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Kinetics of the two-step hydrolysis of triacylglycerol by pancreatic lipases

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Pancreatic lipases catalyze the hydrolysis of triacylglycerol in a sequential manner. First, triacylglycerol is hydrolyzed to 1,2-diacylglycerol, which is subsequently converted to 2-monoacylglycerol. We studied the kinetics of trioleoylglycerol hydrolysis by rabbit and human pancreatic lipases. The products (acylglycerols and fatty acid) were analyzed by extraction from the reaction mixture, separation by thin-layer chromatography, and quantification by capillary gas chromatography. The first-order rate constants of trioleoylglycerol and dioleoylglycerol hydrolysis were calculated showing that both enzymes hydrolyze dioleoylglycerol faster than trioleoylglycerol. Using rabbit pancreatic lipase, we found that deoxycholate enhanced dioleoylglycerol hydrolysis to a higher degree than trioleoylglycerol hydrolysis. Colipase increased both rate constants similarly at high deoxycholate concentrations (35 mM), while at low concentrations (5 mM) a selectivity toward trioleoylglycerol was observed. From the variation of the rate constants with respect to temperature, we calculated the apparent activation energies of trioleoylglycerol and dioleoylglycerol hydrolysis to be 59.8 kJ · mol^{-1} and 53.5 kJ · mol^{-1}, respectively. Upon storage, both rabbit and human pancreatic lipases showed a greater loss of activity toward dioleoylglycerol as compared to trioleoylglycerol, suggesting that different conformational elements of the enzyme molecule are responsible for the interaction with each substrate.

Keywords. Pancreatic lipase; kinetics; rate constants; triacylglycerol; diacylglycerol.

Pancreatic lipases catalyze the hydrolysis of triacylglycerol producing 1,2-diacylglycerol, 2-monoacylglycerol, and fatty acids. Lipases are active at the oil/water interface in heterogeneous reaction systems (Brockman, 1984).

One of the main characteristics of lipolysis by pancreatic lipases is that it takes place in a sequential manner. First, a molecule of triacylglycerol is hydrolyzed yielding one molecule of fatty acid and one molecule of 1,2-diacylglycerol, which is subsequently hydrolyzed to 2-monoacylglycerol and fatty acid. Therefore, 1,2-diacylglycerol constitutes both the initial product of lipolysis and the substrate for the second reaction. Using porcine pancreatic lipase, Constantin et al. (1960) reported a transient accumulation of 1,2-diacylglycerol and a late production of glycerol resulting from the cleavage of 1-monoacylglycerol after isomerization of 2-monoacylglycerol.

A matter of great interest for understanding the whole mechanism of lipolysis, is the kinetics of the hydrolysis of diacylglycerol subsequent to the degradation of triacylglycerol. Despite intense research in the general field of lipases in recent years, the kinetics of this reaction remain unclear. Using trioctanoylglycerol and 1,2-dioctanoylglycerol monolayers, Lagocki et al. (1973) reported that the hydrolysis of the diester to 2-monooctanoylglycerol by porcine pancreatic lipase was slower than hydrolysis of the triester. The latter study was made in the absence of any cofactors and the rates of diacylglycerol and triacylglycerol hydrolysis were measured in separate experiments.

In the present study, we have considered lipolysis to be a common chemical sequential reaction according to the scheme:

$$(acyl)_3Gro \xrightarrow{k_1} (acyl)_2Gro \xrightarrow{k_2} acylGro, \qquad (1)$$

where $(acyl)_3Gro$, $(acyl)_2Gro$ and acylGro represent triacylglycerol, diacylglycerol, and monoacylglycerol, respectively, and k_1 and k_2 are rate constants. By determining the concentrations of the reactants at different time points we were able to calculate k_1 and k_2. This approach has already been applied to the study of kinetics of human milk bile-salt-activated lipase (Wang et al., 1988). We have also investigated the effect of bile salt and colipase (two main cofactors of intestinal lipolysis) on the rates of triacylglycerol and diacylglycerol hydrolysis.

MATERIALS AND METHODS

Materials. Rabbit pancreas acetone powder, trioleoylglycerol, dioleoylglycerol, triheptadecanoylglycerol, heptadecanoic acid, sodium deoxycholate, sodium taurodeoxycholate and silica-gel thin-layer-chromatography (TLC) plates were purchased from Sigma. Colipase was from Boehringer Mannheim. Organic solvents were from Riedel-de Haen. 1,2-Diheptadecanoylglycerol, 1,3-diheptadecanoylglycerol and monoheptadecanoylglycerol were from Larodan.

Lipases. Rabbit pancreatic lipase (RPL) was extracted from 1 g rabbit pancreas acetone powder by continuous stirring in 30 ml 20 mM sodium acetate, pH 4.0, at 4°C for 60 min. After centrifugation at 10000 *g* for 10 min, the pH of the supernatant was adjusted to 5.0 with NaOH. The preparation was loaded onto an 8 cm×2.5 cm CM 52 column already equilibrated at

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Abbreviations. RPL, rabbit pancreatic lipase; HPL, human pancreatic lipase.

