ENZYMATIC "*IN VITRO*" REDUCTIONS OF KETONES. PART 12[1]. REDUCTION OF 1-ALKYL-4-PIPERIDONES IN AN ETHANOL-NAD^+-HLAD-SYSTEM.

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Abstract - The steady state initial rate equation for a ketone-ethanol-NAD^+-HLAD recycling system is checked for 1-*n*-butyl-4-piperidone. All reaction parameters are discussed in order to optimize the reduction of this substrate. For a series of 1-alkyl-4-piperidones the thermodynamic activation parameters for the reduction in this system are determined and discussed.

INTRODUCTION

In a previous paper[2], the kinetics of the HLAD-mediated reduction of cyclohexanone in a coupled-substrate coenzyme recycling system has been elaborated. This recycling system has been proven to be a very practical tool for preparative scale synthesis of chiral alcohols[3,4]. By using this recycling system, a new model for the reduction of ketones by HLAD was constructed from the kinetic and stereochemical study of alkyl-substituted cyclohexanones[5].

Until now cyclic amines have received little attention as potential substrates since they are known to be relatively unreactive[6,7]. In the present paper, the kinetics of the enzymatic reduction of 1-alkyl-4-piperidones are described and compared with the kinetics for the reduction of cyclohexanone.

Thermodynamic activation parameters are given and discussed in view of the new developed reduction model.

1. Study of the kinetic parameters

For the coupled-substrate coenzyme recycling system in this kinetic study, ethanol was used for regenerating the coenzyme in its reduced form. As shown before, with suitable concentrations of enzyme (1.0×10^{-1} unit.ml^{-1}), coenzyme (at least 1.00×10^{-4} M), substrate (1.00×10^{-2} M) and ethanol (1.00×10^{-1} M), the following simplified initial rate equation can eventually be derived. It was developed

and tested with cyclohexanone as a substrate[2].

$$\frac{E_t}{v_0} = \frac{1}{k_{3B}[B]} \quad 1 + \frac{[A']}{K_I} \qquad [1]$$

In this equation E_t is the total HLAD concentration[§], v_0 is the initial reaction rate, [B] is the ketone concentration, [A'] is the ethanol concentration[*], K_I is the dissociation constant of the dead-end complex HLAD-NADH-ethanol and k_{3B} is the rate constant of the following rate determining step:

$$\text{HLAD-NADH} + \text{ketone} + H^+ \xrightarrow[k_{3B}]{} \text{HLAD-NAD}^+ + \text{alcohol}$$

Because of the quite different nature of piperidones compared to cycloalkanones, we reinvestigated the validity of this rate equation in piperidone systems. 1-*n*-Butyl-4-piperidone was chosen as a model substrate for two practical reasons : ease of its isolation by extraction and its sufficient reactivity[7].

In a first experiment, with a constant concentration of substrate, a linear relationship is found between v_0^{-1} and the ethanol concentration in a range from 0.050 M to 1.50 M (Fig. 1). From the slope and the intercept, a value of K_I of 0.18 ± 0.09 M is found, which is in agreement with the K_I value of 0.104 ± 0.011 M found with cyclohexanone as substrate.

A deviation from the linear curve to lower initial rates is found at ethanol concentrations higher than 1.50 M due to denaturation of the enzyme[8]. This was demonstrated by a set of experiments in which a lower reduction rate was found when the ketone addition was delayed.

A linear relationship between v_0^{-1} and $[B]^{-1}$ is found[†], when all the other parameters are constant (Fig. 2).

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§ All experiments were performed under enzyme saturating coenzyme concentration[2]. A concentration of 2.0×10^{-4} M NAD^+ was always used.

* Ethanol is always used in large excess.

† If the steady state initial rate equation is valid the v_0^{-1} versus $[B]^{-1}$ plot has to go through the origin (see Fig. 2). In this case the intersection with the v_0^{-1} axis lies within a range of 2.2 times the standard deviation (σ) of the linear regression.

