Thermodynamic Activity-Based Enzyme Kinetics: Efficient Tool for Nonaqueous Enzymology

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Lipase-catalyzed synthesis reactions must be performed in nonaqueous media (organic solvents or solvent-free systems). The choice of the optimal solvent is usually a fastidious task that necessitates the determination of kinetic parameters in each solvent. The approach used here, to overcome the lack of a model that can predict the kinetics whatever the solvent, consists in the use of thermodynamic activities instead of concentrations of components, and assumes that activity-based kinetic parameters are the same in all solvents. This assumption is discussed, and a solution is proposed which takes into account some observed residual solvent effects. The reaction chosen to test this approach was the esterification of oleic acid with ethanol catalyzed by an immobilized lipase, Lipozyme. For this reaction, the kinetics predicted in various organic solvents and in solvent-free systems is in agreement with the experimental data.

Introduction

There is a growing interest in developing economic and functional processes of enzyme catalysis in nonaqueous media: peptide synthesis by proteases (Kise and Hayakawa, 1991); synthesis of oligosaccharides by invertase (Bieleki and Somiari, 1998) and glucosidase (Laroute and Willemot, 1992); biotransformation of polycyclic aromatic hydrocarbons (Vazquez-Duhalt et al., 1994) and asphaltenes (Fedorak et al., 1993); modification of fats and oils, synthesis of organic compounds, detergent supplements, biodegradable lubricants, enantiomerically pure compounds and analytical procedures using lipases (Björkling et al., 1991; Macrae and Hammond, 1985).

The reactions listed above are thermodynamically impeded in monophasic aqueous systems, but they can be performed in organic media, which favor equilibrium towards synthesis and offer additional advantages: (a) solubilization of hydrophobic compounds that have a serious mass-transfer limitation in aqueous systems; (b) the thermostability of several enzymes is improved in organic solvents (Zaks and Klibanov, 1984); (c) from an industrial point of view, products and enzyme recovery after reaction could be easier by evaporation of the solvents.

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An ideal solvent enables a high initial rate of reaction and an optimal degree of conversion to be obtained, presents low toxicity and flammability and, in the case of synthesis in continuous systems, the solvent must evacuate the water produced by the reaction to avoid biocatalyst deactivation (Colombié et al., 1998). Optimization between these factors would be rendered much easier if a predictive model was available. However, from existing studies, there is no general model to predict the effects of the medium on kinetics (Halling, 1994; Vermue and Tramper, 1995; Yang et al., 1997), on enzyme stability (Zaks and Klibanov, 1984), on enantioselectivity (Nakamura et al., 1995; Parida and Dordick, 1993; Westcott and Klibanov, 1993) and on reactor stability (Colombié et al., 1998). Consequently, optimization of these processes is a hard task and involves determining kinetic parameters for each one of the enzyme-catalyzed reactions in every solvent.

Attempts have been made to establish correlations between activity of the enzyme and different physicochemical properties of the solvents, such as dielectric constant (Affleck et al., 1992; Reslow et al., 1987; Valivety et al., 1991) and hydrophobicity (Laane et al., 1987; Torres et al., 1998), but the validity of these correlations is not general (Janssen et al., 1993; Laroute and Willemot, 1992; van Tol et al., 1995a,b,c; Yang et al., 1997). An interesting macroscopic analysis of nonaqueous enzyme-kinetics was developed by Nurok et al.

(1999) using multiple linear regression. Their model correlates initial rate for lipase and Subtilisin Carlsberg-catalyzed transesterifications in solvents with $\mathrm{Log}\,P > 2.2$, but in this model, concentrations of substrates are not implied, and it is not capable, for instance, of taking into account changes in inhibition vs. the used solvent. A more general approach consists in the use of thermodynamic activities instead of concentrations in kinetic equation. Although this concept was studied as long ago as 1974 by Bell et al., there are few studies using thermodynamic activities in enzyme kinetic equations. These publications discuss the effect of solvent on substrate thermodynamics (Ryu and Dordick, 1992) and the importance of correct kinetic constants for substrate solvation (Reimann et al., 1994; Straathof et al., 1992; van Tol et al., 1992). A more refined theory was developed by van Tol et al., 1995c) according to the transition state theory. The transition state complex is assumed to be in equilibrium with the ground state of the substrate and enzyme, the corresponding thermodynamic equilibrium constant expressed in activities is not affected by the solvent and also the resulting specificity constant (equal to $V_m/([\mathrm{Enzyme}]\cdot K_m)$ in the Michaelis equation) based on activity is independent of the solvent when the following hypotheses are valid: (1) The solvent has not a direct interaction with the active site of the enzyme; (2) The energy of desolvation of the active site must be independent of the nature of the solvent; (3) The solvent does not affect the catalytic mechanism of the enzyme; (4) The extent of solvation of the enzyme species (enzyme, enzyme-substrate complex and transition state complex) should be equal and likewise for their activity coefficients. It implies that the substrate must be completely shielded from the solvent in its complexes with the enzyme.

Kinetic constants based on activity are called "intrinsic parameters," because the solvent does not affect them. When the above premises are valid, values of intrinsic parameters should be equal in all solvents. However, differences between their values in different solvents have been observed (García-Alles and Gotor, 1998; Janssen et al., 1996, 1999; van Tol et al., 1995a,b,c). Different explanations for these remaining differences have been proposed (van Tol et al., 1995c): the solvent may affect the mobility of amino acid residues and therefore the activity of the enzyme or it might compete with substrate for binding to active site. A subsequent approach was made considering a competitive inhibition by the solvent, and an extra parameter was added to the kinetic equation based on activity (van Tol et al., 1995b). The results of this approach gave a best fit of the experimental data in the cases of diisopropylether and 2-butanone, but in the cases of hexane and tetrachloromethane results were not satisfactory. This raises the possibility of other solvent effects on the enzyme. In addition, it is essential to consider that to incorporate an extra parameter involves a greater number of experimental data and a more complex parameter estimation method.

All previous studies using the thermodynamic activities of the substrates in the kinetic equation were performed calculating the intrinsic parameters in each solvent and comparing them. Such an approach, using thermodynamic activity based kinetics, termed here the TABEK approach, has appeared to us to be very promising and the major aim of this work is to contribute to its development. For the fist time we explored a slightly different approach, conserving the activity-based parameters determined in one reference solvent and using them to predict the kinetics in different solvents.

