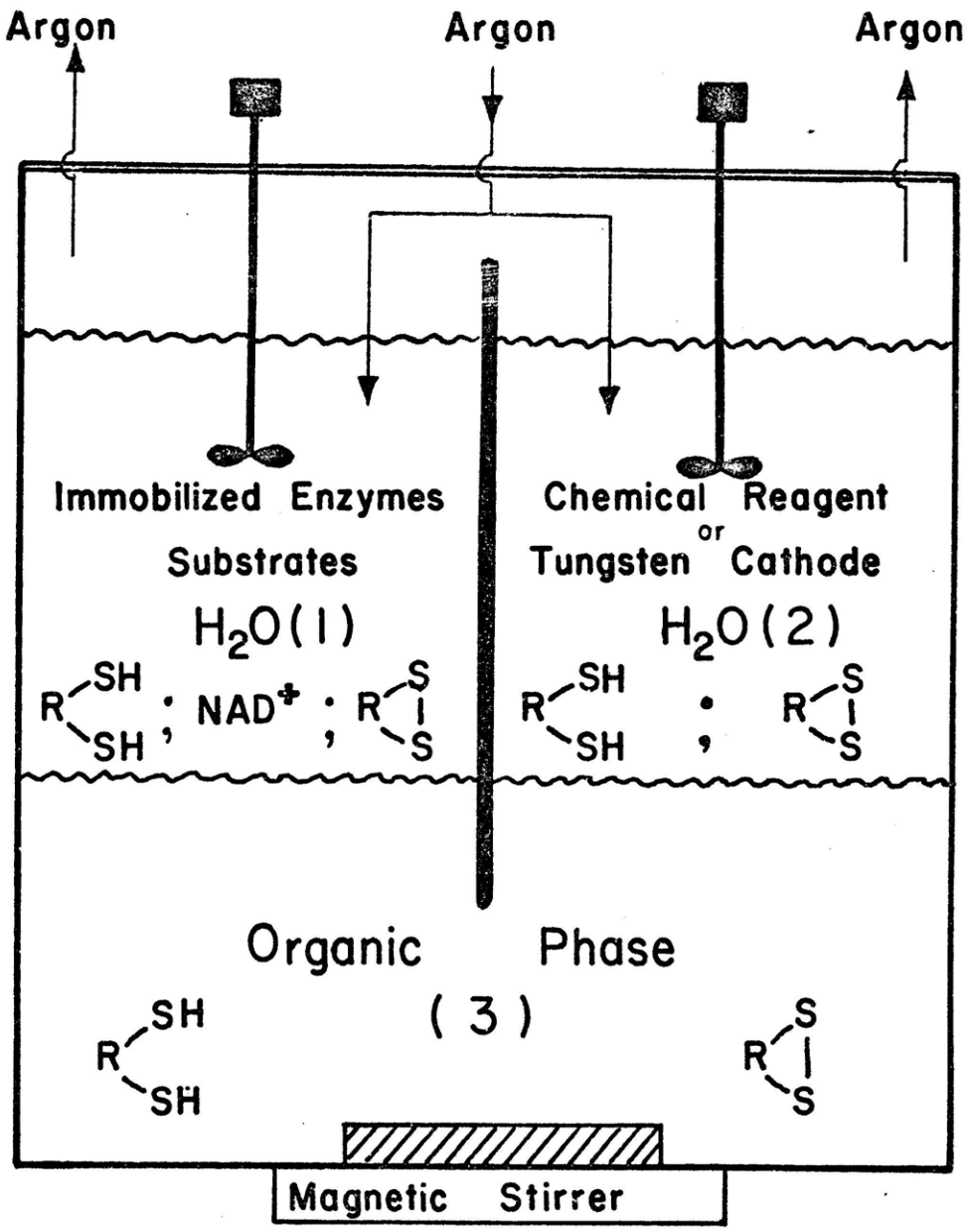
The system couples the enzymatic reduction of $NAD^+$ to NADH (by lipoamide dehydrogenase) and a co-substrate (lipoic acid, lipoamide or 4-hydroxy-1,2-dithiolane) with the oxidation of dithonite or borohydride ion. The coupling is achieved by thiol-disulfide interchange reactions.[7]

## Results and Discussion

Chemical Reductions of Cyclic Disulfides. The proposed recycling scheme required an efficient reduction of cycle disulfides (5-and 6-membered rings) to their corresponding dithiols. Several methods have been demonstrated for the

Scheme 16. Recycling of NADH Based on a Liquid Membrane Redox System.

# Scheme 16


Argon
Argon
Argon
Immobilized Enzymes
Substrates
H2O(1)
R SH SH ; NAD+ ; R S S
Chemical Reagent
or
Tungsten Cathode
H2O(2)
R SH SH ; R S S
Organic Phase
( 3 )
R SH SH
R S S
Magnetic Stirrer

reduction of linear disulfides.[8-10] The reduction of stable cyclic disulfides is more difficult, but can be accomplished quantitatively by using borohydride ion or dithionite. Efforts to reduce cyclic disulfides using other reagents (Formamidine sulfinic acid, Cr(II), Ti(11), $H_3PO_3$) or catalytic hydrogenation with metal catalysts [$CH_3SCo(DMGH)_2Pyr$, W, Pt, Pd/C], and dihydrogen or enzymatically by hydrogenase failed. Sodium dithionite and sodium borohydride are versatile, powerful, and widely used reducing agents. In recent years sodium borohydride and dithionite have been increasingly used for reduction of the disulfide bonds of proteins.[11-13]

