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# Influence of the Support on the Reaction Course of Tributyrin Hydrolysis Catalyzed by Soluble and Immobilized Lipases

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## ABSTRACT

Lipases from different origins have been immobilized in supports chosen by its different aquaphilicity and used as biocatalysts for the hydrolysis of tributyrin. The changes of the concentration of tri-, di-, monobutyrin, glycerol, and butyric acid during the reactions catalyzed by soluble, as well as immobilized, lipases were evaluated by gas chromatography. The experimental data were fitted to a simple kinetic model for the sequential reaction of tributyrin hydrolysis. The calculated apparent rate constants were different for the biocatalysts used and were apparently related to diffusional effects and aquaphilicity of the supports. Maximal yields of dibutyrin were found with the soluble *Candida* lipase, whereas the highest yield of monobutyrin (90%) was obtained with the least aquaphylic derivative (*Candida*-Celite).

**Index Entries:** Aquaphilicity; tributyrin, hydrolysis of; *Candida cilindracea* lipase; lipases, immobilized.

## INTRODUCTION

Lipases (EC 3.1.1.3) comprise a group of enzymes whose biological function is to catalyze the hydrolysis and synthesis of triacylglycerol. For many years, Desnuelle and collaborators (*1*) have carried out detailed studies on the mode of action of lipases, mainly animal pancreatic lipase.

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Later, many lipases from microbial sources have been investigated and found to be promising catalysts for hydrolysis and synthesis of fats and oils (2). Of these, the enzyme from *Candida cylindracea* (now named *C. rugosa*) is an interesting lipase because of its high activity in hydrolytic, as well as in synthetic, reactions. Although it lacks specificity of position in the hydrolysis of triglycerides, it discriminates among different fatty acids (3,4).

Lipases, which act on water insoluble (hydrophobic) substrates, can be adsorbed on hydrophobic surfaces, and this stresses the importance of the hydrophobic/hydrophilic character of the matrix used in the immobilization. Kimura et al. (5) immobilized *C. cylindracea* lipase on different inorganic and organic supports and found that hydrophobic matrices exhibited the highest activity in the hydrolysis of olive oil. A similar behavior of lipase has been reported for synthetic reactions (6–8). Besides, immobilization of enzymes that act on substrates having several bonds susceptible of reaction (lipases, nucleases, carbohydratases, and so on) may create difussional resistances or located gradients that may change the progress of the hydrolysis reaction, compared to the native enzyme. Thus, it has been reported (9) that micrococcal nuclease, after immobilization on a porous matrix (Sepharose), changed from a mainly endonucleolytic DNase to an exonucleolytic one.

In the present work, we have investigated the time course of the hydrolysis of tributyrin, by several immobilized lipases, as a function of the aquaphilicity (7) of the support.

## EXPERIMENTAL

### Materials

*Candida cylindracea* type VII lipase (containing 700 U/mg powder with olive oil as substrate) was a product of Sigma (St. Louis, Missouri, USA). *Mucor miehei* lipase immobilized on Duolite 568, a phenol-formaldehyde macroporous weakly basic anion exchange resin (Lipozyme) (10), and *Candida antarctica* lipase immobilized on a macroporous acrylic resin (SP 382) were kind gifts from Novo Industri A/S (Copenhagen, Denmark). Acrilonitrile for HPLC was purchased from Carlo Erba (Milano, Italy); tributryin from Sigma; diethyl ether from Panreac (Barcelona, Spain); Sepharose CL-6B from Pharmacia (Uppsala, Sweden), and celite for gas-liquid chromatography (30–80 mesh) from BDH (Poledorset, England).