Enzyme. Pancreatic lipase (EC 3.1.1.3).

pH 5.0. Lipase activity was eluted from the column with a NaCl gradient (0−0.3 M) and was precipitated with $(NH_4)_2SO_4$; the 20−50% pellet was collected and suspended in 1 ml 30 mM Tris/HCl, pH 9.0 (buffer A). All subsequent steps were carried out with the aid of this buffer. The enzyme-containing preparation was loaded on a 55 cm×1 cm gel-filtration column (Ultrogel AcA 44). The eluate was passed through two hydrophobic columns: a 4 cm×1.4 cm phenyl-Sepharose column, which did not retain the lipolytic activity, and a 3 cm×1.4 cm octyl-Sepharose column, from which the enzyme activity was eluted with 4 mM sodium taurodeoxycholate in buffer A. Finally, an anion-exchange chromatography column (5 cm×1.4 cm, DEAE-Trisacryl), previously equilibrated with 4 mM taurodeoxycholate in buffer A, was employed. The lipolytic activity was eluted by applying a NaCl gradient (0−0.3 M). Protein was determined according to Bradford (1976).

Human pancreatic lipase (HPL) was purified according to a modified version of De Caro's procedure (Tavridou et al., 1992). The purified enzymes were stored at 4 °C.

Lipase assay. A rapid turbidimetric method (Arzoglou et al., 1989) was employed to assess the lipolytic activity of fractions during the purification procedure. The standard reaction mixture (1 ml) contained 0.4 mM emulsified trioleoylglycerol, 30 mM Tris/HCl, pH 9.0, 0.3 mg/l colipase and 35 mM sodium deoxycholate. The decrease in absorbance was measured at 365 nm. 1 U lipase is defined as the amount of enzyme liberating 1 μmol fatty acid/min.

Preparation of emulsions. The process for obtaining trioleoylglycerol emulsions has already been described (Tavridou et al., 1992). This method produces emulsions of homogeneous particle size (average droplet diameter 2.7 μm with less than 2% of droplets larger than 10 μm). Briefly, a stock emulsion of 10 mM trioleoylglycerol was prepared by adding an appropriate amount to a 12 g/l hydroxypropylmethylcellulose. After shaking by hand, the suspension was sonicated twice for 30 s with a 30-s interval in a Minisonic 4 ultrasonic laboratory homogenizer. A stock emulsion of dioleoylglycerol was prepared in the same way except that it was sonicated in a different apparatus, Soniprep 150, because of the smaller volume needed.

Lipid analysis. Unless otherwise indicated, the reaction mixture for analysis of the components of lipolysis (5 ml) contained 2 mM trioleoylglycerol, 35 mM sodium deoxycholate, 0.3 mg/l colipase, and 30 mM 3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropanesulfonic acid, pH 9.0, at 37 °C. The reaction was initiated by the addition of 10 μg RPL or 2 μg HPL. To quantify trioleoylglycerol, 1,2-dioleoylglycerol, 1,3-dioleoylglycerol, monooleoylglycerol, and oleic acid we followed a procedure consisting of four steps: extraction of lipids from the reaction mixture, separation of lipid classes by TLC, preparation of fatty-acid methyl esters and capillary gas chromatography. Lipid extraction was based on the procedure of Dole (1956). 0.25-ml aliquots were removed from the reaction mixture at several time points and were mixed with 1.25 ml 40:10:1 (by vol.) isopropanol/heptane/0.5 M sulfuric acid containing triheptadecanoylglycerol, 1,2-diheptadecanoylglycerol, 1,3-diheptadecanoylglycerol, monoheptadecanoylglycerol, and heptadecanoic acid as internal standards. After 10 min, 0.5 ml heptane and 0.75 ml water were added for phase separation. The two phases were mixed vigorously for 1 min and the upper phase was removed. Heptane was evaporated under nitrogen and the residue was redissolved in 50 μl 2:1 (by vol.) chloroform/methanol. 7 μl were spotted onto a TLC plate which was then developed with 80:20:1 (by vol.) petroleum ether/diethyl ether/acetic acid. Lipids were made visible under ultraviolet light after spraying with dichlorofluorescein. The retention factor of triacylglycerols was 0.87, fatty acids 0.71, 1,3-diacylglycerols 0.25, 1,2-diacylglycerols 0.16, and monoacylglycerols 0.02. Individual spots were scraped off and incubated in 96:4 (by vol.) methanol/sulfuric acid at 60 °C overnight. The fatty-acid methyl esters thus produced were extracted with petroleum ether. After evaporation under nitrogen, the residue was redissolved in carbon disulfide and 1 μl was injected into a Hewlett Packard 5890 series II gas chromatograph equipped with a 30-m long Carbowax capillary column from Alltech and a flame-ionization detector. The column temperature was 210 °C and the carrier gas was helium at a flow rate of 1.5 ml/min. The retention times of methyl heptadecanoate and methyl oleate were 3.3 min and 4.1 min, respectively. The mass of methyl oleate was calculated by comparing the area under its peak in the chromatogram to that of methyl heptadecanoate. Finally, from the amount of methyl oleate corresponding to each TLC spot, we calculated the amount of trioleoylglycerol, dioleoylglycerol, monooleoylglycerol, and oleic acid in each sample removed from the reaction mixture. In addition, we calculated any amount of glycerol produced by applying the following reasoning: the initial amount of trioleoylglycerol in each sample equals the sum of trioleoylglycerol, dioleoylglycerol, and monooleoylglycerol determined plus any glycerol produced. It also equals one third the sum of all oleoyl groups and oleic acid determined. Therefore, the amount of glycerol can be calculated as the difference between the latter and the sum of trioleoylglycerol, dioleoylglycerol, and monooleoylglycerol.