In this work both reagents were used to reduce cyclic disulfides ($DTT^{ox}$, lipoamide, lipoic acid). The advantages of using borohydride and dithionite are the following: the reduction of the cyclic disulfide compounds is quantitative and both reagents are readily available and relatively inexpensive.[14] There are, however, some important disadvantages. First, the reducing power of these reagents and their stability are strongly pH dependent. Dithionite[15] and borohydride are strong reducing agents in alkaline solutions and in addition borohydride is used as an effective reducing agent in neutral solutions. Both reagents decompose in acidic conditions (pH≤5). Second, it is necessary to use strongly buffered solutions due to rapid pH changes in the presence of those reagents. Third, dithionite solutions are oxidized by atmospheric oxygen[16,17] so air must be rigorously excluded

and the solutions degassed. Finally, dithionite slowly decomposes by a first-order reaction even in alkaline aqueous solutions.[18]

Electrochemical Reduction of Cyclic Disulfides. Cyclic disulfides are quantitatively reduced at tungsten or molybdenum electrodes in neutral, buffered or unbuffered aqueous solutions.[19] Lipoamide, lipoic acid, and $DTT^{ox}$ were reduced to their corresponding dithiols at tungsten cathode at potentials of -0.8 to -1.0 V (vs SCE) and pH 6-7. The electrochemical system was set up in the aqueous solution and employed instead of dithionite (see Scheme 16). The cathodic reduction was performed by a method similar to that described previously.[19] The current efficiencies obtained in their reductions were over 80%

The Enantioselective Synthesis of D- or L-Lactate. The liquid membrane redox system was employed for the synthesis of D- or L-lactates. A representative synthesis of L-lactate is described (Scheme 17). Other synthetic runs were performed under modified conditions and are summarized in Table I.

The enzymatic aqueous phase (1) (see Scheme 16) contained sodium pyruvate, $NAD^+$, 1,3-dithiol-2-propanol as the co-substrate[20] and coimmobilized L-lactate dehydrogenase and lipamide dehydrogenase[21] in 0.1 M Tris-$H_2SO_4$ buffer (pH 7.6). second aqueous phase (2) contained trans-4,5-dihydroxy-

Table 1. The Synthesis of L-Lactate Using the Liquid Membrane Under Different Experimental Conditions

| Cosubstrate | Organic Phase (mM) | Reducing Agent or Tungsten Cathode | $DTT^{ox}$ (mmol) | L-Lactate Obtained in 10 Days (mmol) | e.e. of L-Lactate (%) | $TN_{NAD}$ |
|---|---|---|---|---|---|---|
| DTP(10) | 10 | $NaBH_4$(1%) | 10 | 4 | 82 | 6 |
| DTP(20) | 40 | $NaBH_4$(1%) | 20 | 18 | 80 | 28 |
| Lipoic acid (60) | 40 | $NaBH_4$(1%) | 20 | 16 | 77 | 24 |
| DTP(20) | 40 | $Na_2S_2O_4$(1%) | 20 | 17 | 95 | 34 |
| DTP(20) | 40 | $Na_2S_2O_4$(1%) | 20 | 14 | 95 | 28 |
| DTP(20) | 40 | $Na_2S_2O_4$(1%) | 20 | 11 | 95 | 22 |
| DTP(20) | 40 | $Na_2S_2O_4$(1%) | 20 | 16 | 95 | 32 |
| DTP(20) | 100 | $Na_2S_2O_4$(1%) | 40 | 25 | 95 | 50 |
| DTP(20) | 100 | Tungsten cathode | 40 | 29 | 95 | 58 |
| DTP(20) | 150 | Tungsten cathode | 60 | 40 | 95 | 80 |

All experiments were performed at 25°C following the procedure described in the experimental section. The initial concentration of $NAD^+$ (1 mM) and pyruvate (200 mM, was the same in all the experiments. Initial activities of coimmobilized LipDH (120 - 150 U) and L-LDH (130 - 160 U) were used. The residual activities of the coimmobilized enzymes after 10 days were: LipDH (0- 25 U) and L-LDH (0 - 12 U).

1,2-dithiane (oxidized dithothreitol, $DTT^{ox}$) dissolved in 0.5 M carbonate buffer pH 9.5 that contained sodium dithionite as the reducing agent. o-Dichlorobenzene was used as the organic solvent and contained butane-1,4-dithiol. After seven days about 23% of the pyruvate was converted to L-lactate (Figure 14), and further conversion occured only slowly. The residual activities of L-lactate dehydrogenase and lipoamide dehydrogenase after 7 days were 37% and 47%, respectively of the original immobilized activities. The aggregate residual activity of $NAD^+$ and NADH after 7 days was 55%. The reaction was continued for another 3 days by adding more dithionite to the reagent solution (see Scheme 16). A total of 25 mmol of L-lactate (25% coversion) was produced in 10 days. The residual activities of lipoamide dehydrogenase and L-lactate dehydrogenase after 10 days were 25% and 12%, respectively of the original immobilized activities. The residual aggregate activity of $NAD^+$ and NADH was 42% of the original value. L-lactate (25 mmol) was obtained in 95% enantiomeric purity. The turnover numbers (TN = mol product/mol of cofactor or mol catalyst) were $TN_{NAD} = 50$; $TN_{L\text{-}LDH} = 2.2 \times 10^6$, $TN_{LipDH} = 7.3 \times 10^6$, $TN_{DTP} \sim 3$.

