Enzymatic *in vitro* Reduction of Ketones. Part 8.[1] A New Model for the Reduction of Cyclic Ketones by Horse Liver Alcohol Dehydrogenase. (HLAD)

By **Guy L. Lemière,* Tony A. Van Osselaer, Josef A. Lepoivre,** and **Frank C. Alderweireldt,** Laboratory of Organic Chemistry, University of Antwerp (R.U.C.A.), Groenenborgerlaan 171, B-2020 Antwerp, Belgium

The values for the reaction rate constants of the catalytic step HLAD-NADH + ketone ⟶ HLAD-NAD$^+$ + alcohol in the HLAD-catalysed reduction of cyclic ketones are rationalized in a new model. The fundamental concepts of the model are discussed. An extensive description of the model-building is presented and its predictive value for any possible six-membered ring substrate is shown. A comparison with previous models is given.

HORSE liver alcohol dehydrogenase (HLAD), one of the most extensively studied enzymes,[1-7] exhibits considerable stereospecificity since it shows enantiofacial or diastereofacial differentiation [8] at prochiral carbonyl groups and enantiomer or diastereoisomer differentiation [8] of chiral ketones and alcohols. As a result of its broad constitutional specificity and its narrow stereospecificity it has become a powerful tool for the preparation of alcohols and ketones [7] and for the investigation of structure–reactivity relationships of various substrates. Extensive investigations on the stereochemistry and the relative reduction rates of cyclohexanone derivatives have led to predictive transition-state models for the HLAD-catalysed reactions. One of the most successful, the so called ' diamond lattice model ' introduced by Prelog,[9,10] permits an interpretation of the constitutional specificity and stereochemistry of HLAD for six-membered ring ketones and alcohols.[7,9-12]

Variations of this model, presented by Prelog [9,10] and by Graves,[12] can be considered as refinements of the same concept. Recently an updated model has been presented by Jones [7] (Figure 1). There have been several reviews about how this model is derived and how

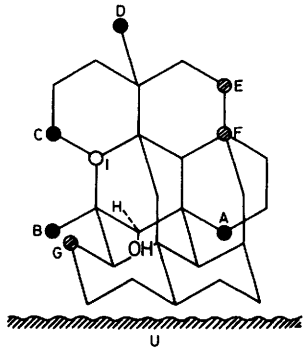

FIGURE 1 Updated diamond lattice model: [7] ●, A—D, forbidden positions; ◍, E—G, and U, undesirable positions; ○, I, newly identified unsatisfactory position

it has to be used.[9,10,12,13] In spite of the great ingenuity and the merit of the ' diamond lattice models ' our present knowledge of the enzyme, its substrate specificity, and transition-state theory is such that it can be refined no further. This may be made clear by mentioning a few features that cannot be handled by this model.

(i) For the construction of the diamond lattice model, cyclohexanone is used as the reference substrate. With this procedure all differences of the reaction rates are automatically ascribed to interactions of the substrate with the enzyme–co-enzyme complex. Consequently the intrinsic reactivity of the substrates is not taken into account. As a result the model cannot rationalize the slow reduction rate of some ketones which fit perfectly in the allowed space of the model.

(ii) The model cannot rationalize the *cis* : *trans* ratios of the 4-alkylcyclohexanols obtained by the reduction of the corresponding 4-alkylcyclohexanones.

(iii) According to this model the substituent in (3*R*)-3-alkylcyclohexanones has to accept an axial position before reduction can occur [11,14,15] This is very unfavourable for bulky substituents and therefore the stereospecificity of the reaction should increase for bulkier groups. We did not observe such an effect. Nevertheless, some rate constants given by Dutler [16,17] do not support this view, but in the recycling system used by his group the fastest reduction rates seem to be levelled off, presumably because the recycling reaction is becoming rate-determining.[18-22]

(iv) The forbidden positions are rather scattered (Figure 1), which is inconsistent with extensive enzyme walls.

Therefore a new transition-state model had to be developed which (a) has to rationalize the intrinsic and the topographic structure–reactivity relationship of the substrates along the whole reaction path; (b) has to rationalize the stereospecificity; (c) must fit with the most recent findings for nucleophilic additions to carbonyl groups; and (d) should not show any incompatibility with recent findings on the tertiary structure of the enzyme molecule.[23,24] In this paper a proposal to meet all these requirements is elaborated and explained.

