Enzymatic *In Vitro* Reduction of Ketones. Part 2.[1] Study of Co-enzyme Recycling in an Ethanol–Cyclohexanone System with HLAD (Horse Liver Alcohol Dehydrogenase)

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The coupled-substrate recycling system is elaborated for a reaction mixture containing cyclohexanone–ethanol (as coupled substrate), the co-enzyme NAD^+, and the enzyme HLAD. All reaction parameters are discussed in order to optimize the reduction of cyclohexanone as a model compound for other reductions, mainly to synthesize chiral compounds. With catalytic amounts of co-enzyme 50 000 recyclings could be realized. A formula for the initial rate of the enzymatic reaction based on a double Theorell–Chance mechanism is given and experimentally checked.

DEHYDROGENASES are biocatalysts able to catalyse the reduction of ketones to secondary alcohols with an impressive stereospecificity. Unfortunately the *in vivo* reduction of unnatural ketones by living micro-organisms is subject to much trial and error.[2] Problems of several kinds are encountered, *e.g.* toxicity of the unnatural substrate for the living organism, degradation of the substrate by several side reactions, and insolubility of the substrate. Most troubles are avoided by *in vitro* reactions with pure enzymes in water–organic solvent media. Several highly purified dehydrogenases are now commercially available so that analytical work can be done on a wide range of substrates. The preparative application however is still limited since stoicheiometric or greater amounts of the reducing agent are necessary. Indeed for this reaction a co-enzyme such as dihydronicotinamide adenine nucleotide (NADH) or the even more expensive dihydronicotinamide adenine dinucleotide

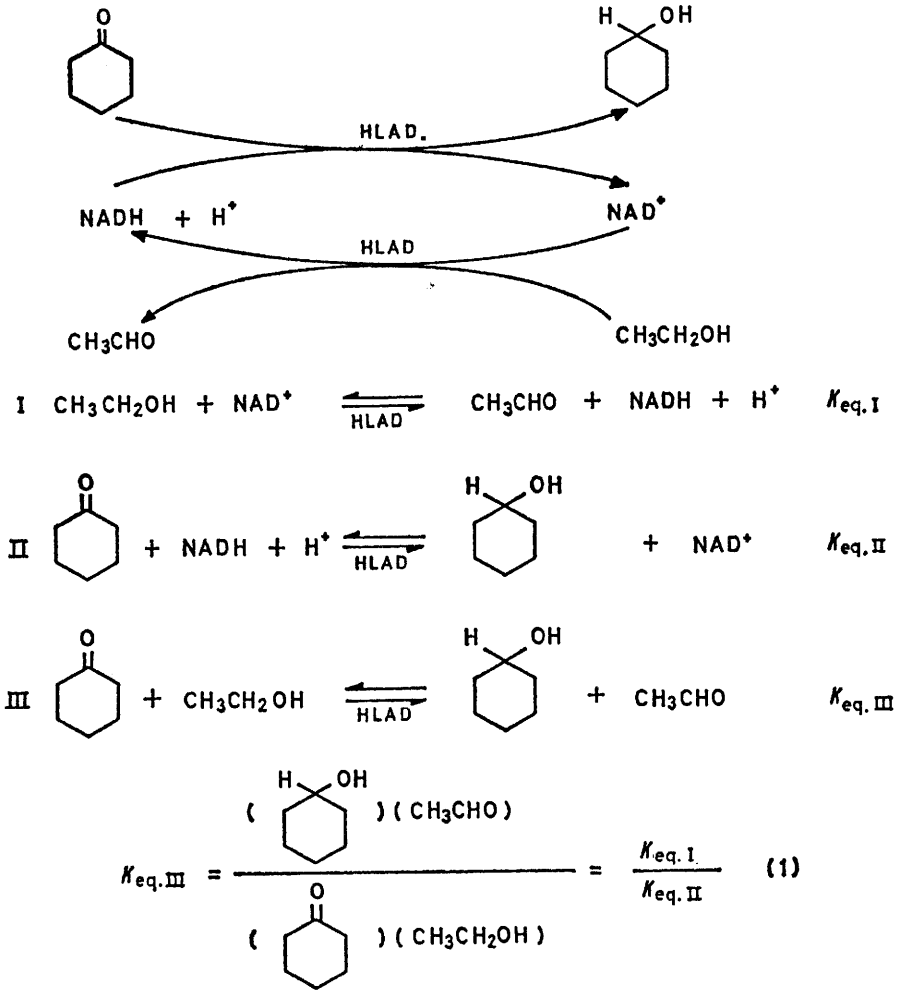
[1] G. L. Lemière, T. A. Van Osselaer, J. A. Lepoivre, and F. C. Alderweireldt, submitted for publication in *Biochim. Biophys. Acta.*

[2] K. Kieslich, *Synthesis*, 1969, **3**, 120; **4**, 147.

phosphate (NADPH) is required. This problem can be overcome by using only catalytic amounts of co-enzyme and recycling the oxidized form. This can be done in three different ways: (a) in a non-enzymatic way, chemically,[3,4] or electrochemically;[5] (b) enzymatically by a coupled-enzyme system;[3,6] or (c) enzymatically by a coupled-substrate system.[3,7] This last procedure, which seems to be very suitable for application on preparative-scale reductions is elaborated in this paper.

