COFACTOR RECYCLING IN LIQUID MEMBRANE-ENZYME SYSTEMS

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SUMMARY

In contrast to other entrapment techniques, hydrocarbon-based liquid surfactant membranes have been shown to effectively retain NADH and NAD^+. The activities of an immobilized yeast alcohol dehydrogenase (ADH) - NAD^+ system and of a coupled cofactor recycling system involving ADH, diaphorase and ferricyanide were examined by determining the extent of both ethanol consumption and acetaldehyde accumulation in the external aqueous solution. The results establish suitability of the liquid membrane system for the immobilization of enzyme systems involving in-situ cofactor regeneration.

Previous work has established that a number of enzymes retain catalytic activity when encapsulated with liquid-surfactant membranes (1-4). Among the purified enzymes which we have investigated are urease, tyrosinase and trypsin and these systems have been carefully examined with respect to catalytic properties and enzyme leakage. The effects of the hydrophobic membrane phase on substrate specificity, product diffusion and kinetic parameters have been examined and the feasibility of recovering the enzyme from the emulsion at the completion of the reaction of interest established. In addition, the successful encapsulation of cell-free homogenates and whole cells with nitrate and nitrite reductase activity has been reported. Thus it is clear from previous results that the liquid-membrane technique represents a viable method for immobilization of enzymes.

Since liquid-membrane encapsulation is a physical entrapment technique, it is eminently suitable for the immobilization of multienzyme systems for which polymer attachment methods are totally unsuitable. However, a number of other physical entrapment techniques, e.g. microencapsulation with permanent membranes, hollow fiber entrapment, use of ultrafiltration

cells, etc. (5), are available, and it is appropriate to ask whether any special characteristics accrue to systems entrapped within a hydrophobic barrier. It is the purpose of this communication to demonstrate that low molecular weight cofactors and enzymatic cofactor regeneration systems can be effectively retained in the liquid membrane system and coupled in situ to other enzymatic reactions. In contrast, retention of dissociable cofactors within conventional entrapment systems generally requires either the use of "tight membranes" which severly restrict diffusion, or prior attachment to macromolecular "carriers" which often results in drastic losses in activity. (6).

EXPERIMENTAL

Enzyme-containing emulsions were prepared according to the general procedure which we have previously described. (1) In all experiments described here, the membrane-forming mixture was composed of 2% Span-80, 3% ENJ-3029 and 95% S-100N. Typically, the scale of the preparation was such that 18 g of this mixture and 15 g of aqueous solution containing enzymes and/or cofactors were used, and emulsification was effected in a specially constructed baffled mixing vessel fitted with a photometrically monitored mechanical stirring system. Enzymatic reactions and leakage tests were carried out batchwise by dispersing the emulsion in an excess of the appropriate aqueous medium via magnetic stirring. The course of the reaction was followed by periodically withdrawing samples of the external aqueous solution, gravity filtering to remove residual emulsion, and then performing the appropriate analysis.

Ethanol determinations were carried out as follows: to 1 ml of 0.05 M Tris buffer, pH 7.6 were added 200 µl of 3 M Tris pH 8.8, 50 µg ADH and 1.3 mg NAD. Fifty µl of the ethanol-containing sample or control were added to initiate the reaction and the formation of NADH followed at 340 nm for at least 8 minutes. Acetaldehyde determinations were carried out as follows: To 1.3 ml of Tris buffer, pH 7.00, were added 50 µg of ADH and 7.6 µg of NADH, and the pH was carefully checked. The absorbance at 340 nm was recorded, 25 µl of the sample was added and the approach to equilibrium monitored at 340 nm.

In the trypsin titration experiments, the emulsion was prepared in the usual fashion, mixed with an equal volume of H_2O, and then centrifuged at 19,500 x g for 40 min., to give three layers. The middle layer was resuspended in phosphate buffer, pH 7.8 and recentrifuged, after which ultrafiltration of the phosphate solution gave the recovered trypsin in about 50% yield.

RESULTS AND DISCUSSION

Before attempting to incorporate well-defined cofactor regenerating and cofactor utilizing enzymes into the liquid membrane system, the extent to which low molecular weight cofactors were retained within the liquid

Table I. NAD^+ and NADH Leakage Tests - In separate experiments 10 ml of an emulsion containing 6 mg of either NAD^+ or NADH was dispersed in 50 ml of Tris buffer, pH 7, and at the times indicated, samples of the external solution were withdrawn and examined spectrophotometrically.