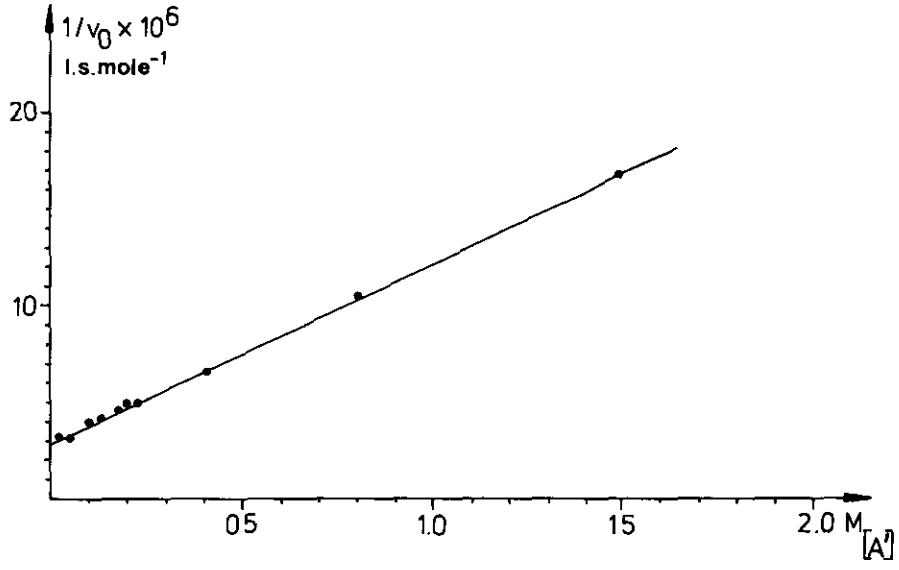

Figure 1 : Dependence of v_0^{-1} on the ethanol concentration [A'] in a coupled-substrate coenzyme recycling system. (Buffer : TRIS-HCl ; pH = 8.9 ; HLAD concentration : 2.0×10^{-1} units.ml^{-1} ; NADH concentration : 2.0×10^{-4} M ; initial ketone concentration : 1.024×10^{-2} M ; temperature : 25°C).

This linearity gives strong evidence that the reduction of 1-*n*-butyl-4-piperidone also proceeds via a double Theorell-Chance mechanism and proves that the simplified initial rate equation is also valid for this heterocyclic derivative.

The influence of ionic strength and pH on the initial rate were also investigated. Ionic strength was found to have little influence on the initial rate in a range from 0.038 to 0.461 (Table 1). Higher ionic strength leads to denaturation of the enzyme.

The pH has a well defined effect on the initial rate of 1-*n*-butyl-4-piperidone (Fig. 3).

The optimum pH for reduction of the heterocyclic derivative lies at pH = 8.9. This is almost two pH units higher than the optimum reduction pH for cyclohexanone (Fig. 3). Below pH = 6 this substrate was not reduced. In order to rationalize these results a detailed knowledge of the behaviour of 1-*n*-butyl-4-piperidone in aqueous solution is necessary. This behaviour is complicated, since besides an

