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The HLADH-Catalyzed Oxidoreduction in a Two-Phase System "Organic Solvent–Moistened Glass Beads"

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A two-phase immobilization method for horse liver alcohol dehydrogenase (HLADH)/NAD(H) is described. According to this method HLADH/NAD(H) is captured in a small volume of aqueous phase adsorbed on a solid support while the substrates, cyclohexanone and cyclopentanol, are dissolved in *n*-hexane. Agitation of the reaction mixture and partial reduction of the water content increase reaction rates. Complete removal of the water from the enzyme preparation, however, destroys its catalytic activity. Partition coefficients and time dependent substrate concentrations in the aqueous phase are measured. The immobilized catalyst is stable through repeated uses for 2 months.

INTRODUCTION

The NAD(H) dependent horse liver alcohol dehydrogenase (HLADH,[1] EC 1.1.1.1) is a very useful catalyst for the preparation of alcohols, ketones, and aldehydes with high enantiomeric purity (*1*). However, its use for preparative scale reactions is restricted by high cost and operational instability. Hence several strategies have been tried out to immobilize both the enzyme and the coenzyme (*2*). Recently Klibanov (*3*) reported a new method in which HLADH and NAD(H) were immobilized as a solid deposit on the surface of glass beads. These beads were, in turn, suspended in an organic solvent containing the substrates. We elaborated a slightly different approach. The biocatalyst was prepared by moistening the glass beads with a buffered solution of HLADH/NAD^+. In our basic procedure, and in contrast to Klibanov's, these beads were not dried and were used as such. In this study a critical evaluation of this procedure is given, using cyclohexanone and cyclopentanol as coupled substrates. *n*-Hexane has been used as the organic phase.

RESULTS AND DISCUSSION

The reactions and equilibria involved in the biphasic system *n*-hexane/Mops buffer (pH 7.0) are shown in Fig. 1.

[1] Abbreviations used: HLADH, horse liver alcohol dehydrogenase; Mops, 4-morpholinopropanesulfonic acid.

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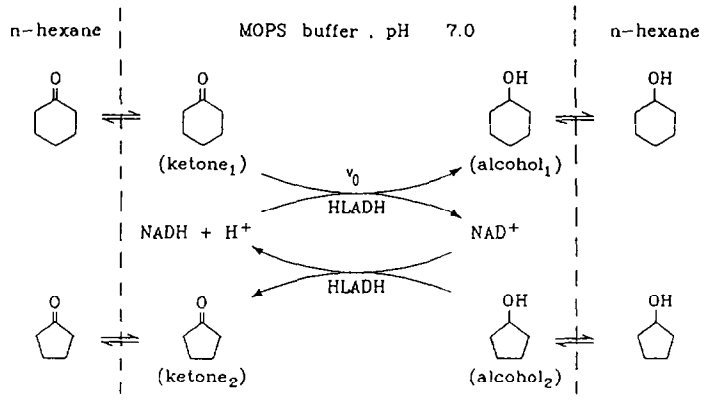

FIG. 1. Reactions and equilibria involved in the biphasic system. v_0 = initial rate of the cyclohexanone reduction step in the buffer solution. $K_{buf} = \frac{[\text{cyclohexanol}]_{buffer} \cdot [\text{cyclopentanone}]_{buffer}}{[\text{cyclohexanone}]_{buffer} \cdot [\text{cyclopentanol}]_{buffer}}$.

A. Reactivity of the Biocatalyst

Initial activities of HLADH/NAD(H) preparations adsorbed on several supports were measured. The results are given in Table 1.

As the initial activities with all these supports are similar, the mechanically more stable glass beads (0.1-mm diameter) were chosen as the support for further experiments.

The overall reaction rate v' has been studied for varying substrate concentrations (Fig. 2).

