Pharmaceutical Research, Vol. 6, No. 6, 1989

Research Article

The Role of Calcium Ions and Bile Salts on the Pancreatic Lipase-Catalyzed Hydrolysis of Triglyceride Emulsions Stabilized with Lecithin

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Received February 2, 1988; accepted January 16, 1989

Lecithin-stabilized triglyceride emulsions are subject to hydrolysis by pancreatic lipase. The time profiles of these reactions are characterized by a lag-phase and a zero-order phase. Lag phases are more pronounced with long-chain triglycerides. Ca^{2+} is effective in reducing the lag-phase and activating lipase. Kinetic analysis of the reactions suggests that, like previous findings by others, taurodeoxycholate (TDC) micellar solutions combine with the lipase–colipase complex to form another catalytically active enzyme form. This enzyme form exhibits reduced activity in the absence of Ca^{2+}. In the presence of Ca^{2+} the mixed micelle–lipase complex becomes more active and opens a new pathway for lipolysis. It is suggested that this enzyme form can bind more easily to interfaces with different physicochemical properties. Under these conditions, Ca^{2+} activates the lipolysis of short-, medium-, and long-chain triglycerides by a similar mechanism. Maximum activities were measured in the presence of approximately 6 m*M* TDC and 30 m*M* Ca^{2+}. The experimental conditions approximate the physiological conditions in the gastrointestinal tract since all of the factors studied here have been reported to be necessary for *in vivo* lipolysis and/or absorption of triglycerides. A mechanistic model for lipolysis in the presence of Ca^{2+} and the bile salt TDC is proposed which accounts for most of the experimental observations in a quantitative manner.

KEY WORDS: pancreatic lipase; lipase; lipolysis; triglycerides; kinetics; mechanism; calcium; bile salts; lecithin; emulsions.

INTRODUCTION

Unlike enzymes acting on soluble substrates, lipolytic enzymes act at the interface between an aqueous medium containing the lipolytic enzyme and the water-immiscible substrate, generally triglycerides (TG). Any compound that can bind or interact with the interface is able, to some extent, to modify and alter the activity of these enzymes. Not surprisingly, the number of compounds that can influence the hydrolysis rate is almost unlimited (1). A major effort in lipolysis research is directed to unraveling the individual mode of action of these effectors, which can be further complicated if cooperative and synergistic effects are present. To simplify this effort, better-defined systems amenable to quantitative scrutiny such as the monolayer approach have been devised. Evidently, these approaches are suited for different mechanistic studies and have shed light into diverse aspects of lipolysis (1,2), particularly the importance of the "interfacial quality" (3,4). However, studies that parallel more closely the physiological conditions during lipolysis are better carried out with phospholipid stabilized emulsions. This work studies the effect of Ca^{2+} and taurodeoxycholate (TDC) on the role of pancreatic lipase hydrolysis of triglyceride emulsions using lecithin as emulsifier. A fat emulsion, Intralipid 10% (IL-10), was employed as a standard emulsion because it is readily available and relatively well characterized (5). The long-range purpose of this work is to understand how drugs, dissolved in triglycerides, are released and absorbed from the gastrointestinal tract. There is ample evidence that very lipophilic, low-water-soluble drugs such as mitotane (*o,p'*-DDD) are more effectively absorbed from the gastrointestinal tract when administered in metabolizable triglycerides (6).

MATERIALS AND METHODS

Chemicals

The triglycerides, tributyrin (TB; Grade I, 99%) and trioctanoin (TO; 99%), were from Sigma Chemical Co., St. Louis, Mo. Anhydrous taurodeoxycholic acid (TDC), as its sodium salt, L-α-phosphatidylcholine (lecithin; 60% from egg yolk) and pancreatic lipase from porcine pancreas, Type II (crude preparation with approximately 30% protein, which combines the lipase–colipase complex), were also obtained from Sigma Chemical Co., St. Louis, Mo. The refined medium-chain oil, Neobee M5 (mainly trioctanoin), was from

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R. P. Scherer, Clearwater, Fla. The 10% i.v. fat emulsions, IL-10, were from Cutter Medical (Miles Laboratories). Calcium chloride dihydrate and glycerol, spectral quality, were from Matheson Coleman and Bell, Norwood, Ohio. All other chemicals were reagent grade. Solutions were prepared using double-distilled deionized water.

Emulsions

All emulsions were prepared to match closely the composition of the IL-10 fat emulsion. The emulsions, prepared by weight, consisted of 10% oil, 1.2% lecithin, 2.25% glycerol, and water. Solid lecithin was suspended in warm water with continuous stirring to obtain a homogeneous suspension. Glycerol and oil were then added with continuous stirring to give a very crude emulsion. Finally, water was added to adjust the desired composition. This crude emulsion was then passed, four to six times, through a hand homogenizer to obtain a finer emulsion. Finally, the hand-homogenized emulsion was placed in an ice bath and sonicated in an ultrasonifier (Branson Sonic Power Co., Model W-350) for 6 to 8 min (20-KHz, 350-W output). Invariably, these final emulsions appeared homogeneous under visual inspection. The particle size of stored emulsions (except for the tributyrin emulsion) kept under refrigeration for several months was determined when a Nicomp Model 370 submicron particle size analyzer (HIAC/Royco Instruments) became available. The particle size of 8- to 9-month-old emulsions was determined and compared with the particle size of freshly prepared emulsions. It was found that they did not differ more than a tenth of a micron. Average particle sizes were 0.2 to 0.3 μm. Although kinetic studies were carried out with only freshly prepared emulsions, no noticeable differences in the reaction rates were observed with emulsions kept under refrigeration for over a month.