Elsewhere [1] rate equations valid for several possible reaction parameters is given in order to examine possible correlations with equilibrium and reaction rate and to optimize the recycling process for preparative purposes.

Cyclohexanone is taken as a prototype of a substrate to be reduced for some practical and theoretical reasons. The reaction mixture cyclohexanol–cyclohexanone can easily be extracted from the water solution and quantitatively analysed by gas–liquid chromatography. Furthermore, cyclohexanone is a cyclic ketone which is known to be easily reduced by HLAD.



SCHEME 1

reaction mechanisms of coupled-substrate recycling systems are elaborated. In this work the reduction of cyclohexanone with ethanol as coupled substrate, horse liver alcohol dehydrogenase (HLAD) and NAD(H) is chosen as a model system. An extensive study of the

Ethanol as the natural and inexpensive second substrate can be added in large quantities in order to obtain a favourable equilibrium position and to increase the solubility of the cyclohexanone (or other substrates). Furthermore the equilibrium constant of the ethanol–acetaldehyde redox system is well known.

The choice of horse liver alcohol dehydrogenase

[3] J. B. Jones and J. F. Beck, in ' Applications of Biochemical Systems in Organic Chemistry,' Part 1, ed. A. Weissberger, Techniques of Chemistry vol. X, J. Wiley and Sons, New York, 1976, p. 107.

[4] A. J. Irwin and J. B. Jones, *J. Amer. Chem. Soc.*, 1976, **98**, 8476; J. B. Jones and K. E. Taylor, *Canad. J. Chem.*, 1976, **54**, 2974; K. E. Taylor and J. B. Jones, *J. Amer. Chem. Soc.*, 1976, **98**, 5689; H. B. Goodbrand and J. B. Jones, *J.C.S. Chem. Comm.*, 1977, 469.

[5] M. Aizawa, S. Suzuki, and M. Kubo, *Biochim. Biophys. Acta*, 1976, **444**, 886; R. J. Day, S. J. Kinsey, E. T. Seo, H. Weliky, and H. P. Sibouman, *Trans. N.Y. Acad. Sci.*, 1972, 588.

[6] J. Campbell and T. Ming Swi Chang, *Biochem. Biophys. Res. Comm.*, 1976, **69**, 562.

[7] A. R. Battersby, J. Staunton, and H. R. Wiltshire, *J.C.S. Perkin I*, 1975, 1156.

(HLAD) is due to its remarkable reactivity for the reduction of a wide range of ketones.[3] Furthermore the kinetics of the HLAD-catalysed single reactions are so extensively described in the literature that this enzyme is very suitable for using recycling systems and for checking the corresponding mechanisms as elaborated in our previous article.[1]

RESULTS AND DISCUSSION

(1) *The Reaction Equilibrium.*—The overall equilibrium of the present substrate-coupled recycling system depends only on the free energies of the two alcohols and the corresponding carbonyl compounds as may be seen from Scheme 1. The overall equilibrium constant $K_{eq.III}$ is given by equation (1). As may be expected from this equation the value of $K_{eq.III}$ was found to be independent of pH over a range from 6 to 9 (Table 1). At pH values below 6 the enzyme is rapidly inactivated.[8] At pH 10 the reaction did not reach equilibrium even after one week. A variation of ionic strength between 0.028 and 0.44 did not influence the yield of cyclohexanol (Table 1).

An equilibrium constant $K_{eq.III}$ of $(2.4 \pm 0.1) \times 10^{-2}$ was obtained as a mean value from 16 experiments. It is almost temperature independent (Table 2). A free enthalpy ΔG°_{III} of only 9.2 kJ/mol (2.2 kcal/mol) is found. The ΔG° value for the partial equilibria eq. I (see Scheme 1) is known:[9b] $\Delta G^\circ_I = 63.7$ kJ/mol (15.2 kcal/mol). Combining these ΔG° values ΔG°_{II} and $K_{eq.II}$ can be calculated and are found to be −54.4 kJ/mol (−13.0 kcal/mol) and 2.7×10^{-10} mol l^{-1}. This result is compared with similar K_{eq} values from the literature in Table 3. Since the exact reaction conditions are not always given in the literature it is difficult to make a good comparison. The above mentioned value for ΔG°_{II} can now be combined with the ΔG° value for the reaction $NAD^+ + H_2 \rightleftharpoons NADH + H^+$, for which 22.1 kJ/mol (5.3 kcal/mol) is given.[9b] A result of −32.2 kJ/mol (−7.7 kcal/mol) is found as ΔG° value for the reaction cyclohexanone + $H_2 \rightleftharpoons$ cyclohexanol. This is in good agreement with values found in the literature for the same reaction, but obtained by non-enzymatic reactions. For the Oppenauer oxidation[10] and for the catalytic hydrogenation[11] values of respectively −32.2 kJ/mol (−7.7 kcal/mol) and −31.4 kJ/mol (−7.5 kcal/mol) are reported.