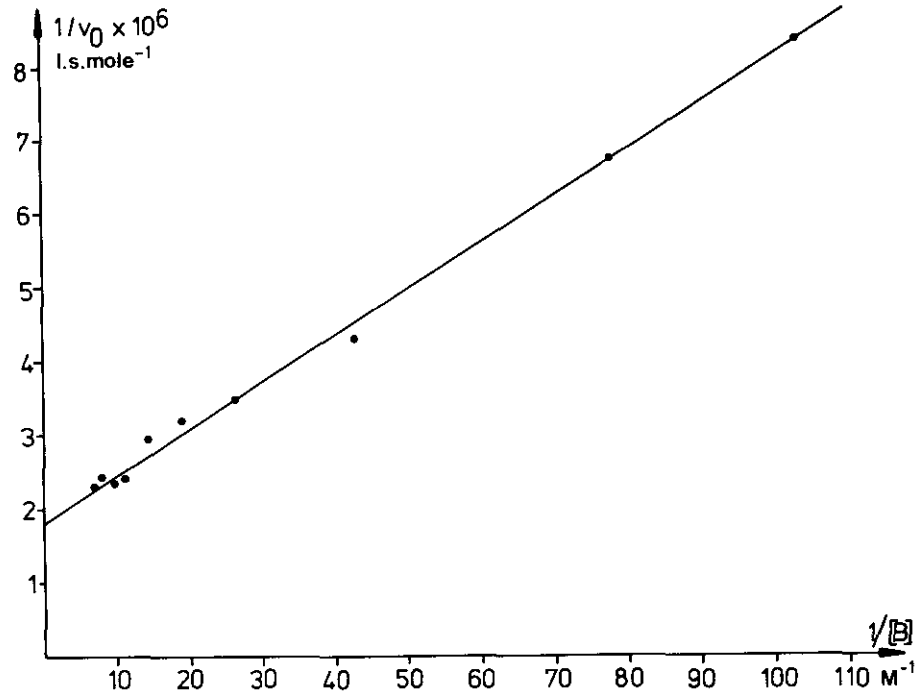

Figure 2 : Dependence of v_0^{-1} on the ketone concentration $[B]^{-1}$ in a coupled-substrate coenzyme recycling system. (Buffer TRIS-HCl ; HLAD concentration : 2.0×10^{-1} units.ml^{-1} ; NADH concentration : 2.0×10^{-4} M ; ethanol concentration : 1.0×10^{-1} M ; temperature : 25°C).

acid-base equilibrium there also exists a keto-diol equilibrium in the aqueous solution[9] (Scheme 1). In a study of these equilibria with ^{1}H-NMR spectroscopy a clear pH-dependence is revealed (see Fig. 4). In solutions with pH higher than 8.9, the unprotonated 1-methyl-4-piperidone* is partly hydrated for 25%. Below pH≈7.5 the fully protonated base is strongly hydrated for about 85%. An analoguous hydration shift was found for the 1-*n*-butyl derivative.

This equilibrium shift can in part explain the lower reaction rates below pH = 8.9. The effective concentration of ketone decreases with decreasing pH and consequently so does the initial rate.

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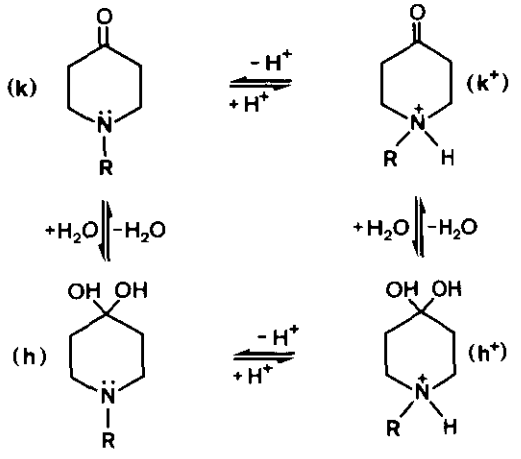
* 1-Methyl-4-piperidone was used in this experiment, since this product is more soluble in water than the 1-*n*-butyl derivative.

Table 1 : Influence of the ionic strength on the initial rate v_0 in a coupled-substrate coenzyme recycling system. (HLAD concentration : 2.0×10^{-1} units.ml^{-1} ; NADH concentration : 2.0×10^{-4} M ; buffer : TRIS-HCl ; temperature : 25°C ; presision of pH : ± 0.04)

pH		concentration ketone ($\times 10^{-3}$ M)	concentration ethanol ($\times 10^{-1}$ M)	v_0 ($\times 10^{-8}$ mole.l^{-1}.s^{-1})
8.81	0.038	9.77	10.22	9.0
8.96	0.077	9.77	10.15	12.1
9.03	0.154	9.98	10.15	9.6
9.02	0.345	10.06	10.35	7.6
9.05	0.461	9.66	10.01	9.8
9.06	0.692	9.82	10.00	5.2
9.08	0.961	9.98	10.08	5.9

Below pH ≈ 6, the substrate completely appears in the nitrogen protonated form. Since no reaction occurs below this pH, it can be concluded that the nitrogen protonated keto form (k^+ : 15%) cannot be reduced by HLAD. This matter will be further discussed in the last chapter.



Scheme 1. Equilibria of 4-piperidones in aqueous solution.

