Kinetic properties of human milk bile salt-activated lipase: studies using long chain triacylglycerol as substrate

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Abstract Studies on the hydrodynamic properties of human milk bile salt-activated lipase (BAL) indicated that it is a monomer with molecular weight of 107,000. The presence of taurocholate (1 mM) did not lead to an association of the enzyme. The enzyme had a basal activity with trioctanoylglycerol and with shorter chain, but not with longer chain, monoacid triacylglycerols. Based on kinetic analyses, we suggest that the BAL-catalyzed lipolysis of long-chain triacylglycerol can be described to follow a compulsory sequential mechanism. The initial interaction of BAL with the activator (taurocholate) leads to a conformational change of the enzyme which facilitates the further interaction with the long chain triacylglycerol substrate in forming the enzyme–bile salt–substrate ternary complex. We also suggest that the binding of BAL with substrate involves direct interaction of the active site with the fatty acyl-chain of the triacylglycerol rather than with nonspecific hydrophobic interactions at the emulsion interface. **—Wang, C-S., and D. M. Lee.** Kinetic properties of human milk bile salt-activated lipase: studies using long chain triacylglycerol as substrate. *J. Lipid Res.* 1985. **26:** 824–830.

Supplementary key words lipase • taurocholate • trioleoylglycerol • enzyme kinetics • reaction mechanism

Bile salt-activated lipase (BAL) represents the major lipolytic activity in human milk (1–3). The presence of BAL in human milk seems to play an important role in early life by providing an additional enzymatic activity that can supplement the infant's pancreatic juice in the task of hydrolyzing the long chain triacylglycerols contained in milk (3). Our recent comparative studies (4), as well as the studies by Bläckberg et al. (5), indicate that human milk BAL is similar to but not identical to human pancreatic carboxylesterase. These studies indicate that they share a similar amino acid composition, with proline (13 mol %) as the major amino acid constituent. Both enzymes also have the same N-terminal amino acid, alanine (6, 7). Despite differences in the tissue origin of these two enzymes, it is interesting to note that both enzymes require bile salt as the activator for the full expression of their activities; in addition, both have a common physiological site (intestinal lumen) for expression of the activity.

Because of the functional importance and availability of purified human milk BAL (6), we have continued studies on the structure and functional properties of this enzyme. In addition, the study of this enzyme could be used as a model for the less accessible human pancreatic carboxylesterase. Previously, we have reported the kinetic property of BAL using water-soluble *p*-nitrophenyl acetate as substrate (1). In this study, we wish to describe the kinetic property of the enzyme using water-insoluble long chain triacylglycerol as substrate. The results indicated that bile salt was an essential activator of the enzyme for the digestion of long chain triacylglycerols. The BAL-catalyzed reaction follows a compulsory sequential mechanism with binding to bile salt first, followed by binding of the long chain triacylglycerol substrate. This is in contrast to results observed for the BAL-catalyzed reaction with water-soluble substrate in which the formation of an enzyme–bile salt–substrate complex was found to be via a random pathway. The difference in the mechanism of the BAL-catalyzed reaction due to the fatty acyl-chain length effect of the substrates and the physiological role of this enzyme have also been discussed.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise stated, all chemicals were purchased from Sigma. The [carboxyl-^{14}]taurocholate (60.9 μCi/μmol) and glycerol tri[1-^{14}C]oleate (56 μCi/μmol) were obtained from Amersham. Monoacid triacylglycerols with different fatty acid chain length were obtained from Nu-Chek-Prep. The purification of human milk BAL was performed as described previously (5).

Abbreviation: BAL, bile salt-activated lipase.