TABLE 1

Influence of pH and ion strength on the equilibrium constant $K_{eq.III}$ (HLAD concn. 0.1 unit/ml)

pH	Buffer	μ	Ethanol * (mol l^{-1})	Cyclohexanone * (10^{-3} mol l^{-1})	% Cyclohexanol at equilibrium	$K_{eq.III}$ (35 °C) (10^{-2})
5.97	Phosphate	0.12	0.515	5.0	74.9	2.2
6.44	Phosphate	0.16	0.515	5.0	77.2	2.6
6.44	Phosphate	0.16	0.515	5.0	75.4	2.3
6.96	Phosphate	0.22	0.515	5.0	76.5	2.4
7.45	Phosphate	0.27	0.515	5.0	76.6	2.5
7.22	Phosphate	0.03	0.500	10.0	64.2	2.3
7.12	Phosphate	0.06	0.500	10.0	63.9	2.4
7.02	Phosphate	0.11	0.500	10.0	64.4	2.3
6.84	Phosphate	0.44	0.500	10.0	64.3	2.2
6.97	Phosphate	0.22	0.500	20.0	51.4	2.2
7.00	Phosphate–Titrisol	0.29	0.500	5.0	74.7	2.2
6.00	Citrate–NaOH–Titrisol	0.16	0.500	5.0	73.4	2.2
8.00	Borate–HCl–Titrisol	0.06	0.500	5.0	75.7	2.4
9.00	Boric acid–KCl–NaOH–Titrisol	0.07	0.500	5.0	76.8	2.5
6.95	Tris–HCl	0.05	0.500	5.0	76.7	2.5
6.95	Tris–HCl	0.05	0.500	5.0	76.8	2.5 †
						$\langle K_{eq} \rangle = (2.4 \pm 0.1) \times 10^{-2}$

* Initial concentrations. † Initial concentration of NAD^+ 2×10^{-5} mol l^{-1}; in all other experiments 2×10^{-4} mol l^{-1}.

Using Tris–HCl buffers with gradually increasing concentrations from 0.05 to 0.4 mol l^{-1} the yield of cyclohexanol gradually rises above the yield predicted by the above calculated equilibrium constant (Table 4). This is most probably due to the formation of a Schiff base between the free amine group of tris-hydroxymethyl-

TABLE 2

Influence of the temperature on the equilibrium constant $K_{eq.III}$. [Concentration of HLAD: 0.1 unit/ml. Initial concentrations: ethanol 0.500 mol l^{-1}, cyclohexanone 0.020 mol l^{-1}, NAD^+ 2×10^{-4} mol l^{-1}, phosphate buffer 0.10 mol l^{-1} (pH 7, $\mu = 0.22$)]

Temp. (°C)	Yield of cyclohexanol at equilibrium (%)	$K_{eq.III}$ ($\times 10^{-2}$)
10	50.8	2.1
15	50.9	2.2
20	51.7	2.3
25	52.2	2.3
30	51.4	2.2
35	52.6	2.4
40	53.4	2.5
45	52.7	2.4
50	52.8	2.4
55	53.2	2.5

[8] C. H. Blomquist, *Arch. Biochem. Biophys.*, 1967, **122**, 24.
[9] (*a*) K. I. Bäcklin, *Acta Chem. Scand.*, 1958, **12**, 1279; (*b*) K. Burton, *Biochem. J.*, 1974, **143**, 365.
[10] H. Adkins, R. M. Elofson, A. G. Rossow, and C. C. Robinson, *J. Amer. Chem. Soc.*, 1949, **71**, 3622.
[11] G. Waldvogel, Dissertation 3681, E.T.H. Zürich, 1965.

aminomethane and acetaldehyde.[12] This shift of the cyclohexanol yield can be combined with the equilibrium

TABLE 3

Literature values of $K_{eq.II}$

$K_{eq.II}$ (mol l^{-1})	Medium	Temp.	Ref.
$(2.5 \pm 0.5) \times 10^{-9}$	pH 8.8	37 °C	7
5.5×10^{-9}	Phosphate	R.T.	*a*
3.6×10^{-10}	0.1M-KPP, pH 7.6		*b*
9.0×10^{-10}	Tris–HCl, pH 8.4	25 °C	13
3.4×10^{-10} / 4.0×10^{-10}	0.1M-Sodium glycyl-glycinate, pH 8.6	25 °C	16*f*

[a] A. D. Merritt and G. M. Tomkins, *J. Biol. Chem.*, 1959, **234**, 2778. [b] R. Mislin, Dissertation 4169, E.T.H. Zurich, 1968.

constant $K_{eq.III}$ and the $pK_a = 8.2$ of the Tris–HCl buffer [13] and consequently a rough calculation of the

TABLE 4

Influence of the Tris–HCl buffer concentration on the yield of cyclohexanol. [Concentration of HLAD: 0.1 unit/ml, initial concentration NAD^+: 2×10^{-4} mol l^{-1}, temp. 35 °C]

pH	Tris * ($\times 10^{-2}$ mol l^{-1})	Ethanol * (mol l^{-1})	Cyclohexanone * ($\times 10^{-3}$ mol l^{-1})	Yield of cyclohexanol at equilibrium % Exptl.	Calc.†
6.95	5.0	0.515	5.0	77.9	75.8
6.95	5.0	0.515	5.0	76.8	75.8
7.90	5.0	0.515	5.0	90.5	75.8
8.35	5.0	0.515	5.0	93.5	75.8
8.87	5.0	0.515	5.0	94.3	75.8
8.85	5.0	0.500	50.0	60.7	37.9
8.91	10.0	0.500	50.0	76.6	37.9
9.14	20.0	0.500	50.0	84.9	37.9
9.42	40.0	0.500	50.0	90.4	37.9

* Initial concentrations. † Calculated from $K_{eq.III}$.