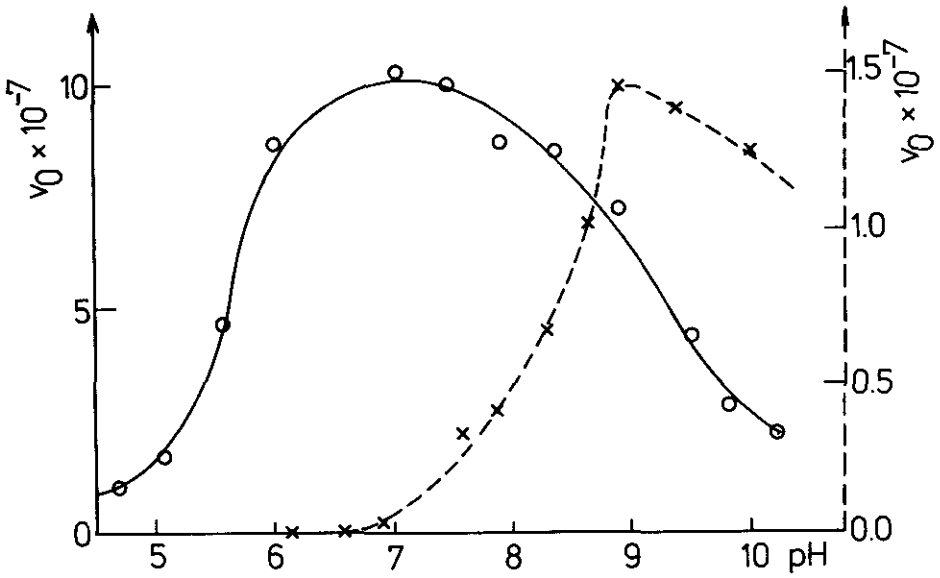


Figure 3 : Dependence of the initial rate v_0 (mole.l^{-1}.s^{-1}) on the pH in a coupled-substrate coenzyme recycling system.

O Cyclohexanone : HLAD concentration : 1.0×10^{-1} unit.ml^{-1} ; initial concentrations : ethanol : 0.515 M ; cyclohexanone : 5.0×10^{-3} M and NAD^+ : 2.0×10^{-4} M ; temperature : 35°C.

X 1-*n*-Butyl-4-piperidone : HLAD concentration : 2.0×10^{-1} units.ml^{-1} ; initial concentrations : ethanol : 1.0×10^{-1} M ; ketone : 1.0×10^{-2} M and NAD^+ : 2.0×10^{-4} M ; temperature : 25°C.

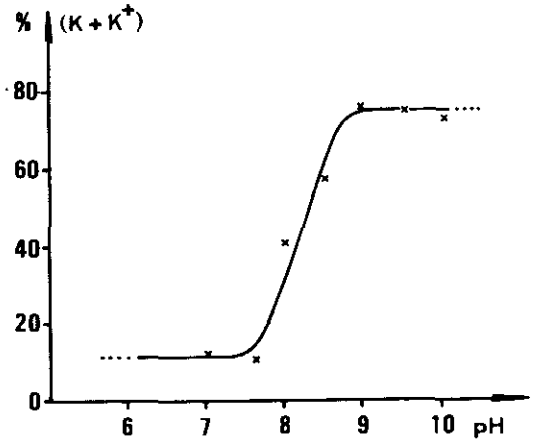


Figure 4 : Percentage of 1-methyl-4-piperidone present in the *ketone form as function of pH*, calculated from ^{1}H-NMR measurements.

2. The reaction yield

The reaction yield as a function of pH was studied (Table 2). Between pH = 7.4 and pH = 10.0 a yield of 97-99% of 1-*n*-butyl-4-hydroxypiperidine was found after two weeks. These high yields show that the equilibrium in the reduction of 1-alkylpiperidones lies more to the alcohol side than found for the reduction of cyclohexanone[2].

Since the nitrogen in the hydroxypiperidine is more basic than in the piperidone (respectively pK_a 9.6 versus 8.6), the reaction equilibrium will indeed be favourably shifted in this pH-region. Between pH = 6 and pH = 7 only a low reduction yield is obtained. Obviously the reaction rate is too low and the end point is not reached. Below pH = 6, no alcohol was found after two weeks, even with high concentrations of enzyme (1.0 unit.ml^{-1}).