The hydration of the immobilized catalyst has been described as one of the crucial parameters to control since it defines the conformation of the protein. As we have used a commercial immobilized enzyme Lipozyme, all tested solvents were hydrated at the same water activity in order to have the same enzyme conformation and to compare their effects suitably (Adlercreutz, 1991; Marty et al., 1992). The quantity of water added to solvents was calculated in order to obtain an optimal 0.55 water activity adsorbed on the catalyst (see Material and Methods section).

Thus, in the present study we propose to use the TABEK approach, which is based on intrinsic parameters determined only in a suitable arbitrarily chosen solvent. Our objective is to predict initial rates in a number of organic solvents without previous determination of all kinetic parameters. This method would reduce considerably the number of experiments required and facilitate the choice of the optimal solvent and of the most favorable reaction conditions.

Materials and Methods

Chemicals and solvents

Immobilized lipase (EC.3.1.1.3), Lipozyme, 200 U/g from *R. miehei*, supported on macroporous anionic resin beads, was kindly provided by Novo Industry, Denmark. Oleic acid (70%), absolute ethanol, hexane (95%), heptane (99%), cyclohexane (99%), and isooctane (99.5%), were purchased from Prolabo, France. 5-methyl-2-hexanone (98%) was purchased from Fluka, Switzerland.

Initial rate determination

Ethyl oleate synthesis was carried out in a glass tube with 10-mL solution containing oleic acid (200 mM) and ethanol (50–1,000 mM) and 30 mg of Lipozyme in the cases of reactions with solvent. In the case of free solvent reactions the total volume was 5 mL and the quantity of enzyme was 100 mg. All reactions were performed at 40°C and magnetically stirred at 500 rpm to overcome external diffusional limitations. Samples were taken from the tube at various times and analyzed by HPLC, equipped with a Varian R1 refractometer. Standard curves had been previously produced enabling the concentrations of the residual acid and of the ester product to be followed with time. Reactions never exceeded 15% of total conversion in order to respect the hypothesis of initial kinetics.

Determination of the activity coefficients of the substrates

Activity coefficients were determined using the UNIFAC group contribution method based on vapor-liquid equilibria (Freudenslund et al., 1977). The activity coefficients were based on a mol fraction scale with reference to an ideal solution as defined by Raoult's law, since in this way the equilibrium constant is independent of the solvent, which is obviously needed in our approach. The PhoPhy v. 2.1 software from ProSim S.A. (Toulouse, France) was used with a FORTRAN program for UNIFAC calculations.

Determination of intrinsic parameters in n-hexane

Parameter estimation was done by the general reduced gradient method (GRG2) (Lasdon and Smith, 1992), using Microsoft Excel solver. A set of 90 experimental data in *n*-hexane (with concentration of oleic acid varying from 5 to 200 mM and ethanol concentration varying from 8 to 750 mM) was used. The variation of the activity-based kinetic parameters was constrained between zero and one for the reason that the activity coefficients were based on a mol fraction scale.

Medium polarity

The logarithm of the partition coefficient octanol-water (LogP) was adopted as the polarity scale for this work. All LogP used are experimental values taken from Hansch (1995). In the case of cosolvent mixtures, the LogP value was calculated from LogP values of pure solvents as follows (Laane et al., 1987)

$$\mathrm{LogP}_{\mathrm{mix}} = x_1 \mathrm{LogP}_1 + x_2 \mathrm{LogP}_2$$

where x_1 and x_2 are the mol fractions of solvents 1 and 2, respectively.

Solvent hydration

In order to adjust the water activity in the initial reaction system to the optimal value of 0.55 for the Lipozyme, the quantity of water added to the reaction media (Table 1) was calculated by the method described in Condoret et al. (1997), and we used the UNIFAC model to estimate the activity coefficients. Because, in the case of hydrophobic solvents (hexane, 90% hexane + 10% 5-methyl-2-hexanone, cyclohexane, heptane, isooctane), the necessary amount of water predicted by UNIFAC exceeded the maximal solubility, which is a well-known limitation of UNIFAC, we made an adjustment based on the experimental water adsorption isotherm for the hexane. Given that the water solubility in hexane, cyclohexane, heptane and isooctane is similar, the quantity of water added to these media was the same.

Results and Discussion

The reaction model chosen was the esterification of oleic acid with ethanol catalyzed by Lipozyme. It follows a Ping-Pong mechanism in *n*-hexane with competitive inhibition by the alcohol (Chulalaksananukul et al., 1990). The equation for initial kinetics is

$$V_i = \frac{V_m[\mathrm{Oleic}][\mathrm{Eth}]}{Km_{\mathrm{Oleic}}[\mathrm{Eth}]\left(1+\frac{[\mathrm{Eth}]}{K_i}\right) + Km_{\mathrm{Eth}}[\mathrm{Oleic}] + [\mathrm{Oleic}][\mathrm{Eth}]} \quad (1)$$

where V_i is the initial reaction rate, V_m is the maximum initial velocity, and [Oleic] and [Eth] are the initial concentration of oleic acid and ethanol, respectively; Km_{Oleic} and Km_{Eth} are the affinity constants for oleic acid and ethanol, respectively, and K_i is the inhibition constant of the ethanol.

According to our initial considerations, the activity-based kinetic parameters should have a constant value in all media. Consequently, it is viable to predict initial rates in different solvents from one set of kinetic constants determined in any solvent by fitting of the parameters contained in the equation based on activity

$$V_i = \frac{V_m a_{\mathrm{Oleic}} a_{\mathrm{Eth}}}{Km_{\mathrm{Oleic}} a_{\mathrm{Eth}}\left(1+\frac{a_{\mathrm{Eth}}}{K_i}\right) + Km_{\mathrm{Eth}} a_{\mathrm{Oleic}} + a_{\mathrm{Oleic}} a_{\mathrm{Eth}}} \quad (2)$$

where V_i is the initial reaction rate, a_{Oleic} and a_{Eth} are the initial thermodynamic activities of oleic acid and ethanol, respectively. Kinetic parameters have the same significance as in Eq. 1, but they are based on activity in Eq. 2.