For a few concentrations of the same substrates the reaction rates in homoge-

TABLE 1

Influence of Support Type on Reaction Rate v'

Support	Reaction rate (10^{-6} mol/h · unit)	Support	Reaction rate (10^{-6} mol/h · unit)
Glass beads, 0.10 mm	3.0	Cellulose	3.0
Glass beads, 0.17 mm	3.6	Silica gel 60	1.8
Glass beads, 0.30 mm	2.1	Kieselguhr G	3.8
Glass beads, 0.50 mm	4.0	Aluminum oxide HF_{254}	2.9
Glass beads, 1.00 mm	2.7	Aluminum oxide G	5.3
Quartz	3.3	Sephadex G-25	3.2
Molecular sieves, 3A	4.7	Sephadex G-50	3.6
Molecular sieves, 4A	1.0	Sephadex G-100	4.6
Molecular sieves, 5A	2.7	Sephadex G-200	4.2
Chromosorb P AW	3.0	Acetyl cellulose	3.8

Note. v', Quantity of cyclohexanol formed in the *n*-hexane layer after 1 h of reaction. Organic phase, 1.00 ml *n*-hexane, 0.100 M initial concentration of cyclohexanone and cyclopentanol. Aqueous phase, 0.250 ml Mops buffer containing 1.0 unit HLADH and 0.5 mg NAD^+.

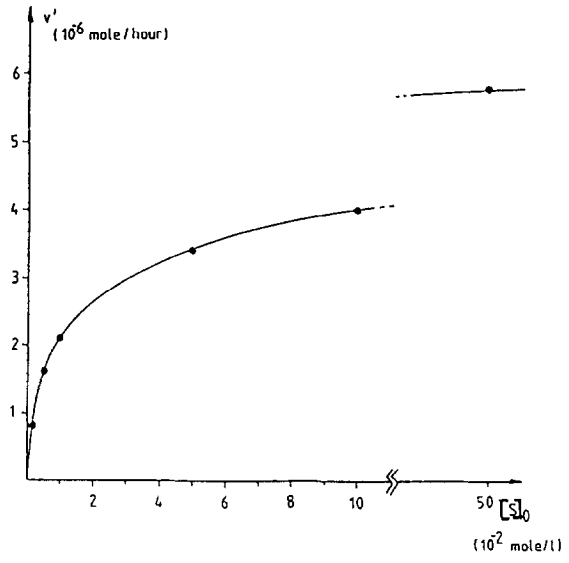

FIG. 2. Influence of initial substrate concentration on the reaction rate v'. v' = quantity of cyclohexanol formed after 1 h of reaction in *n*-hexane. $[S]_o$ = initial concentration of cyclohexanone/cyclopentanol in *n*-hexane or homogeneous medium. Organic phase, 10.0 ml *n*-hexane. Aqueous phase, 0.200 ml Mops buffer containing 1.0 unit HLADH adsorbed on 1.00 g glass beads. Homogeneous medium, 1.0 unit HLADH in 10 ml Mops buffer.

neous aqueous medium were determined as well. A comparison between these results shows a sevenfold decrease in the biphasic medium at a concentration of 10 mM for both substrates and a threefold decrease at 100 mM. This decrease is not surprising as the substrate concentrations in the aqueous phase depend on the partition of the substrates between the two phases and on the diffusion rates. Therefore partition coefficients for substrates and products were determined (Fig. 3).

The diffusion rate was checked only for the substrates cyclohexanone and cyclopentanol. The results, shown in Fig. 4, confirm that diffusion is relatively slow and that reduced activity can be ascribed to lower substrate concentrations.

The combination of these effects makes it very difficult to formulate a theoretical expression for the reaction rate in this two-phase system. Nevertheless, the shape of the curve in Fig. 2 can be explained by assuming that the reaction rate equation, elaborated for the coupled substrates cyclohexanone and ethanol (Eq. [1], (*4*)), is also valid in this system:

$$\frac{E_t}{v_0} = \frac{1}{k'_{3a} \cdot [A']} + \frac{1}{k'_{3b} \cdot [B]} \cdot \left(1 + \frac{[A']}{K_I}\right), \qquad [1]$$

where E_t is the total enzyme concentration, v_0 is the initial reaction rate, k'_{3a} is the reaction rate constant of the enzymatic recycling step HLADH-NAD^+ + $alcohol_2$ → HLADH-NADH + $ketone_2$, k_{3b} is the reaction rate constant of the enzymatic reduction step HLADH-NADH + $ketone_1$ → HLADH-NAD^+ + $alcohol_1$, [A'] is

