# THE RECYCLING OF $NAD^+$ (FREE AND IMMOBILIZED) WITHIN SEMIPERMEABLE AQUEOUS MICROCAPSULES CONTAINING A MULTI-ENZYME SYSTEM

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SUMMARY: Semipermeable aqueous collodion microcapsules were prepared containing both yeast alcohol dehydrogenase (EC 1.1.1.1) and malic dehydrogenase (EC 1.1.1.37). These microcapsules exhibited both enzymic activities in good amount in the ratio 3:1 with respect to malic dehydrogenase : alcohol dehydrogenase.

Both $NAD^+$ and NADH were successfully cycled within the microcapsules by employing the included enzyme activities acting sequentially. A soluble, immobilized $NAD^+$ derivative was also recycled within the semipermeable microcapsules.

INTRODUCTION: To date, most of the situations in which immobilized enzymes have been demonstrated to be practical propositions have involved rather simple enzyme systems. This is so because more complex enzyme systems usually require a supply of coenzymes. Recently, there have been several attempts to improve economics and availability in the use of coenzymes (1-3). Expensive and labile coenzymes have been generated from cheaper and more stable precursors (4), while the cyclic regeneration of coenzymes has been demonstrated in several different situations (3,5,6).

Microcapsules can be prepared containing a great variety of entrapped enzymes (7). Because of this, microcapsules appear to present an ideal immobilized enzyme system to achieve the cyclic regeneration of coenzymes. Recently, the cyclic regeneration of ATP between its fully phosphorylated and its dephosphorylated form was demonstrated using an encapsulated multi-enzyme system (1).

MATERIALS AND METHODS: Alcohol dehydrogenase (EC 1.1.1.1) from yeast (YADH) (321 units/mg); malic dehydrogenase (EC 1.1.1.37) from beef heart (MDH) (4,500 units/mg); and type 1 glutamic oxaloacetic transaminase (EC 2.6.1.1) from pig heart were all obtained from Sigma Chemical Co.,



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St. Louis, Mo., U.S.A. Grade III $NAD^+$ from yeast; Grade III NADH (disodium salt) from yeast; and Grade I cis-oxaloacetic acid were all obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-glutamic acid hydrochloride and L-malic acid were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A.

Preparation of semipermeable microcapsules containing YADH and MDH: YADH and MDH were immobilized simultaneously by the procedure of microencapsulation within collodion membranes (8,9), employing the updated procedure (7). 2.8 mg of YADH and 0.22 mg of MDH were dissolved in 2.5 ml of the hemoglobin solution prior to the generation of the microcapsules (1). The microcapsules were then exposed to a cross-linking procedure using glutaraldehyde, which was a modification of that previously described by Chang (10). The microcapsules were collected via centrifugation and were suspended in a total volume of 25 ml by the addition of 0.1M borate, pH 8.5, and the suspension cooled to 4°C. 50 µl of 50% (w/w) glutaraldehyde was added, and the mixture agitated at 4°C for 30 minutes, at which time the microcapsules were recovered by centrifugation and washed three times with 0.1M-Tris, pH 8.5. The cross-linked microcapsules were then washed five times with saline, and were finally suspended in 0.1M-$NaH_2PO_4$, pH 8.0 (50 ml total volume) and were stored in this form at 4°C.

Preparation of semipermeable microcapsules containing YADH, MDH, and polyethyleneimine-$NAD^+$: A water soluble $NAD^+$ derivative was prepared by attaching $NAD^+$ to polyethyleneimine by the method of Wykes et al. (3). The final precipitate of the polyethyleneimine-$NAD^+$ adduct was redissolved in 10 ml 0.05M-sodium acetate, pH 5.5 containing 2M NaCl, and stored in this form at 4°C. The appropriate amount of polyethyleneimine-$NAD^+$ to be encapsulated was precipitated with 1.0M-$NaH_2PO_4$, pH 6.0, and was then redissolved in the 10g% (w/v) hemoglobin solution (along with YADH and MDH), prior to the initial emulsification step. The rest of the microencapsulation procedure was as described previously (7). Two loadings of

polyethyleneimine-$NAD^+$ were encapsulated. The high loading contained 5.8 μ moles reducible $NAD^+$, and the low loading contained 0.28 μ moles reducible $NAD^+$.