TABLE 5

Influence of the NAD^+ concentration on the yield of cyclohexanol and the co-enzyme recycling number. [Concentration of HLAD: 0.1 unit/ml. Initial concentrations: ethanol: 0.515 mol l^{-1}, cyclohexanone: 5.0×10^{-3} mol l^{-1}, and tris–HCl buffer: 0.050 mol l^{-1} (pH 8.87, $\mu = 0.05$), temp. 35 °C]

NAD^+ * (mol l^{-1})	Yield of cyclohexanol after 7 days	Co-enzyme recycling
2×10^{-4}	94.3	23
2×10^{-5}	94.8	237
2×10^{-6}	94.3	2 357
2×10^{-7}	64.1	16 025
2×10^{-8}	22.6	56 500

* Initial concentration.

equilibrium constant for the reaction between acetaldehyde and Tris can be made:

$$K_{add} = \frac{\text{(Schiff base)}}{\text{(Tris) (Acetaldehyde)}} = (145 \pm 30)\ \text{l mol}^{-1}$$

Since $K_{eq.III}$ is also independent of the NAD^+ concentration quite low co-enzyme concentrations can be used and this, in turn, results in high co-enzyme recycling numbers (Table 5). Nevertheless with an enzyme concentration of 10^{-6} to 10^{-7} mol l^{-1} an NAD^+ concentration of 10^{-6} mol l^{-1} is a practical lower limit. Below this limit the reaction very sharply slows down (see below), because the enzyme is no longer saturated with co-enzyme. Although at such low co-enzyme concentrations (2×10^{-8} mol l^{-1}) very high numbers of NAD^+-recyclings are obtained (50 000) these conditions are not recommended, not only because of the very large and unpractical reaction times required, but also because of the deterioration of the enzyme or the co-enzyme which prevents the equilibrium being reached.

An increase in the ethanol concentration from 0.05 to 3 mol l^{-1} results in a predictable increase of the yield of cyclohexanol (Table 6). In an ethanol concentration of

TABLE 6

Influence of the ethanol concentration on the yield of cyclohexanol at equilibrium. [Concentration of HLAD: 0.1 unit/ml. Initial concentrations: cyclohexanone: left-hand part of Table: 0.020 mol l^{-1}, right-hand part of Table: 0.0050 mol l^{-1}; NAD^+: 2×10^{-4} mol l^{-1}, and Tris–HCl buffer: 0.050 mol l^{-1} (pH 8.87, $\mu = 0.05$); temp. 35 °C]

Ethanol * (M)	Yield of cyclohexanol % Expt.	Calc.‡	Ethanol * (M)	Yield of cyclohexanol % Expt.	Calc.‡
0.25	72.2	69.5	0.05	70.6	69.4
0.50	80.0	79.4	0.10	79.4	79.6
0.75	84.6	84.3	0.25	89.8	90.0
1.00	86.7	87.2	0.52	94.3	94.4
1.50	88.4	90.6	1.03	95.9	97.0
2.00	91.7	92.6	2.06	95.6	98.5
2.50	93.5	93.9	4.12	79.7	
3.00	92.8				

* Initial concentration. ‡ Calculated from $K_{eq.III}$ and K_{add}.

4 mol l^{-1} a considerable lower yield of cyclohexanol was obtained. Clearly the very high ethanol concentration inhibits the reaction by the formation of a dead-end

TABLE 7

Influence of the cyclohexanone concentration on the yield of cyclohexanol. [Concentration of HLAD: 0.1 unit/ml. Initial concentrations: ethanol: 0.515 mol l^{-1}, NAD^+: 2×10^{-4} mol l^{-1}, and Tris–HCl buffer: 0.050 mol l^{-1} (pH 8.87, $\mu = 0.05$); temp.: 35 °C]

Cyclohexanone * (mol l^{-1})	Yield of cyclohexanol at equilbrium (%) Expt.	Calc.‡
2.5×10^{-3}	96.4	97.3
5.0×10^{-3}	94.3	94.4
1.00×10^{-2}	89.0	89.1
2.50×10^{-2}	77.5	75.8
5.00×10^{-2}	60.7	60.4
1.000×10^{-1}	52.2	44.2

* Initial concentrations. ‡ Calculated from $K_{eq.III}$ and K_{add}.

complex (see below) and also by denaturation of the enzyme.[14]

By variation of the cyclohexanone concentration from

[12] M. D. Hurwitz, *Chem. Abs.*, 1952, **46**, 8146.
[13] S. M. Rapoport and H. J. Raderecht, 'Physiologisch-Chemisches Praktikum,' VEB Verlag Volk und Gesundheid, Berlin, 1972, p. 467.
[14] M. Dixon and E. C. Webb, 'Enzymes,' Longmans, Green and Co. Ltd., London, 1964.

2.5×10^{-3} to 1.0×10^{-1} mol l^{-1} the yield of cyclohexanol varies as expected (Table 7).