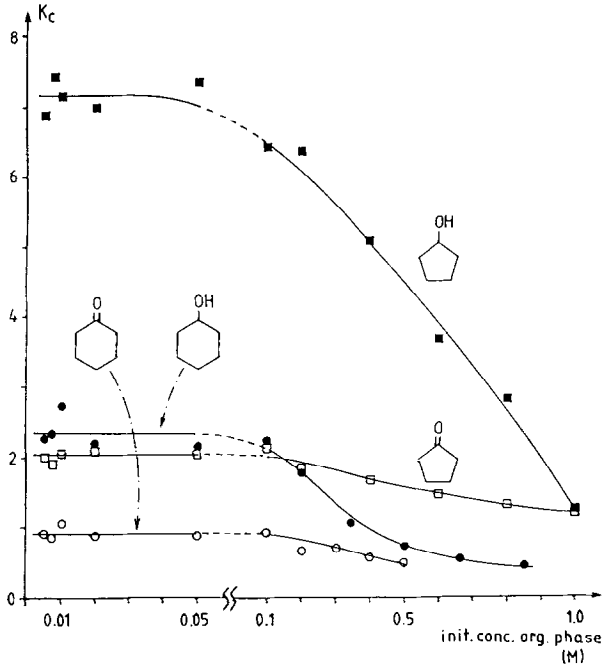

FIG. 3. Partition coefficients $K_c^X = \dfrac{\text{concn in Mops buffer}}{\text{concn in } n\text{-hexane}}$.

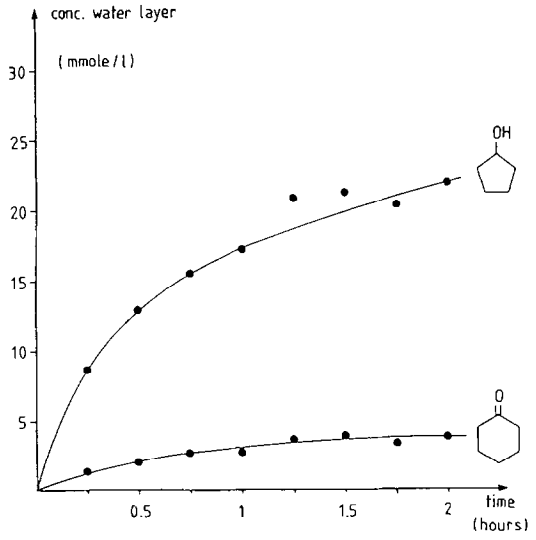

FIG. 4. Concentration variation of substrates in the aqueous phase as a function of time. Aqueous phase, 0.200 ml Mops buffer adsorbed on 1.00 g glass beads. Organic phase, 1.00 ml *n*-hexane, 10.0 mM initial concentration of cyclohexanone and cyclopentanol.

the concentration of cosubstrate (alcohol$_2$), [B] is the concentration of substrate (ketone$_1$), and K_I is the dissociation constant of the dead-end complex HLADH-NADH–alcohol$_2$.

By introducing the experimental conditions $[B]_w = f_B \cdot K_c^B \cdot [B]_o$, $[A]_w = f_A \cdot K_c^A \cdot [A]_o$, and $[A]_o = [B]_o = [S]_o$ (K_c^A, K_c^B = partition coefficients, f_B, f_A = correction factor for slow diffusion), Eq. [1] can be transformed to an equation of the general form $v' = a_1/(a_2 + a_3/[S]_o)$.

The shape of the curve in Fig. 2 is in agreement with this equation.