### Methods

Covalent immobilization of *C. cylindracea*, through its amino groups on Sepharose CL-6B activated with 2,3-epoxi-1-propanol, was carried out as previously described (11). After 30 min of reaction at 25°C between

enzyme and support, reduction was effected with sodium borohydride. The immobilized preparation contained 31 mg lipase/mL Sepharose gel ("Candida-Seph"). For adsorption of the enzyme on Celite ("Candida-C"), 100 mg lipase powder were dissolved in 5 mL of 0.1*M* phosphate buffer, pH 7.2, 0.1*M* NaCl. After 1 h stirring at room temperature, 500 mg of the support were added, and the stirring continued for 2 h. Then, 5 mL of cold (−15°C) acetone were slowly added with stirring. The immobilized enzyme was filtered, washed with 2×5 mL acetone, dried 30 min *in vacuo*, and stored in a closed vial at 4°C.

The aquaphilicity, as defined by Reslow et al. (7) (L water in the matrix/L water in diisopropyl ether phase in contact with the solid), was determined in Candida-C, SP 382, and Lipozyme. The immobilized enzymes were washed with acetone, dried under reduced pressure, and used for determination of aquaphilicity by the procedure in ref. (7), or for reaction with tributyrin.

Hydrolysis of tributyrin was carried out as follows: 0.82 g tributyrin were added to 38 mL 0.1*M* $Na_2HP0_4$, pH 8.0, containing 0.1*M* NaCl and acetonitrile (3% v/v). The reaction was carried out at 30°C with stirring and was started by addition of the biocatalyst. The pH was maintained at 8.0 with conc. NaOH solution. At intervals, 2 mL aliquots were drawn, mixed with an equal volume of diethyl ether, and shaken vigorously for 10 min. The concentrations of mono-, di-, and triglyceride were measured using a Shimadzu GC-R1A gas chromatograph provided with FID. The organic phase (0.1 μL) were injected in a 3.2 mm×1.8 m methyl silicone column (3% SP-2100 on 100/120 Supelcoport from Supelco, Gland, Switzerland); after 5 min at 65°C, the temperature was raised to 220°C at 10°C/min. Glycerol and butyric acid in the aqueous phase were analyzed using a 3.2 mm× 2.3 m Porapak Q column at 240°C and a FID detector.

## RESULTS

The support materials for immobilization of lipases used in this work are quite different regarding hydrophobic-hydrophilic properties. The aquaphilicity of three immobilized enzyme preparations appear in Table 1. As expected, Celite, a matrix increasingly used in investigations with lipases, yields the derivative with lowest aquaphilia, 0.17, similar to the value (0.36) obtained by Reslow et al. (7) for the support alone, but using more drastic drying procedures. The aquaphilicity of the Candida-Seph derivative was not determined because of the inability of this hydrophilic gel to recover its structure after drying. However, according to Reslow et al. (7), Sephadex is one of the most aquaphilic supprots ($A_q$ = 12.3). Therefore, Sepharose 6B gels that contain approximately 94% water, much more than Sephadex gels, must have higher $A_q$ values than the other support listed in Table 1.

Table 1
Aquaphilicity of Immobilized Enzyme Derivatives

| Immobilized enzyme | Aq |
|---|---|
| Candida-C | 0.17 |
| SP 382 | 0.67 |
| Lipozyme | 1.31 |
| Candida-Seph | nd[a] |

[a]nd: not determined.