TIME (min)	TOTAL LEAKAGE NAD^+ (%)	TOTAL LEAKAGE NADH (%)
0	0	0
10	0	0
25	0	0
45	1.3	1.4
65	0.1	0
125	0.5	0

membrane was determined. In these experiments NAD^+ - and NADH - containing emulsions were dispersed in appropriate aqueous buffers and the external solution periodically assayed for cofactor leakage. As is evident from Table I, the extent of leakage is very small over the time period normally used for our liquid membrane experiments. In other experiments, we have found that the extent of leakage per-se does not increase significantly with time but the absolute integrity of the particular emulsions we use begins to decrease after several hours, due to the high stirring rate and small reactor size used in our experiments. Mohan and Li (4) have reported good long term retention of nitrate and nitrite reduction activity using an emulsion of somewhat different formulation and different reaction conditions.

Typical data which are obtained when emulsions containing various amounts of ADH and NAD^+ are dispersed in aqueous solutions of ethanol are shown in Figure 1. In these experiments, the ethanol concentration in the external solution was determined at various times by enzymatic assay with ADH as described in the Experimental Section. In the presence

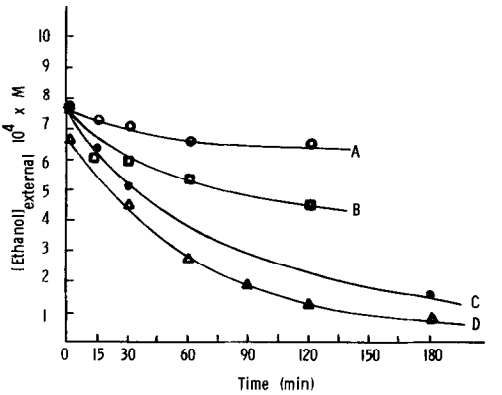


Figure 1. Ethanol Comsumption in ADH-NAD^+ Systems. The emulsion phase contained (per ml): A, 0.125 mg ADH, 0.65 mg NAD^+; B, 0.5 mg ADH, 2 mg NAD^+; C, 0.475 mg ADH, 1.5 mg NAD^+, 0.75 mg ferricyanide, 25 μg diaphorase; D, 0.49 mg ADH, 0.6 mg NAD, 0.8 mg ferricyanide, 25 μg diaphorase. Ten ml (expt. A) or 20 ml (expt B,C,D) of emulsion were dispersed in 50 ml ethanol solution to run the reaction.

of 0.5 M Tris in alkaline solution, the acetaldehyde product is trapped and the ADH reaction driven to completion, and thus the amount of NADH formed is a measure of the initial ethanol concentration. (7,8). It is evident from curves A and B of Figure 1 that both the rate and the extent of ethanol consumption from the external solution increase as the amounts of ADH and NAD^+ in the emulsion phase are raised. In the absence of NAD^+ or ADH, the extent of ethanol disappearance from the external solution into the emulsion phase is negligibly small. It is thus clear that the inherently low permeability of the polar ethanol molecule through the liquid membrane is enhanced by the incorporation of an ethanol-consuming system within the emulsion phase.

If the extent of ethanol loss from the external solution depends on the extent of its enzymatic oxidation within the membrane, then recycling of the NADH produced during the oxidation reaction back to NAD^+ should enhance ethanol diffusion by driving the ADH reaction in the direction of acetaldehyde formation. Accordingly, diaphorase and ferricyanide, in addition to ADH and NAD^+, were incorporated into a liquid membrane emulsion and the

results obtained when emulsions of this type are dispersed in aqueous solutions of ethanol are shown in Figure 1, curves C and D. It is apparent that cofactor recycling has greatly enhanced the rate and extent of ethanol consumption, even though the amount of NAD^+ present in the emulsions was considerably less than that in experiment B.