In Table 2 are presented the activity-based kinetic parameters for our model determined in *n*-hexane, which will serve to predict initial rates in other solvents. In the numerical identification procedure the variation in the activity-based kinetic parameters was constrained between 0 and 1 for the reason that the activity coefficients were based on a mol fraction scale. Considering the values of V_m, in concentration and in activity, it appears very positive that numerical identification of these two distinct approaches has led to close values. The ethanol affinity constant reaches 1, the maximum value possible. This is not surprising since the value of this concentration-based parameter (600 mM) is considerable, proving the low affinity of the enzyme for the ethanol.

Figure 1 shows that for solvents with similar polarity to *n*-hexane the prediction of kinetics appears to be satisfactory

Table 1. Water Added to Initial Reaction Media

Solvent	Water Added (g/L)						
	Ethanol Concentration (mM)						
	50	100	200	300	500	750	1,000
5-Methyl-2-hexanone (5M2H)	7.0	7.0	7.5	8.0	8.5	9.5	10.5
25% Hexane + 75% 5M2H	5.4	5.5	5.9	6.0	6.8	7.5	8.5
50% Hexane + 50% 5M2H	3.9	4.0	4.2	4.4	5.0	5.5	6.4
75% Hexane + 25% 5M2H	2.2	2.4	2.6	2.7	3.1	3.6	4.3
90% Hexane + 10% 5M2H	0.50	0.50	0.60	0.70	0.80	0.90	1.1
Hexane, Heptane, Cyclohexane, Isooctane*	0.40	0.40	0.43	0.47	0.57	0.73	0.83

*Since the water solubility of hydrophobic solvents is similar, the quantity of water added is the same.

Table 2. Concentration and Activity-Based Kinetic Parameters for Lipozyme-Catalyzed Esterification of Oleic Acid with Ethanol in *n*-Hexane Hydrated at $a_w = 0.55$

Kinetic Parameter	Value in Conc. (Eq. 1)	Value in Activity (Eq. 2)
V_m	45.6 mmol/(min·g)	49.8 mmol/(min·g)
Km_{Oleic}	450 mM	0.51 mmol/mmol
Km_{Eth}	600 mM	1 mmol/mmol
K_i	60 mM	0.52 mmol/mmol

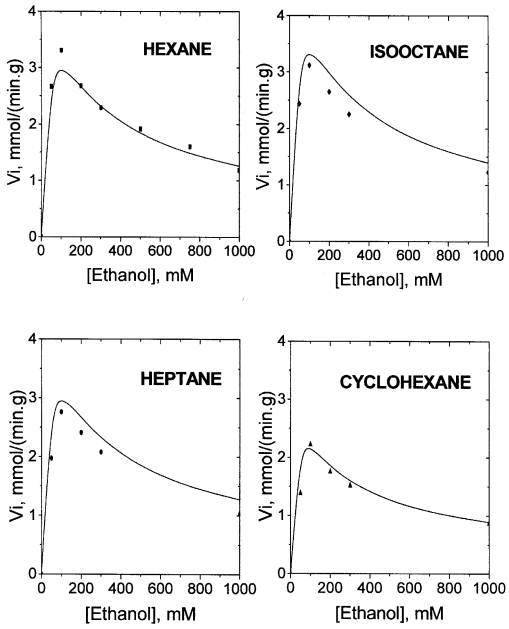

Figure 1. Initial rate as a function of ethanol concentration in hydrophobic solvents.

[Oleic acid] = 200 mM, $a_w = 0.55$. Symbols, experimental data; curves, calculated using Eq. 2 and activity-based parameters of Table 2.

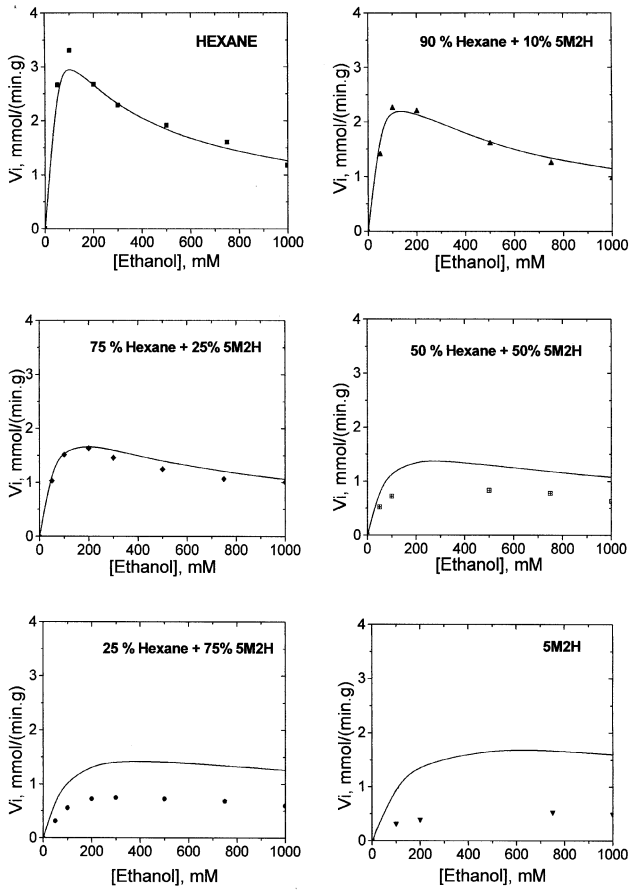

Figure 2. Initial rate as a function of ethanol concentration in different mixtures of *n*-hexane and 5-methyl-2-hexanone (5M2H) hydrated at $a_w = 0.55$.

Percentages are based on volume. [Oleic acid] = 200 mM. Symbols, experimental data; curves, calculated with Eq. 2 and activity-based parameters of Table 2.

and correctly accounts for the alcohol inhibition effects. For these solvents, the optimal concentration in ethanol (at which the initial rate is maximal) is approximately 100 mM at [Oleic acid] = 200 mM, as predicted by the model. Therefore, the TABEK approach, in spite of small deviations from the experimental value of the initial rate, can be used to estimate the most favorable reaction conditions. The maximal activity was observed in isooctane as well as in hexane. As isooctane is less toxic than hexane (Johnson and Whim, 1996), it could be considered as an alternative for industrial applications.

Kinetics obtained in less hydrophobic media are presented in Figure 2. Different mixtures of *n*-hexane (Log $P = 3.90$) and 5-methyl-2-hexanone (5M2H, Log $P = 1.88$) were tested to observe if model deviation increases with the solvent polarity.