The Stability of the Coimmobilized Enzymes and of NAD(H) in the Liquid Membrane System. The stability of coimmobilized L-lactate dehydrogenase or D-lactate dehydrogenase and lipoamide dehydrogenase was monitored under operating

conditions of the liquid membrane (Figure 15). D- and L-lactate dehydrogenases are somewhat less stable than lipoamide dehydrogenase but all three immobilized enzymes lose half of their original activity in 5-7 days. The inactivation of the immobilized enzyme in this liquid-membrane system is probably due to the decomposition of the polymeric gel in the aqueous phase and at the interface with the organic solvent. The chlorinated hydrocarbons (o-dichlorobenzene and chlorobenzene) are slightly soluble in the organic aqueous buffer (Tris-$H_2SO_4$). This solubility contributes to the inactivation of the gel-immobilized enzymes. After 10 days the residual activities of the coimmobilized lipoamide dehydrogenase and L-lactate dehydrogenase were 25% and 12%, respectively of the initial immobilized activities. In other experiments, in which methylene chloride or tetrachloroethane were used both enzymes lost their full activity after 6-8 days.

The polyacrylamide gel immobilization method is apparently not suitable for the kind of application described here. A different immobilization method (glutaraldehyde cross linking, glass beads) might improve the stability of enzymes in aqueous phases that contain low concentrations of organic solvents.

The concentrations of $NAD^+$ and NADH in the enzymatic reactor were monitored during the synthesis of L-lactate. The aggregate activities of $NAD^+$ and NADH after 7 days were about 55% of their original activities. Similar residual

Scheme 17. The Synthesis of L-Lactate Using NADH Recycling Based on a Liquid Membrane Redox System.

# Scheme 17

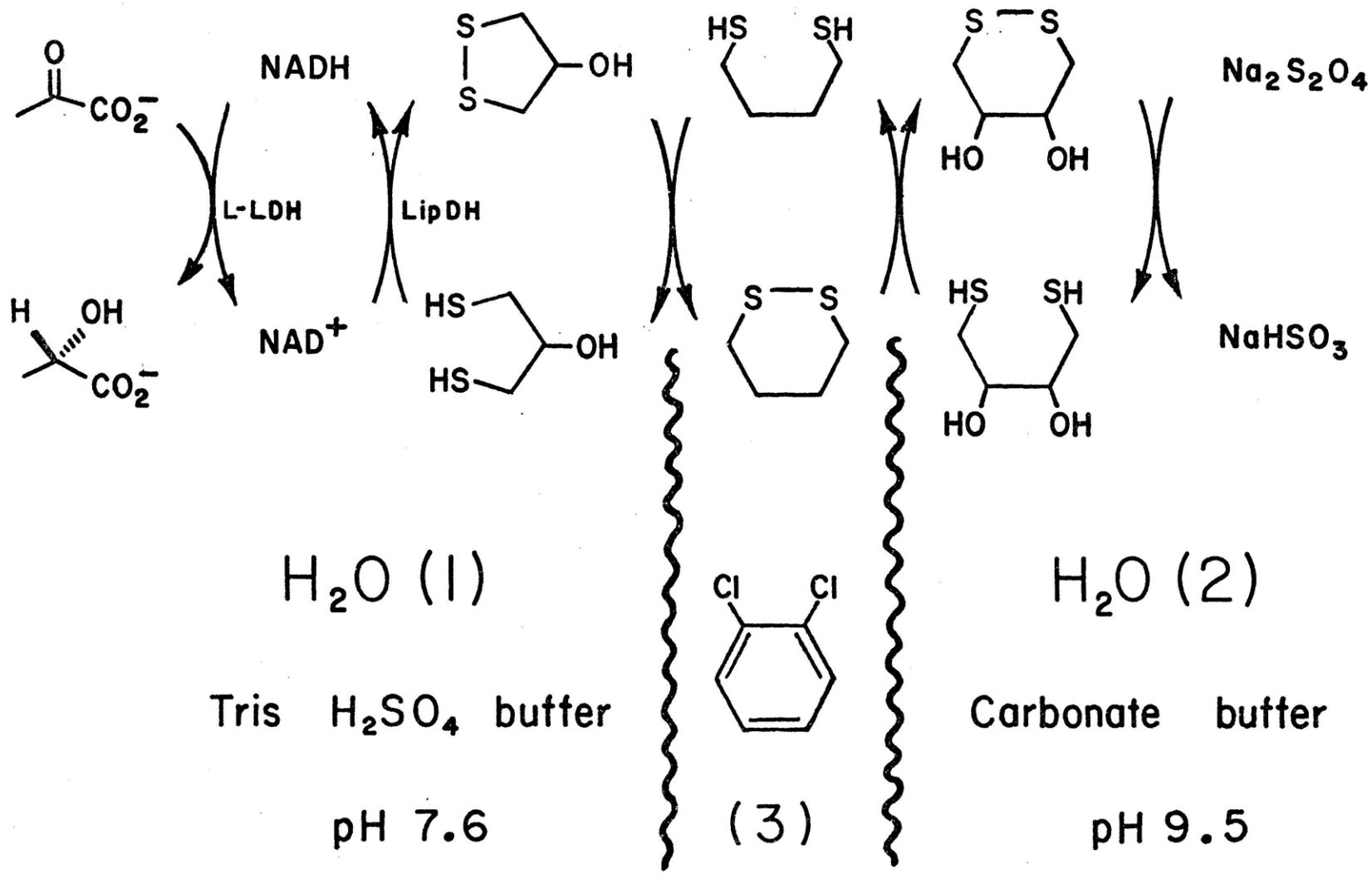
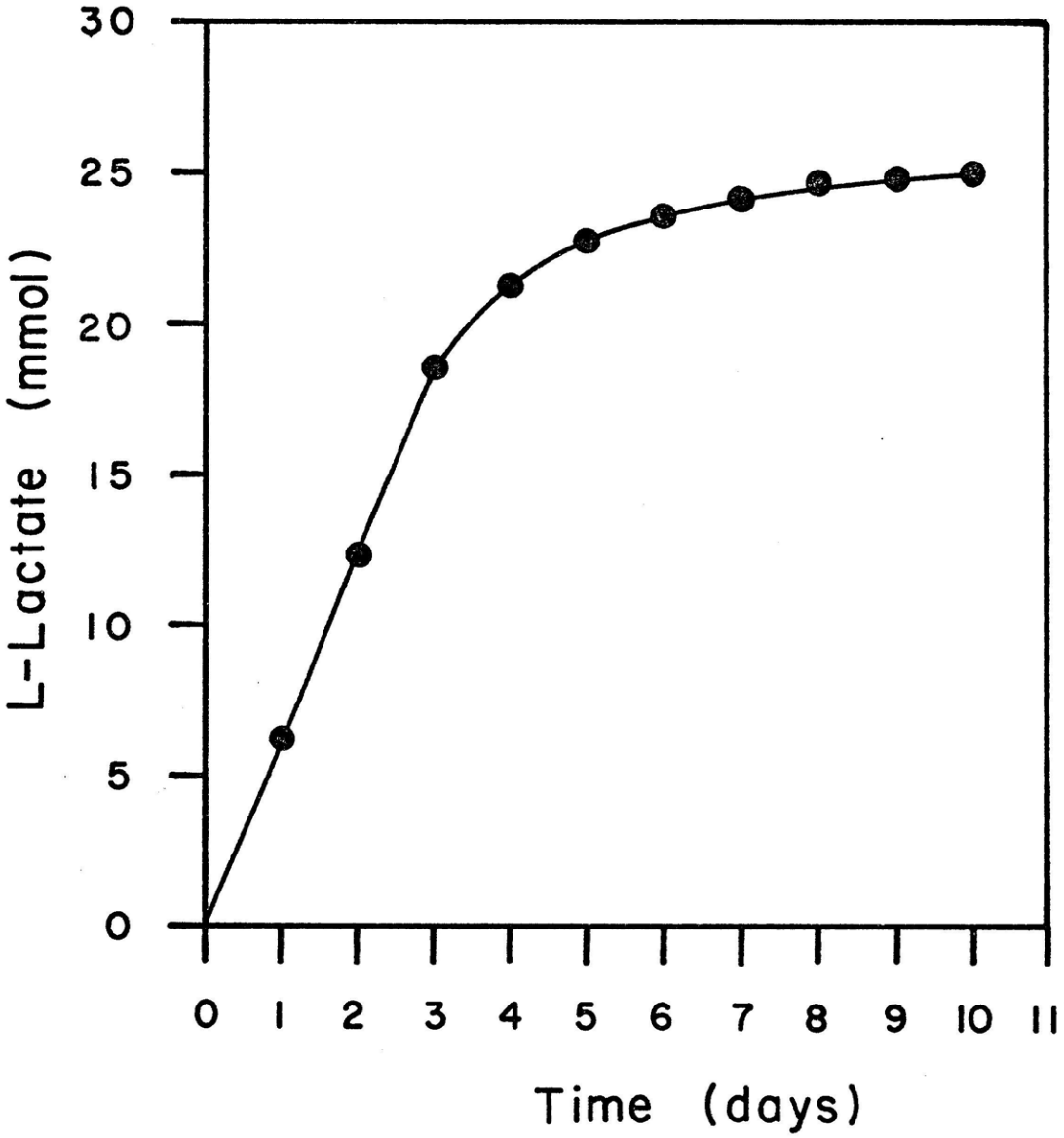

