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Kinetic study on lipase-catalyzed esterification in organic solvents

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A twin inhibition is observed for the esterification reaction between ethanol and isovaleric acid using immobilized lipase from *Rhizomucor miehei* in hexane and in mixed solvent system. The observed bi-substrate inhibition pattern follows a Ping-Pong Bi-Bi mechanism with dead-end inhibition of enzyme by both the substrates. An increase in K_m value for alcohol in mixed solvent (0.645 *M*) than in hexane (0.256 *M*), indicates that the enhanced solvation of ethanol in mixed solvent results in lower degree of inhibition.

Keywords: Eesterification, ethanol, isovaleric acid, immobilized lipase, *Rhizomucor miehei*

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A number of theoretical models have been proposed to explain the kinetic studies of esterification reactions in organic solvents[1-4]. Most of these studies deal with lipase catalyzing reactions of long chain fatty acids. It has been shown that, the lipase from *Rhizomucor miehei* and lipase B from *Candida antarctica* follows a Ping-Pong Bi Bi kinetics with competitive inhibition by the acyl acceptor or donor[5,6,19]. The choice of solvent has an important bearing while esterifying polar substrate due to variation in solubility[7]. Ethyl alcohol being a polar substrate, strips off the essential layer around the enzyme surface, thus leading to lower esterification yields[8]. In such cases, a mixed solvent system can be used to increase the solubility of the polar substrate[9], as the solubility of substrate is known to change with a change in the polarity of the reaction medium[3]. Most of the esterification and transesterification models reported to date are based on the application of simple Michaelis-Menten kinetics, wherein only short chain alcohol and long chain acid substrates are considered. While the alcohol or acid inhibition is thoroughly studied, the inhibition by both the substrates are rarely studied. Ethyl isovalerate (3-methylbutanoic acid ethyl ester) is a flavour compound and possess apple/fruity flavour and is used in food and pharmaceutical industry[10]. There are not many reports on lipase-catalyzed kinetics of ethyl isovalerate in organic solvents compared to other low molecular weight esters like acetates, propionates and butyrates.

The present investigation focused on the influence of the reaction media on kinetics of lipase-catalyzed synthesis of ethyl isovalerate using lipase from *Rhizomucor miehei* in *n*-hexane and in mixed solvent system [*n*-hexane-diethyl ether, 85:15 (%, v/v)].

The schematic representation of reaction is as shown in **Chart I**.

The mixed solvent system was used mainly to increase the solubility of ethanol. Of the various co-solvents tested, higher reaction rate was found when diethyl ether was used with *n*-hexane in the ratio 15: 85 (%, v/v). Thus, this ratio was chosen for esterification reaction[11].

Results and Discussion

Effect of ezyme concentration

The esterification of ethanol and isovaleric acid (0.2 *M*) as a function of enzyme concentration is shown in **Figure 1**. The results indicate a linear increase in reaction rates with an increase in enzyme concentration in *n*-hexane and in mixed solvent system[*n*-hexane-diethyl ether, 85:15 (%v/v). The reaction rate was 0.150 and 0.158 μ mol/min/mg enzyme at 2 g/L of enzyme, which increased to 0.543 and 0.578 μ mol/min/mg enzyme at 15 g/L enzyme in

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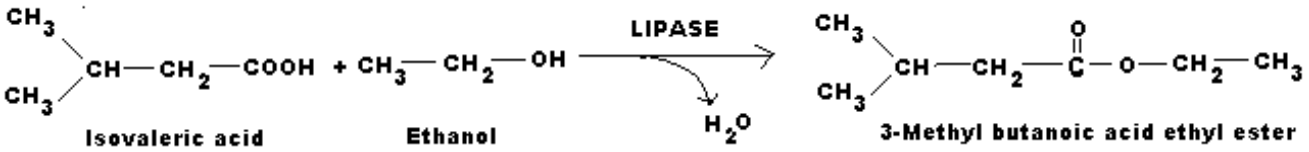