A disadvantage of all these supports is clogging of the moistened material. This effect reduces the contact surface between the phases, resulting in lower diffusion rates and consequently lower reactivities. In order to minimize this drawback several experimental modifications were tested. We tried to enhance the diffusion rate by rotating the test tubes and by drying the biocatalyst to various extents. Both modifications resulted in higher reaction rates. Mostly a two- to fourfold increase was found. The highest reaction rates were obtained when 30 to 70% of the aqueous phase was removed. This is in good accordance with the results of Klibanov (*3*). Complete drying of the biocatalyst however (less than 2% water remaining) resulted in a complete loss of activity. The activity was partially restored by remoistening the beads. In comparison with nondried enzyme preparations, 20% of the activity could be restored. This proves that the biocatalyst works only in a real biphasic solvent–water system. Therefore, it is of utmost importance to prevent stripping off of the water layer, by presaturating the organic phase with water.

Further, a twofold increase of the reaction rate was obtained when the glass beads were spread out in an Erlenmeyer flask (1-mm layer) instead of in test tubes.

Compared with Klibanov's results for the substrates 2-methylvaleraldehyde and ethanol (*3*), the activity per unit enzyme of our preparations is substantially better. Although the inherent reactivity of our substrates is lower, at least a 10-fold higher activity is reached. It is true that the turnover number is much lower but as, in our opinion, the reactivity is the most important feature of the enzyme preparation, the approach described in this article offers better opportunities.

A few other qualitative remarks can also be given. From a kinetic point of view the best cosubstrate A′ is the one giving a concentration in the aqueous phase high enough to assure a smooth recycling (cf. first term of Eq. [1]) without inhibition effects (cf. last term of Eq. [1]). From a preparative point of view, a cosubstrate has to be chosen which gives a favorable overall equilibrium.

B. The Overall Equilibrium

The overall equilibrium constant K_{biph} can be correlated with K_{buf} (Fig. 1) and the partition coefficients K_c:

$$K_{biph} = K_{buf} \cdot \frac{(1 + 1/a \cdot K_c^{CHOL}) \cdot (1 + 1/a \cdot K_c^{CPON})}{(1 + 1/a \cdot K_c^{CHON}) \cdot (1 + 1/a \cdot K_c^{CPOL})} \qquad [2]$$

with

$$K_c^X = \frac{[X]_{water}}{[X]_{org}} \qquad a = \frac{V_{water}}{V_{org}}$$

CHOL = cyclohexanol, CHON = cyclohexanone,
CPOL = cyclopentanol, CPON = cyclopentanone.

A similar formula has been derived by Martinek (*5*), for enzymatic reactions in reversed micelle systems.

In homogeneous aqueous medium the equilibrium constant K_{buf} for the reaction cyclohexanone + cyclopentanol $\rightleftharpoons$ cyclohexanol + cyclopentanone was determined to be 61 (*6*). From the K_c-values for substrate concentrations of 100 mM (Fig. 3) and a volume ratio of the two phases of 1/5, a K_{biph} of 58 can be calculated. This favorable equilibrium was an important reason to choose these substances as the model substrates.

For cyclohexanone and ethanol the equilibrium constant K_{buf} is equal to 0.024 (*4*). This unfavorable equilibrium can be circumvented only by the use of a large excess of ethanol. Since the partition coefficient of ethanol is very large, the concentration of ethanol in the tiny aqueous layer becomes very high, which causes a strong inhibition of the enzyme (last term of Eq. [1]) or even denaturation. For this reason cyclopentanol is definitely chosen as the cosubstrate, although its K_I-value of 0.007 M is less favorable than that of ethanol (K_I = 0.103 M (*4*)).

C. Stability of the Biocatalyst

The stability of the biocatalyst was tested by reusing the enzyme preparation regularly. After 15 experiments of several hours each, spread over a period of 2 months, 50% of the activity was remaining. During the intervals between the experiments the biocatalyst was kept under *n*-hexane. When the biocatalyst was in permanent contact with a 0.100 M substrate solution, the activity gradually dropped to 10–20% after 1 month. Higher substrate concentrations up to 0.5 M were found to be even more deleterious. This is obvious since the cyclopentanol concentration in the buffer is then more than 1.0 M or approximately 10%. This leads to a considerable inhibition of the enzyme and to denaturation in the long run. We also found that the more active, partially dried enzyme preparations, lose their activity much faster than preparations which were not dried.