Good correlation is obtained up to the mixture with 75% hexane, but after that, deviation increased progressively with the addition of 5M2H. Nevertheless, it is evident that this deviation concerns the absolute value of initial rate whereas the shape of experimental and predicted curves is similar. In particular, the model enables the decrease in ethanol inhibition to be predicted. Indeed, with a polar solvent, the ethanol activity coefficient decreases, leading to low inhibition. It is possible that inaccuracies in UNIFAC predictions had contributed to these deviations. Even so, the tendencies of deviation from ideality predicted by UNIFAC must be right. Indeed, from the values of Table 3, at a given ethanol concentration, we can observe that oleic acid exhibits similar activity coefficient in all hydrophobic solvents, and this is also true for ethanol. In Table 3, when medium polarity increases (due to the increase in 5M2B fraction), ethanol activity coefficient decreases, as expected. When we plotted ethanol activity vs. predicted initial rate for the mixtures hexane-5M2H, the same ethanol optimal activity (ethanol activity giving the optimal enzyme activity) was observed for all percentages (data not shown), contrasting with the large variation in ethanol optimal concentration observed in Figure 2. This leads to the assumption that the estimation of the activity coefficients is correct.

In the case of solvent effects nonaccounted for our model (called residual solvent effects), the validity of the hypotheses of the model has to be analyzed. The X-ray structures of subtilisin Carlsberg (Fitzpatrick et al., 1993, 1994; Schmitke et al., 1997, 1998b) and chymotripsin (Yennawar et al., 1994) were studied in various organic solvents, and it was shown that the overall structure is basically the same as in water, but in some cases solvent molecules were found in the active site. Consequently, hypothesis 1 that assumes no direct interaction of the solvent with the active site might be questioned.

If the substrate is partially exposed to the solvent when complexed with the enzyme, the interactions with the solvent are not the same in all forms of the enzyme, and, therefore, hypothesis 4 might also be open to criticism. If one solvent

Table 3. Activity Coefficients for Oleic Acid (γOL) and Ethanol (γETH) in Solvents and Their Mixtures Hydrated at $a_w = 0.55$, [Oleic acid] = 200 mM

	Solvent							
	Hexane		Heptane		Cyclohexane		Isooctane	
[Eth] mM	γOL	γETH	γOL	γETH	γOL	γETH	γOL	γETH
50	2.68	18.83	2.61	17.36	2.21	22.33	2.47	15.23
100	2.47	16.70	2.41	15.42	2.03	19.59	2.28	13.48
200	2.16	13.59	2.11	12.59	1.77	15.69	1.99	10.96
300	1.93	11.44	1.89	10.62	1.59	13.06	1.79	9.26
500	1.63	8.70	1.61	8.11	1.35	9.78	1.54	7.11
750	1.41	6.71	1.39	6.28	1.17	7.47	1.35	5.59
1,000	1.26	5.49	1.26	5.16	1.06	6.07	1.23	4.66

	Solvent Mixtures Hexane-5M2H									
	0% Hexane		25% Heptane		50% Hexane		75% Hexane		90% Hexane	
[Eth] mM	γOL	γETH	γOL	γETH	γOL	γETH	γOL	γETH	γOL	γETH
50	1.09	2.35	0.89	2.92	0.88	4.03	1.18	6.76	1.77	11.19
100	1.09	2.33	0.89	2.88	0.87	3.94	1.15	6.48	1.69	10.42
200	1.09	2.28	0.88	2.79	0.86	3.76	1.09	5.98	1.55	9.15
300	1.09	2.24	0.88	2.72	0.84	3.60	1.05	5.55	1.44	8.15
500	1.08	2.16	0.87	2.57	0.82	3.32	0.98	4.86	1.28	6.69
750	1.08	2.06	0.86	2.42	0.80	3.04	0.91	4.21	1.14	5.47
1,000	1.09	1.98	0.86	2.29	0.78	2.80	0.87	3.72	1.05	4.65

better solvates the substrate partially exposed to the solvent in the enzyme-substrate complex, it causes a slightly lower relative energy of the enzyme-substrate complexes and the intrinsic parameter V_m becomes larger or K_m becomes smaller (Reimann et al., 1994).

Another point to be possibly considered is also the possible interaction of solvents with the enzyme solid-support itself which might cause reactant partition. However, nonimmobilized enzymes being insoluble in organic solvents, catalysis has to be realized anyway in heterogeneous medium.

Since it is not possible to formally take into account the above described solvent effects in the TABEK model and based on the fact that this model appears to fail to predict the value of the initial velocity rather than the shape of the curve, we propose to replace the intrinsic maximum initial velocity parameter V_m in Eq. 2 by an analogous solvent-adapted parameter V_m^*, which includes this residual solvent effects. V_m^* can be easily determined with one experimental datum using Eq. 2 and the rest of the intrinsic parameters determined in *n*-hexane. Thus, for each solvent, an experimental measurement of the initial velocity at [Ethanol] = 100 mM was realized to identify the maximum initial velocity corrected by solvent effects (V_m^*). These maximum initial velocities (Table 4) will be used with the rest of the activity-based parameters of Table 2 to predict kinetics in all media.

Results with this "solvent-adapted" model are given in Figure 3. A good agreement between experimental data and predicted curves was obtained. This signifies now that it is possible to reduce drastically the number of experiments to determine kinetic parameters in various solvents. As a result, the choice of the optimal solvent and the most favorable reaction conditions can be made easily.

With the aim of obtaining a completely predictive model, we tried to find a correlation for our variable parameter V_m^* with a number of solvent properties. As in previous works (Laane et al., 1987; Valivety et al., 1991), the best results were obtained with the popular Log$P$ value of the solvent. All Log$P$ values used are experimental values taken from Hansh (1995). No simple correlation of V_m^* as a function of Log$P$ can be deduced (Figure 4). Therefore, when we segregated the experimental points for the mixtures *n*-hexane + 5M2H, a clear linear correlation was observed. This agrees with the results of Yang et al. (1997), where a good correlation between Log$P$ or dielectric constant and the reaction rate was found for a given cosolvent mixture, but there was no clear relationship when different cosolvent mixtures were compared.