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Enzyme assay

The lipase assay was performed with Triton X-100-emulsified trioleoylglycerol as substrate (8) in a final assay mixture of 200 μl in 50 mM NH_4OH-HCl buffer, pH 8.5, containing 60 mg/ml bovine serum albumin, 10 mM trioleoylglycerol, 0.1 μCi/μmol of glycerol [1-^{14}C]trioleate (0.2 μCi per assay tube), 2.5 mg/ml Triton X-100, 20 mM taurocholate, and 20 μl of the enzyme solution. All assays were performed in triplicate. The incubation was performed at 37°C in a water bath shaker for 1 hr. The reaction was terminated by adding 3.2 ml of chloroform–heptane–methanol 5:4:5.6 (by vol) and 1 ml of 0.2 N NaOH. After centrifugation, 1.2 ml of the top layer was mixed with 10 ml of Instagel and radioactivity was measured in a Packard liquid scintillation counter.

Binding studies

The binding of BAL to Triton X-100-emulsified trioleoylglycerol was performed using a modified procedure described previously (9). The binding assay mixture (final volume 1 ml) contained 60 mg/ml bovine serum albumin, 10 mM emulsified trioleoylglycerol, in a series of tubes with BAL concentrations ranging from 60 to 700 nM. In addition to the above, tubes in a second series also contained 40 mM taurocholate. The tubes were incubated at 0°C and 37°C for 4 min and then centrifuged for 4 min at 2°C in a microcentrifuge at 13,500 *g*. Tubes were punctured with a No. 25 needle and the infranatant solutions were collected in separate tubes and assayed for lipase activity. Fractional free enzyme was calculated by dividing the BAL activity remaining in the infranatant solutions of the experimental samples by the total lipase activity of the respective control samples.

Binding of taurocholate to bovine serum albumin and trioleoylglycerol

The binding of taurocholate to bovine serum albumin and trioleoylglycerol emulsion was performed by using a 50-ml Diaflo cell (Amicon) with YM5 membrane. The binding assay mixture contained 50 mM NH_4OH-HCl buffer, pH 8.5, with 1 mM and 10 mM of [carboxyl-^{14}C]-taurocholate (0.01 μCi/ml) and in the presence and absence of bovine serum albumin (1 mM) and Triton X-100 emulsified trioleoylglycerol (10 mM) as described in **Table 1**. Each assay mixture was stirred within the cell at 25°C for 10 min and then the filtrate was collected by using N_2 at 10 lb/in^2 pressure. The initial three portions of 5 ml each of the filtrate were returned to the cell. After this step for achieving equilibration, 0.2-ml samples in triplicate from the solution in the Diaflo cell and from the filtrate (1 ml) were taken for measuring the radioactivity. The former represents the total, while the latter represents the free form of the taurocholate concentration. Since the YM5 membrane has a sieving effect with molecular weight cutoff of 5,000, the free form contains both the monomeric and small micellar form of taurocholate with molecular weight of less than 5,000. Further distinction of these monomeric and micellar forms of taurocholate in the filtrate was not attempted.

Basal activity of BAL with monoacid triacylglycerols as substrate

In a final volume of 3 ml, the lipolysis mixture contained 1 mg/ml of BAL, 60 mg/ml of bovine serum albumin in 50 mM NH_4OH-HCl buffer, pH 8.5. The substrates, trihexanoyl-, triheptanoyl-, trinonanoyl-, tridecanoyl-, and tridodecanoylglycerol concentrations were 0.2 mM and each monoacid triacylglycerol was incubated individually with the enzyme. The extent of lipolysis was measured by the disappearance of the intact triacylglycerol after the incubation of the substrate in the assay mixture for 10 min at 37°C. Triplicate samples (0.5 ml) were removed from each assay mixture and added to 4 ml of n-heptane-isopropanol 3:7 (v/v) containing 50 μg of cholesteryl butyrate as internal standard for gas-liquid chromatography analyses. After being acidified with 2.5 ml of 0.033 N H_2SO_4, the mixture was vortexed for 30

TABLE 1. Effect of trioleoylglycerol and albumin on the concentration of dialyzable form of taurocholate[a]