Measurement of enzymic activities: The enzymic activities of the microcapsules were determined by the procedure described earlier (1). All reactions were carried out at 27°C and were initiated by the addition of 0.5 ml of the microcapsule suspension. The total reaction volume in each case was 2.55 ml. Blank assays were performed by adding 0.5 ml of 0.1M-$NaH_2PO_4$ instead of the microcapsule suspension to initiate the reaction.

Enzymatic cycling of free $NAD^+$ or NADH with semipermeable aqueous microcapsules: The enzymatic recycling of $NAD^+$ or NADH was achieved by employing the YADH and MDH activities of the microcapsules sequentially. The recycling system employed for $NAD^+$ is shown schematically in Fig. 2 (a), and is referred to as Cycle I. In this cycling system, reactions were terminated after the desired reaction time, by heating the reaction mixture at 100°C for 5 minutes. This served the dual purpose of quickly stopping the reaction by inactivating the enzymes, and also destroying any unreacted oxaloacetic acid (5). The production of malic acid indicated the functioning of both the enzymes inside the microcapsules, and this was followed by a modification of the procedure described by Kato et al. (5) (Fig. 2 (a)). After termination of the cycling reaction, the reaction mixture was centrifuged, and 0.5 ml of the supernatant was used in the assay for malic acid. The enzymatic recycling of NADH was achieved by a method identical to that described previously by Cycle I, except that NADH was used instead of $NAD^+$. This system is known as Cycle II and is shown schematically in Fig. 3 (a).

Controls for Cycles I and II were performed by blocking the cycles by omitting the non-coenzyme substrate of the second acting enzyme in each cycle.

Enzymatic cycling of polyethyleneimine-$NAD^+$ within semipermeable aqueous microcapsules: Polyethyleneimine-$NAD^+$ was recycled between its oxidized

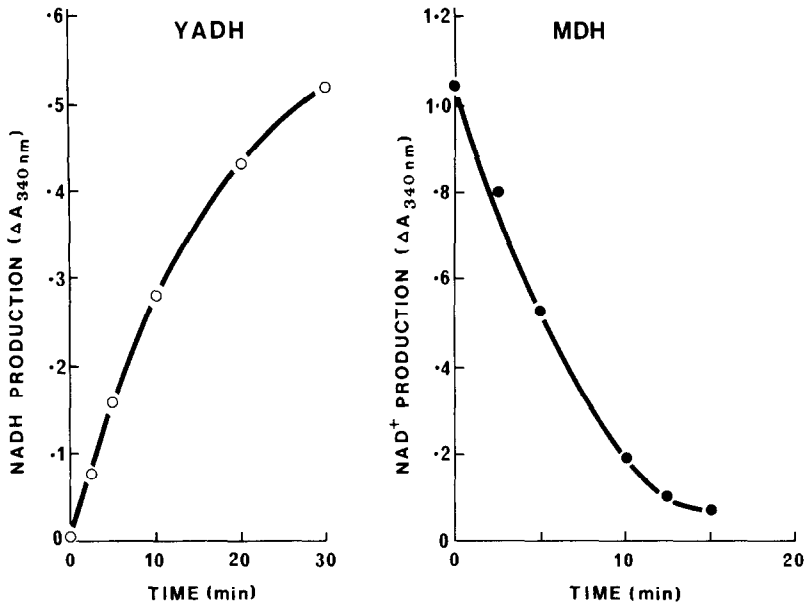


Figure 1. The individual enzyme activities of the microcapsules. The YADH activity was determined singly by agitating the microcapsules in the presence of 0.21M-ethanol; 0.19mM-$NAD^+$; and 0.1M-$NaH_2PO_4$, pH 8.0. The MDH activity was determined singly be agitating in the presence of 0.981mM-oxaloacetic acid; 0.196mM-NADH; and 0.1M-$NaH_2PO_4$, pH 8.0. (•)-MDH activity. (o)-YADH activity. The change in absorbance at 340 nm was a measure of the enzymic activities.

and reduced state by employing the cycling system described previously for Cycle I, but in this case employing the microcapsules containing ADH, YADH, and the polyethyleneimine-$NAD^+$ derivative. The buffer used in this case was 0.1M-Tris, pH 8.0.