Table 1. Purification steps of rabbit pancreatic lipase.

Step	Activity	Protein	Specific activity	Purification	Recovery
	U	mg	U/mg	-fold	%
Extraction	814	54	15	1	100
CM 52	592	12	49	3	72
Ultrogel AcA 44	493	1.6	308	20	60
Phenyl-Sepharose	358	0.8	448	30	44
Octyl-Sepharose	255	0.4	637	42	31
DEAE-Trisacryl	200	0.3	667	44	24

Determination of rate constants. Assuming first-order kinetics for the sequential reaction 1, the equations providing the concentrations of $(acyl)_3Gro$, $(acyl)_2Gro$, and acylGro during the course of the reaction are (Roberts, 1977):

$$[(acyl)_3Gro] = [(acyl)_3Gro]_0\, e^{-k_1 t}, \quad (2)$$

$$[(acyl)_2Gro] = [(acyl)_3Gro]_0 \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}), \quad (3)$$

$$[acylGro] = [(acyl)_3Gro]_0 \left(1 - \frac{k_2 e^{-k_1 t} - k_1 e^{-k_2 t}}{k_2 - k_1}\right). \quad (4)$$

$[(acyl)_3Gro]_0$ is the initial concentration of $(acyl)_3Gro$, and the initial concentrations of $(acyl)_2Gro$ and acylGro are zero. k_1 was determined by fitting the experimentally determined concentrations of trioleoylglycerol to Eqn (2) with the aid of the MicroCal Origin program (MicroCal Software, Northampton, MA). Using this value, and fitting the experimentally determined concentrations of dioleoylglycerol to Eqn (3), we calculated k_2. All results reported are representative of at least two experiments.

RESULTS

Data on the purification of RPL are summarized in Table 1. The enzyme preparation obtained appears as one band after SDS/PAGE (Fig. 1); its molecular mass was estimated to be

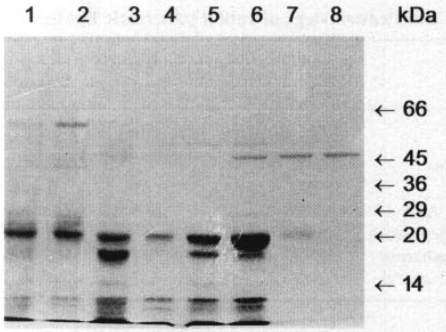

Fig. 1. SDS/PAGE (10% polyacrylamide) patterns (silver staining) of rabbit pancreatic lipase at various purification steps. Lane 1, initial extract; lane 2, preparation after adjusting to pH 5.0; lane 3, fractions containing lipolytic activity after CM 52; lane 4, ammonium sulfate precipitate; lanes 5–8, fractions after Ultrogel AcA 44, phenyl-Sepharose, octyl-Sepharose, and DEAE-Trisacryl chromatographies.

46 kDa. The procedure described results in a 24% yield and a 44-fold enrichment compared to the initial extract.

Kinetic analysis of lipolysis by pancreatic lipases. Fig. 2A and B presents the time course of trioleoylglycerol hydrolysis by RPL and HPL, respectively. Trioleoylglycerol, 1,2-dioleoylglycerol and monooleoylglycerol are expressed as molar fractions of the initial trioleoylglycerol concentration. No production of 1,3-dioleoylglycerol or glycerol was observed. With both enzymes one can note that, as the concentration of trioleoylglycerol decreased, there was a transient accumulation of 1,2-dioleoylglycerol and a continuous increase in monooleoylglycerol concentration. This profile is typical of sequential reactions with rate constants of the same order of magnitude. Plots of $\ln[(acyl)_3Gro]$ versus time (data not shown) were linear for both RPL and HPL ($r > 0.99$), indicating that trioleoylglycerol hydrolysis is first order with respect to trioleoylglycerol. The slopes of these curves yield the experimental rate constant, k_1, for RPL and HPL. However, this methodology is not applicable to the determination of k_2 since the concentration of 1,2-dioleoylglycerol throughout lipolysis depends on both its hydrolysis and production rate. Therefore, another approach is necessary.