Chart I

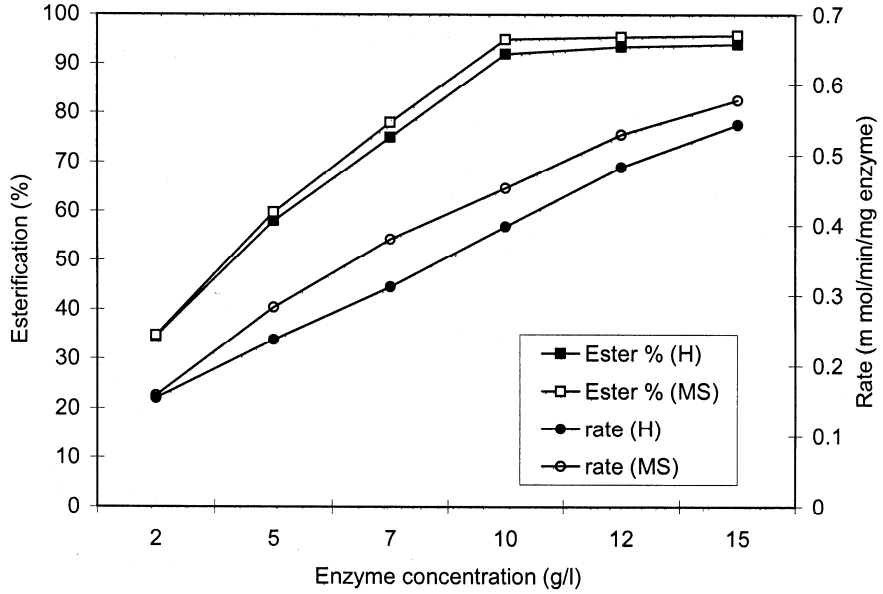


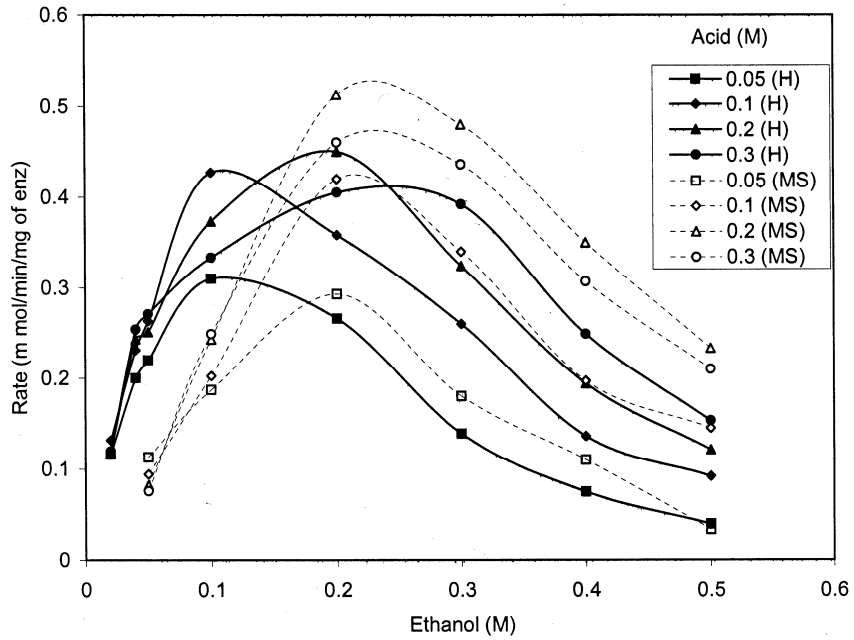
Figure 1 — Reaction rate of esterification as a function of enzyme concentration at 0.2 *M* substrate concentration (equimolar ratio) and 40°C in *n*-hexane (H) and in mixed solvent (MS).

n-hexane and mixed solvent system, respectively. The rate of reaction was higher in mixed solvent system than in *n*-hexane. The percent esterification linearly increased upto 10 g/L enzyme concentration and remained constant upto 15 g/L enzyme concentration in both the systems. Thus, an enzyme concentration of 10 g/L was chosen for the determination of kinetic parameters.