In order to test for acetaldehyde accumulation, the external solution was assayed with ADH and NADH as described in the Experimental Section. At the pH of the assay (7.0), the value of K_{eq} for ethanol oxidation by ADH is 7.5×10^{-12} (9). It can be readily calculated that in the presence of $[NADH]_o = 10^{-5}$ M, acetaldehyde concentrations in the range of 10^{-6} to 1.45×10^{-5} M can be determined by simply following the decrease in NADH absorbance caused by addition of the sample. In the range of $[acetaldehyde]_o$, $= 10^{-6}$ M, the aldehyde is quantitatively converted to alcohol with a corresponding oxidation of an equivalent amount of NADH (10% of $[NADH]_o$), while in the range of $[acetaldehyde]_o = 10^{-5}$ M, the assay begins to deviate from strict proportionality. Accordingly, our sample sizes were adjusted to keep the concentration of added acetaldehyde in the assay mixture within the linear range.

Figure 2 shows the results obtained when an emulsion containing the ADH-diaphorase system was dispersed in an aqueous solution of ethanol, and the external solution monitored for both ethanol and acetaldehyde. It is evident that acetaldehyde is indeed formed and does accumulate in the external solution. Inspection of Figure 2 reveals that about 20% of the ethanol is unaccounted for, indicating some accumulation of ethanol and/or acetaldehyde in the emulsion phase. Taken together, the data in hand establish that the coupled enzyme process outlined in Scheme 1, which involves cofactor recyling, is occuring in the liquid membrane system.

From the point of view of enzyme utilization, the incorporation of cofactor recycling catalysts in liquid membrane systems offers the unique advantage of reversibility - i.e. the emulsion can be broken and the enzymes

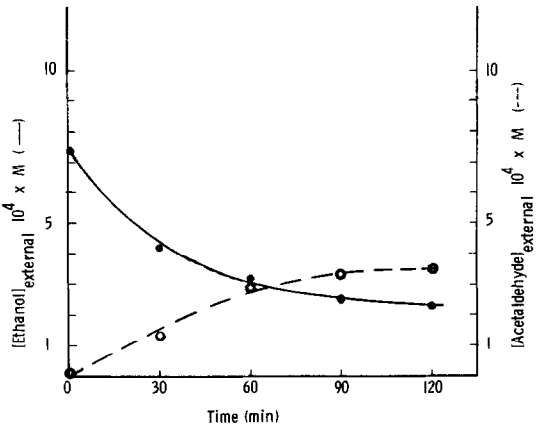
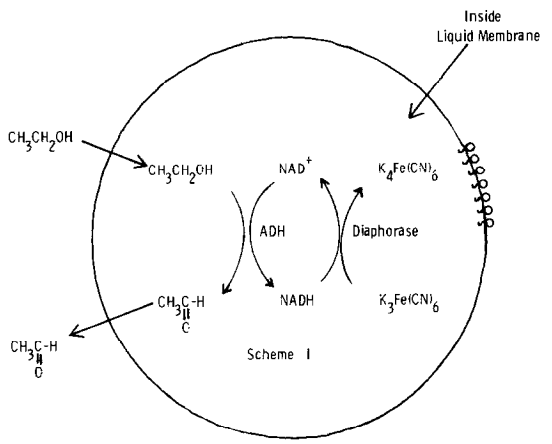


Figure 2. Cofactor Recycling System. The emulsion contained 0.475 mg/ml ADH, 1.5 mg/ml NAD^+, 0.75 mg/ml ferricyanide and 25 μg/ml diaphorase.



recovered at the completion of the process. In this regard, a critical question is how much irreversible denaturation has occurred during the emulsification and recovery processes. In order to answer this question, trypsin-containing emulsions were prepared and then broken, and the starting and recovered enzyme titrated with p-nitrophenyl-p'-guanidinobenzoate hydrochloride (10). In three separate experiments with different trypsin preparations the number of active sites per mol "protein" (based on $E_{1\%}^{280} = 14.3$) for the starting

and recovered trypsin were, respectively, 0.74 and 0.62; 0.36 and 0.27; 0.34 and 0.29. Thus, it is evident that gross denaturation of the starting trypsin does not occur during the emulsification and disruption processes.

In considering the special properties of liquid membrane systems, it seems likely that oxygenase enzymes might exhibit enhanced physical stability or radically altered reactivity when associated with a hydrophobic microenvironment, since these enzymes are known to be membrane-bound in the cell, and their reactivity can be influenced by hydrophobic interactions (11,12). By utilizing cofactor recycling systems, this possibility can now be more fully explored.

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