Based on the first-order dependence of trioleoylglycerol hydrolysis on trioleoylglycerol concentration, we made the assumption that hydrolysis of 1,2-dioleoylglycerol also obeyed first-order kinetics with respect to 1,2-dioleoylglycerol concentration. The kinetic equations describing two first-order reactions in sequence are Eqns (2–4). Fitting the experimental values of the relative concentration of trioleoylglycerol during hydrolysis by RPL to Eqn (2), we determined k_1 to be 0.0276 ± 0.0004 min^{-1} (estimate ± standard error throughout). Using this value and fitting the experimental values of the relative concentration of 1,2-dioleoylglycerol to Eqn (3) we found $k_2$to be 0.2430 ± 0.0246 min^{-1}. The curves of Fig. 2A show the time courses produced from Eqns (2–4) when k_1 and k_2 are replaced by the values determined above. The coefficients of variation between the experimental and theoretical values were 1.7% for $(acyl)_3Gro$, 22.0% for $(acyl)_2Gro$, and 4.0% for acylGro.

The ratio of k_2 to k_1 is 8.8 and equals the ratio of the respective reaction rates if the concentrations of trioleoylglycerol and 1,2-dioleoylglycerol are equal. Thus, under the particular experimental conditions, RPL hydrolyzes dioleoylglycerol approximately ninefold faster than trioleoylglycerol. The half-lives of trioleoylglycerol and dioleoylglycerol derived from the above rate constants ($\ln 2/k$) are 25.1 min and 2.9 min, respectively.

Following the above steps in the case of HPL, we obtained values of $k_1 = 0.0130 \pm 0.0002$ min^{-1} and $k_2 = 0.0360 \pm 0.0022$ min^{-1} indicating that HPL hydrolyzes 1,2-dioleoylglycerol 2.8-fold faster than trioleoylglycerol. In agreement with Fig. 2A, the curves of Fig. 2B represent the theoretical time courses. The coefficients of variation between the experimental and theoretical values were 1.2% for $(acyl)_3Gro$, 8.7% for $(acyl)_2Gro$ and 15.3% for acylGro. The half-lives of trioleoylglycerol and 1,2-dioleoylglycerol were 53.3 min and 19.3 min, respectively.

k_2 can also be calculated from Eqn (4). The values determined in this way for both RPL and HPL were similar to the ones derived from Eqn (3), differing by 4.5% and 3.3%, respectively.

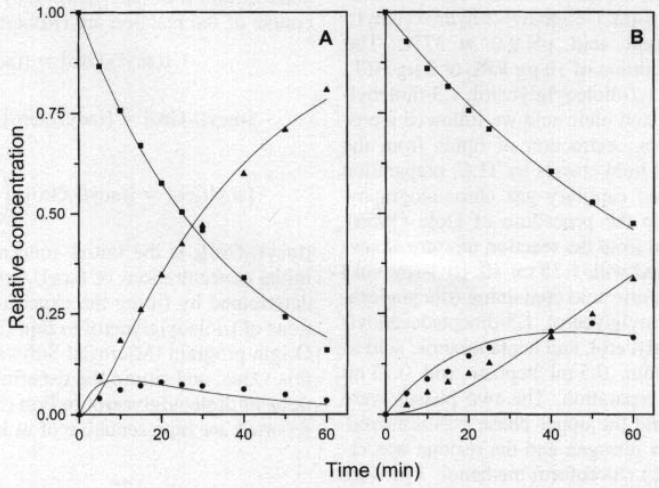

Fig. 2. Time course of hydrolysis of trioleoylglycerol (2 mM) by pancreatic lipases at pH 9.0 and 37°C, in the presence of 35 mM sodium deoxycholate and 0.3 mg/l colipase. (A) Rabbit pancreatic lipase, 2 mg/l. (B) Human pancreatic lipase, 0.4 mg/l. Data are representative of two independent experiments. Symbols indicate the experimentally determined relative concentrations of trioleoylglycerol (■), 1,2-dioleoylglycerol (●), and monooleoylglycerol (▲), whereas curves correspond to concentrations calculated from Eqns (2–4) after determination of the rate constants by fitting the experimental data.

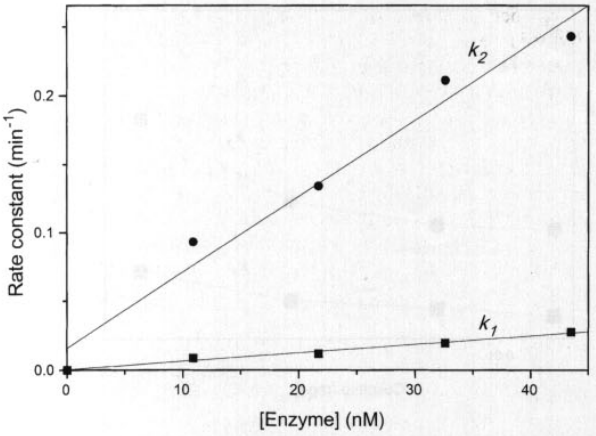

Fig. 3. Effect of rabbit pancreatic lipase concentration on k_1 and k_2. Reactions were performed with an initial concentration of 2 mM trioleoylglycerol at pH 9.0 and 37 °C, in the presence of 35 mM sodium deoxycholate and 0.3 mg/l colipase.