Kinetics and Mechanism.—It is widely accepted that the Theorell–Chance mechanism as shown in Scheme 1 sufficiently describes the kinetics of the HLAD-catalysed reactions.[18-20,25-29] This mechanism assumes a compulsory binding order with no kinetically significant ternary complexes or binary enzyme–ketone and enzyme–alcohol complexes.

Only the reaction rate of step (2) is determined by the structure of the ketones. The dissociation of the

enzyme–NAD^+ complex in the forward (k_2'), and the enzyme–NADH complex in the backward reaction (k_2) are slow steps which are generally, at least in part, rate-limiting.[18-22]

$$E + NADH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}NADH \quad (1)$$

$$E\text{-}NADH + R\text{-}CO\text{-}R' \underset{k_3'B}{\overset{k_3B}{\rightleftharpoons}} E\text{-}NAD^+ + R\text{-}CHOH\text{-}R' \quad (2)$$

$$E\text{-}NAD^+ \underset{k'_1}{\overset{k'_2}{\rightleftharpoons}} E + NAD^+ \quad (3)$$

SCHEME 1

The catalytic step [k_{3B} in (2) and (5)] with ketones can be made rate-determining under steady-state conditions by the addition of a sufficient quantity of ethanol, as a co-enzyme recycling substrate. In such a system [30,31] the enzyme–co-enzyme complex never dissociates and is alternately reduced and oxidised by ethanol and the ketone, respectively. However an abortive enzyme–NADH–ethanol complex is then formed (K_I) [30] and must

$$E\text{-}NAD^+ + CH_3CH_2OH \underset{k_{3A}}{\overset{k'_{3A}}{\rightleftharpoons}} E\text{-}NADH + CH_3CHO \quad (4)$$

$$E\text{-}NADH + R\text{-}CO\text{-}R' \underset{k'_{3B}}{\overset{k_{3B}}{\rightleftharpoons}} E\text{-}NAD^+ + R\text{-}CHOH\text{-}R' \quad (5)$$

$$E\text{-}NADH + CH_3CH_2OH \overset{K_I}{\rightleftharpoons} E\text{-}NADH\text{-}CH_3CH_2OH \quad (6)$$

SCHEME 2

be taken into account [reaction (6)]. Under these conditions the co-enzyme recycling system has a very simple initial steady-state reaction rate equation,[30] from which k_{3B} can easily be calculated [equation (7)]. Values of

$$\frac{E_t}{v_0} = \frac{1}{k_{3B}(\text{R-CO-R}')}\left(1 + \frac{(CH_3CH_2OH)}{K_I}\right) \quad (7)$$

k_{3B} for the reduction of cycloalkanones, 2-, 3-, and 4-alkylcyclohexanones, *gem*-dimethylcyclohexanones, and polycyclic alkanones were published earlier.[1,2]

Comparison with $NaBH_4$ *Reductions.*—For the construction of our new model we tried to distinguish the inherent reactivity of the substrates from rate-decreasing or -increasing effects of the HLAD–NAD(H) complex. Therefore the reaction rate constants k_{3B} for the HLAD-catalysed reductions are compared with k for the $NaBH_4$ reduction. The $NaBH_4$ reduction is a good reference reaction [32] for several reasons.

The HLAD-catalysed reduction and the $NaBH_4$ reduction can be considered as hydride reduction reactions,[32,33] the 1,4-dihydronicotinamide moiety of the co-enzyme acting as hydride donor,[33] and Zn^{2+} ion as the Lewis acid, complexing the carbonyl oxygen atom.[6,32,34-36]

$NaBH_4$ is a relatively small reagent. Therefore differences of the $NaBH_4$ reduction rate can be ascribed mainly to short-range steric interactions or to differences in intrinsic reactivity of the carbonyl group. This is why we can safely assume that marked differences in the relative reaction rates between $NaBH_4$ reduction and enzymatic reduction are due to specific effects from the enzyme–co-enzyme complex.

The reaction-rate constants k_{3B} [equation (5)] and k, the rate constant of the first and rate-limiting reaction step [37] of the $NaBH_4$ reduction, R-CO-R' + BH_4^- ⟶ $BH_3(OCHRR')^-$, can safely be compared since both characterize a bimolecular reaction.