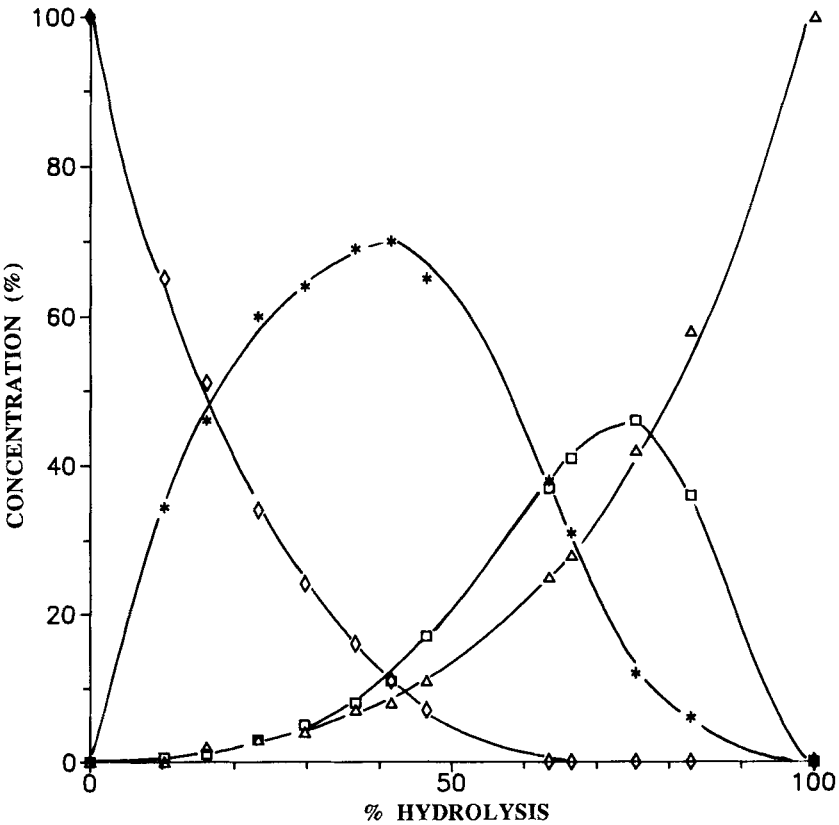


Fig. 1. Variation in the composition of the reaction mixture as a function of the extent of hydrolysis. The initial concentration of tributyrin was 68 m*M*. For reaction conditions, *see* the Methods section. Biocatalyst: 0.125 g *Candida* lipase powder. ◇, TG: *, DG; □, MG; △, glycerol. Fifty percent hydrolysis was obtained in 2 h.

Figures 1 to 6 present the curves of product distribution vs percent tributyrin hydrolysis calculated as a function of the amount of burytric acid produced. The reactions were carried out at 30°C and pH 8.0 using several biocatalysts, *viz.*, lipase from *C. cylindracea* in the absence and presence of 100 m*M* $Ca^{2+}$, Candida-Seph, Lypozyme, SP 382, and Candida-C.