(2) *The Initial Rate.*—In a previous paper [1] the following steady-state initial rate equation for coupled-substrate recycling systems were elaborated:

$$\frac{1}{v_0} = \frac{K'_a}{V_{2a}} \cdot \frac{1}{A'} + \frac{K_b}{V_{1b}} \cdot \frac{1}{B}\left(1 + \frac{A'}{K_I}\right) + \left(\frac{1}{V_{2a}} - \frac{1}{k_2 E_t}\right) + \left(\frac{1}{V_{1b}} - \frac{1}{k'_2 E_t}\right) \quad (2)$$

When rate constants are introduced in equation (2), assuming a Theorell–Chance mechanism [15] for both single reactions, the equation becomes:

$$\frac{E_t}{v_0} = \frac{1}{k'_{3A} \cdot A'} + \frac{1}{k_{3B} \cdot B}\left(1 + \frac{A'}{K_I}\right) \quad (3)$$

This equation is further simplified to:

$$\frac{E_t}{v_0} = \frac{1}{k_{3B} \cdot B}\left(1 + \frac{A'}{K_I}\right) \quad (4)$$

when working with high concentrations of coupled substate A'.

This simple initial rate equation is applicable to HLAD since it is well known that this enzyme follows a Theorell–Chance mechanism.[16] Moreover the validity

$$E + O \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} EO$$

$$EO + A' \underset{k_{2A}}{\overset{k'_{3A}}{\rightleftharpoons}} ER + A$$

$$ER + B \underset{k'_{3B}}{\overset{k_{3B}}{\rightleftharpoons}} EO + B'$$

$$ER \underset{k_1}{\overset{k_2}{\rightleftharpoons}} E + R$$

$$ER + A' \overset{K_I}{\rightleftharpoons} ERA'$$

SCHEME 2

E = enzyme, R = NADH, O = NAD^+, A = acetaldehyde, A' = ethanol, B = cyclohexanone, B' = cyclohexanol, ER and EO = binary complexes, ERA' = ternary dead-end inhibition complex.

$K_I = \frac{(ER)\ (A')}{(ERA')}$: dissociation constant of the inhibition complex.

of this double Theorell–Chance mechanism is experimentally confirmed further on.

TABLE 8

Literature values * of the rate constants of Scheme 2

Rate constants (23.5 °C)	Phosphate buffer (pH 7, $\mu = 0.1$)	Phosphate–glycerine buffer (pH 9, $\mu = 0.1$)
k'_1/ l mol^{-1} s^{-1}	0.525×10^6	0.508×10^6
k'_2/s^{-1}	74	8.15
k'_{3A}/ l mol^{-1} s^{-1}	0.0122×10^6	0.0085×10^6
k_{3B}/ l mol^{-1} s^{-1}	0.0035×10^6 †	
k_2/s^{-1}	3.12	4.9

* H. Theorell and J. S. McKinley-McKee.[16b] † K. Dalziel and F. M. Dickinson, *Biochem. J.*, 1966, **100**, 491.

In Scheme 2 all the reactions involved are explained. In Table 8 values for the rate constants are given as found in the literature.

In a first experiment with a constant concentration of cyclohexanone (B), a constant and high concentration of ethanol (A') and varying concentrations of co-enzyme (O), the initial rate was constant above co-enzyme concentration of 10^{-6} mol l^{-1} but dropped abruptly below this concentration (Figure 1). This could be expected

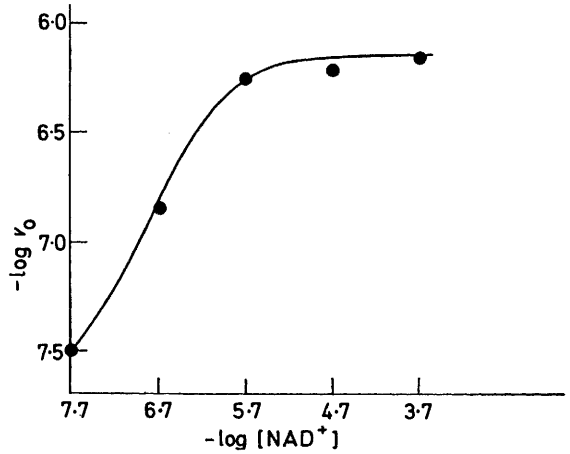


FIGURE 1 Dependence of the initial rate v_0/mol l^{-1} s^{-1} on the NAD^+ concentration (mol l^{-1}). Concentration of HLAD, 0.1 unit/ml. Initial concentrations: ethanol 0.515 mol l^{-1}, cyclohexanone 5.0×10^{-3} mol l^{-1}, and Tris–HCl buffer 0.050 mol l^{-1} (pH 8.85, $\mu = 0.05$); temperature 35 °C

from the given values of k_2 and k_1' (Table 8). Equation (4) is clearly only valid for co-enzyme concentrations higher than 10^{-6} mol l^{-1} (see Appendix). A plot of v_0^{-1} *vs.* $(O)^{-1}$ gives a linear correlation as expected.