$NaBH_4$ reduction in propan-2-ol has been very extensively investigated and many reference k values are available in the literature.[38]

The Complexation-induced Conformational Perturbation Concept.—The model of Chérest and Felkin [39,40] is generally accepted as the most up to date transition-state model for hydride reduction of carbonyl groups. In contrast to older models [41-46] it invokes only one type of transition state to explain the reduction of acyclic as well as cyclic, and unhindered as well as hindered, ketones. A recent modification of this model by Doyle [47] provides for the important role of the Lewis acid in complexing the carbonyl oxygen atom.[48] In cyclohexanone rings this complexation creates a torsional strain between the Lewis acid and the equatorial 2- and 6-substituents of the ketone. By this interaction the chair form is no longer the most stable conformation in the transition state. This results in a flattening of the cyclohexanone ring around the carbonyl group, which is called *complexation-induced conformational perturbation.* The increased torsional strain between C-2 and -3, and C-5 and -6 is compensated by the decrease in the torsional strain created by the complexing reagent. The enzymatic reducibility of ketones can now be explained in a similar way.

The New Model.—In the model for HLAD-catalysed reduction of ketones proposed in this paper, the cyclohexanone ring is completely flattened (C-1, -2, -3, -5, and -6 in one plane). We realize that this is an oversimplification since the extent of ring flattening is dependent on several factors such as the flexibility of the ketone, the degree of association of the complexing agent, and the nature and size of the 2- and 6-substituents.[47] Nevertheless an appreciable flattening can be expected since the catalytic Zn atom with its polypeptide ligands is a very bulky Lewis acid.

Assuming that the dihydronicotinamide ring of the co-enzyme is positioned below * the flattened cyclohexanone ring, the substrate still can be oriented with C-4 upward or downward. A superposition of these two possibilities forms the core of the new model (Figure 2).

In the following discussion the numbering of the ring carbon atoms is the same as in Figure 2. C-4 will have the number 4 if oriented upward, and 4' if oriented downward. Furthermore substituents below the flattened cyclohexanone ring are called α-substituents and the others β-substituents (Figure 3), since the adjectives equatorial and axial are sometimes ambiguous.

* An O=C · · · H angle of 90 or 107° [49] is not essential for the model.

In the model presented here 4-, 3-,* and 2-alkylcyclohexanones will be merged. Each one is oriented towards the reducing co-enzyme in such a way that experimentally formed alcohols are obtained when the

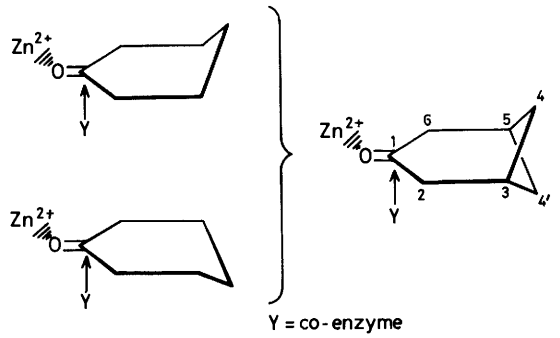

FIGURE 2 Core of the new model

hydride shift occurs, *i.e. cis*- and *trans*-4-alkylcyclohexanol, (1*S*,3*S*)- and (1*S*,3*R*)-3-alkylcyclohexanol, and (1*S*,2*S*)-2-alkylcyclohexanol † (Figure 4). By merging all the orientations shown in Figure 4 a picture is obtained (Figure 5) showing an allowed (2α, 3α, 3β, 4α, 4'β) and a forbidden side (2β, 5α,‡ 5β,‡ 6α, 6β) of the cyclohexanone ring.

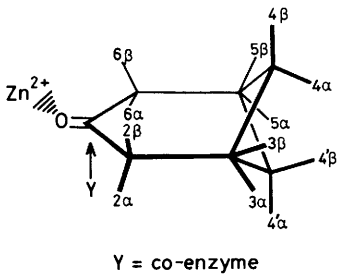

FIGURE 3 Specification of the substituents

This procedure of determining ' allowed ' and ' forbidden ' zones around the active site and of localising these zones in the substrate gives a qualitative picture in the first instance. However, our present knowledge of experimental data concerning rate constants and thermodynamic parameters [1,2] allows us to do more, *i.e.* to elaborate a quantitative model. The starting point is to calculate the intrinsic effect of the substituent. For the reasons mentioned above the effects of a substituent on the $NaBH_4$ reduction rates can be taken as a measure of this intrinsic influence.