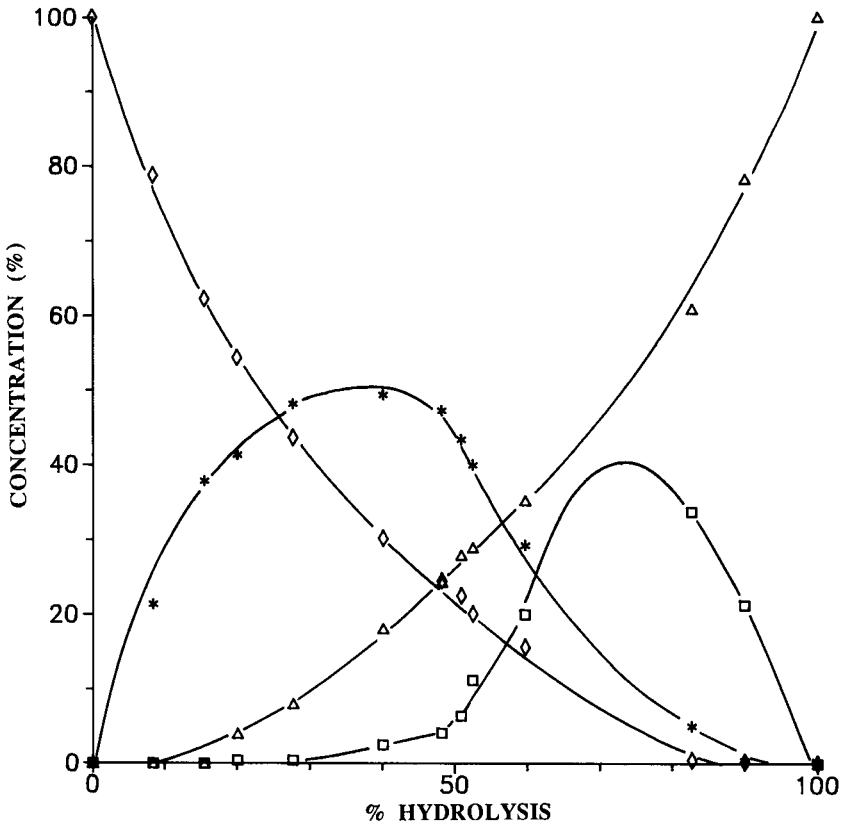


Fig. 2. Conditions the same as in Fig. 1, except that $CaCl_2$ (0.588 g) was present in the reaction in a molarity half that of fatty acids liberated in the total hydrolysis of 68 m*M* tributyrin. Fifty percent hydrolysis was obtained in 1.5 h.

The curves of dibutyrin and monobutyrin concentration are clearly different depending on the state of the lipase (soluble or immobilized) and the type of support used. From these differences, it can be inferred that the course of the reaction is affected by the state of the biocatalyst. However, the presence of $Ca^{2+}$ (Figs. 1 and 2) does not exert a profound influence on the pattern of hydrolysis: the main difference is that glycerol appears at lower percentages of total hydrolysis in the presence of $Ca^{2+}$. Comparing the reactions with immobilized lipases (Figs. 3–6), several comments are warranted. The concentration of dibutryin in the curves of Figs. 3, 5, and 6 is very small, indicating that its hydrolysis is very fast. Using the preparation of Candida-Celite, the concentration of monobutyrin reaches 90% (Fig. 6), suggesting that, in this case, the hydrolysis of this monoglyceride is the rate-limiting step in the conversion of tributyrin to glycerol. It is also interesting to comment the appearance of glycerol: in Figs. 3 and 4, its production does not show lag phase, whereas in Fig. 6, it only appears when a 40% of the hydrolysis has been attained.

The hydrolysis of triglycerides in water solutions can be described by a sequence of irreversible reactions