RESULTS AND DISCUSSION. Enzymic activities: The individual enzyme activities of the microcapsules, assayed as described, can be seen in Fig. 1. The microcapsules displayed both YADH and MDH activities in good amount, in the ratio 3:1, MDH:YADH under the conditions employed.

Free coenzyme recycling within semipermeable microcapsules: The YADH and the MDH activities of the microcapsules were employed sequentially in order to recycle free $NAD^+$ and NADH, and the result of this can be seen in Figure 2 (b) and Fig. 3 (b) respectively. In the case of Cycle I, the formation of malate indicated the functioning of both enzymes, and hence

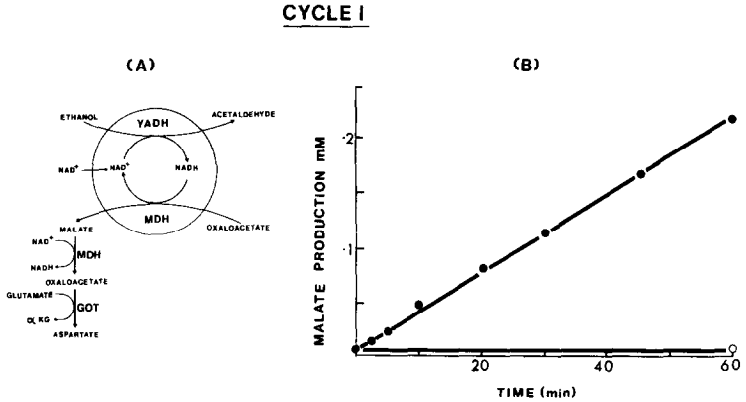


Figure 2. (a) Schematic representation of Cycle I, and the assay for produced malate. Cycle I was achieved by suspending the microcapsules in the presence of 0.0196mM-$NAD^+$; 0.217M-ethanol; 0.981mM-oxaloacetic acid; and 0.1M-$NaH_2PO_4$, pH 8.0. The malate produced was assayed as described in the text. Thus, 0.5 ml supernatant was added to 1.5 ml $NAD^+$ (2mM); 0.5 ml 30mM-glutamic acid; and 1.0 ml 0.1M-glycine/NaOH, pH 10.0. The change in absorbance at 340 nm after the addition of 5 µl glutamate oxaloacetate transaminase and 25 µl MDH was noted (after equilibration). This procedure was standardized by subjecting known amounts of malic acid to the same assay.

(b) The time course of Cycle I, showing the formation of malate with time. (●)-Complete Cycle I; (o)-Blocked Cycle I (oxaloacetate omitted). All reactions are drawn unidirectional for simplicity.

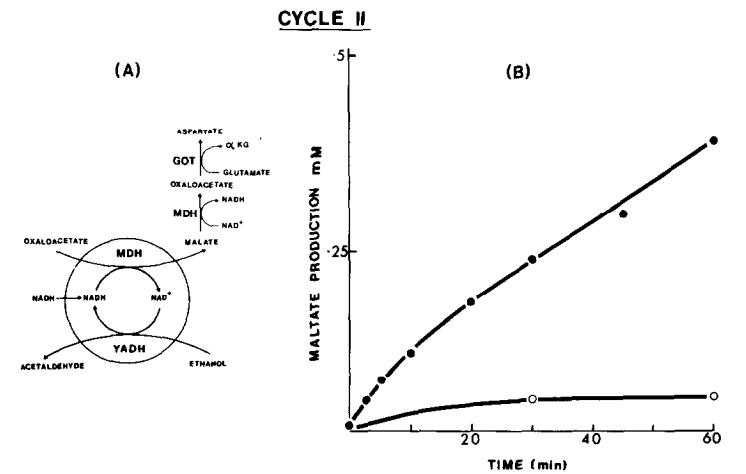


Figure 3. (a) Schematic representation of Cycle II, and the assay for produced malate. Cycle II was achieved in a similar fashion to Cycle I, except that 0.0196mM-NADH was used instead of $NAD^+$. The malate produced was assayed as described in Fig. 2.