Kinetics

The lipolysis of the emulsions were followed by continuously titrating the liberated fatty acids (FA) with a Brinkmann pH-stat (Dosimat 665, pH-Meter 632 and Impulsomat 614). The time dependence of the reaction was recorded on a Brinkmann BR-500 strip-chart recorder. A Haake FE water bath was used to circulate water through the jacketed reaction vessel in order to keep the temperature constant at 25°C. In a typical kinetic run an aliquot of the triglyceride emulsion was mixed with measured volumes of 0.1 *M* $CaCl_2$, 30.0 m*M* TDC, and 0.2 *M* NaCl solutions to give the desired concentrations in a final volume of 5.0 ml. This mixture was brought to the pH of the reaction, 8.5–8.6, with the addition of 0.020 *M* NaOH solution. The reaction was started with the addition of the pancreatic lipase solution (30 to 50 μl) and followed continuously for different lengths of time. Vigorous stirring of the reaction mixture was maintained during the kinetic run using a magnetic stirrer. The oil concentration is reported as the percentage (w/v) of oil in the final volume before addition of the enzyme.

The pancreatic lipase solution was routinely prepared by dissolving 250 mg of the commercial preparation in 5.0 ml of cold water. The solution was stirred until all soluble material went into solution (about 15 min). The insoluble material was separated by centrifugation, 15 min in a benchtop centrifuge, and the supernatant collected and kept at 4°C during the kinetic runs. Only freshly prepared solutions were used for the kinetic studies. A gradual loss of lipase activity was noticed when the solutions were assayed for activity after more than 1 day. Lipase activities were determined with TB and olive oil emulsions in the presence of 30 m*M* Ca^{2+}, pH 8.5, with and without lecithin. When lecithin was the emulsifier, the emulsions were prepared as indicated above; otherwise the oil and water were mixed vigorously to form a crude emulsion and then sonicated as above. These emulsions were unstable and were used immediately after preparation. Lipase activity with olive oil, in the absence of lecithin and TDC, was 160 U/mg of protein (the lipase preparation used in these studies indicates that the lipase activity under similar conditions, pH 7.7, is 135 U/mg protein). With a lecithin-stabilized olive oil emulsion the activities in the presence of 6.0 m*M* TDC were 1420 and 960 U/mg protein at 37 and 25°C, respectively. With TB, in the absence of lecithin and TDC, the activity was 995 U/mg protein at 37°C. The highest activities were obtained with lecithin-stabilized TB emulsions in the presence of 6.0 m*M* TDC. Activities at 37 and 25°C were 2600 and 1850 U/mg protein, respectively. One unit is defined as the amount of lipase that liberates 1 μequiv of FA from a TG per hr at the temperature and pH specified. Lipase activities reported in this study refer to TB emulsions in the presence of 30 m*M* Ca^{2+}, 60 m*M* TDC, pH 8.5, at 25°C. These activities are approximately two orders of magnitude lower than for pure pancreatic lipase (sp act, 4.2×10^5), however, since pancreatic lipase acts on water-insoluble substrates, most of the contaminant proteins should have little or no effect on TG lipolysis. The effect of any contaminating phospholipase was not determined, but based on Borgström's work (7), it should be manifested in a reduction in the lag phase and not in the final steady-state rate (see Results). The majority of the results presented here refers to the linear, or zero-order, phase (see Time Dependence of the Reaction and Activation Phase, under Results). Zero-order rate constants (initial rates) were calculated from the slopes of the product versus time dependencies following the lag phase. Part of the error in the estimation of these slopes arises from the manual calculation and the difficulty in assessing the end of the activation phase. Nevertheless, reproducibility in the slopes was normally better than 5%.

RESULTS

Effect of Lipase and "Substrate" Concentration on the Rate of Lipolysis

Initial rates of lipolysis for all substrates studied increased proportionally to the lipase concentration under all conditions examined. Hydrolysis rates of IL-10 displayed saturation kinetics with respect to total oil (substrate) concentration. The results under saturating Ca^{2+} concentrations, with and without TDC, are displayed in Fig. 1a. The saturation data do not obey the typical Michaelis–Menten equation. The increase in rate with concentration is faster than expected from a square hyperbola. The substrate dependence in the presence of TDC and Ca^{2+} shows a fast increase in rate with the first additions of oil. A small inhibition at high substrate concentrations is observed in this