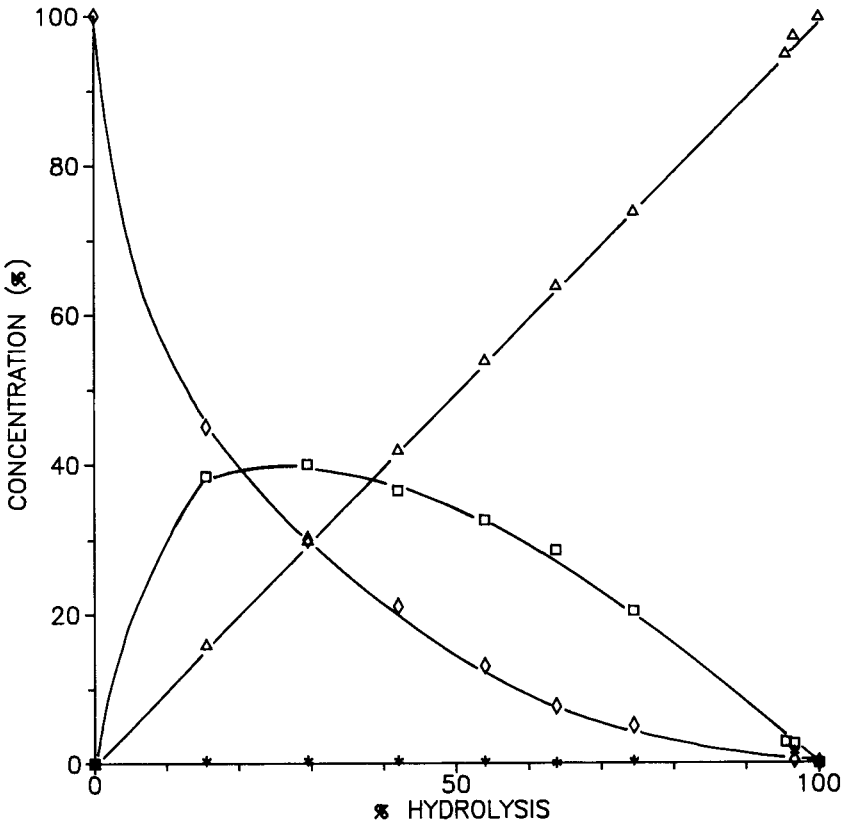


Fig. 3. Conditions the same as in Fig. 1, except that the biocatalyst was 6 mL Candida-Sepharose gel. Fifty percent hydrolysis was obtained in 5 h.

$$TG \xrightarrow{k_1} DG \xrightarrow{k_2} MG \xrightarrow{k_3} Glycerol \quad (1)$$

where TG, DG, and MG stand for triacylglycerol, diacylglycerol, and monoacylglycerol, respectively, and, $k_1$, $k_2$, and $k_3$ are the apparent first-order rate constants of hydrolysis. Integration of the rate equations for the reactions in [1] allows to express the concentrations of TG, DG, and MG as a function of time

$$[TG] = [TG]_0 \exp(-k_1 t) \quad (2)$$

$$[DG] = \frac{k_1 [TG]_0}{(k_2 - k_1)} \left( \exp(-k_1 t) - \exp(-k_2 t) \right) \quad (3)$$

$$[MG] = \frac{k_1 k_2 [TG]_0}{(k_1 - k_2)(k_2 - k_3)(k_3 - k_1)} \times \left( (k_3 - k_2) \exp(-k_1 t) + (k_1 - k_3) \exp(-k_2 t) + (k_2 - k_1) \exp(-k_3 t) \right) \quad (4)$$

where $[TG]_0$ represents the initial triacylglycerol concentration.

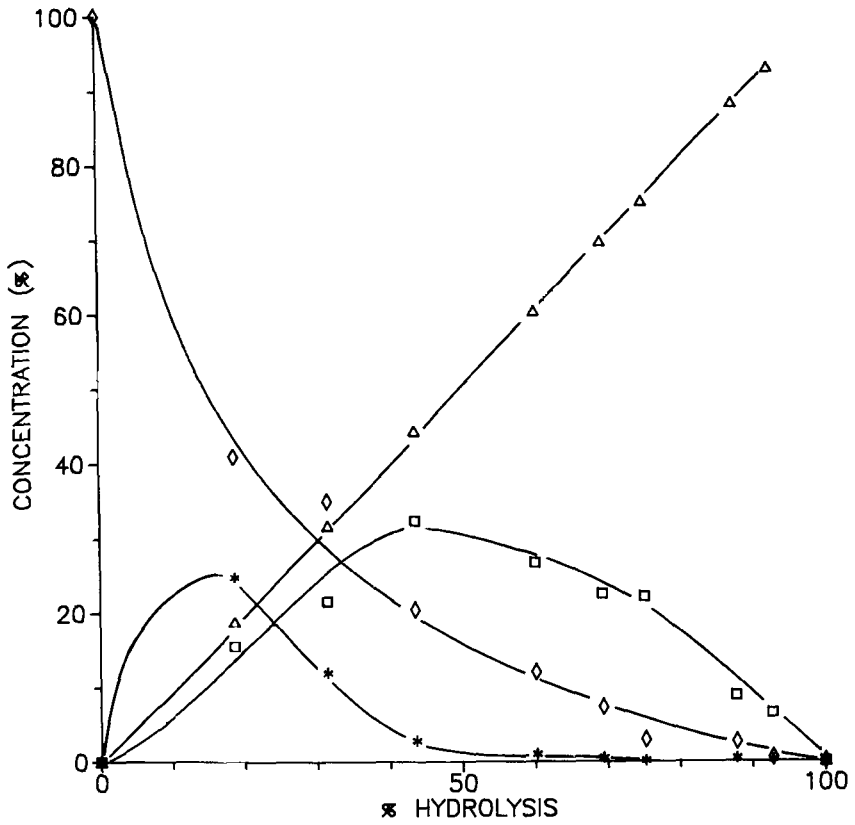


Fig. 4. Biocatalyst: 4 g Lipozyme. All conditions the same as in Fig. 1. Fifty percent hydrolysis was obtained in 13 min.