Determination of rate constants

The determination of the kinetic parameters was performed by initial rate analysis. **Figure 2a** shows the variation of the reaction rates as a function of ethanol concentration (from 0.02 to 0.5 *M*) for various concentrations of isovaleric acid (0.05, 0.1, 0.2 and 0.3 *M*) using 10 g/L enzyme concentration in *n*-hexane and mixed solvent. The results indicate that the reaction rates increased with an increase in acid concentration. At low acid concentration (0.05 and 0.1 *M*), the reaction rate increased upto 0.1 *M* ethanol concentration and a decrease in reaction rate with a further increase in ethanol concentration upto 0.5 *M* was observed in *n*-hexane. However, the reaction rate continued to increase upto 0.2 *M* ethanol concentration at an acid concentration of 0.2 and 0.3 *M*, which then gradually decreased with a further increase in ethanol in *n*-hexane. When the reactions were carried out in mixed solvent system, the reaction rate increased with an increase in ethanol concentration upto 0.2 *M*. A decrease in reaction rate was observed with a further increase in ethanol concentration (0.5 *M*) at all acid concentrations (0.05, 0.1, 0.2 and 0.3 *M*) tested. These results indicate that the lipase activity is affected at higher ethanol concentration, which could be due to polar nature of ethanol[12]. The shift in higher reaction rate profile observed from 0.1 *M* in *n*-hexane to 0.2 *M* in mixed solvent system at relatively higher substrate concentrations could be due to enhanced solvation of ethanol (increased solubility), which in turn could be due to a slight increase in media polarity. The reaction rate was highest at 0.2 *M* ethanol and isovaleric acid concentration.

The results depicted in **Figure 2b** show the dependence of reaction rate as a function of isovaleric acid concentration at different ethanol concentrations (0.05, 0.1, 0.2 and 0.3 *M*) in *n*-hexane and in mixed solvent system. In *n*-hexane, the rate of a reaction increased upto 0.1 *M* acid concentration and



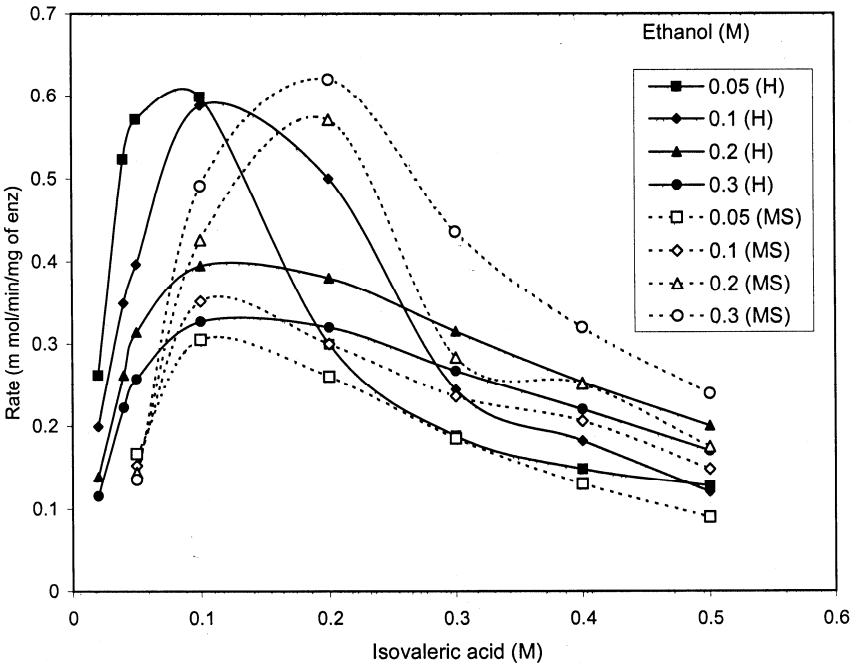


Figure 2 — (a). Reaction rate of esterification as a function of ethanol concentration at varying concentrations of isovaleric acid in *n*-hexane (H) and in mixed solvent (MS) at 40°C and 10 g/Lenzyme concentration.
(b) Reaction rate of esterification as a function of isovaleric acid concentration at varying concentrations of ethanol in *n*-hexane (H) and in mixed solvent (MS) at 40°C and 10 g/Lenzyme concentration.