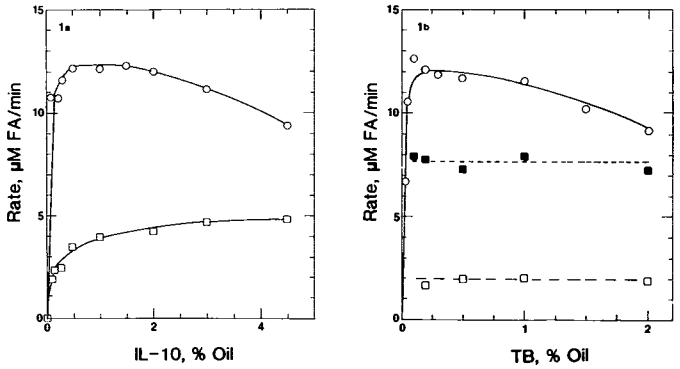


Fig. 1. Observed dependencies of the zero-order rates of lipolysis of IL- 10 (a) and TB (b) emulsions on the total oil concentration. (a) One thousand four hundred units of lipase (50 μl, 0.75 mg protein); Ca^{2+} = 30 m*M*; TDC = 0.0 m*M* (□) and 6 m*M* (○). (b) Eight hundred forty units of lipase (30 μl, 0.475 mg protein); Ca^{2+} = 30 m*M* and TDC = 6.0 m*M* (○); Ca^{2+} = 0.0 m*M* and TDC = 0.0 m*M* (■); Ca^{2+} = 0.0 m*M* and enough TDC to saturate the system at each oil concentration and inhibit lipase action (□). *T* = 25°C, pH 8.5.

case. This apparent substrate inhibition at high oil concentrations has a different origin and it is discussed below. Similar results were obtained with TO and Neobee-M5. Data for TB are shown in Fig. 1b.

Stoichiometry of the Lipase-Catalyzed Hydrolysis of Triglyceride Emulsions with Varying FA Chain Lengths

The hydrolysis of IL-10, TO, Neobee-M5, and TB was followed to completion to calculate the number of liberated FA from each TG (Table I). IL-10 lipolysis showed that release of the first two FA was much faster than the release of the third FA. The release of the third FA from pancreatic lipase hydrolysis of IL-10 probably involves an intramolecular transesterification step (1,8) of the 2-glyceride to the 1- or 3-monoglyceride, a facile step at pH 8.5, followed by lipolysis. In the absence of a lag phase, the linear part of the reaction extended for approximately 30 to 50% of the total reaction. A 100% reaction is considered to be the release of the number of FA indicated in Table I. Only two FA could be quantitated during the hydrolysis of TO (Table I). Similar results were obtained with Neobee-M5, where it was assumed, for calculation purposes, that this oil is composed solely of TO. Finally, hydrolysis of TB emulsions resulted in the production of only one equivalent of butyric acid (Table I). If other FA are produced, their rate of formation is much slower than the hydrolysis rate of the third FA in IL-10. It is of interest to note that the hydrolysis of TO and TB emulsions resulted in clear solutions at the end of the reaction. These results suggest that the observed change in stoichiometry with change in FA chain length is not due to a particular order of specificity by lipase but probably to the fact that the diglyceride derived from TB and the monoglyceride from TO are polar compounds soluble in the aqueous micellar medium employed. Substrates in solution can be hydrolyzed by lipase but at a greatly reduced rate (1,2).

Time Dependence of the Reaction and Activation Phase

The time dependence of the reactions, particularly in the absence of TDC, are characterized by three distinct phases. (a) In the activation phase or lag phase, the rate of

Table I. Stoichiometry of the Lipase-Catalyzed Hydrolysis of Different Triglyceride Emulsions[a]

	TB C-4		Neobee-M5 C-8	TO C-8	IL-10 C-18, C-20
μmol TG	16.5		10.5	10.5	27.5
μmol OH	16.8		20.0	20.0	82.0
$^{-}$OH/TG	1.02		1.91	1.91	2.98
μmol TG	33.0	33.0	52.5	21.0	
μmol OH	35.0	32.0	98.0	42.0	
OH/TG	1.06	0.97	1.87	2	
μmol TG	82.0	82.0	105.0	31.5	
μmol OH	78.0	81.0	212.0	62.0	
OH/TG	0.95	0.99	2.02	1.97	
FA/TG	1	1	2	2	3

[a] μmol TG, μmol of TG at the beginning of the reaction; μmol OH, total μmol of hydroxide consumed at the end of the reaction; FA/TG, the apparent number of fatty acids released from a TG molecule under the experimental conditions of this study. Reaction conditions: *T* = 25°C, pH 8.5, 840 to 1400 U of lipase, Ca^{2+} = 30 m*M*, and TDC = 6 m*M* per run.

the reaction increases continuously until a maximum rate is reached (7,9). (b) In the zero-order or initial-rate phase, the rate of FA production is linear with time. Linear kinetics were observed for up to 30–50% based on the observed number of FA released (see stoichiometry discussion). (c) The final phase is characterized by a continuous decrease in rate until it stops when all the possible FA have been released or when the rate of FA formation becomes negligible compared with the zero-order rate.

The presence of an activation phase and a lag phase depended on the nature of the TG and the experimental conditions used. Qualitatively, long-chain TG had a more pronounced and longer lag phase than medium- or short-chain TG. Lag phases decreased with calcium concentration. Figure 2a shows the dependence of the lag phase on the reciprocal of Ca^{2+} concentration for the lipolysis of IL-10 (2%). A good linear dependence is obtained when the data are plotted in this manner (intercept, -0.003 ± 1.004; $r = 0.966$). The lag time for these reactions decreased with increasing enzyme concentration (data not shown). In contrast with the results obtained with IL-10 and other long-chain fatty acid triglycerides (10), the lipase-catalyzed hydrolysis of TO and Neobee-M5 did not show a lag phase in the absence of TDC. Lag phases were observed in the presence of TDC depending on whether Ca^{2+} was present and on the TDC concentration. The TDC dependence in the inset in Fig. 3 shows a large increase in the lag phase with an initial addition of TDC. Further increases in TDC concentration produced a maximum followed by a rapid decrease in the lag phase until the lag phase disappeared completely.