The concentration of reaction products (ordinate values in Figs. 1-6) were tabulated as a function of the time required to attain the percentage of hydrolysis indicated in the abcissa and were fitted to Eqs. [2-4] by least-squares, nonlinear analysis (*12,13*). First, by fitting the results to Eqs. [2], [3], and [4], the orders of magnitude of $k_1$, $k_2$, and $k_3$, respectively, were evaluated. Then, we used an iterative procedure to find the collection of rate constants values that best fit the experimental values. In all cases, the fittings were satisfactory up to about 75% of tributyrin hydrolysis. At higher degrees of hydrolysis, the experimental concentrations of products were consistently lower than expected from theoretical calculations. In this context, it should be mentioned that, when aliquots of the soluble enzyme ($\pm Ca^{2+}$, Figs. 1 and 2) were removed from the reaction vessel and tested for tributyrin hydrolase activity, inactivation was observed after 3 h of continuous reaction (not shown), which explains the deviation observed at high conversions for these preparations. However, the immobilized lipases under study proved to be stable during the whole reaction. Difussional constraints in the porous network of the sup-

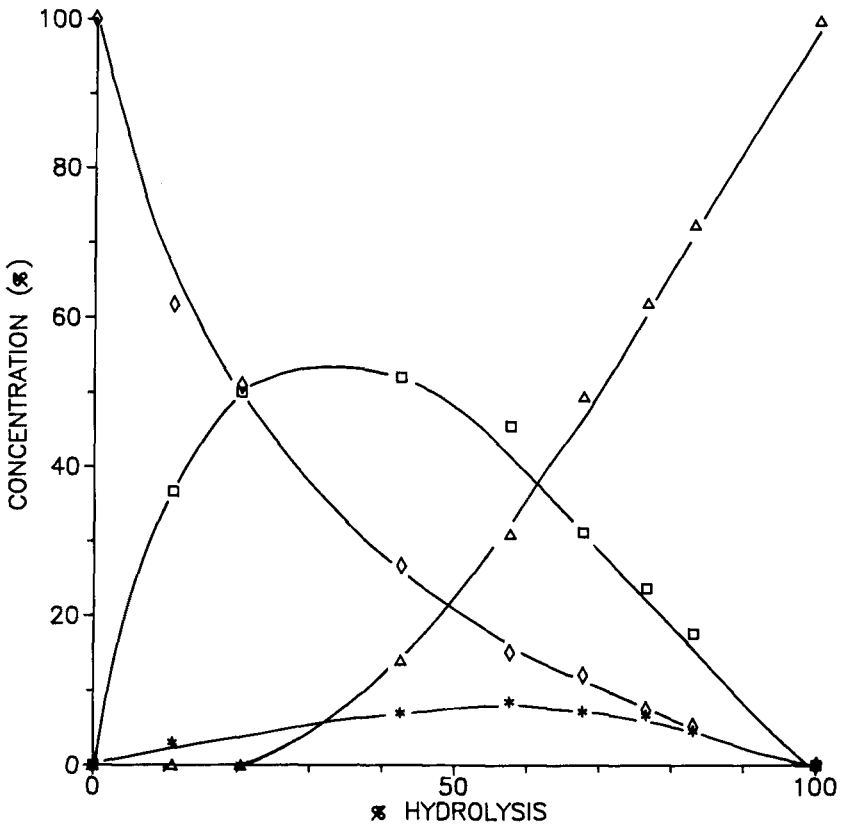


Fig. 5. Conditions the same as in Fig. 1. Biocatalyst: 400 mg SP 382. Fifty percent hydrolysis was obtained in 12 min.

ports could contribute to the observed deviation from the theoretical behavior with these biocatalysts.