Solvent polarity is indeed a determining factor in biocatalyst activity, but it is not the only one and it is difficult to correlate a solvent polarity parameter directly with a kinetic parameter. These more sophisticated methods for predicting V_m^* as a function of solvent properties do not give better results and, for this reason, it is preferable to adapt the TABEK model by including residual solvent effects in a variable parameter V_m^* determined simply with one experimental datum. The model with the experimentally determined V_m^* is

Table 4. Solvent-Adapted Maximum Initial Velocity Parameter Values (V_m^*) for Lipozyme-Catalyzed Esterification of Oleic Acid with Ethanol in Different Solvents Hydrated at $a_w = 0.55$

Solvent	V_m^* mmol/(m·g)
5M2H	15.2
25% Hex + 75% 5M2H	26.1
50% Hex + 50% 5M2H	30.6
75% Hex + 25% 5M2H	47.3
90% Hex + 10% 5M2H	52.3
n-Hexane	49.8
n-Heptane	42.6
Cyclohexane	45.5
Isooctane	42.8

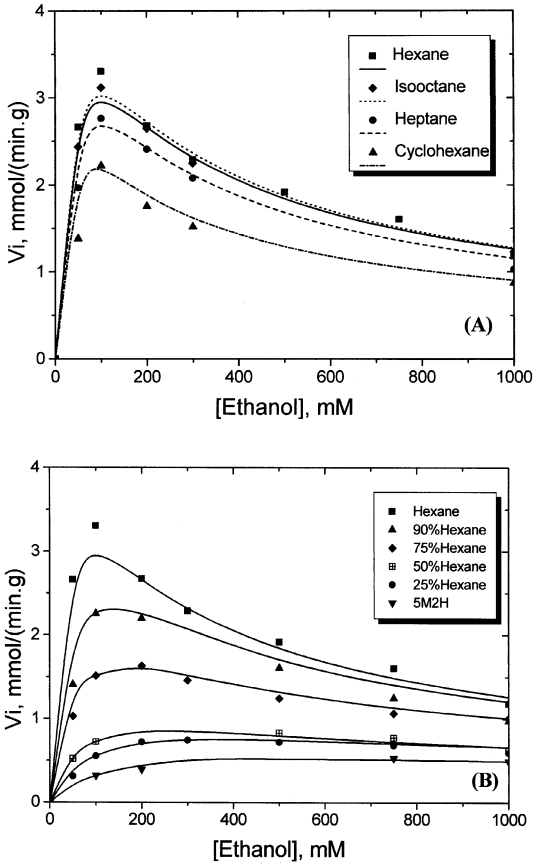

Figure 3. Kinetics prediction with corrected V_m^* in: (A) hydrophobic solvents; (B) different mixtures of *n*-hexane and 5-methyl-2-hexanone (5M2H).

[Oleic acid] = 200 mM, $a_w = 0.55$. Percentages are based on volume. Symbols, experimental data; solid lines, calculated with Eq. 2 and activity-based parameters of Table 2, except V_m which was replaced by V_m^* of Table 4.

simple and effective, so we consider that looking for a correlation to predict V_m^* theoretically is only justified in the case of mixtures of solvents, where a good correlation is possible.

Solvent-free media

In the case of a solvent-free system, we may consider each medium with different molar ratio of substrates as a different "solvent" (such as, LogP varies from 3.73 to 1.35 for molar ratios [Ethanol]/[Oleic acid] of 1 and 4, respectively). This would seem to imply the need to determine experimentally the V_m^* for every molar ratio and not for a single ratio. However, if we consider the solvent-free system as a "cosolvent" mixture, a similar linear correlation between LogP and V_m^* must be obtained. Given that the molar fraction of substances in our solvent-free system is linearly proportional to the LogP value of the medium, a linear correlation should be observed if we plot V_m^* as function of the oleic acid molar fraction (Figure 5). To find V_m^* as a function of the oleic acid molar fraction, we have taken the equation of the straight line connecting two points at [Ethanol]/[Oleic acid] = 1 ($X_{\text{oleic acid}} = 0.5$) and 4 ($X_{\text{oleic acid}} = 2$).

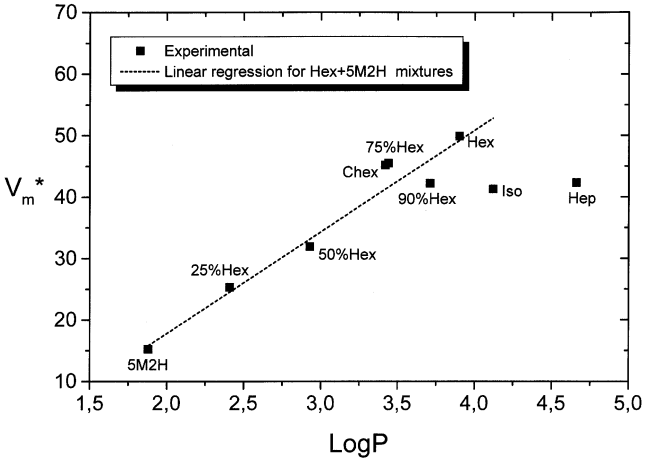

Figure 4. Corrected V_m^* as a function of solvent LogP values.

[Oleic acid] = 200 mM, $a_w = 0.55$. All LogP values for pure solvents are experimental values from Hansch (1995). For cosolvent mixtures LogP was calculated from LogP of pure solvents as proposed by Laane et al. (1987). Solvents are: 5M2H, 5-methyl-2-hexanone; Hex, hexane; *x* %Hex, hexane + 5-methyl-2-hexanone mixture where *x* is the hexane percentage based on volume; Chex, cyclohexane; Iso, isooctane; Hep, heptane.

We have perferred a correlation with the substrate mol fraction to a correlation as a function of LogP because in this way we save from searching LogP values, which vary in the different bibliographic references and are often unavailable or doubtfully estimated, as is the case of LogP for the oleic acid.

The V_m^* values calculated with the correlation from Figure 5 were used with the rest of the activity-based parameters to predict the kinetics of the solvent-free system. These results are presented in Figure 6 and compared with the results obtained from a kinetic model based on concentration.