Dependence of Lipolysis on Ca^{2+} Concentration in the Absence of TDC

The effect of Ca^{2+} concentration on the initial rates of TG lipolysis varied depending on the length of the triglyceride's FA side chain. IL-10 displayed a sigmoidal saturation dependence on Ca^{2+} concentration (Fig. 2b). In the absence of added Ca^{2+}, no lipolysis was observed. Concentrations in the 30 to 40 m*M* range were required to achieve maximal rates, independent of the oil concentration. A relatively short lag phase was still observed under these experimental conditions (Fig. 2a).

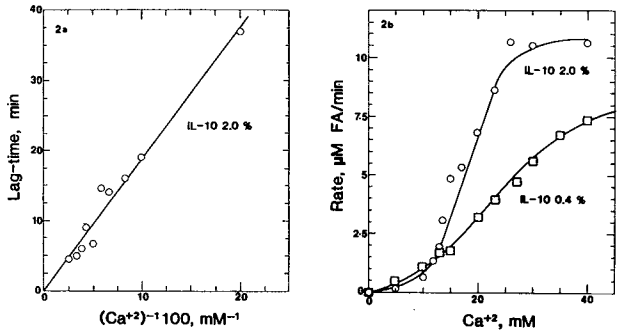


Fig. 2. (a) Observed lag-phase times versus the reciprocal of the Ca^{2+} concentration during lipolysis of IL-10 emulsions (1400 U of lipase, 50 μl, 0.75 mg protein), T = 25°C, pH 8.5. Observed lag phases were proportional to the lipase concentration. The solid line was calculated from a linear least-squares regression to the data. Intercept = -0.003 ± 1.004, $r = 0.966$. (b) Calcium concentration dependence of the observed zero-order rates during lipolysis of IL-10 emulsions in the absence of TDC: 1400 U of lipase (50 μl, 0.75 mg protein), T = 25°C, pH 8.5. IL-10 concentrations are indicated.

The hydrolysis of Neobee-M5 and TO (medium-chain TG) was described by apparent exponential kinetics. In the absence of added Ca^{2+}, the reaction proceeds to only a small extent, approximately 20–25%, and is rapidly inhibited, k(inh), by the products. Total amounts of product formed were proportional to Ca^{2+} and oil concentrations present in the system (Table II). In contrast, initial rates of TB hydrolysis were independent of the Ca^{2+} concentration. In all cases, the extent of reaction showing linear time dependence, increased with the Ca^{2+} concentration.

Dependence of Lipolysis on TDC Concentration in the Presence of Ca^{2+}

TDC effect on the hydrolysis rate of long-chain TG was studied under saturating calcium concentrations, 30 m*M*. Figure 3 shows the effect of increasing TDC concentration on the rate of hydrolysis of IL-10 and TO. There appears to be a moderate increase in hydrolysis rate up to about 0.5 m*M* TDC. In the 0.5 to 1.5 m*M* range the rate of lipolysis levels off and then increases again following typical saturation behavior. This unusual dependence manifested by the "break" or discontinuity in the TDC dependence was reproducible using different enzyme preparations, emulsions, and TG concentrations. Interestingly, the maximum seen in the inset in Fig. 3 for the lag-time dependence on TDC concentration occurs in this discontinuity range.

Dependence of Lipolysis on TDC Concentration in the Absence of Ca^{2+}

TB was the only substrate examined whose hydrolysis rate was independent of Ca^{2+} concentration. Therefore, it was the only substrate where the effect of TDC could be isolated. This represents the only example where "inhibition" by a bile salt on the lipolysis of a TG was observed (Fig. 4). Also, Fig. 4 shows that initial rates were independent of TB concentration before the addition of TDC (see y intercepts) and after saturation with respect to TDC (Table III). A linear relationship between the TDC concentration needed to inhibit the enzyme, as reflected by the reduced lipolysis rate, and the amount of TB emulsion present was noted. Similarly, a linear relationship was noted between the TDC concentration at the inflection point (Fig. 4) and the TB concentration. The TDC concentration at the inflection point, TDC(ip), can be derived from Eq. (1) (see Discussion) and is given by $TDC(ip) = \sqrt[N]{(N-1)K/(N+1)}$, where K (see Table III for definition) is the apparent dissociation constant for the TDC micelles (30). This type of inhibition differs from the well-known inhibition by TDC below its critical micellar concentration (CMC) observed with TG (11–14,26).