Although k_2 is higher than k_1 with both lipases studied, the rate of trioleoylglycerol hydrolysis is higher than that of dioleoylglycerol hydrolysis throughout most of the reaction period because the concentration of trioleoylglycerol is far greater than that of dioleoylglycerol. This relationship between the rates is reversed when $k_1[(acyl)_3Gro] = k_2[(acyl)_2Gro]$. By replacing $[(acyl)_3Gro]$ and $[(acyl)_2Gro]$ with the mathematical expressions of Eqn (2) and Eqn (3) one can calculate that the reversal takes place at 10.1 min for RPL and 44.3 min for HPL.

To establish that the defined parameters, k_1 and k_2, are reliable markers of lipase activity, we checked their dependence on enzyme concentration. Fig. 3 shows the values of k_1 and k_2 in the presence of different RPL concentrations. Both rate constants exhibited a linear increase with increasing enzyme concentrations ($r = 0.99$); therefore these rate constants reflect enzymic catalysis. Moreover, the linearity indicates a first-order dependence of the rates of trioleoylglycerol and dioleoylglycerol hydrolysis on enzyme concentration. The slopes of the lines of Fig. 3 are the second-order rate constants, $k_{(acyl)_3Gro}$ and $k_{(acyl)_2Gro}$:

$$-\frac{d[(acyl)_3Gro]}{dt} = k_{(acyl)_3Gro}[E][(acyl)_3Gro], \quad (5)$$

$$\frac{d[(acyl)_2Gro]}{dt} = k_{(acyl)_3Gro}[E][(acyl)_3Gro] - k_{(acyl)_2Gro}[E][(acyl)_2Gro]. \quad (6)$$

The values of these constants were $k_{(acyl)_3Gro} = 6.1\times10^5\,M^{-1}\cdot min^{-1}$ and $k_{(acyl)_2Gro} = 5.6\times10^6\,M^{-1}\cdot min^{-1}$.

Having determined the rate constant of dioleoylglycerol hydrolysis occurring sequentially to trioleoylglycerol hydrolysis, we determined the rate constant in the presence of 1,2-dioleoylglycerol alone. For this purpose, using RPL, we substituted 1,2-dioleoylglycerol for trioleoylglycerol in the reaction mixture. Again, hydrolysis was first order with respect to 1,2-dioleoylglycerol. When we fitted the data to the following equation:

$$[(acyl)_2Gro] = [(acyl)_2Gro]_0\, e^{-k_2^* t}, \quad (7)$$

we found k_2^* to be 83% of k_2 determined under identical conditions.

Effect of deoxycholate on k_1 and k_2. Fig. 4A shows how the two rate constants changed when the deoxycholate concentration in the reaction mixture was decreased from 35 mM to 5 mM. Both k_1 and k_2 decreased, but not to the same extent; while k_1 decreased nearly 11-fold, k_2 underwent a 33-fold decrease. Consequently, the ratio, k_2/k_1, decreased from 9, with 35 mM deoxycholate, to 3, in the presence of 5 mM deoxycholate (Fig. 4B). These data suggest that deoxycholate enhances hydrolysis of dioleoylglycerol preferentially compared to the hydrolysis of trioleoylglycerol.

Effect of colipase on k_1 and k_2. Fig. 5 depicts the effect of colipase concentration on k_1 and k_2 at two deoxycholate concentrations, 35 mM and 5 mM. Both k_1 and k_2 decreased when we decreased the colipase concentration from 0.3 mg/l to 0.006 mg/l; in the presence of 35 mM deoxycholate, k_1 and k_2 decreased 4.3-fold and 4.7-fold, respectively, whereas at 5 mM deoxycholate

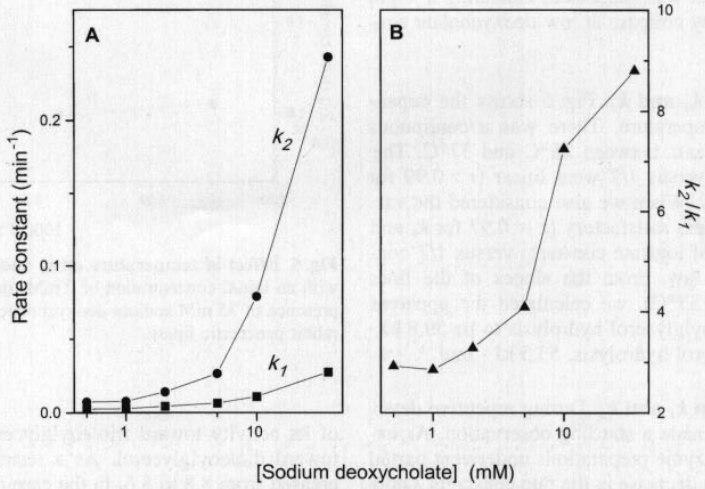

Fig. 4. Effect of deoxycholate concentration on k_1 and k_2 (A), and k_2/k_1 (B) (semilogarithmic plots). Reactions were performed with an initial concentration of 2 mM trioleoylglycerol at pH 9.0 and 37 °C in the presence of 0.3 mg/l colipase and 2 mg/l rabbit pancreatic lipase.