The V_m value used in the concentration-based calculations for the solvent-free media corresponds to the value deter-

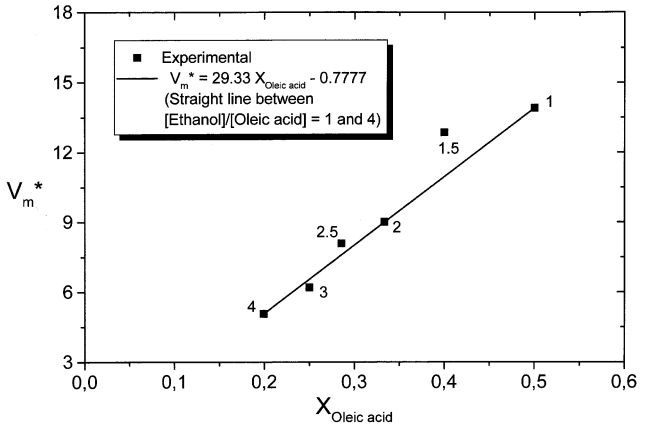

Figure 5. Corrected V_m^* in function of oleic acid molar fraction for different solvent-free media.

Numbers near points represent the molar ratio [Ethanol]/[Oleic acid].

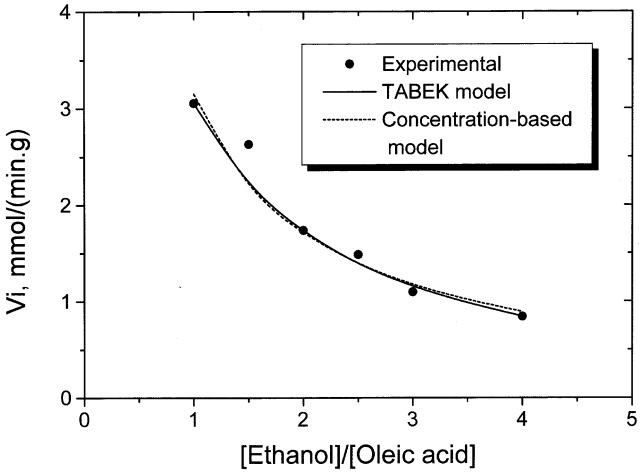

Figure 6. Kinetics prediction for different solvent-free media as a function of substrates molar ratio.

The curves were calculated using Eqs. 1 (concentration) and 2 (activity), and parameters of Table 2, except V_m which was replaced by $V_m = 28$ mmol/(min·g) for the concentration-based model and V_m^* calculated for each molar ratio with the correlation of Figure 5 for the activity-based model.

mined in *n*-hexane not hydrated [28 mmol/(min·g)], due to the fact that solvent-free experiments were performed without previous addition of water. This value of V_m was taken as a constant in the concentration-based model for all [Ethanol]/[Oleic acid] molar ratios, while in the activity-based model a different V_m^* was calculated from the correlation of the Figure 5 for each [Ethanol]/[Oleic acid] molar ratio.

Amazingly, concentration and activity-based models give practically the same results. However, according to the concentration-based model, a decrease in the initial rate is due to inhibition, while in the TABEK model it is the decrease in the V_m^* value which is responsible for this effect.

We can observe the evolution of activity coefficients before and at the end of reaction in solvent-free media in Table 5.

Table 5. Activity Coefficients for Oleic Acid (γOL), Ethanol (γETH), Ethyl Oleate (γESTER), and Water (γW) in Solvent-Free Media before Reaction and at Equilibrium*

Molar Ratio (Ethanol/ Oleic Acid)	[Oleic Acid], M	[Ethanol], M	$X_{\text{oleic acid}}$	X_{ethanol}	γOL	γETH
1.0	2.66	2.66	0.50	0.50	1.12	1.41
1.5	2.47	3.71	0.40	0.60	1.22	1.32
2.0	2.31	4.62	0.33	0.67	1.33	1.26
2.5	2.19	5.48	0.29	0.71	1.44	1.21
3.0	2.03	6.09	0.25	0.75	1.55	1.18
4.0	1.81	7.27	0.20	0.80	1.78	1.13

Molar Ratio (Ethanol/Oleic Acid)	Conv. at Equilib.	γOL	γETH	γESTER	γW
1.0	86	0.98	3.07	1.05	24.6
1.5	87	0.92	2.17	1.31	16.8
2.0	89	0.94	1.65	1.66	12.0
2.5	90	1.03	1.40	2.12	9.41
3.0	92	1.16	1.31	2.71	8.16
4.0	96	1.51	1.17	4.39	6.29

* Water partition between reaction media and enzymatic support was considered in activity coefficient calculations.

Logically, an increase in the polarity of the medium has led to an increase in the activity coefficients of nonpolar substrates (oleic acid and ester), and on the contrary, to a decrease in the activity coefficients of polar solutes (ethanol and water).

There is no reason that kinetic parameters determined in concentration in *n*-hexane correlate experiments in completely different media without solvent, where there is no buffering effect in respect to the ratio of substrates, the water partition and the physical properties of the medium. Given that the TABEK approach predicts correctly ethanol inhibition in the cases of hexane + 5M2H mixtures, and that the concentration-based model cannot, we can assume that in the case of solvent-free media, the concentration-based model accuracy is merely coincidental.

Conclusion

In this study, a recent approach for enzymatic kinetics, pioneered by van Tol et al. (1995c), was considered and designated here as the thermodynamic activity-based kinetics (TABEK) approach. It has been clearly shown to provide useful predictions for kinetics in different solvents and in solvent-free media. The idea of using the thermodynamic activity of components instead of their concentrations in the kinetic equations is valuable if hypotheses defined by van Tol et al. (1995c) are valid, as mentioned in the introduction. It appeared here that hypotheses 1 and 4 that suppose no direct interaction of the solvent with the enzyme or enzyme-substrate complexes have to be questioned, and the possibility of solvent interaction with the enzymatic support has to be considered. For the reason that the solvent effects on the structure of the enzyme and on the enzymatic support cannot mathematically be taken into account in the TABEK model, we had to implement a "solvent-adapted" parameter for the maximum initial velocity (V_m) to account for observed deviations.

In this work the TABEK approach has also been successfully tested in the case of solvent-free reaction media (with the same restriction concerning the V_m parameter), but in this case a linear variation of this adapted parameter, as a function of the composition, has been derived. In such media this approach may be of great help because, when reactions are performed at various molar ratios of substrates, an inducement of great variations in the concentration of components and no buffering by a solvent are expected. This solvent buffering effect appears to act mainly through the constant nature of the activity coefficients that are included in the parameters of concentration-dependent kinetic equations and are responsible for their variation with the nature of the solvent.

In all cases studied here (pure solvent, cosolvent mixture, solvent-free media), the number of experiments required to predict and describe the kinetic evolution of the system, is considerably reduced, compared to a conventional concentration-based approach.