Dependence of Lipolysis on Ca^{2+} in the Presence of TDC

Although Ca^{2+} appeared not be required for the hydrolysis of TB emulsions, particularly in the absence of bile salts, it was a potent activator in their presence. Ca^{2+} reversed the inhibition by TDC and increased the hydrolysis

Table II. Inhibition of TO Lipolysis in the Presence of Ca^{2+} [a]

% oil	Ca^{2+} (m*M*)	*k*(inh) (min^{-1})	±SD	% Rxn	μmol FA released
0.25	0	1.9×10^{-2}	1.5×10^{-3}	25	13.0
0.50	0	3.1×10^{-2}	5.0×10^{-3}	24	24.3
1.00	0	2.0×10^{-2}	6.9×10^{-4}	22	45.6
1.50	0	2.2×10^{-2}	1.3×10^{-3}	19	61.0
0.50	10	3.0×10^{-2}	7.6×10^{-4}	33	
0.50	20	2.3×10^{-2}	1.3×10^{-3}	42	
0.50	30	1.7×10^{-2}	9.5×10^{-4}	57	
0.50	40	1.3×10^{-2}	9.0×10^{-4}	80	

[a] Apparent first-order inhibition constant, *k*(inh). Reactions carried out in the absence of TDC. Conditions: 25°C, pH 8.5, and 1400 U of lipase (50 μl, 0.75 mg protein).

rate by almost one order of magnitude. The dependence on Ca^{2+} concentration was characterized by a hyperbolic saturation profile (Fig. 5). Dissociation constants from the enzyme "complex" were calculated by fitting the data to the kinetic model shown in Scheme II (discussed later). The effect of Ca^{2+} on the rate of hydrolysis of medium- and long-chain TG was qualitatively the same (Fig. 5). The results are summarized in Table IV.

DISCUSSION

Two decades ago, Klein *et al.* (15) showed that lecithin inhibits the lipase-catalyzed hydrolysis of TG. Recently, there has been renewed interest in understanding the mechanism of inhibition by phospholipids (7,16–21) and other effectors of lipases (22,23). Recent reports have shown that in the presence of phospholipids and bile salts, the lipase–colipase complex does not bind to the interface of TG (7,18). The substantial lag phase that precedes the steady-state rate of hydrolysis of TG has been attributed to this lack of binding to the interface (7,9,18). The continuous increase in the instantaneous rate of product formation should be considered as a pre-steady-state phenomenon that reflects a number of continuous changes at the interface. In general terms, they represent changes in enzyme affinity for its, so-called, supersubstrate (1,22), before a new steady state is established. Since most exogenous effectors of lipase, under the majority of *in vitro* reaction conditions, do not change their concentration as a function of time in the activation phase, and since most events in solution (binding, complexation, adsorption, etc.) are much faster than the observed activation phases, it can be concluded that activation is triggered by a change in product concentration. This conclusion is in agreement with other work where it was shown that partial hydrolysis of phospholipids by phospholipase A2 or addition of FA could restore lipase activity (7,21,24). Up to now, most studies have dealt with the role of lipase effectors in the activation phase, while their effects under steady-state conditions have not been well characterized. Interestingly, these conditions are expected to prevail in the natural physiological environment of the gastrointestinal tract.

Borgström (7) looked at the effect of bile salts, Ca^{2+},

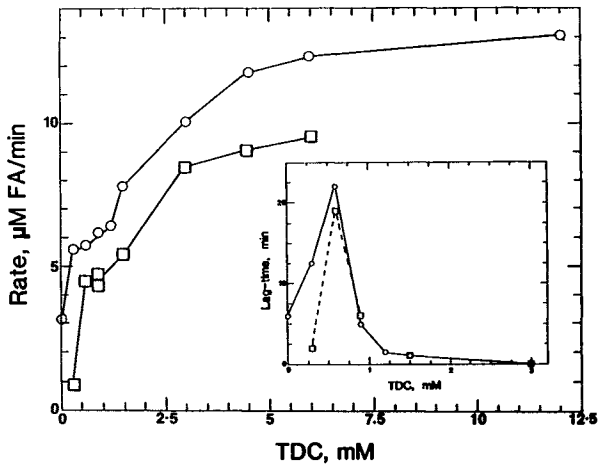


Fig. 3. TDC concentration dependence on the zero-order rates of TG [IL-10, 0.5% (○) and TO, 0.5% (□)] lipolysis in the presence of a saturating Ca^{2+} concentration. The observed dependence was independent of the TG concentration (not included here). One thousand four hundred units of lipase (50 μl, 0.75 mg protein), T = 25°C, pH 8.5, 30 m*M* Ca^{2+}. Inset: Observed lag-phase times versus TDC concentration during lipolysis of 0.5% IL-10 and 0.5% (○) TO (□) emulsions.