CONCLUSION

The results clearly support the utility of this two-phase procedure for the simultaneous immobilization of HLADH and NAD(H). Reaction rates are sufficiently high but for large scale reactions the technology should be improved to optimize the diffusion of compounds and the stability of the biocatalyst. It has been shown that for further elaboration of two-phase enzyme/cofactor immobilization procedures a preliminary study must be made of the partition coefficients of all compounds involved, two-phase equilibrium rates, and diffusion rates in order to

make an optimum choice of cosubstrate, concentrations, and experimental conditions.

EXPERIMENTAL

Materials

HLADH (200-unit packages, A-6128) and NAD^+, (grade III, 98%, N-7004) were from Sigma. Glass beads (ϕ = 0.1 to 0.11 mm) came from B. Braun. Cyclohexanone (Merck, 822269), cyclohexanol (Merck, 2891), cyclopentanone (Fluka, 29760), cyclopentanol (Fluka, 29750), CS_2 p.a. (Ferak, 01455), *n*-hexane (Merck, 4368), and mesitylene (Fluka, 63910) were tested by GLC and further used as such. $(NH_4)_2SO_4$ (Merck, 1216), $HClO_4$ (Merck, 519), and Mops (Janssen, 17.263.94) were also commercially available.

Methods

Preparation and activity of the immobilized biocatalyst. HLADH (3.0 mg (= 5 Sigma units)/ml and NAD^+ (3.0 mg/ml) were dissolved in Mops buffer (0.050 M, pH 7.0). Of this solution 0.200 ml was added per gram of glass beads. The biocatalyst preparations were used as such or dried partially at a pressure of 1 mm Hg, during 3 to 15 min. The reaction is started by adding the *n*-hexane solution of cyclohexanone, cyclopentanol, and mesitylene in *n*-hexane. After various reaction times samples were taken from the *n*-hexane layer and diluted with CS_2 in order to obtain a cyclohexanone concentration of approximately 5×10^{-3} M. From these solutions 0.005-ml aliquots were analyzed on a Varian Vista 4600 chromatograph equipped with a Carbowax 20M (3% on Chromosorb GHP 100/120, 3 m × 3 mm) column. Mesitylene was used as the internal standard.

Activity in homogeneous aqueous medium. Cyclohexanone, cyclopentanol, NAD^+, HLADH, and Mops buffer (0.050 M, pH 7.0) were mixed in a test tube and thermostatted at 25°C; final concentrations: cyclohexanone and cyclopentanol, 0.100 or 0.0100 M; NAD^+, 2×10^{-4} M; HLADH, 0.10 Sigma units/ml. The reaction was started by addition of HLADH. After various time intervals 1.0-ml samples of the reaction mixture were mixed consecutively with 0.05 ml $HClO_4$, 0.6 g $(NH_4)_2SO_4$, and 2.0 ml CS_2. After stirring and centrifugation the CS_2 extracts were analyzed by GLC (see above).

Determination of partition coefficients. Solutions of cyclohexanone (0.0050, 0.0075, 0.010, 0.020, 0.050, 0.10, 0.20, 0.30, 0.40, 0.50 M), cyclohexanol (0.0050, 0.0075, 0.010, 0.020, 0.050, 0.17, 0.34, 0.51, 0.68, 0.85 M), cyclopentanol, and cyclopentanone (0.0050, 0.0075, 0.010, 0.020, 0.050, 0.20, 0.40, 0.60, 0.80, 1.0 M) were prepared in *n*-hexane, containing mesitylene as internal standard. The solutions were analyzed by GLC. Portions of 1.00 ml of the respective solutions were equilibrated with 0.200 ml of Mops buffer adsorbed on 1.00 g of glass beads. After 1 day the *n*-hexane layers were analyzed again on GLC and partition coefficients were calculated from the concentration decreases.

Determination of stability. The biocatalyst was prepared and tested as de-

scribed before (substrate concentrations, 0.100 M). After every experiment the *n*-hexane layer was completely removed. The glass beads were washed with *n*-hexane, presaturated with water, until the wash layer contained no substrates anymore (GLC). Then the biocatalyst was stored under *n*-hexane or was brought in contact with fresh substrate solution.

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