Indeed, once intrinsic parameters are determined in one reference medium, only one extra experimental point is needed to adapt the model. We have used here *n*-hexane as the reference solvent, but this choice was arbitrary and was made because many studies, as well as our own laboratory

know-how, related to this solvent. Hindsight would indicate that a less hydrophobic medium would have brought better accuracy in the determination of these intrinsic parameters.

To investigate in more detail the direct interactions of the solvent with the enzyme and substrates and to give an understanding of the solvent-adapted parameter V_m^*, the use of molecular modeling methods would be very useful. This will be the direction of new research axis in our laboratory.

Finally, the TABEK approach has been tested here on a specific reaction, the lipase-catalyzed esterification. We are confident that, because of its thermodynamic background, it could be successfully tested against other reactions catalyzed by lipases (transesterification, interesterification, amidation) or by other enzymes (peptide synthesis by proteases, synthesis of oligosaccharides by invertase and glucosidase) in order to generalize its validity.

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Literature Cited

Adlercreutz, P., "On the Importance of the Support Material for Enzymatic Synthesis in Organic Media: Support Effects at Controlled Water Activity," *Eur. J. Biochem.*, **199**, 609 (1991).

Affleck, R., C. A. Haynes, and D. Clarck, "Solvent Effects on Protein Dynamics," *Proc. Nat. Acad. Sci. USA*, **89**, 5167 (1992).

Bell, R. P., J. E. Critchlow, and M. I. Page, "Ground State and Transition State Effects in the Acylation of Alpha-Chymotrypsin in Organic Solvent-Water Mixtures," *J. Chem. Soc. Pekin II 1974,* 66 (1974).

Bielecki, S., and L. R. Somiari, "Oligosaccharide Synthesis by Invertase in Organic Media Containing SDS," *Biotechnol. Lett.*, **20**, 287 (1998).

Björkling, F., S. E. Godtfredson, and O. Kirk, "The Future Impact of Industrial Lipases," *Trends Biotechnol.*, **9**, 360 (1991).

Chulalaksananukul, W., J. S. Condoret, P. Delorme, and R. M. Willemot, "Kinetic Study of Esterification by Immobilized Lipase in *n*-Hexane," *FEBS Lett.*, **276**, 181 (1990).

Colombié, S., J. T. Russel, J. S. Condoret, and A. Marty, "Water Activity Control: a Way to Improve the Efficiency of Continuous Lipase Esterification," *Biotechnol. Bioeng.*, **60**, 362 (1998).

Condoret, J. S., S. Vankan, X. Joulia, and A. Marty, "Prediction of Water Adsorption Curves for Heterogeneous Biocatalysis in Organic and Supercritical Solvents," *Chem. Eng. Sci.*, **52**, 213 (1997).

Fedorak, P. M., K. M. Semple, and R. Vazquez-Duhalt, "Chloroperoxidase-Mediated Modifications of Petroporphyrins and Asphaltenes," *Enzyme Microb. Technol.*, **15**, 429 (1993).

Fitzpatrick, P. A., D. Ringe, and A. M. Klibanov, "X-Ray Structure of Cross-Linked Subtilisin Carlsberg in Water vs. Acetonitrile," *Biochem. Biophys. Res. Commun.*, **198**, 75 (1994).

Fitzpatrick, P. A., A. C. U. Steinmetz, D. Ringe, and A. M. Klibanov, "Enzyme Crystal Structure in Neat Organic Solvent," *Proc. Natl. Acad. Sci. USA*, **90**, 8653 (1993).

Fredenslund, A., J. Gmehling, and P. Rasmussen, *Vapor-liquid Equilibria using UNIFAC*, Elsevier, New York (1977).

García-Alles, L. F., and V. Gotor, "Alcohol Inhibition and Specificity Studies of Lipase B from Candida Antarctica in Organic-Solvents," *Biotechnol. Bioeng.*, **59**, 163 (1998).

Halling, P. J., "Solvent Selection for Biocatalysis in Mainly Organic Systems: Predictions of Effects on Equilibrium Position," *Biotechnol. Bioeng.*, **35**, 691 (1990).

Halling, P. J., "Thermodynamic Predictions for Biocatalysis in Nonconventional Media: Theory, Tests, and Recommendations for Experimental Design and Analysis," *Enzyme Microb. Technol.*, **16**, 178 (1994).

Hansch, C., *Exploring QSAR,* Amer. Chem. Soc., Washington, DC (1995).

Janssen, A. E. M., B. J. Sjursnes, A. V. Varkurov, and P. J. Halling, "Kinetics of Lipase-Catalyzed Esterification in Organic Media: Correct Model and Solvent Effects on Parameters," *Enzyme Microb. Technol.*, **24**, 463 (1999).

Janssen, A. E. M., A. M. Vaidya, and P. J. Halling, "Substrate Specificity and Kinetics of Cetida Rugosa Lipase in Organic Media," *Enzyme Microb. Technol.*, **18**, 340 (1996).

Janssen, A. E. M., A. van der Padt, H. M. van Sonsbeek, and K. van't Riet, "The Effect of Organic Solvents on the Equilibrium Position of Enzymatic Acylglycerol Synthesis," *Biotechnol. Bioeng.*, **41**, 95 (1993).

Johnson, P. G., and B. B. Whim, *Directory of Solvents*, Blackie Academic & Professional, Chapman & Hall, London (1996).

Kise, H., and A. Hayakawa, "Immobilization of Proteases to Porous Chitosan Beads and their Catalysis for Ester and Peptide Synthesis in Organic Solvents," *Enzyme Microb. Technol.*, **13**, 584 (1991).

Laane, C., S. Boeren, K. Vos, and C. Veeger, "Rules for Optimization of Biocatalysis in Organic Solvents," *Biotechnol. Bioeng.*, **30**, 81 (1987).

Laroute, V., and R. M. Willemot, "Effect of Organic Solvents on Stability of Two Glycosidases and on Glucoamylase-Catalyzed Oligosaccharide Synthesis," *Enzyme Microb. Technol.*, **14**, 528 (1992).

Lasdon, L. S., and S. Smith, "Solving Sparse Nonlinear Programs Using GRG," *J. Computing*, **4**, 2 (1992).

Macrae, A. R., and R. C. Hammond, "Present and Future Applications in Lipases," *Biotechnol. Genet. Eng. Rev.*, **3**, 193 (1985).