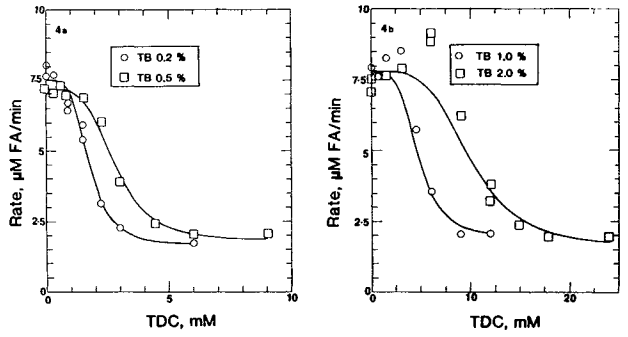


Fig. 4. (a) TDC concentration dependence on the zero-order rates of tributyrin lipolysis in the absence of Ca^{2+}. The TB concentrations are indicated. Observed initial rates in the absence of TDC ($V_1 \approx$ 7.3–7.9 μmol FA/min) and in the presence of saturating TDC concentrations ($V_2 \approx$ 1.4–1.9 μmol FA/min) are independent of TB concentration (see Table III). The solid lines are theoretical lines calculated from a fit of the data to Eq. (1) with N, the number of TDC "micelles" (30), equal to 4. The observed "humps" in dependencies in (b) were not included during the fit to Eq. (1). The reason for these humps was not considered in the model in Scheme I. Eight hundred forty units of lipase (30 μl, 0.45 mg protein), T = 25°C, pH 8.5.

Table III. Inhibition of Tributyrin Lipolysis by TDC[a]

% oil	N	$V_1 \pm$ SD	$V_2 \pm$ SD	$K \pm$ SD, mM^N [$K_T K_M / K_S$, Eq. (1)]	Root[K]
0.2	3	7.7 ± 0.2	1.4 ± 0.4	5.6 ± 1.2	1.78
	4	7.5 ± 0.2	1.7 ± 0.3	9.6 ± 2.5	1.76
0.5	3	7.3 ± 0.2	1.5 ± 0.3	24.7 ± 5.6	2.91
	4	7.2 ± 0.1	1.8 ± 0.1	62.7 ± 9.1	2.81
1.0	3	7.7 ± 0.2	1.7 ± 0.5	149 ± 44	5.30
	4	7.7 ± 0.3	1.9 ± 0.4	497 ± 151	4.72
2.0	3	8.0 ± 0.3	1.2 ± 0.5	1000 ± 319	10.00
	4	7.7 ± 0.2	1.6 ± 0.3	10000 ± 2445	10.00

[a] V_1 and V_2 are initial and final velocities (μmol FA/min), respectively. K is the apparent dissociation constant for the TDC "micelle." N is the number of TDC molecules/micelles assumed to be present. Root[K] is the Nth root of the dissociation constant K (mM^{-1}). See text for explanations. Reactions carried out in the absence of Ca^{2+}. Conditions: 25°C, pH 8.5, and 840 U of lipase (30 μl, 0.45 mg protein).

pH, and FA on the lag phase of lipase-catalyzed hydrolysis of Intralipid. Our results are in good agreement from a qualitative standpoint since the experimental conditions were not identical for both studies. However, the effect of any contaminating phospholipase in reducing the lag time at the onset of lipolysis in our systems has not yet been resolved. Based on Borgström's results (7), however, a decrease in the observed lag phase is expected, but not a change in the final steady-state rate. We have extended some of those studies and found that, in principle, it will be possible to reduce the lag phase completely, regardless of the presence or absence of phospholipases, with a high enough Ca^{2+} concentration (Fig. 2a).

Of greater interest was the recognition that the break point in the lag-phase dependence on TDC concentration occurs around the critical micelle concentration (CMC) of the bile salt (25) (Fig. 3). These results suggest that molecular dispersed solutions of bile salts can bind to the interface and hinder lipase binding. In agreement with other observations, it appears that micellar solutions of TDC are able to solubilize the phospholipid at the interface (16,24,27,28) and restore lipase binding. However, these results do not explain the effect of Ca^{2+} on the lag phase and the role of other effectors under the steady-state rate conditions. For example, the IL-10 hydrolysis shows a threefold increase in the lag phase with almost no change in the steady-state rate with increasing IL-10 concentration. In addition, lag phases are dependent on the triglyceride side chain. In this regard, the absence of a lag phase with TO is consistent with the results of Pieroni and Verger (29), who have shown that lipase–colipase binding to TO films, but not the rate of hydrolysis, is relatively independent on the amount of lecithin present. These results are consistent with Verger's model for lipase action where binding at the interface is fast and reversible (4). Bound enzyme can partition into solution (desorption) or undergo a conformational change (or penetration) into an active form capable of turning over the substrate. Enzyme effectors can alter this partition ratio as well as the intrinsic reactivity of the "effective enzyme complex." Based on this model, the increase in lag phase with IL-10 concentration results from a change in the partition ratio that favors the unbound enzyme [probably due to differences in the free phospholipid concentration that derives from the original IL-10 emulsion (7)]. However, as the reaction progresses and small amounts of product are formed, this ratio changes until all the enzyme is bound and the same final steady-state rate is observed.

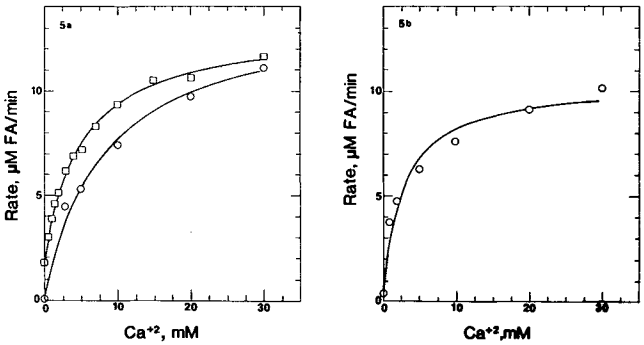


Fig. 5. Observed and calculated saturation dependencies of the zero-order rate of lipolysis of TG emulsions on the Ca^{2+} concentration. The solid lines were drawn using the parameters calculated from a nonlinear fit of the data to Eq. (4). Reaction carried out in the presence of 6.0 mM TDC, 25°C, pH 8.5. (a) 0.2% TB (□) and 840 U of lipase (30 μl, 0.45 mg protein); 1.0% IL-10 (○) and 1400 U of lipase (50 μl, 0.75 mg protein). (b) 1.0% TO (○) and 1400 U of lipase (50 μl, 0.75 mg protein).