NADH
NAD+
L-LDH
LipDH
$Na_2S_2O_4$
$NaHSO_3$
$H_2O$ (1)
$H_2O$ (2)
Tris $H_2SO_4$ buffer
pH 7.6
(3)
Carbonate buffer
pH 9.5

Figure 14. The Synthesis of L-Lactate.

L-lactate was determined enzymatically as described. Sodium dithionite (1% solution) was used as the reducing agent. The following concentrations were used. Butane-1,4-dithiol (0.1 M), $DTT^{ox}$ (40 mM), $NAD^{+}$ (1.0 mM), DTP (20 mM). Coimmobilized LipDH (152 U) and L-LDH (156 U) were used.

# Figure 14



activities (54-66%) were obtained in other reactors. After 10 days the aggregate activities of NAD and NADH were ∿ 41% of the initial aggregate activities. The decomposition of the nicotinamide cofactors ($NAD^+$, NADH) in the described liquid membrane system is analogous to their decomposition in the formate/formate dehydrogenase method

Characterization of the Liquid Membrane Redox System. Control experiments, determination of partition coefficients of the thiols and disulfides involved, and experiments in modified liquid membrane reactions were carried out to characterize the system and to obtain a better understanding of how the system operates. First, it was important to establish that the reagent (dithionite) is not carried over mechanically from one aqueous phase to the other. Control experiments were done in which methylene blue attached to dextran was dissolved in one of the two aqueous phases that were separated by an organic layer (o-dichlorobenzene, chlorobenzene or 1:1 mixture of both). All three phases were stirred and the system was observed for 4 days.