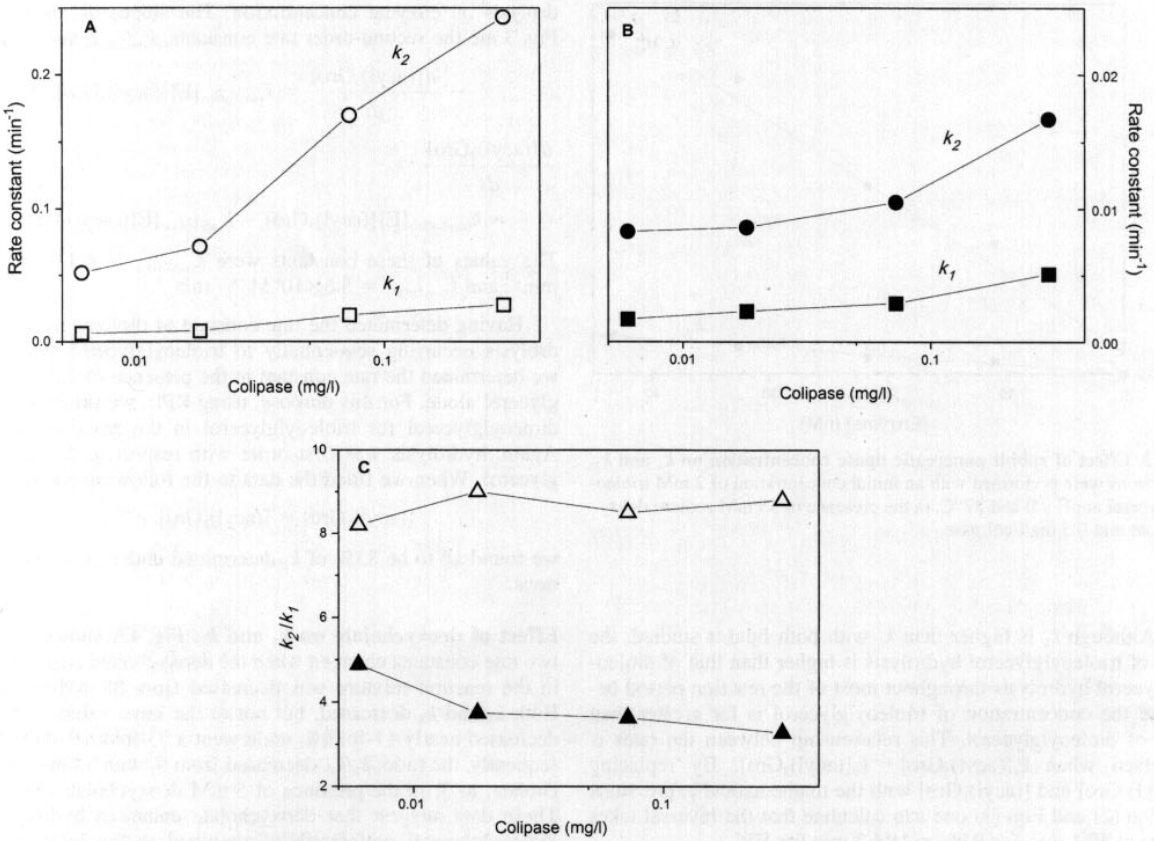

Fig. 5. Effect of colipase concentration on k_1 and k_2 (semilogarithmic plots). Reactions were performed with an initial concentration of 2 mM trioleoylglycerol at pH 9.0 and 37 °C in the presence of 2 mg/l rabbit pancreatic lipase and (A) 35 mM sodium deoxycholate or (B) 5 mM deoxycholate. *C*, ratio of k_2 and k_1 at 35 mM (△) and 5 mM deoxycholate (▲).

the corresponding values were 3.0 and 2.0. The ratios of k_2 to k_1 (Fig. 5C) remained relatively unchanged with 35 mM deoxycholate, with values in the range 8.2−9, suggesting a similar effect of colipase on trioleoylglycerol and dioleoylglycerol hydrolysis. At 5 mM deoxycholate, k_2/k_1 increased from 3.3 to 4.9 as the colipase concentration was decreased, indicating a slight selective stimulation of k_1 by colipase at low deoxycholate concentrations.