Table 2 : Influence of pH on the reaction yield in a coupled-substrate enzyme recycling system.
(Ethanol concentration : 1.0×10^{-1} M ; HLAD concentration : 2.0×10^{-1} units.ml^{-1} ; NAD^+ concentration : 2.0×10^{-4} M ; temperature : 25°C).

pH	buffer	1-*n*-butyl-4-piperidone (10^{-3} M)	% alcohol	
			1 week	2 weeks
5.34	phosphate-NaOH	10.00	no reaction	
5.75	phosphate-NaOH	10.00	no reaction	
6.04	phosphate	10.00	no reaction	
6.66	phosphate	10.00	13	16
6.96	phosphate	10.18	36	57
7.43	phosphate	10.15	90	98
7.79	TRIS-HCl	10.12	91	98
8.31	TRIS-HCl	10.16	94	97
8.72	TRIS-HCl	10.09	97	97
8.90	glycine-NaOH	10.12	98	99
9.44	glycine-NaOH	10.12	98	99
10.05	glycine-NaOH	10.15	97	99

3. Thermodynamic parameters

Using equation [1] , k_{3B} values for the HLAD-catalyzed reduction of 1-alkyl-4-piperidones are determined at six different temperatures between 0°C and 45°C. The corresponding E_A-values have been calculated from the Arrhenius-plots and the activation parameters G and S are calculated from the Eyring formula[11].

In order to calculate these parameters, the exact knowledge of the ketone-hydrate equilibrium of these substrates at pH = 8.9 and at these six different temperatures was necessary. The percentage of ketone form (k) was calculated and found from ^{13}C-NMR measurements to be 40% ± 4% for 1-methyl, 1-*n*-propyl and 1-*n*-butyl-4-piperidone at pH = 8.9 and T = 25°C (Table 3).

Table 3 : Percentage of the ketone form (k) of four different 1-alkyl-4-piperidones at different temperatures and at pH = 8.9 .

1-alkyl-4-piperidone	% ketone form (k)	temperature (°C)
1-*methyl*	16±4	5
	32±4	20
	49±4	36
	57±4	45
1-*n*-propyl	40±4	25
1-*n*-butyl	40±4	25
1-isopropyl	36±4	25

pH dependence for other 1-alkyl-4-piperidones with longer alkyl chains could not be examined since these products were not enough soluble in water to obtain good spectra even after 7000 accumulations. It is assumed that the ketone-hydrate equilibrium is about the same for all the enzymatically examined 1-alkyl-4-piperidones. Only for the 1-isopropylderivative a slightly lower percentage of 36% ketone form (k) was found.

The ketone-diol equilibrium for 1-methyl-4-piperidone is shown to be temperature-dependent. An increase of 1% ketone per °C was found. The same temperature dependence was assumed for the ketone-diol equilibrium in other 1-alkyl-4-piperidones.

In analogy with the reduction of 4- and 3-alkylcyclohexanones[3] chain lengthening of the alkylsubstituent increases the reduction rate (Table 4).

Furthermore, also here an isoenthalpic isokinetic relationship is found for the *n*-alkylpiperidones. Thus, all these substrates go through one type of transition

Table 4 : Comparison between the kinetic parameters for the reduction of 1-alkyl-4-piperidones and 4-alkylcyclohexanones. ($\sigma_{\Delta H^{\ddagger}} = \pm 3$ kJ.mole^{-1} ; $\sigma_{\Delta S^{\ddagger}} = \pm 10$ J.mole^{-1}.K^{-1}) (HLAD concentration : 2.0 x 10^{-1} units.ml^{-1} ; NAD^{+} concentration : 2.0 x 10^{-4} M ; initial ethanol concentration : 1.0 x 10^{-1} M ; buffer : TRIS-HCl ; pH = 8.9 ; temperature : 25°C)