decreased with a further increase in isovaleric acid concentration upto 0.5 *M*. The rate of reaction was higher at low ethanol concentration (0.598 and 0.588 μ mol/min/mg enzyme for 0.05 and 0.1 *M*, respectively). However, at higher concentrations of ethanol, the reaction rate was much lower (0.395 and 0.327 μ mol/min/mg enzyme, for 0.2 and 0.3 *M* ethanol, respectively). When the reactions were carried out in mixed solvent system, though, the trend observed was similar, the higher reaction rates were observed at 0.2 and 0.3 *M* ethanol concentration (0.571 and 0.620 μ mol/min/mg enzyme,

respectively). This increase in reaction rate could probably be due to the enhanced solubility of ethanol in mixed solvent system. However, a decrease in reaction rate at higher acid concentration (>0.3 *M*) in both the systems indicates a profound acid inhibition. The inhibitory effect of the acid could be the result of modification of enzyme at the interface, which could in turn be due to presence of higher acid concentration (increased hydrophobicity) at the vicinity of lipase and displacement of ethanol from the active site[2]. The decreases in reaction rate at higher concentrations (upto 0.5 *M*) of ethanol and isovaleric acid (**Figures 2a,b**) indicate the twin inhibition of *Rhizomucor miehei* lipase by ethanol and isovaleric acid.

Figures 3a, b show the double reciprocal plot of reaction rate *versus* ethanol and isovaleric acid concentrations. At low substrate concentrations (0.05 and 0.1 *M*) the appearance of parallel lines can be seen with an increase in slope. A decrease in intercept with an increase in substrate concentration (0.2 and 0.3 *M*) is also evident. These results suggest a typical Ping-Pong Bi-Bi mechanism with dead-end inhibition by alcohol and acid. The inhibition pattern was similar in both the reaction systems (*n*-hexane and in mixed solvent system). The reaction rate equation for this mechanism with inhibition by both the substrates