Effect of Ca^{2+} on the Post-Lag-Phase Rate of Lipolysis

The nonlinear increase in the rate of hydrolysis of IL-10 with Ca^{2+} concentration (Fig. 2b) indicates a more complicated Ca^{2+} dependence, probably involving more than one

Table IV. Apparent Dissociation Constants for Ca^{2+} from Calcium-Bound Lipase[a]

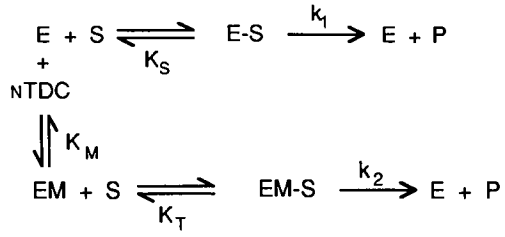
Oil	% oil	$K(Ca^{2+})$, mM ± SD	Y-YHAT
TB	0.2	5.1 ± 0.3	0.999
TB	0.5	4.6 ± 0.4	0.999
TB	2.0	5.9 ± 0.3	1.000
TO	1.0	3.1 ± 1.0	0.989
Neobee-M5	2.0	5.1 ± 0.4	1.000
IL-10	1.0	8.5 ± 1.3	0.995

[a] $K(Ca^{2+}) = K_{Ca}K_b/K_T$ and was calculated from a nonlinear fit of the data to Eq. (4) (Scheme II). Y-YHAT is the correlation of the nonlinear regression. See text for details.

Ca^{2+} ion in more than one role acting in a cooperative fashion. The decrease in the lag phase for IL-10 lipolysis with Ca^{2+} concentration (Fig. 2a) suggests that a Ca^{2+} ion is involved in the activation phase promoting the formation of the active enzyme form. This may be represented by "penetration" of the enzyme or a conformational change that situates the enzyme and substrate in closer proximity to facilitate catalysis. Since the general shape of the sigmoidal saturation curve is independent of the IL-10 concentration (Fig. 2b), it suggests that any other involvement of Ca^{2+} in the reaction, including the possibility of removal of the product as calcium soaps, occurs through some sort of binding to the enzyme–substrate complex or altering the interface with the resulting change in the binding or spatial configuration of one of the enzyme forms.

Effect of TDC on the Rate of Hydrolysis of Triglycerides

Observed changes in the rate of hydrolysis of TB emulsions with TDC (Fig. 4) reflect a shift in the equilibrium distribution of enzyme forms. The analysis of these kinetic data suggests a model in which a catalytically active enzyme form, E, can bind reversibly to the TDC "micelle" (30) to give a new, although catalytically less active, enzyme form EM (see Scheme I). The observed dependencies in Fig. 4



Scheme 1

were better described by a model where the number of TDC micelles, N, in the equilibrium is four (Table III). The rate law for this model, assuming saturation with respect to substrate ($S \gg K_M$ and $S \gg K_T$), is given by Eq. (1), where S represents the substrate concentration (oil), K_T and K_S are the dissociation constants from the enzyme substrate complexes EM-S and E-S, respectively, K_M is the dissociation constant from the micellar enzyme form EM, and e_o is the total enzyme concentration.

$$\frac{V}{e_0} = \frac{k_1(K_T K_M/K_S) + k_2[\mathrm{TDC}]^N}{(K_T K_M/K_S) + [\mathrm{TDC}]^N} \quad (1)$$

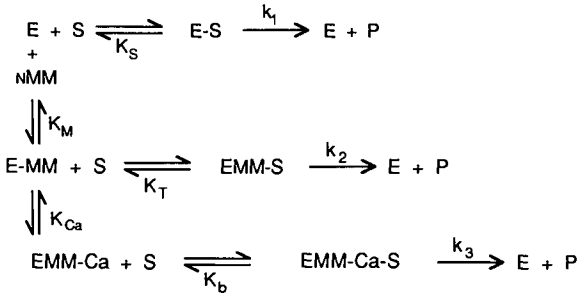
Several workers have accumulated evidence for an enzyme complex of this sort (18,30–34). However, the linear dependence observed with emulsion concentration (see above and Table III) indicates that a constituent of the emulsion is also involved in the formation of this enzyme complex, Eq. (2). A likely candidate is the phospholipid (PL) because other evidence indicates that mixed-micelle (MM) formation has a significant influence on lipase binding and activity (7,17–20,31–34).

$$N\mathrm{TDC} + M\mathrm{PL} \rightleftharpoons N\mathrm{MM}$$
$$N\mathrm{MM} + \mathrm{E} \rightleftharpoons \mathrm{E\text{–}MM} \quad (2)$$

It appears, then, that a "lipase–colipase–bile salt–phospholipid" mixed-micelle complex (E–MM) behaves as a different catalytically active enzyme form. It is important to note that all these enzyme complex constituents are present during lipolysis under physiological conditions (28,33–35). The unexpected lower activity with a mixed-micelle complex (Fig. 4) is due to the absence of another cofactor, namely, Ca^{2+} (see below).