(b) The time course of Cycle II, showing the formation of malate with time. (●)-Complete Cycle II; (o)-Blocked Cycle II (ethanol omitted). All reactions are drawn unidirectional for simplicity.

the cycling of the coenzyme. The production of malate was found to be linear throughout the length of the experiment, and indicated a rate of reaction equal to the production of 0.064 μ moles malate/h. The lower line of Fig. 2 (b) was the result obtained when Cycle I was blocked by the omission of oxaloacetic acid from the reaction mixture, and thus represents the reaction blank. As expected, the reaction blank was zero for this cycle. When Cycle II was blocked by the omission of ethanol from the reaction mixture, only a very small amount of malate was produced as would be expected. This is indicated by the lower line in Fig. 3 (b). Nearly ten times as much malate was produced when the substrate ethanol was added to complete the cycle. The upper line in Fig. 3 (b) indicates the cycling of NADH by the microencapsulated bi-enzyme system. Initially, the measured rate of coenzyme cycling was greater for Cycle II than for Cycle I, but after about the first third of the cycling reaction, the rate of cycling was similar for Cycles I and II. This was presumably due initially to the higher MDH activity of the microcapsules, and later to the rate limiting effect of the YADH activity.

The overall rate of cycling for the cycles discussed here could have been increased substantially by several factors. Firstly, more enzyme could have been encapsulated initially. Secondly, the encapsulated activity would have been further increased by carrying out the immobilization procedures, and cross-linking steps in the presence of either a substrate or a competitive inhibitor for the enzyme(s), hence, maximizing the immobilized activity. Thirdly, higher substrate concentrations could have been employed, and lastly, the ratio of the individual enzymes could have been optimized. This work however, was not concerned with the miaximization of the cycling rate. We have attempted to establish the feasibility of using microencapsulated multi-enzyme systems as vehicles to achieve the cyclic regeneration of free $NAD^+$/NADH.

Recycling of polyethyleneimine-$NAD^+$ within semipermeable microcapsules: Fig. 4 shows the production of malate with time when the microcapsules

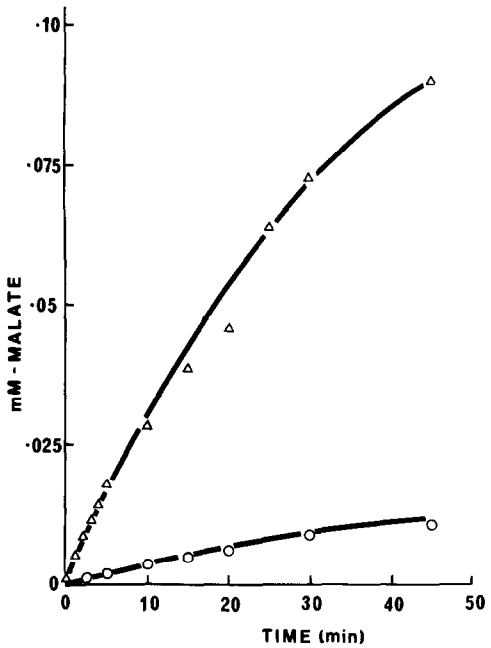


Figure 4. The enzymatic recycling of polyethyleneimine-$NAD^+$ within microcapsules containing YADH, MDH, and two different polyethylenimine-$NAD^+$ loadings. (o)-Low loading; (Δ)-High loading. Experimental details are given in the text. The production of malate with time indicates the functioning of the cycle.

containing YADH, MDH, and polyethyleneimine-$NAD^+$ were exposed to the cycling conditions described previously for Cycle I. From Fig. 4, it can be seen that the partially self-sufficient system functioned well, however, the amounts of malate produced by both the low and high loadings are perhaps lower than would be expected from the initial reducible $NAD^+$ concentrations employed. However, diffusional restrictions within the microcapsules may be responsible for this. In any event, the production of malate demands that the immobilized $NAD^+$ had been recycled, and work is now proceeding in order to achieve a more quantitative approach, and to optimize this and other recycling processes.

In conclusion, the cyclic processes described in this work appear to function well in the recycling of $NAD^+$ and NADH within semipermeable

aqueous microcapsules. Similar results were reported recently for ATP and ADP (1). Thus, it appears that microcapsules containing the appropriate cycling systems, are useful entities in situations where it is desired to cyclically regenerate coenzymes (free or immobilized).

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