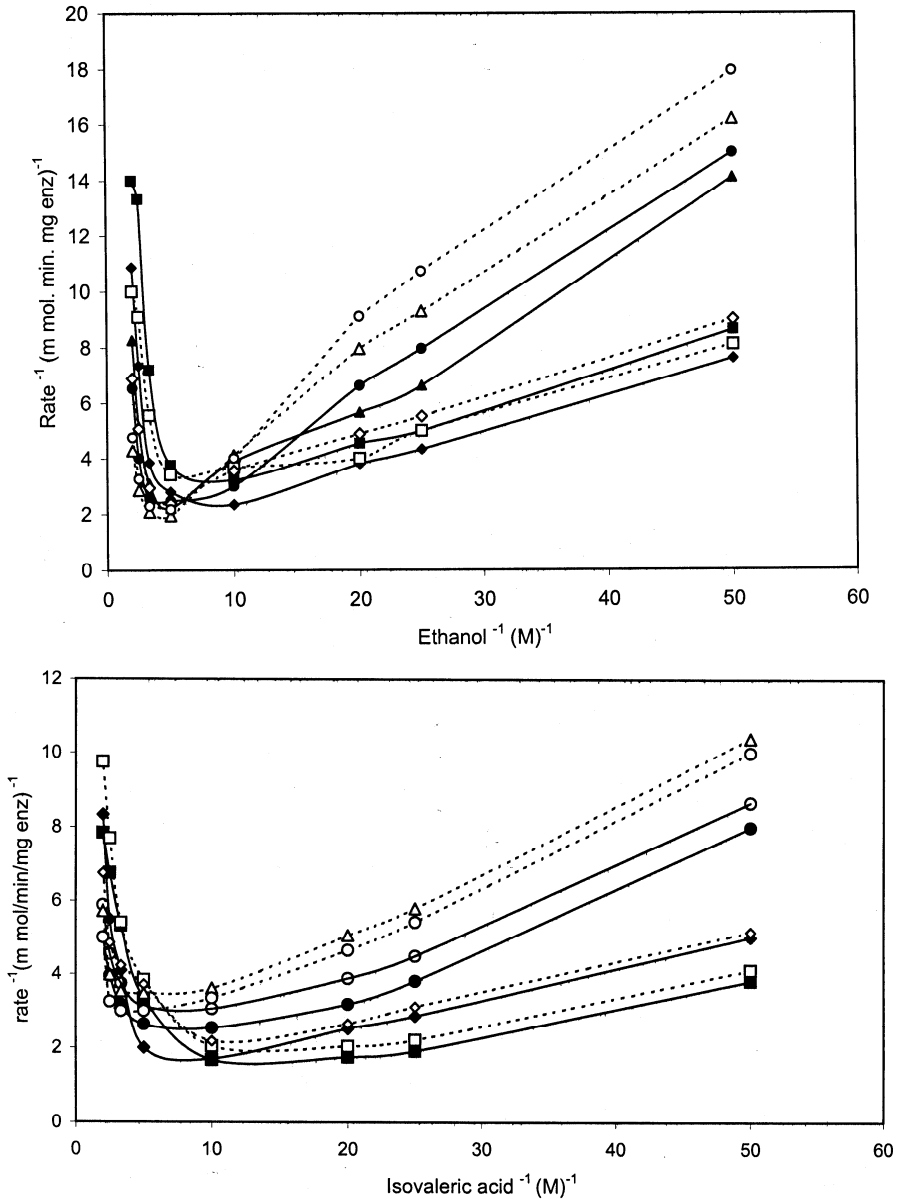


Figure 3 - (a). Double reciprocal plot of reaction rate *versus* ethanol concentration, (b). Reaction rate *versus* isovaleric acid concentration. Legends, as given in **Figure 2**.

is given as[13]:

$$V = \frac{V\max[A][B]}{[A][B] + K_A[B]\left\{1 + \frac{[B]}{K_iB}\right\} + K_B[A]\left\{1 + \frac{[A]}{K_iA}\right\}}$$

where V is initial reaction rate; V_{max} is the maximum reaction rate; A is acid concentration and B, alcohol concentration; K_A, K_B are binding constants of acid and alcohol; $K_{i\,A}$, $K_{i\,B}$ are inhibitory constants of acid and alcohol.

In this study, esterification between short chain acid and alcohol has been chosen, and values of rate constants were incorporated into a Ping-Pong Bi-Bi mechanism with inhibition by both the substrates. The observed inhibitory patterns in the present study appear to be different from the reported esterification kinetics, which indicate inhibition by short chain alcohols and long chain acids but not by short chain acids[5,14,15]. The inhibition of esterification reaction by short chain alcohols is well documented[4,7,15-17].

The alcohol inhibition observed in present study is in concurrence with these observations. The inhibition by acid substrate can be explained by the hypothesis that the acyl enzyme intermediate, forms a dead-end complex, thus the transfer of acyl moiety to alcohol is inhibited[13] as shown in **Figure 4**. In this reaction sequence, enzyme (E) combines with acyl donor (A) to form enzyme acyl complex (E.A). Soon after the formation of E.A complex, the first product, water (P) will be released. Then the second substrate alcohol (B) is attached to form acyl enzyme alcohol complex (E.Ac.B). Later E.Ac.B complex will be dissociated into free enzyme (E) and ester product (Q). The irreversible binding of either acid (E.B) or alcohol (E.Ac.A) to enzyme leads to dead-end inhibition (which are inactive).

The estimated kinetic values are given in **Table I**. While comparing the kinetic values of esterification in *n*-hexane and mixed solvent systems, there is a large difference in values of K_m and K_i for both the substrates, where K_m value of isovaleric acid is almost similar in both the systems (mixed solvent than in *n*-hexane) and K_m of alcohol is 2.5 folds higher in mixed solvent system than in *n*-hexane. The increase in K_m for alcohol might be due to better solvation of ethanol in mixed solvent system (increase in polarity of the media), thus leading to a lower degree of inhibition by ethanol.

Similarly, the K_i value of alcohol in mixed solvent system is one-fold higher than that in hexane, which again indicates a lesser degree of inhibition by ethanol. These findings are in good agreement with the results reported by Otamiri *et al*[9]. These results indicate that the mixed solvent system could be a better choice for the esterification of polar substrates like ethanol for higher esterification rates. However, the increase in K_m value of alcohol in mixed solvent system (0.645 *M*) than in *n*-hexane (0.256 *M*) could not be explained totally by the differences in solvation of the substrates. It could also be due to interaction of mixed solvent with the active site of lipase more strongly than that of *n*-hexane, thereby increase in the K_m value of the alcohol as a competitive inhibitor[6] and such competitive inhibition by solvent has been reported[18].