Table 2 lists the calculated values of rate constants, and Table 3 presents the ratios $k_1/k_2$ and $k_2/k_3$. The values of these ratios are related, respectively, to the maximum yields of [DB] and [MB] experimentally found. Maximum yield to dibutryrin range from 70% for soluble *Candida* lipase to 0.4% for the same lipase immobilized on Sepharose. The yields of monobutyrylglycerol are almost similar (30–50%), with the 90% value found with the *Candida*-C excepted.

## DISCUSSION

Wang et al. (*12,13*) investigated the kinetics of acylglycerol sequential hydrolysis by human milk lipoprotein lipase and calculated the values of the pseudo-first-order rate constants for the hydrolysis of trioleylglycerol. These authors found that the enzyme has decreasing reactivity in the

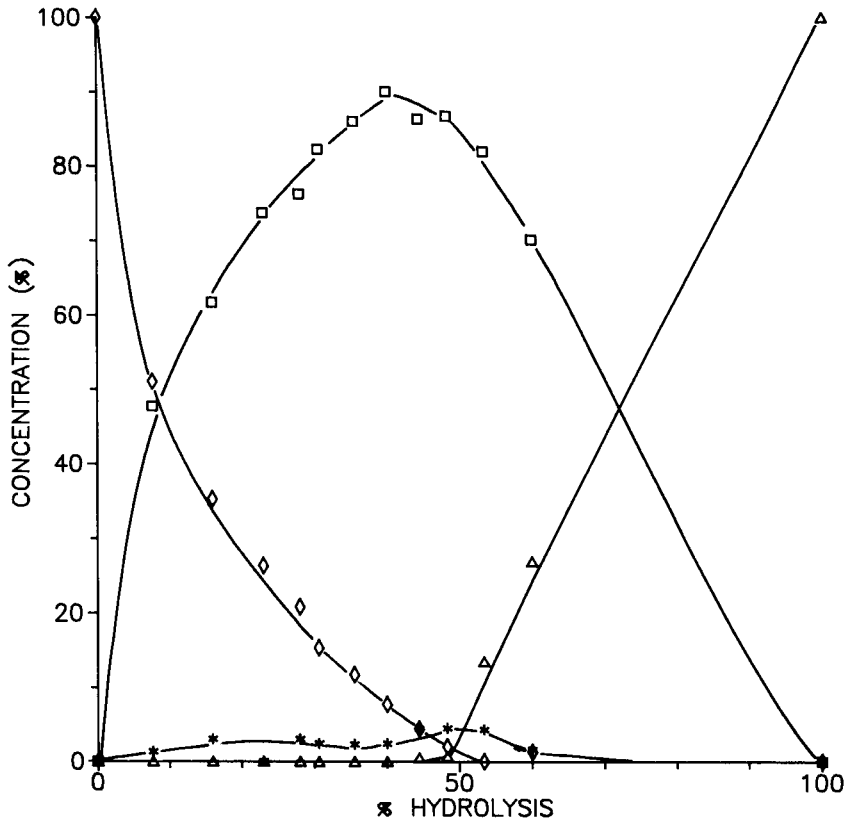


Fig. 6. Conditions the same as in Fig. 1, except that 400 mg Candida-Celite were added. Fifty percent hydrolysis was obtained in 2 h.