The size differences in the reagents can be regarded as a more specific effect of the enzyme–co-enzyme complex,

* 3- and 4-alkylcyclohexanones probably do not have exactly the same orientation in the activated complex.[2] However, superposition of these substrates for the purposes of model building is believed to be a sufficient approximation.

† Only the stereoisomeric alcohols which are formed under initial rate conditions are considered.

‡ Position 5 is not forbidden in an absolute sense, since under certain conditions differing from our initial rate conditions, a very slow reaction is observed with a methyl group at position 5.[1,14–16]

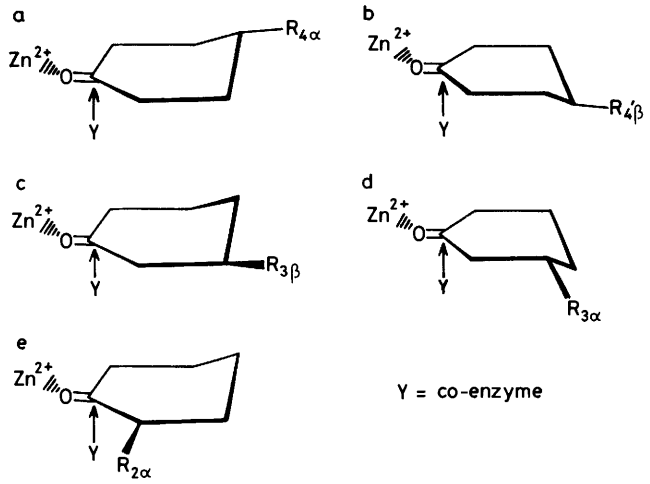

FIGURE 4 Alkylcyclohexanones as reduced by HLAD

since this complex can be considered as one unit in determining the topology of the active site.

In the Table the ' calculated ' total rate for the enzymatic reduction is obtained by multiplying the k_s/k_o ratio of the $NaBH_4$ reduction rate of the substituted *versus* the unsubstituted cyclohexanone by 524 l mol^{-1} s^{-1},[2] *i.e.* the rate for the enzymatic reduction of cyclohexanone. This indeed gives the overall calculated ' intrinsic ' rate for the enzymatic reaction. For the

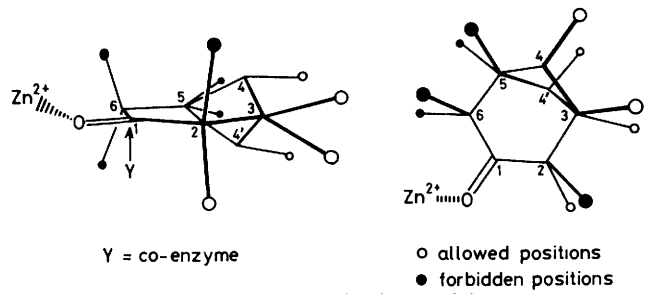

FIGURE 5 Qualitative model

partial rates of formation of *cis* and *trans* isomeric alcohols the *cis* : *trans* ratio for the $NaBH_4$ reduction is used. These figures are then compared with the observed partial rates to obtain the net topographic effect of the enzyme–co-enzyme system.

The figures for $NaBH_4$ reduction show a slight acceleration for the 4-methyl derivative, and for the 3- and 2-methyl derivative a gradual decrease is observed as the substituent is closer to the reaction site (Table, k_s/k_o values).

As can be seen from the Table the observed total rate of reduction of 4-methylcyclohexanone is considerably lower than the calculated total rate. This decreased reaction rate is almost totally caused by a 3.6-fold decrease of the rate of formation of the *trans*-alcohol, starting from conformation a in Figure 4. Obviously the 4α-position is hindered and possibly also C-4 itself in conformation 4.