Marty, A., W. Chulalaksananukul, R. M. Willemot, and J. S. Condoret, "Kinetics of Lipase-Catalyzed Esterification in Supercritical CO_2," *Biotechnol. Bioeng.*, **39**, 273 (1992).

Nakamura, K., M. Kinoshita, and A. Ohno, "Structure Solvent Affects Enantioselectivity of Lipase-Catalyzed Transesterification," *Tetrahedron*, **51**, 8799 (1995).

Nurok, D., R. M. Keyle, B. B. Muhoberac, M. C. Frost, P. Hajdu, D. H. Robertson, S. V. Kamat, and A. J. Russell, "Study of Enzyme-Catalyzed Reactions in Organic Solvents Using Multiple Linear Regression," *J. Mol. Catal. B Enzym.*, **7**, 273 (1999).

Parida, S., and J. S. Dordick, "Tailoring Lipase Specificity by Solvent and Substrate Chemistries," *J. Org. Chem.*, **58**, 3238 (1993).

Reimann, A., D. A. Robb, and P. J. Halling, "Solvation of CBZ-Amino Acid Nitrophenyl Esters in Organic Media and the Kinetics of their Transesterification by Subtilisin," *Biotechnol. Bioeng.*, **43**, 1081 (1994).

Reslow, M., P. Adlercreutz, and B. Mattiasson, "Organic Solvents for Organic Synthesis: 1. Optimization Parameters for a Chymotrypsin Catalyzed Process," *Appl. Microbiol. Biotechnol.*, **26**, 1 (1987).

Ryu, K., and J. S. Dordick, "How Do Organic Solvents Affect Peroxidase Structure and Function?," *Biochemistry*, **31**, 2588 (1992).

Schmitke, J. L., L. J. Stern, and A. M. Klibanov, "The Crystal Structure of Subtilisin Carlsberg in Anhydrous Dioxane and its Comparison with those in Water and Acetonitrile," *Proc. Nat. Acad. Sci. USA*, **94**, 4250 (1997).

Schmitke, J. L., L. J. Stern, and A. M. Klibanov, "Comparison of X-Ray Crystal Structures of an Acyl-Enzyme Intermediate of Subtilisin Carlsberg Formed in Anhydrous Acetonitrile and in Water," *Proc. Nat. Acad. Sci. USA*, **95**, 12918 (1998a).

Schmitke, J. L., L. J. Stern, and A. M. Klibanov, "Organic Solvent Binding to Crystalline Subtilisin in Mostly Aqueous Media and in the Neat Solvents," *Biochem. Biophys. Res. Commun.*, **248**, 273 (1998b).

Straathof, A., J. L. L. Rakels, and J. J. Heijnen, "Enzyme Kinetics in Monophasic and Biphasic Aqueous-Organic Systems," *Biocatalysis in Non-Conventional Media*, J. Tramper, M. H. Vermue, H. H. Beeftink, and U. von Stockar, eds., Elsevier, Amsterdam, pp. 137–144 (1992).

Torres, E., B. Siminovich, E. Barzana, and R. Vazquez-Duhalt, "Thermodynamic Hydrophobicity of Aqueous Mixtures of Water-

Miscible Organic-Solvents Predicts Peroxidase-Activity," *J. Mol. Catal. B Enzym.*, **4**, 155 (1998).
Valivety, R. H., G. A. Johnston, C. J. Suckling, and P. J. Halling, "Solvent Effects on Biocatalysis in Organic Systems: Equilibrium Position and Rates of Lipase Catalyzed Esterification," *Biotechnol. Bioeng.*, **38**, 1137 (1991).
van Tol, J. B. A., J. B. Odhental, J. A. Jongejan, and J. A. Duine, "Relation of Enzymatic Reaction Rate and Hydrophobicity of the Solvent," *Biocatalysis in Non-Conventional Media*, J. Tramper, M. H. Vermue, H. H. Beeftink, and U. von Stockar, eds., Elsevier, Amsterdam, pp. 229–235 (1992).
van Tol, J. B. A., J. A. Jongejan, J. A. Duine, H. G. T. Kierkels, E. F. T. Gelad, F. Mosterd, W. J. J. van der Tweel, and J. Kamphuis, "Thermodynamic et Kinetic Parameters of Lipase-Catalyzed Ester Hydrolysis in Biphasic Systems with Varying Organic Solvents," *Biotechnol. Bioeng.*, **48**, 179 (1995a).
van Tol, J. B. A., D. E. Kraayveld, J. A. Jongejan, and J. A. Duine, "The Catalytic Performance of Pig Pancreas Lipase in Enantioselective Transesterification in Organic Solvents," *Biocatal. Biotrans.*, **12**, 119 (1995b).
van Tol, J. B. A., R. M. M. Stevens, W. J. Veldhuizen, J. A. Jongejan, and J. A. Duine, "Do Organic Solvents Affect the Catalytic Properties of Lipase? Intrinsic Kinetic Parameters of Lipases in Ester Hydrolysis and Formation in Various Organic Solvents," *Biotechnol. Bioeng.*, **47**, 71 (1995c).
Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak, "Lignin Peroxidase Oxidation of Aromatic Compounds in Systems Containing Organic Solvents," *Appl. Environ. Microbiol.*, **60**, 459 (1994).
Vermue, M. H., and J. Tramper, "Biocatalysis in Nonconventional Media: Medium Engineering Aspects (Technical Report)," *Pure Appl. Chem.*, **67**, 346 (1995).
Westcott, C. R., and A. M. Klibanov, "Solvent Variation Inverts Substrate Specificity of an Enzyme," *J. Amer. Chem. Soc.*, **115**, 1629 (1993).
Yang, F., T. W. Weber, J. Gainer, and G. Carta, "Synthesis of Lovastantin with Immobilized Candida Rugosa Lipase in Organic Solvents: Effects of Reaction Conditions on Initial Rates," *Biotechnol. Bioeng.*, **56**(6), 671 (1997).
Yennawar, N. H., H. P. Yennawar, and G. K. Farber, "X-Ray Crystal Structure of γ-Chymotrypsin in Hexane," *Biochemistry*, **33**, 7326 (1994).
Zaks, A., and A. M. Klibanov, "Enzymatic Catalysis in Organic Media at 100° C," *Science*, **224**, 1249 (1984).

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