Effect of temperature on k_1 and k_2. Fig. 6 shows the dependence of k_1 and k_2 on temperature. There was a continuous increase of both rate constants between 25 °C and 37 °C. The plots of log(rate constant) versus $1/T$ were linear ($r > 0.99$ for both k_1 and k_2) at 25−33 °C. When we also considered the values at 37 °C, linearity was less satisfactory ($r = 0.97$ for k_1 and 0.96 for k_2). The linearity of log(rate constant) versus $1/T$ conforms with the Arrhenius law. From the slopes of the lines (based on the data at 25−33 °C), we calculated the apparent activation energy for trioleoylglycerol hydrolysis to be 59.8 kJ · mol^{-1} and for dioleoylglycerol hydrolysis, 53.5 kJ · mol^{-1}.

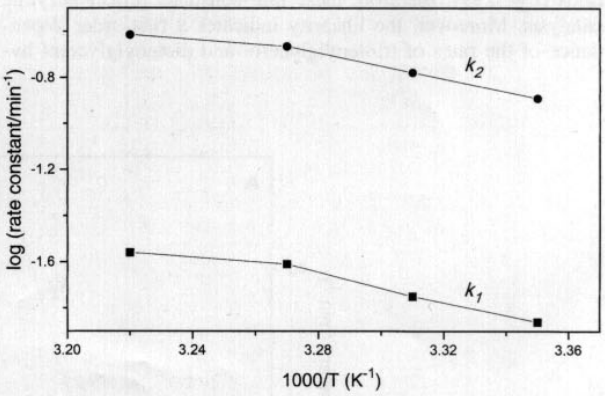

Fig. 6. Effect of temperature on k_1 and k_2. Reactions were performed with an initial concentration of 2 mM trioleoylglycerol at pH 9.0 in the presence of 35 mM sodium deoxycholate, 0.3 mg/l colipase, and 2 mg/l rabbit pancreatic lipase.

Effect of enzyme ageing on k_1 and k_2. During repetitive determinations of k_1 and k_2 we made a startling observation. As expected, upon ageing, our enzyme preparations underwent partial inactivation manifested by a decrease in the rate constants. Quite unexpectedly, though, the relative decreases of k_1 and k_2 were different. Four months after its purification, RPL had lost 60% of its activity toward trioleoylglycerol and 74% of its activity toward dioleoylglycerol. As a result, the ratio of k_2 to k_1 decreased from 8.8 to 5.6. In the case of HPL, the effect was even more pronounced. Three months of ageing resulted in a loss of 28% of its activity toward trioleoylglycerol and 78% of its activ-

ity toward dioleoylglycerol, and k_2/k_1 decreased from 2.8 to 0.9. This finding necessitated the use of fresh (up to one-month old) enzyme preparations.

DISCUSSION

Pancreatic lipase has already been purified and characterized from various species. The gene sequence of RPL was revealed recently (Aleman-Gomez et al., 1992), although no purification of the enzyme was reported. Following the procedure described in the Materials and Methods section we purified this enzyme to apparent homogeneity and studied some of its physical properties. The molecular mass determined is consistent with the sequence of the gene. The degree of purification and the final yield are comparable to those of pancreatic lipases from other sources. We also purified HPL according to an established procedure for comparison to RPL.

We have studied the action of both lipases against emulsified triacylglycerol. The time courses obtained show similarities with the previously studied porcine pancreatic lipase, i.e. exponential decay of triacylglycerol and transient accumulation of 1,2-diacylglycerol as well as no production of 1,3-diacylglycerol or glycerol. Having determined the changes in the relative concentrations of the components of lipolysis with time and by applying the calculus of sequential reactions to the data of Fig. 2, we determined the first-order rate constants for the hydrolysis of triacylglycerol and diacylglycerol. Our results indicate that the kinetic equations of sequential reactions provide a satisfactory description of the whole process of lipolysis.

The approach of sequential-reaction kinetics has also been followed in the case of other lipolytic enzymes. Wang et al. (1988) used the kinetic equations for sequential reactions to investigate the effect of taurocholate on human milk bile-salt-activated lipase. Muderhwa et al. (1992) studied the exchange of ^{18}O between water and fatty acids catalyzed by carboxylester lipase. We therefore believe that kinetic equations for sequential reactions are useful tools in studying lipolytic reactions.

We have calculated the rate constant of dioleoylglycerol hydrolysis (k_2) to be higher than that of trioleoylglycerol hydrolysis (k_1) with both lipases tested. Additionally, when dioleoylglycerol alone was the substrate for pancreatic lipase the rate constant determined was similar to k_2, remaining higher than k_1. This finding contrasts with the report of Lagocki et al. (1973) on the faster hydrolysis of trioctanoylglycerol compared to dioctanoylglycerol by porcine pancreatic lipase. That study employed the monolayer technique in the absence of bile salts, which we show to favor the hydrolysis of diacylglycerol in the present study. The nature of the acyl groups attached to glycerol, pH, ionic strength, etc. could also modulate the ratio of k_2 to k_1. Further studies are therefore needed to determine the factors regulating this ratio.