The HLAD-catalysed reduction of (3*S*)- and (3*R*)-3-

	$NaBH_4$		HLAD k_{3B} (l mol^{-1} s^{-1})					
			Calculated			Observed		
	cis : *trans*	k_s/k_o	*cis*	*trans*	Total	*cis*	*trans*	Total
CHN †								524 [2]
4-Me-CHN	14 : 86 [50]	1.25 [51]	92	563	655	110 [2]	150 [2]	258 [2]
3-Me-CHN (3*S*)	14 : 86 [50]	0.76 [51]	342	56	398	0	276 [2]	276
3-Me-CHN (3*R*)			342	56	398	5.1 [2]	0	5.1
2-Me-CHN (2*S*)	30 : 70 [52]	0.19 [51]	30	70	100	0	6.6 [1]	6.6
2-Me-CHN (2*R*)			30	70	100	0	0	0

† CHN = cyclohexanone.

methylcyclohexanone gives pure (1*S*,3*S*)-*trans*-3-methylcyclohexanol (Figure 4d) and (1*S*,3*R*)-*cis*-3-methylcyclohexanol (Figure 4c) respectively. It is known that the introduction of a 3-methyl group in cyclohexanone decreases the rate of the $NaBH_4$ reduction to *ca.* 0.76 of the original value.[51] Assuming that this effect also occurs in the enzymatic reaction and comparing the calculated values with the experimental reaction rates (see Table), it is found that the (3*S*)-isomer is reduced 4.9 times faster than calculated and the (3*R*)-isomer 67 times slower. Clearly the 3α-position is not only an allowed position but also a favoured one, whereas the 3β-position is strongly hindered.

The HLAD-catalysed reduction of (2*S*)-2-methylcyclohexanone to pure (1*S*,2*S*)-*trans*-2-methylcyclohexanol (Figure 4e) is 11 times slower than the calculated rate. The (2*R*)-isomer is not reduced under initial rate conditions. This means that the 2α-position is hindered and the 2β-position is forbidden.*

Summarizing the conclusions for the rate of reduction of 4-, 3-, and 2-methylcyclohexanone a semiquantitative picture can be given (Figure 6). Increasing hindrance is

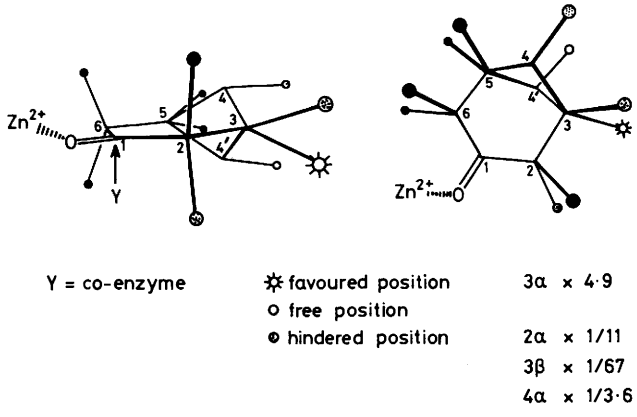

FIGURE 6 Semiquantitative model

observed on going from the 4α- to the 3β- and further to the 2β-position. On the other side, the 4′β-position is free, the 3α-position seems to be catalytically favoured, and the 2α-position is hindered.

Combination of these results with those above suggests that during enzymatic reduction a rear wall screens the C(5) and (6), and an upper wall is responsible for the increasing hindrance at positions 4′α, 3β, and 2β.

* Position 2β is not forbidden in an absolute sense, since a very slow reduction rate of 2,2-dimethylcyclohexanone is found.[1]

An additional feature is observed by studying cyclohexanone derivatives with an n-alkyl homologous series. For all allowed positions an increase in reaction velocity is observed when the alkyl substituent is sufficiently long. This is not found in $NaBH_4$ reductions. The increase starts from the n-propyl substituent for positions 3α, 3β, and 4′β, and from the n-butyl substituent for positions 4α and 2α. The accelerations are ascribed to hydro-

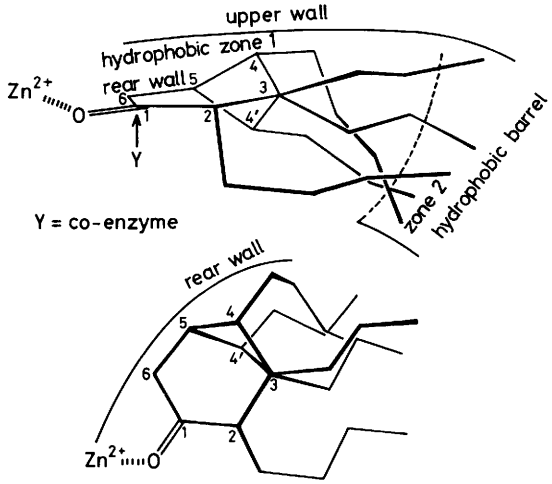

FIGURE 7 The new model

phobic interactions between the alkyl aubstituent and a remote hydrophobic zone of the enzyme.[1]