order dioleylglycerol > trioleylglycerol > monooleylglycerol. In the present study, we have calculated the value of the same constants for the hydrolysis of tributyrin by soluble (with or without $Ca^{2+}$) and immobilized lipases (Table 2). The ratios $k_1/k_2$ and $k_2/k_3$ give an interesting insight into the mechanism of the sequential hydrolytic reaction. Several conclusions can be advanced: (a) Using *C. cylindracea* lipase, the addition of $Ca^{2+}$ does not substantially affect $k_1/k_2$, but increases $k_3$. In other words, the cation facilitates total hydrolysis to glycerol; in fact, maximum yields to DB and MB decrease from 70 to 46% to 49 and 34%, respectively. With porcine pancreatic lipase, Desnuelle and coworkers (*1*) found a different effect of $Ca^{2+}$ in the hydrolysis of triolein. The presence of the cation produces an enrichment of the reaction mixture in monoolein; and (b) Although in the case of the soluble enzyme $k_1 > k_2$, after immobilization, $k_1$ becomes smaller (2- to 150-fold) than $k_2$. This effect could be owing to the use of porous supports that hamper the difussion of the diacylglycerol out of the pores, thus facilitating its hydrolysis because of a higher local concentration of substrate. Indeed, the $k_1/k_2$ ratio is lowest in the case of the support (Sephar-

Table 2
Pseudo-First-Oder Rate Constants for the Sequential Hydrolysis of Tributyrin

| Lipase | $10^3 \cdot k_1$ | $10^3 \cdot k_2$ | $10^3 \cdot k_3$ |
|---|---|---|---|
| | | ($min^{-1}$) | |
| Candida | 33.6 | 6.6 | 4.8 |
| Candida, $Ca^{2+}$ | 21.6 | 11.4 | 180.0 |
| Candida-Seph | 12.3 | 1800.0 | 10.2 |
| Lipozyme | 272.4 | 546.0 | 660.0 |
| SP 382 | 100.8 | 2340.0 | 0.6 |
| Candida-C | 67.2 | 2700.0 | <0.6 |

Table 3
Maximum Yields to Dibutyrin and Monobutyrin

| Lipase | $k_1/k_2$ | $\%DG_{max}$, time[a] | $k_2/k_3$ | $\%MG_{max}$, time |
|---|---|---|---|---|
| Candida | 5.1 | 70 (1 h) | 1.4 | 46 (23 h) |
| Candida, $Ca^{2+}$ | 1.9 | 49 (1 h) | 0.063 | 34 (27 h) |
| Candida-Seph | 0.0068 | <1 (-) | 180 | 40 (2.5 h) |
| Lipozyme | 0.50 | 25 (3 min) | 0.83 | 32 (10 min) |
| SP 382 | 0.040 | 8 (-) | 3900 | 52 (10 min) |
| Candida-C | 0.025 | 4 (-) | >5000 | 90 (1.4 h) |

[a]In parenthesis, the time required to attain this maximum concentration.

ose) with the smallest pores, about 135 nm average pore diameter (*14*). In this regard, Mattiason and Mosbach demonstrated (*15*) that when three enzymes acting in sequence were coimmobilized on the same particle, such preparations were more efficient than the corresponding system using soluble enzymes.

The maximum yield in dibutyrin depends directly upon $k_1/k_2$. Therefore, from our data, we conclude that in order to obtain the diacylglycerol from the triglyceride, the use of the soluble enzyme, without $Ca^{2+}$, is preferable to the use of immobilized lipases.

The influence of the support aquaphilicity on the time course of the reaction of trybutyrin hydrolysis is illustrated in Table 3 by the values of the ratio $k_2/k_3$, which is around 1 for the soluble *Candida* lipase. In the immobilized enzymes, $k_2/k_3$ seems to be related to the aquaphilicity. So, for a very aquaphilic support like Sepharose, this ratio is about 200, whereas in the case of the least aquaphilic one, Celite, this ratio is higher than 5000. Matrices of high to moderate aquaphilicity that increase $k_3$ favor the hydrolysis to glycerol (Table 3). In fact, Candida-Seph derivatives have been found to be poor biocatalysts for the synthesis of acylglycerols (*16*). On the other hand, since the maximal yield to monoacylgly-

cerol depends on the value $k_2/k_3$, the best supports for obtaining the monoester from triglycerides will be those of low aquaphilicity, e.g., Celite. *Candida*-celite lipase gives a maximum yield to monobutyrin of 90% (Table 3).

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