ketone	k_{3B} (1)	E_A (2)	$\Delta H^{\ddagger}$ (2)	$\Delta S^{\ddagger}$ (3)	$\Delta G^{\ddagger}$ (2)
cyclohexanone	524 (4)	36	34	-79	58
1-*n*-propyl-4-piperidone	9	65	62	-18	68
4-*n*-propylcyclohexanone	374 (4)	38	35	-77	58
1-*n*-butyl-4-piperidone	34	65	62	-7	64
4-*n*-butylcyclohexanone	1016 (4)	38	36	-67	56
1-*n*-pentyl-4-piperidone	95	64	62	-1	62
4-*n*-pentylcyclohexanone	2455 (4)	38	36	-60	54
1-*n*-hexyl-4-piperidone	126	65	63	+5	61
1-isopropyl-4-piperidone	2	77	75	+12	71
4-isopropylcyclohexanone	135 (4)	35	33	-92	61
1-isobutyl-4-piperidone	88	58	56	-21	62
4-isobutylcyclohexanone	491 (4)	38	35	-75	58
1-isopentyl-4-piperidone	26	65	62	-9	65

(1) in l.katal^{-1}.s^{-1}.

(2) in kJ.mole^{-1}.

(3) in J.mole^{-1}.K^{-1}.

(4) k_{3B} at 25°C in TRIS-buffer with pH = 8.5 (see reference 2).

state[12]. 1-Alkyl-4-piperidones with branched alkyl groups do not fit well in this relationship, especially when the branching point is near the piperidone ring.

By comparison of the individual k_{3B} values of the 1-*n*-alkyl-4-piperidones with those of the corresponding 4-*n*-alkylcyclohexanones it can be seen that insertion of a nitrogen atom on the 4-place reduces the rate with a factor of 25*. This is in sharp contrast to the $NaBH_4$ reduction where 1-methyl-4-piperidone is reduced 10 times faster than cyclohexanone[13]. Consequently the very low enzymatic reactivity can hardly be due to the intrinsic reducibility of the piperidones.

A comparative study of the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values reveals that this rate reduction is due to a quite constant increase of about 26 $kJ.mole^{-1}$ of $\Delta H^{\ddagger}$, partially compensated by an overall increase of about 59 $J.mole^{-1}.K^{-1}$ of $\Delta S^{\ddagger}$. This suggests that the landing of the substrate in the active site of the enzyme demands more energy for piperidones than for cyclohexanones. This can be ascribed to differences in solvation. A shell of water molecules must be removed from the nitrogen atom in order to fit the molecule in the hydrophobic active site, which explains the $\Delta H^{\ddagger}$ value increase. Also $\Delta S^{\ddagger}$ variations are induced by desolvation. Since more ordered water molecules are turned into disorder for the 4-piperidones, the observed increase of $\Delta S^{\ddagger}$ is also accounted for.

The results also fit well with the recently proposed transition state model[5] (see Fig. 5) where hydrophobic interaction with zone 2 of the model account for the acceleration observed for chain lenghthening.

The flattened piperidone ring is situated in the hydrophobic zone 1, the nitrogen atom occupying positions 4 or 4'. In position 4 the polar nitrogen is very near the hydrophobic wall. This is unfavourable for a protonated nitrogen atom. The free base (pH > 6) most probably is reduced with its nitrogen atom in position 4', which is farther away from the hydrophobic wall. When the nitrogen atom is protonated (pH < 6), even position 4' is so unfavourable that no reduction occurs.

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* The k_{3B}-values of the 1-*n*-alkyl-4-piperidones are first multiplied with a factor of 1.31. This factor reflects the enzyme activity difference between pH = 8.5 and pH = 8.9 (see Table 4).