The aqueous methylene blue solution on one side of the liquid membrane system was not transferred to the other aqueous phase (double distilled $H_2O$), which remained colorless. Second, it was important to establish where the thiol-disulfide interchange reaction takes place. Without the presence of butane-1,4-dithiol the conversion of pyruvate to

Figure 15. Stability of Coimmobilized L-Lactate Dehydrogenase or D-Lactate Dehydrogenase and Lipoamide Dehydrogenase Under Operating Conditions Using o-Dichlorobenzene as the Organic Phase.

(●) Lipoamide Dehydrogenase

(▲) D-Lactate Dehydrogenase

(■) L-Lactate Dehdrogenase

Assays of the immobilized enzymes were performed according to the procedures described in experimental.

# Figure 15

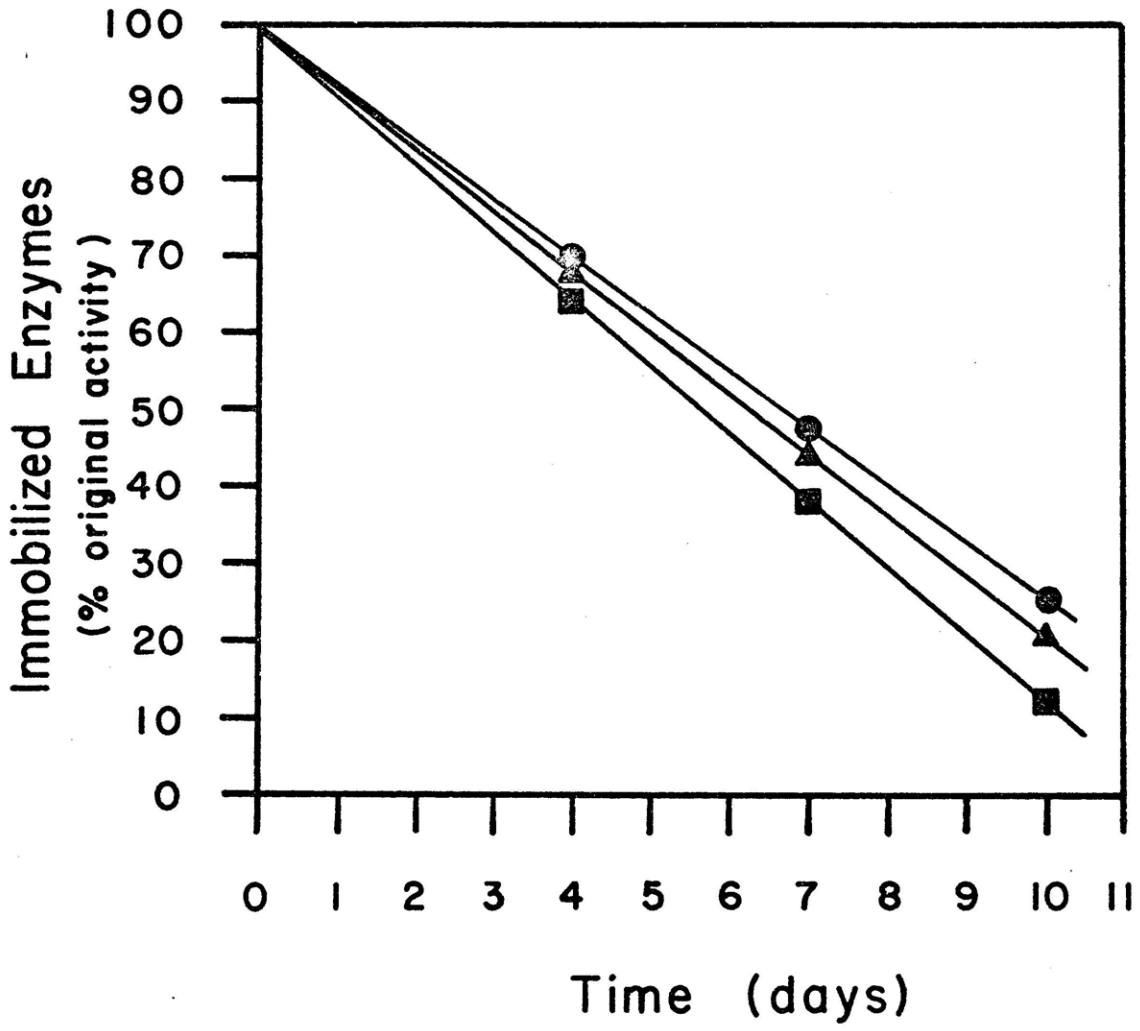
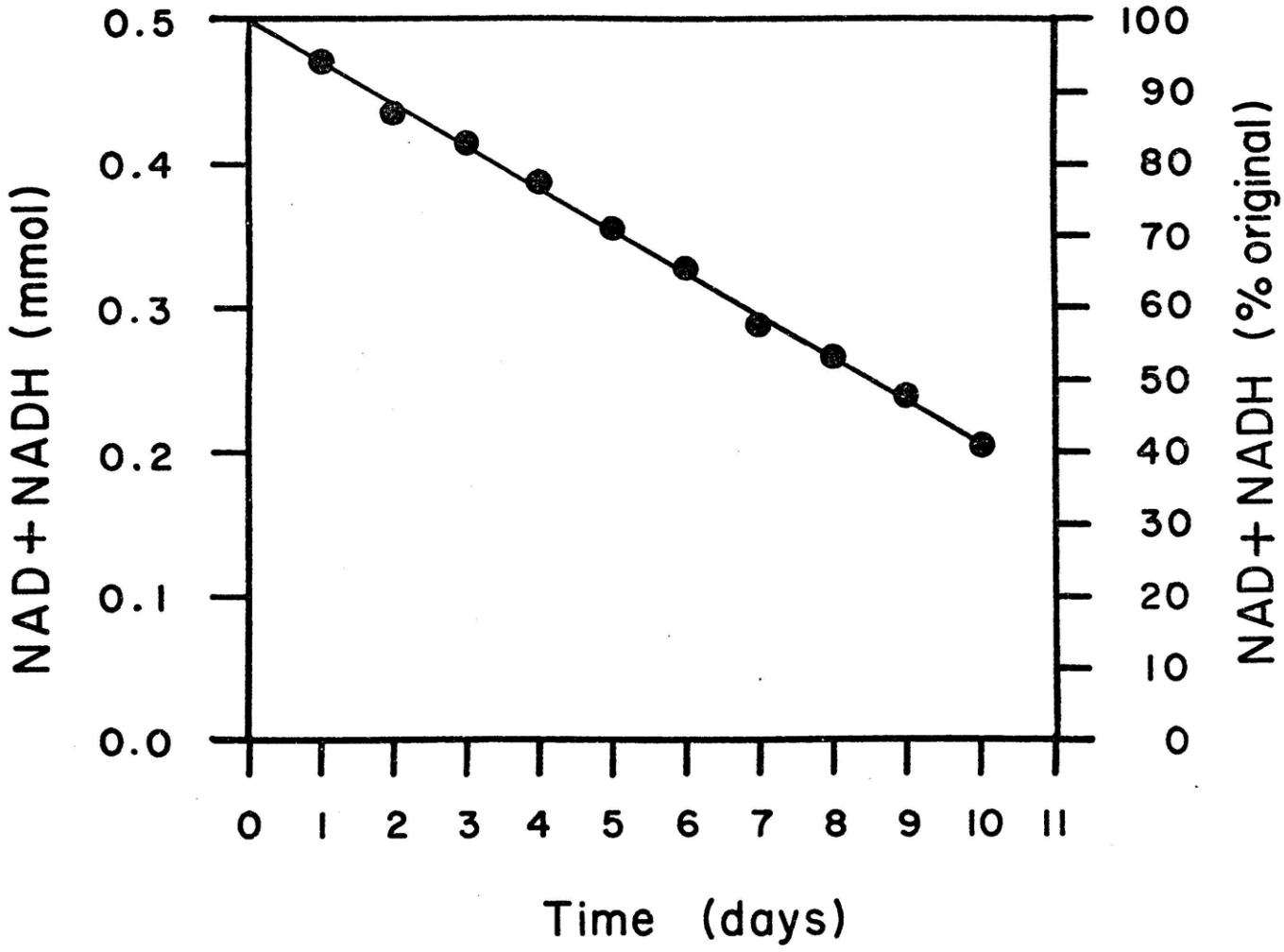