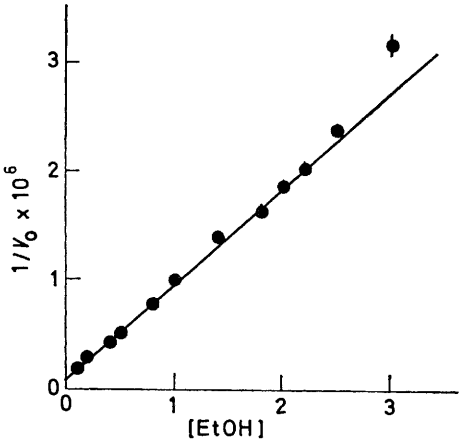


FIGURE 2 Dependence of the initial rate v_0 (mol l^{-1} s^{-1}) on the ethanol concentration (mol l^{-1}). Concentration of HLAD, 0.1 unit/ml. Initial concentrations: cyclohexanone 5.0×10^{-3} mol l^{-1}, NAD^+ 2×10^{-4} mol l^{-1}, and Tris–HCl buffer 0.050 mol l^{-1} (pH 8.5, $\mu = 0.05$), temperature 35 °C

Using varying ethanol concentrations (A') and constant concentrations for all other reagents a linear

[15] K. Dalziel, *Acta Chem. Scand.*, 1957, **11**, 1706.

[16] (*a*) H. Theorell and B. Chance, *Acta Chem. Scand.*, 1951, **5**, 1127; (*b*) H. Theorell and J. S. McKinley-McKee, *Acta Chem. Scand.*, 1961, **15**, 1797; (*c*) K. Dalziel, *Biochem. J.*, 1962, **84**, 244; (*d*) K. Dalziel, *J. Biol. Chem.*, 1963, **238**, 2850; (*e*) K. Dalziel and F. M. Dickinson, *Biochem. J.*, 1966, **100**, 34; (*f*) G. R. Ainslie and W. W. Cleland, *J. Biol. Chem.*, 1972, **247**, 946.

correlation is found between v_0^{-1} and (A') in the range 0.05 to 2.2 mol l^{-1} (Figure 2). The initial reaction rate slows down by the formation of the dead-end complex HLAD–NADH–ethanol. From the slope and the intercept we find values for K_I and $E_t \cdot k_{3B}$ of 0.103 mol l^{-1} at 35 °C (Theorell:[17] 0.040 mol l^{-1} at 23.5 °C) and 1.1×10^{-3} s^{-1}, respectively. At ethanol concentrations higher than 2.2M a deviation from the linear correlation to lower initial rates due to denaturation[14] of the enzyme is observed.

Experiments with varying cyclohexanone concentrations (B), all other parameters being kept constant, give linear plots of v_0^{-1} *vs.* $(B)^{-1}$ and v_0 *vs.* (B) (Figure 3). This is strong evidence for the double Theorell–Chance mechanism since only for this mechanism the last two

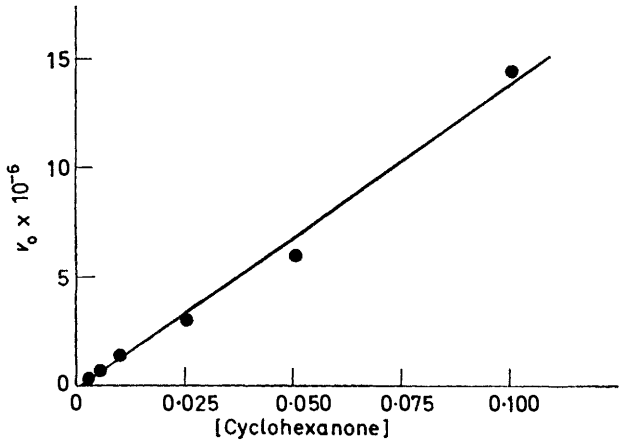


FIGURE 3 Dependence of the initial rate v_0 (mol l^{-1} s^{-1}) on the cyclohexanone concentration (mol l^{-1}). Concentration of HLAD 0.1 unit/ml. Initial concentrations: ethanol 0.515 mol l^{-1}, NAD^+ 2×10^{-4} mol l^{-1}, and Tris–HCl buffer 0.050 mol l^{-1} (pH 8.85, $\mu = 0.05$); temperature 35 °C

terms of equation (2) $(1/V_{2a} - 1/k_2E_t)$ and $(1/V_{1b} - 1/k'_2E_t)$ vanish. The experimental slopes of both linear plots are in good agreement with the slopes of calculated from the above given $E_t \cdot k_{3B}$ and K_1 values. It is noteworthy that the cyclohexanone does not cause any saturation effect in this recycling system up to a concentration of 0.1 mol l^{-1}, although in the single reaction a value of K_M of 1.9×10^{-3} mol l^{-1}, as determined by normal Michaelis–Menten kinetics, is found.[18]

The linear dependency of the rate on enzyme concentration E_t was checked between 0.1 and 0.01 units/ml (Figure 4).

The influence of temperature on the initial rate was studied from 0 to 80 °C. A good correlation between ln v_0 and $1/T$ is found up to 60 °C. Above this temperature the reaction slows down by thermal denaturtion of the enzyme (Figure 5). Although the reaction conditions were not the same (Tris–HCl buffer pH = 8.5, $\mu = 0.05$, 3% ethanol) the thermal stability found corresponds with the stability reported by Theorell[19] (phosphate buffer pH = 7.0, $\mu = 0.1$, 0% ethanol).

[17] H. Theorell and J. S. McKinley-McKee, *Acta Chem. Scand.*, 1961, **15**, 1811, 1834.
[18] A. D. Winer, *Acta Chem. Scand.*, 1958, **12**, 1695.