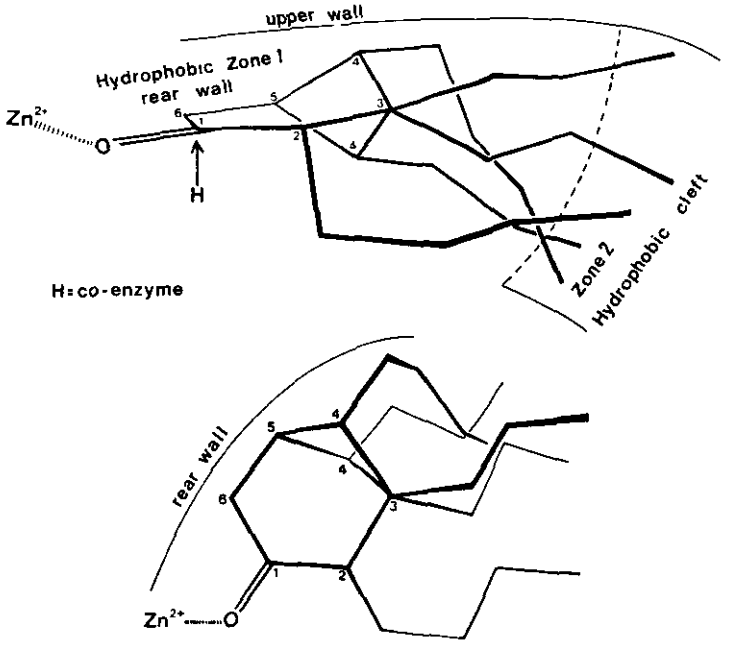

Figure 5 : New model for the reduction of cyclic ketones with HLAD.

CONCLUSIONS

The following conclusions can be made from the reduction experiments of 1-alkyl-4-piperidones in the coupled-substrate coenzyme recycling system : (1) Only the free amine form is reduced. The pH of the medium must be maintained around or above the pK_a-value of the piperidones. The low reactivity can be ascribed to the strong solvation of these substrates. (2) The simplified steady state initial rate equation [1] described for isocyclic ketones, is also valid for the reduction of 1-alkyl-4-piperidones. (3) For 1-*n*-alkyl-4-piperidones one isokinetic relationship is found. This indicates that all derivatives pass through one type of transition state. The isokinetic relationship is isoenthalpic. This means the alkyl substituents only influence the reaction rate by changing $\Delta S^{\ddagger}$. This is in agreement with the reduction model described for isocyclic ketones[3,5].

EXPERIMENTAL

Materials. HLAD was purchased from SIGMA (340-L2) in packages of 200 units. Before use 200 units of HLAD were dissolved in 100 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. Ketones were synthesized according to the method of Ruzicka et al.[14] Their purity was checked with G.L.C. Ethanol p.a. from MERCK was directly used for stock solutions. Buffers were prepared as described by Gomori[15] and controlled on pH at the different used temperatures.

Methods. Depending on the desired concentrations of reagents, portions of buffered stock solutions of HLAD, ethanol and NAD^+ were mixed in a thermostatic vessel and diluted with buffer to 9 ml. The reaction was started by adding 1 ml of the ketone stock solution.

G.L.C. analysis. After various reaction times 1 ml of samples were withdrawn from the reaction mixture, added to 0.7 g $(NH_4)_2SO_4$ (UCB 4859) with 50 µl $HClO_4$ p.a. (MERCK 519) and extracted with 2 ml of $CHCl_3$ p.a. (MERCK 2445). The $CHCl_3$ extracts were analyzed on a VARIAN 3700 gas chromatograph equipped with a 1.04% Carbowax 20M/Chromosorb W (1.5 x 2 mm) column of 1.5 m and a VARIAN CDS 111 integrator. All samples were injected three times and average results were calculated.

N.M.R. spectra. The ^{1}H-NMR spectra were recorded on a JEOL-PS-100 spectrometer operating at 100 MHz. The probe temperature was 31 ± 2°C. The ^{13}C-NMR spectra were recorded on a JEOL-FX-100 spectrometer equipped with a T.I.-E.C.-100 computer for operation in the Fourier-Transform mode. Noise decoupled spectra were obtained under elimination of N.O.E. and relaxation time effects by accumulating 8k interferograms; spectral width 6250 Hz, 25° flip angle (6 µs) and 3 s repetition time. The carbonyl carbon of the ketone form (k) (δ = 214.9 ppm) of 1-*n*-butyl-4-piperidone in water between pH = 7 and 9, is not only separated from the hydrate carbon (δ = 91.6 ppm), but is also separated from the carbonyl carbon of the ketone form (k^+) (δ = 213.2 ppm). Simple peak integration reveals the concentration of the ketone form (k), the only form which is reduced by HLAD.

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