Immobilized Enzymes
(% original activity)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
Time (days)

Figure 16. Stability of $NAD^+$ and NADH in the Aqueous Phase of the Liquid Membrane System Under Operating Conditions.

The concentrations of $NAD^+$ and NADH were determined according to the assays described in experimental.

# Figure 16


0.5
0.4
0.3
0.2
0.1
0.0
NAD+NADH (mmol)
100
90
80
70
60
50
40
30
20
10
0
NAD+NADH (% original)
0 1 2 3 4 5 6 7 8 9 10 11
Time (days)

L-lactate practically does not occur. On the other hand, butane-1,4-dithiol is insoluble in aqueous solution (partition coefficient <0.01, between o-chlorobenzene and $H_2O$). Therefore, it is reasonable to assume that the thiol-disulfide interchange takes place at the interface of the described system. Third, the productivity of the method is low (Figure 14). In other experiments (Figure 17) the efficiency was improved by employing the cathodic reduction of cyclic disulfides. Use of the electrochemical system allowed the reaction to be carried out at pH 6-7. This decrease in pH improved the mass transfer of the reduced product (DTT) from the aqueous to the organic phase. Higher concentrations of butane-1,4-dithiol (80 - 150 m$\underline{M}$) in the organic phase and of $DTT^{ox}$ (40 - 60 m$\underline{M}$, very close to saturation) in the aqueous solution improved the productivity of the system by 20 - 30% (Figure 17). The most efficient system (Table I, last experiment) was achieved by employing the electrochemical system and by using high concentrations of butane-1,4-dithiol and $DTT^{ox}$. Substituting $\underline{o}$-dichlorobenzene with other chlorinated hydrocarbons (chlorobenzene, $CH_2Cl_2$) or a mixture of them did not improve the efficiency of the systems. Some of these solvents ($CH_2Cl_2$, $CH_3CCl_3$) were more soluble in the aqueous phase and enhanced the inactivation of the coimmobilized enzymes. A few experiments were done with a "reverse" liquid membrane, where toluene, xylene, or decane were used as the organic layer on the top of the two aqueous sections. This modified liquid membrane

Figure 17. The Synthesis of L-Lactate Using the Liquid Membrane Redox System Under Different Conditions.

The syntheses described were performed at 25°C under the same $NAD^+$ (1.0 m$\underline{M}$), and DTP (20 m$\underline{M}$) concentrations (500 mL 0.1 $\underline{M}$ Tris-$H_2O_4$, pH 7.6). The coimmobilized enzymes were: LipDH (120 - 150 U) and L-LDH (130 - 160 U). The organic phase in all the experiments was o-dichlorobenzene (400 mL). The product (L-lactate) was determined enzymatically according to the procedure described in the experimental section.