The good linear correlation between ln v_0 and $1/T$ over a temperature range of 0—60 °C allows the calculation of

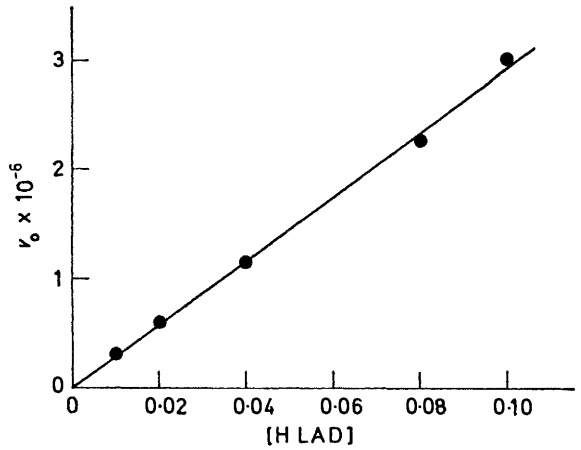


FIGURE 4 Dependence of the initial rate v_0 (mol l^{-1} s^{-1}) on the HLAD concentration (unit/ml). Initial concentrations: ethanol 0.20 mol l^{-1}, cyclohexanone 0.010 mol l^{-1}, NAD^+ 2×10^{-4} mol l^{-1}, and Tris–HCl buffer 0.050 mol l^{-1} (pH 8.5, $\mu = 0.05$); temperature 35 °C

some thermodynamic quantities. These will be presented elsewhere.

Finally the influence of pH and ionic strength on the initial rate were investigated. It was found that the ionic strength has little influence on the initial rate (Table 9). In contrast the pH has a well defined effect (Figure 6). A maximal initial rate was found in a phosphate buffer of pH = 7. Below pH = 6, the initial rate abruptly slows down by inactivation of the enzyme.[9] In a more alkaline solution the initial rate also slows down, though not so dramatically.

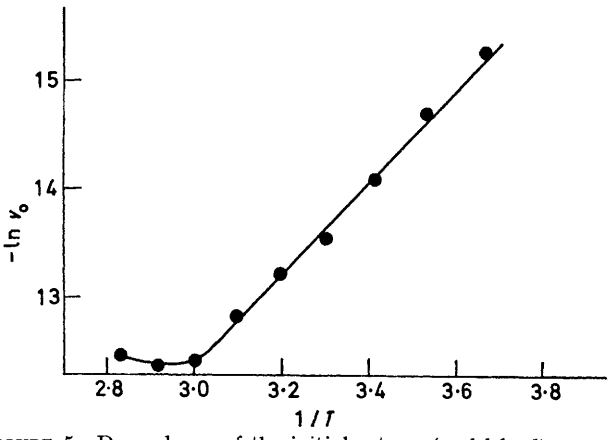


FIGURE 5 Dependence of the initial rate v_0 (mol l^{-1} s^{-1}) on the reaction temperature (K). Concentration of HLAD 0.1 unit/ml. Initial concentrations: ethanol 0.50 mol l^{-1}, cyclohexanone 0.010 mol l^{-1}, NAD^+ 2×10^{-4} mol l^{-1}, and Tris–HCl buffer 0.050 mol l^{-1} (pH 8.5, $\mu = 0.05$)

Conclusion.—The validity of the initial rate equation (2), which was elaborated in a previous paper[1] and which is valid for several substrate-coupled recycling

[19] T. Yonetani and H. Theorell, *Arch. Biochem. Biophys.*, 1962, **99**, 433; H. Theorell and K. Tatemoto, *ibid.*, 1971, **143**, 354.

systems with different mechanisms, is checked experimentally for horse liver alcohol dehydrogenase with

TABLE 9

Influence of the ion strength μ on the initial rate v_0. [Concentration of HLAD: 0.1 unit/ml. Initial concentrations: ethanol 0.50 mol l^{-1}, cyclohexanone 0.010 mol l^{-1}, and NAD^+ 2×10^{-4} mol l^{-1}; temp. 35 °C]

Phosphate buffer		
μ	pH	$10^6 v_0$/mol s^{-1}
0.028	7.22	2.18
0.055	7.12	2.23
0.111	7.02	2.22
0.290	7.00	2.37
0.444	6.84	2.46

cyclohexanone and ethanol as coupled substrate. The excellent linear correlation between the cyclohexanone concentration and the initial rate gives strong evidence for a double Theorell–Chance mechanism, which is the simplest mechanism considered in the previous paper.[1] The results for the cyclohexanone–cyclohexanol equilib-

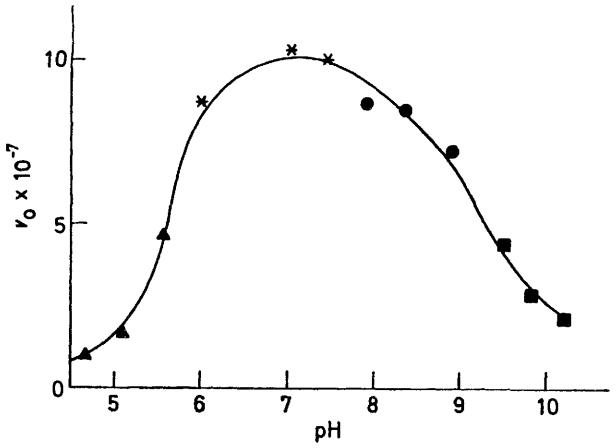


FIGURE 6 Dependence of the initial rate v_0 (mol l^{-1} s^{-1}) on the pH. Concentration of HLAD 0.1 unit/ml. Initial concentrations: ethanol 0.515 mol l^{-1}, cyclohexanone 5.0×10^{-3} mol l^{-1}, and NAD^+ 2×10^{-4} mol l^{-1}; temperature 35 °C. ▲ Phthalate–NaOH buffer, * phosphate buffer, ● Tris–HCl buffer, and ■ glycine–NaOH buffer

rium and the reaction rate are in good agreement with literature values.