All features are collected in the model shown in Figure 7. Two superimposed flattened cyclohexanone rings as discussed above are represented in the hydrophobic binding zone 1.[1] At the forbidden positions 5α and β, 6α and β, and 2β* no alkyl groups are present. At the other positions n-alkyl groups are represented as reaching the hydrophobic zone 2 at the right front side with the third or fourth carbon atom. The 4′β-, 3α-, and 3β-n-alkyl groups are in a zig-zag conformation and the substituents at position 4α and 2α are presented in *gauche* conformations between C(1′) and (2′). This is done to move them away from the hindering upper wall and the NADH molecule. Furthermore this brings all the alkyl groups together in the hydrophobic zone 2. These all-staggered conformations may be an oversimplification. Nevertheless, extended instead of coiled alkyl chains are consistent with the observed rate accelerations upon hydrophobic binding. In Figure 7 there are upper and rear walls. The upper wall is responsible for the increasing hindrance at positions 4α, 3β, and 2β. The

rear wall screens C(5) and (6). The lower front side is totally free for the alkyl substituents. No dihydronicotinamide ring or any other part of the co-enzyme is represented since our study gives no direct information about the relative orientation of substrate and co-enzyme. Nevertheless, the co-enzyme is certainly not directly under the cyclohexanone ring, since this space can be occupied by bulky alkyl groups.

The model developed here from stereochemical and kinetic data is in good agreement with the geometry of the substrate binding pocket of HLAD as revealed by *X*-ray analysis.[23,24] The rear and the upper wall correspond to the bottom and the side of a cleft. At the bottom of this cleft the catalytic zinc atom which complexes the carbonyl oxygen atom of the substrate is situated. The hydrophobic zone 2 corresponds to a hydrophobic barrel which is completely lined with non-polar amino-acid residues.

There is a great deal of correspondence between the above model and the picture developed by Dutler,[14,16,17] but there are also a few important differences in conception. In our model the cyclohexanone ring is flattened according to the principle of complexation-induced conformational perturbation [47] whereas Dutler considers ideal chair conformations. In contrast to Dutler, we only need alkylcyclohexanones with equatorial substituents for the construction of our model, and all our stereochemical and kinetic results can be ascribed exclusively to substrate–enzyme interactions. If axial conformers really are involved in the enzymatic reduction one would expect increasing stereospecificity of the reaction with increasing bulk of the alkyl group. Such stereospecificity is not observed; on the contrary, a remarkably constant ratio of the reduction rates of (3*S*)- and (3*R*)-3-alkylcyclohexanones is observed.[2]

Conclusions.—We believe that, with some precautions, this new model for the reduction of ketones by HLAD, built up from a kinetic and stereochemical study of alkyl-substituted cyclohexanones, can also be used for other ketones such as halogenated cyclohexanones,[53] cycloalkanones,[54] and heterocyclic [55] and even acyclic ketones.[56] For simple cycloalkanones a good correlation between enzymatic and $NaBH_4$ reductions can be expected.[54] However when polar groups are present, *e.g.* in heterocyclic ketones such as piperidones,[55] additional interactions of these groups can result in highly different rates of reduction. Interactions between solvated heteroatoms and hydrophobic zones of the enzyme can dramatically decrease the reaction rate.

The configurations of the alcohols from ketones formed under initial rate conditions by the ethanol-coupled co-enzyme recycling system can be predicted by locating the ketones in a flattened conformation in the model. When the initial rate conditions are not fulfilled other stereoisomers can be found.

The reaction rate relative to cyclohexanone can be estimated by evaluating the effect of parts of the substrate molecule occupying unfavourable or favourable positions. This can only be done successfully if the intrinsic reactivity of the ketone relative to cyclohexanone is known from the $NaBH_4$ reduction.

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