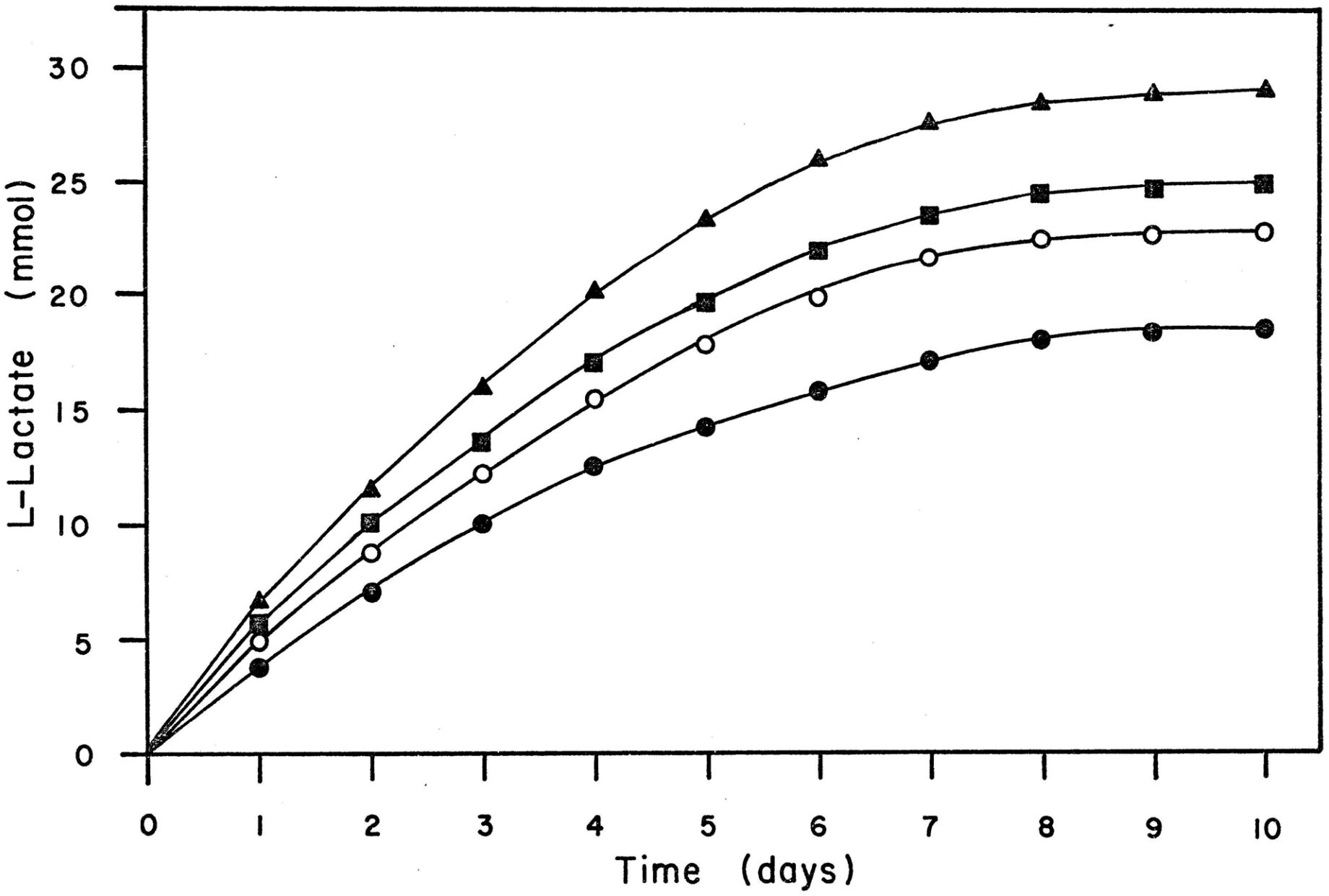
(●) Butane - 1,4-thiol (40 m$\underline{M}$), $DTT^{ox}$ (20 m$\underline{M}$), dithionite (1% solution, 500 mL carbonate buffer, pH 9.5)

(o) Butane-1,4-(40 m$\underline{M}$), $DTT^{ox}$ (20 m$\underline{M}$), electrochemical reduction (tungsten cathode, 10 ft wire, 0.03 in. diameter)

(■) Butane - 1,4-thiol (0.1 $\underline{M}$), $DTT^{ox}$ (40 m$\underline{M}$), dithionite solution)

(▲) Butane - 1,4-thiol (0.1 $\underline{M}$), $DTT^{ox}$ (40 m$\underline{M}$), electrochemical reduction (tungsten cathode 10 ft wire, 0.030 in. in diameter and a constant potential of -0.9 V vs. SCE).

Figure 17



system had low productivities (< 1 mmol L-lactate produced/ day).

The results suggest that when dithionite is used as the reducing agent, the rate-limiting step in the system is the thiol-disulfide interchange that takes place at the interface of the organic aqueous phase that contains the reagent. Indeed, this may be the case since DTT once formed by the reduction, is in the ionized form and the partition coefficient (as measured with Ellman's reagent) is exceedingly low (< 0.05). On the other hand, when the electrochemical system is employed, the rate determining step is the thiol-disulfide interchange reaction that takes place at the interface between the organic layer and the aqueous solution that contains the enzymes and the cosubstrate. The oxidized form of DTP (4-hydroxy 1,2-dithiolane) remains basically in the aqueous phase.