Some recommendations for preparative reduction of ketones with HLAD in a coupled-substrate co-enzyme recycling system can now be given. (1) Reductions are best performed in Tris–HCl buffer at pH 8—9. In phosphate buffer pH 7 the initial rate is slightly higher, but in Tris–HCl buffer much higher yields of alcohol are obtained by trapping of the acetaldehyde formed during the reaction. (2) Reaction temperatures up to 50 °C can be used. (3) A co-enzyme concentration of 10^{-6}M-NAD^+ is convenient since higher concentrations give no increase in the reaction rate. (4) A small excess of ethanol can be used. High ethanol concentrations are not recommended since the reaction rate slows with increasing ethanol concentration. Increase in yield is facilitated with higher Tris–buffer concentrations.

In the above given optimal conditions preparative stereospecific reductions can be carried out with at least a ten-fold improvement of recycling number compared with the usually described systems and, consequently, with a proportional economy of co-enzyme. Moreover, ethanol as a reductant has the advantages of being both quite harmless and still increasing the solubility of the substrate.

Although this system was primarily developed for practical preparative scale stereospecific reductions, it can also be used for theoretical studies as will be described elsewhere.

APPENDIX

Equation (2) which is elaborated in another paper [1] for a number of possible reaction mechanisms is only valid for concentrations of co-enzyme high enough for saturating the enzyme. Also equations (3) and (4) derived from equation (2), assuming a Theorell–Chance mechanism for both single reactions, are only valid for enzyme-saturating co-enzyme concentrations.

A full steady-state rate equation for non-saturating co-enzyme concentrations is generally too complex for use. In the case of a double Theorell–Chance mechanism, however, a relatively simple initial rate equation (3′) can be derived, using the King–Altman procedure [20] and introducing the initial conditions A = B′ = R = 0. This equation

$$\frac{E_t}{v_0} = \frac{1}{k'_{3A} \cdot A'}\left(1 + \frac{k'_2}{k'_1 \cdot O}\right) + \frac{1}{k_{3B} \cdot B}\left(1 + \frac{k_2}{k'_1 \cdot O} + \frac{A'}{K_I}\right) + \frac{k_2}{k'_{3A}k_{3B} \cdot A' \cdot B}\left(1 + \frac{k_2'}{k'_1 \cdot O}\right) \quad (3')$$

(3′) is the more elaborate form of equation (3) valid for non-saturating co-enzyme concentrations. For high concentrations of coupled substrate ethanol (A′) equation (3′) simplifies to (4′) which is the more elaborate form of equation (4):

$$\frac{E_t}{v_0} = \frac{1}{k_{3B} \cdot B}\left(1 + \frac{k_2}{k_1'O'} + \frac{A'}{K_I}\right) \quad (4')$$

EXPERIMENTAL

Materials.—HLAD, crystallized and lyophilized, was purchased from Sigma (340–L2) in packages of 20 units. Before use 20 units of HLAD were dissolved in 10 ml of buffer and divided into 0.5 ml (1 unit) portions. The portions not directly used were stored under liquid nitrogen. NAD^+, grade III (98%) from Sigma (N-7004), was dissolved in buffer immediately before use. Cyclohexanone from UCB was fractionated before use and its purity controlled by g.l.c. Ethanol, p.a. from Merck (983), was directly used for stock solutions. Buffers were prepared as described by Gomori [21] and controlled on pH at 25 °C. Also Puffer Titrisol solutions from Merck (9886–9890) were used.

Methods.—The enzymatic reaction. Depending on the desired concentrations of reagents, portions of buffered stock solutions of cyclohexanone, ethanol, and HLAD were

[20] E. G. King and C. Altman, *J. Phys. Chem.*, 1956, **60**, 1975.
[21] G. Gomori, in 'Methods in Enzymology,' eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1955, vol. 1, p. 138.

mixed in a thermostatted reaction vessel and diluted with buffer to 9 ml. The reaction was started by adding 1 ml of a NAD^+ stock solution.

G.l.c. analysis. After various reaction times 1 ml samples were withdrawn from the reaction mixture, added to 0.7 g of $(NH_4)_2SO_4$ (UCB 4859) with 50 μl of $HClO_4$ p.a. (Merck 519) and extracted with 2 ml of CS_2 p.a. (Merck 2214). The CS_2 extracts were analysed on a Varian 1840–41 gas chromatograph equipped with a 6% Carbowax 1500/Chromosorb W column (6 ft × ¼ in), a Varian Aerograph autosampler 8 000, and a Varian CDS 111 integrator. All samples were injected three times and average results were calculated.

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