Effect of Ca^{2+} and TDC on the Rate of Lipolysis of Triglyceride Emulsions

In contrast to the effect of Ca^{2+} discussed above or in other reports (11,36), it was found that Ca^{2+} was a powerful activator of lipase (Fig. 5). Activation of the hydrolysis rate was general for all the triglyceride emulsions independent of the FA side chain. The dependence on Ca^{2+} concentration indicates that Ca^{2+} binds to the enzyme in a saturable process opening a parallel pathway for lipolysis. Scheme II



Scheme II

complements the model shown in Scheme I and includes the effect of Ca^{2+}. The rate law for this model, assuming saturation with respect to substrate, is given by Eq. (3).

$$\frac{V}{e_0} = \frac{k_1(K_T K_M/K_S) + [\mathrm{TDC}]^N \{k_2 + k_3([Ca^{2+}]/K_{Ca})\,(K_T/K_b)\}}{(K_T K_M/K_S) + [\mathrm{TDC}]^N \{1 + ([Ca^{2+}]/K_{Ca})\,(K_T/K_b)\}} \quad (3)$$

Under saturating TDC concentrations Eq. (3) becomes Eq. (4).

$$\frac{V}{e_0} = \frac{k_2(K_{Ca}K_b/K_T) + k_3[Ca^{2+}]}{(K_{Ca}K_b/K_T) + [Ca^{2+}]} \quad (4)$$

Scheme II includes a new active enzyme form, EMM–Ca, and a new enzyme–substrate complex that incorporates Ca^{2+}. The new enzyme–substrate complex, EMM-Ca–S, can turn over the substrate at a higher rate than EMM–S. The dissociation constants from EMM-Ca–S to EMM–Ca and from EMM–Ca to EMM are given by K_b and K_{Ca}, respectively. Binding of Ca^{2+} to EMM–S to form EMM-Ca–S is an alternative mechanism that cannot be distinguished kinetically but that hopefully, can be sorted out by changing the nature of the substrate. It would be expected that Ca^{2+} affinity for the different enzyme–substrate complexes is not the same. The dissociation constants for Ca^{2+} (K_{Ca}) were calculated from nonlinear fits to Eq. (4) and are indicated in Table IV.

The dissociation constants for Ca^{2+} were found to be basically independent of the substrate concentration and the nature of the triglyceride emulsion. These two observations favor the mechanism proposed in Scheme II and suggest that the catalytic entity also contains Ca^{2+} bound to the effective enzyme complex. Lipase hydrolysis of medium- and long-chain triglycerides occurs at approximately the same rate under these conditions (TDC = 6.0 m*M*, Ca^{2+} = 30 m*M*) (10). That should come as no surprise from an enzyme whose biological function is to hydrolyze a large variety of TG. Only under these optimum conditions is the rate of TB hydrolysis twice as fast as the rate of hydrolysis of long-chain TG. This is also in agreement with other studies (1). The Ca^{2+} concentration required to activate lipase fully is somewhat higher than what is expected to be found in the intestinal contents. The average Ca^{2+} concentration in the lumen appears to be diet dependent and is suggested to be about 7 m*M* (37). However, even if the diet does not provide the Ca^{2+} concentration required to active lipase fully, the bile, particularly the gallbladder bile, is rich in Ca^{2+} [about 11 m*M* (38)] and can provide the "microenvironment" necessary to activate lipase. Furthermore, Ca^{2+} appears to play some other important roles in lipolysis, such as the complexation with bile (39) to form a liquid crystalline phase (40) in which FA are soluble and probably transported to the gastrointestinal membrane to facilitate absorption. In this regard, it should be mentioned that no calcium soap precipitates are formed when TDC is present, even when the concentration of FA produced is higher than the total TDC concentration. It is known that FA soaps can form micelles (41), or alternatively, they can be solubilized and combined with the TDC micelles to form mixed micelles (25,28), which could also exert an effect on lipase action. The exact role of these different species in solution is not known and deserves further exploration.

Conclusions

The role of Ca^{2+} in the hydrolysis of TG has been shown to be dependent on the physical state and the nature of the substrate. It has been shown that Ca^{2+} is an activator of lipase regardless of the nature of the TG. The kinetic analysis of the hydrolysis of TG emulsions stabilized with lecithin suggests that there are several pathways for lipolysis. This results from the fact that lipase can be catalytically active in different forms depending on the medium. A lipase complex formed with TDC–phospholipid mixed micelle and Ca^{2+} appears to be the catalytically most effective enzyme complex. Based on our kinetic results, the pseudophysiological conditions employed with substrates, lipase effectors, and the pancreatic lipase preparation, and information gathered from the literature, it is concluded that this enzyme form represents the most likely physiologically active lipase complex.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant GM-33387 and a grant from R. P. Scherer Corporation.

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