## Concluding Remarks

The liquid membrane redox system for the recycling of NADH described here is less efficient than the formate/formate dehydrogenase or the coupled electrochemical/enzymatic systems that were described in the previous chapters and its productivity is less. Much better partition coefficients of the cosubstrates (in their reduced and oxidized forms) are needed. Obtaining better partition coefficients will require the syntheses of 1,3-dithiol compounds that have good partition coefficients (> 0.5) between the aqueous phases and the immiscible organic solvent and in addition will be effective substrates (low $K_m$ and high $V_{max}$ values). The use of a different enzyme immobilization method would also be beneficial to this liquid membrane system, if it proved to yield more stable enzymatic activities.

## Experimental

General and Materials. Lipoamide dehydrogenase (EC 1.6.4.3) from porcine heart (suspension in 3.2 M $(NH_4)_2SO_4$ solution 20 U/mL) or from torula yeast (stable lypophilized powder, ∿ 35 U/mg protein), L-lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle (lyophilized, salt free powder, 1200 U/mg), D-lactate dehydrogenase (EC 1.1.1 28) from lactobacillus leichmannii (suspension in 3.2 M $(NH_4)_2SO_4$ solution, 350 U/mg protein(, DL-6,8-thioctic acid (DL-α-lipoic acid, oxidized form), DL-6,8-thioctic acid amide (DL-dipoamide), trans-4,5-dihydroxy-1,2-dithiane (oxided DTT), DTT, NAD, were obtained from Sigma Chem. Co. 1,3-dithiol-2-propanol (DTP), sodium borohydride, sodium dithionite and 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) were obtained from Aldrich Chem. Co. Spectrophotmetric measurements were carried out on a Perkin Elmer Model 552 spectrometer. pH measurements were done using a Radiometer PH M62 standard potentiometer. Water was doubly distilled using Corning AG-1B still.

Enzyme Assays: All assays were performed spectrophotometrically at 340 nm in the thermostatically controlled sample at 25°C. D- and L-lactate dehydrogenase were assayed according to procedures described in Bergmeyer.[22] Lipoamide dehydrogenase was assayed by a modified procedure described by Massey. The substrate was 1,3-dithiol-2-propanol. The concentrations in the assay mixture were: 70 mM Tris-$H_2SO_4$

buffer (0.1 M, pH 7.8), 0.1 mM EDTA, 0.5 mM NAD, 20 mM, 1,3-dithiol-2-propanol (the dithiol was distiled, and kept under argon atmosphere before use) and lipoamide dehydrogenase (0.5 U/mL in the assay mixture).

Enzymatic Determination of $NAD^+$ and NADH. $NAD^+$ and NADH were determined enzymatically according to the procedures described.[23]

Enzymatic Determination of D- and L-Lactate. D- and L-lactates were determined enzymatically according to the procedures described.[24]

Coimmobilization of L-Lactate Dehydrogenase or D-Lactate Dehydrogenase and Lipoamide Dehydrogenase. PAN-1000 (2.5 g) was dissolved at room temperature in 10 mL of 0.3 M Hepes buffer (pH 7.6) that contained lipoic acid (40 mM), NAD (10 mM), NADH (5 mM) and pyruvate (20 mM). After 1-2 min of vigorous stirring all the polymer dissolved. DTT (0.1 mL 0.1 mL, 0.3 M solution) was added immediately followed by the addition of 1 mL of the enzyme's solution. The enzyme solution contained 380 U of lipoamide dehydrogenase and 310 U of L-lactate dehydrogenase (or 300 - 350 U of D-lactate dehydrogenase). Triethylenetetramine (0.75 mL of 0.5 M solution) was added. Within 2-3 min, the solution became a transparent gel, which was allowed to stand for 60 min at room temperature under argon. The gel was ground in a mortar for about 10 - 15

min with periodic additions of aliquots (2 mL) of washing solution (50 mM Hepes buffer, pH 7.6, degassed). The gel suspension was transferred to a centrifuge tube (sealed with serum stopper) and separated from the washing solution by centrifugation (∿ 5 min at 3000 rpm). The gel suspension was assayed for activity.

Synthesis of L-Lactate Using Dithionite as the Reducing Agent. A representative reaction was carried out at 25°C in a 3 L beaker that was divided to two sections by a flat piece of glass forming a U shape within the beaker (Scheme 16) A long magnetic stirring bar was placed at the bottom of the beaker. o-Dichlorobenzene (400 mL) that contained butanedithiol (0.1 M) was added first. Sodium pyruvate (11 g, 100 mmol), DTP (0.94 mL, 10 mmol), NAD (0.33 g, 0.5 mmol) were dissolved in 500 mL of Tris-$H_2SO_4$ buffer (0.1 M, pH 7.6) and the solution adjusted to pH 7.6. Lipoamide dehydrogenase (LipDH, 152 U) and L-lactate dehydrogenase (L-LDH, 156 U), coimmobilized in crosslinked polyacrylamide gel (PAN-1000, 30 mL of gel) were added. The aqueous solution that contained the reagent was prepared by dissolving sodium dithionite (1%) and $DTT^{ox}$ (3.0 g, 40 mM) in 500 mL of carbonate buffer (0.5 M, pH 9.5, degassed and kept under argon). The two aqueous solutions were added carefully to the two sides of the divided beaker. All three solutions were stirred by using mechanical and magnetic stirrers.

The reactor was kept under argon and the reaction was followed by enzymatic assays of L-lactate and NAD(H). Each day during the 10 days of the reaction, dithionite (1% solution) was added. No further reaction was observed at 10 days. The aqueous solution that contained the product and the gel was transferred by a cannula into a beaker. The gel was separated by contrifugation, and assayed for the residual enzymatic activities. The supernatant was assayed to determine the concentrations of L-lactate and NAD(H).

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