Lipolysis can be divided into three events. These include substrate partitioning to the lipid/water interface, enzyme partitioning, and catalysis at the interface (Brockman, 1984). Therefore, the second-order rate constants $k_{(acyl)_3Gro}$ and $k_{(acyl)_2Gro}$ that we determined should be considered as apparent rate constants of the process. The difference between $k_{(acyl)_3Gro}$ and $k_{(acyl)_2Gro}$ will correspond to differences in the interaction between lipase and substrate if triacylglycerol and diacylglycerol are topologically equivalent in the heterogeneous reaction mixture, meaning that, after being produced, diacylglycerols do not leave the interface but are arranged on it together with the remaining triacylglycerols. This has been verified by other researchers. Patton and Carey (1979) showed that diacylglycerol formed during fat digestion by human pancreatic lipase, in the presence of bile salts and colipase, remains in the oil phase while monoacylglycerol enters the aqueous phase. Lagocki et al. (1973) studied the hydrolysis of trioctanoylglycerol and the partition of 1,2-dioctanoylglycerol between the lipid and the aqueous phase. They concluded that the diester remains on the surface, while both 2-monooctanoylglycerol and octanoic acid enter the aqueous phase. Finally, Scow et al. (1979) indicated that diacylglycerol formed by the action of lipoprotein lipase remains and spreads at the interface. Consequently, the assumption of topological equivalence between triacylglycerol and diacylglycerol seems to hold and the relationship between $k_{(acyl)_3Gro}$ and $k_{(acyl)_2Gro}$ may reflect the relationship between the true rate constants of the reactions.

Bile salts and colipase are main cofactors of pancreatic lipases. Their presence is known to increase rates of lipolysis with no distinction made between the effects on each of the two reactions. After validation of the kinetic scheme described by the sequential-reaction model, investigation on the effect of these cofactors on each reaction was feasible. We found deoxycholate and colipase to enhance both trioleoylglycerol and dioleoylglycerol hydrolysis, as manifested by the increase in k_1 and k_2 (Fig. 4A, Fig. 5A and B). It was, therefore, of interest to investigate whether deoxycholate and colipase exerted any selective influence on trioleoylglycerol or dioleoylglycerol hydrolysis. Indeed, deoxycholate enhances dioleoylglycerol hydrolysis selectively resulting in an increase of k_2/k_1 (Fig. 4B). In contrast, colipase (Fig. 5C) does not alter k_2/k_1 considerably at high concentrations of bile salt. At low concentrations of bile salt, however, colipase seems to favor trioleoylglycerol hydrolysis.

Several mechanisms for the influence of bile salts on pancreatic lipase activity have been proposed. These include stabilization of the enzyme molecule (Momsen and Brockman, 1976), removal of the fatty acids produced from the interface (Borgstrom, 1964), modulation of the adsorption of lipase to the lipid/water interface (Lairon et al., 1978, 1980), regulation of the conformational status of substrate at the interface (Momsen et al., 1979). Bile salts could also regulate the partitioning of the substrate between the lipid bulk phase and the surface phase and the conformation of lipase at the interface. The effect of bile salts on stabilization of the enzyme, removal of the products, and adsorption of the enzyme is not expected to alter k_1 or k_2 selectively. Therefore, the differential effect of deoxycholate on the rate constants should be attributed to selective alteration of the interaction between enzyme and trioleoylglycerol or dioleoylglycerol or of the partitioning of the two substrates. At present we are unable to distinguish between these possibilities.

Colipase, however, is believed to anchor pancreatic lipase (Verger et al., 1977) to the lipid/water interface. It also protects lipase from interfacial denaturation (Momsen and Brockman, 1976). According to these findings, colipase would be expected to alter k_1 and k_2 uniformly. This is what we found at high concentration of deoxycholate. There is no obvious explanation for the preferential effect of colipase on k_1 at low deoxycholate concentration.

From the variation of k_1 and k_2 with respect to temperature (Fig. 6), we calculated the apparent activation energies for trioleoylglycerol and dioleoylglycerol hydrolysis according to the Arrhenius law. These energies include the true activation energy of each reaction, the energy of adsorption of lipase to the interface and the energy of translocation of the substrate from the bulk lipid phase to the interface. Considering the topological equivalence of triacylglycerol and diacylglycerol, we may assume that the second and third of the above factors contribute equally to the apparent activation energies. Consequently, the difference in the experimentally determined values may reflect the difference in the true activation energies of triacylglycerol and diacylglycerol hydrolysis.

The main findings of the present study are summarized as follows: (a) RPL hydrolyzes dioleoylglycerol faster than trioleoylglycerol; (b) deoxycholate enhances dioleoylglycerol hydrolysis preferentially; (c) colipase leads to a similar increase in the rate of both reactions at high deoxycholate concentration in contrast to a slight selectivity toward trioleoylglycerol at low deoxycholate concentration; (d) upon storage the enzyme suffers a greater loss of activity toward dioleoylglycerol as compared to trioleoylglycerol; (e) kinetic properties and stability patterns appear to be very similar between RPL and HPL. Overall, our results indicate intrinsic differences in the hydrolysis of triacylglycerol and diacylglycerol by pancreatic lipases. Further studies are needed to elucidate the exact molecular events responsible for these differences.

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