The present investigation shows that the esterification kinetics in both system follows a Ping-Pong Bi Bi mechanism with competitive inhibition by ethanol and isovaleric acid. The maximum rate of a reaction was almost three-folds higher in mixed solvent system than in *n*-hexane. The increase in polarity of the media resulted in increased reaction rates and reduced the ethanol inhibition.

Materials

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3) from *Rhizomucor miehei* supported on macro porous anionic resin beads was from Boehringer

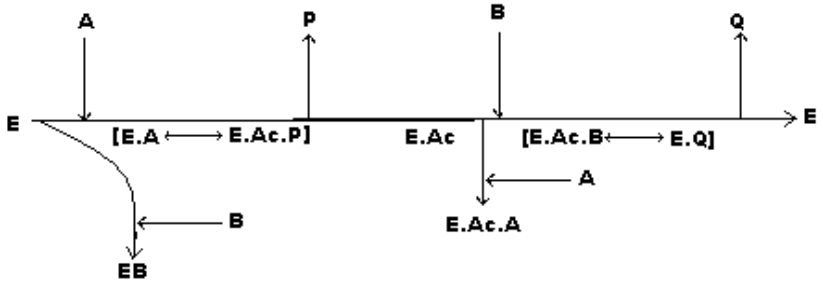


Figure 4 — Schematic representation of the Ping-Pong Bi-Bi mechanism with competition inhibition by both alcohol and acid substrates. E, A, B, P, Q and Ac represents enzyme, acid, alcohol, water, ester and acyl group respectively. E-B and E.Ac. A are the dead-end inhibition complexes of enzyme with alcohol and acid respectively.

Table I — Estimated kinetic parameters in *n*-hexane and in mixed solvent system

Parameter	*n*-Hexane	Mixed solvent [a]
$K_{m\ (acid)}$	0.296	0.281
$K_{m\ (alcohol)}$	0.256	0.645
$K_{i\ (acid)}$	1.62	0.192
$K_{i\ (alcohol)}$	0.0987	0.190
$V_{(max)}$	2.637	7.723

[a]*n*-hexane/diethyl ether (85:15, %, v/v).V_{max}: μ mol/min/mg enzyme; K_m, K_i: Molar (*M*)

Mannheim, Germany. The hydrolytic activity of lipase was 15000 U/g using tributyrin as the substrate at *p*H 7 and 37 °C (ref. 19).

Water content of lipase

Catalytic activity depends on the water content present at the enzyme surface[20]. The water content of *Rhizomucor miehei* lipase has been estimated as described elsewhere[19] and it is 8.5%, which is sufficient for catalytic activity and further addition of water was not attempted.

Substrate and Chemicals

Isovaleric acid and tributyrin were obtained from Aldrich Chemicals Ltd (Milwaukee, USA). Ethanol, *n*-hexane and diethyl ether and molecular sieves (3Å) were purchased from SD Fine Chemicals Ltd (Mumbai, India). The solvents were distilled and solvents and substrates were dried over molecular sieves prior to use.

Experimental Section

Esterification reactions were carried out in a stoppered conical flask (100 mL) with 10 mL working volume of *n*-hexane or mixed solvent (*n*-hexane/ diethyl ether at a ratio of 85:15, %, v/v). Substrates were dissolved in solvent and incubated with requisite quantities of enzyme on a rotary shaker at 150 rpm (Remi Instruments, Mumbai, India, Model No. CIS-24) at 40 °C for 24 hr. Initial rate was measured from linear portion of the curve (triplicate). Samples were withdrawn at regular intervals of time.

Gas chromatographic analysis (GC)

The aliquots of reaction mixture were analyzed using GC-14 B (Shimadzu Corporation, Tokyo, Japan) equipped with FID detector and Carbowax 20-M column (3 m length, 3.175 mm i.d.). The oven temperature was kept at 100°C (isothermal), the injection port and detector temperatures were maintained at 200°C and 250°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. The sample volume of 50 μL was dissolved in 2 mL of *n*-hexane containing 10 μg of internal standard (*n*-octanol), 1 μL of this sample was injected to column. Peak areas were computed using chromatopac C-R6A integrator.

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