Additive	Filtrate Taurocholate Concentration[b]	
	Total Taurocholate Concentration 1 mM[c]	Total Taurocholate Concentration 10 mM[c]
	%	
1. No addition	97.0 ± 0.2	87.7 ± 3.2
2. Bovine serum albumin (1 mM)	11.3 ± 0.6	35.5 ± 0.7
3. Trioleoylglycerol (1 mM)	78.3 ± 1.8	76.3 ± 2.6
4. Trioleoylglycerol (10 mM) + bovine serum albumin (1 mM)	6.2 ± 0.2	35.4 ± 1.2

[a] Ultrafiltration was performed using YM5 membrane with a molecular weight cutoff of 5000 and in 50 mM NH_4OH-HCl buffer, pH 8.5.
[b] The taurocholate concentration in the filtrate (mean ± SD, n = 3), expressed in percent of total taurocholate concentration.
[c] The taurocholate concentration in the Diaflo cell.

sec; the organic phase containing the triacylglycerols and the internal standard was transferred to a 3-ml conical tube and the solvent was evaporated under nitrogen. The residue was redissolved in 100 μl of n-hexane and 2-μl aliquots were injected into the gas chromatograph. The pattern of separation of triacylglycerols has been shown previously (10).

Analytical ultracentrifugal analyses

Ultracentrifugal analyses were carried out in a Spinco Model E ultracentrifuge equipped with a phase schlieren diaphragm and interference optics, as well as an automatic temperature control unit. The sedimentation rate was determined at constant temperature (20°C) using a rotor speed of 52,000 rpm. The sedimentation coefficient was calculated as described by Schachman (11). The molecular weight of BAL was estimated by the sedimentation equilibrium procedure of Yphantis (12), using a rotor speed of 17,000 rpm. The enzyme was dissolved in 5 mM NH_4OH-HCl solution (0.5 mg/ml), pH 8.5, and dialyzed in 1 liter of the same buffer for 48 hr at 4°C. The partial specific volume of BAL was calculated to be 0.727 ml/g based on the amino acid composition and carbohydrate composition of the enzyme (1).

Other methods

Protein content in samples was determined as described previously (13). Bovine serum albumin was used as the standard. Linear regression analyses were performed with a Hewlett-Packard HP-97 programmable calculator.

RESULTS

Effect of taurocholate on BAL sedimentation rate and molecular weight

Because of the glycoprotein nature of BAL (1), its molecular weight cannot be accurately measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; therefore, in the present study we performed analytical ultracentrifugaton in order to determine the molecular weight of the enzyme and to examine its hydrodynamic properties.

By the sedimentation rate analysis, the enzyme was characterized by a single, symmetrical boundary (**Fig. 1**) with an apparent sedimentaton coefficient of 4.44 S in the absence and 4.34 S in the presence of taurocholate (1 mM). Similarly, in the absence and presence of taurocholate, the respective molecular weights of 105,000 and 107,000 were determined by sedimentation equilibrium analyses. These results indicated that taurocholate did not alter the molecular size of the enzyme. Based on the approximate molecular weight of 125,000 of the subunit of BAL by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1), the results suggested that the intact BAL is composed of a single polypeptide chain.

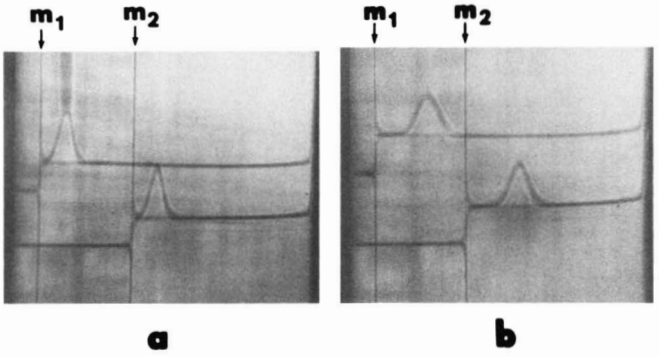

Fig. 1. The schlieren patterns of BAL. The samples (5 mg/ml) were dissolved in 5 mM NH_4OH-HCL buffer, pH 8.5, and in the presence (upper pattern) and absence (lower pattern) of 1 mM taurocholate. The arrows m_1 and m_2 are the meniscus of the upper and lower cell, respectively. Exposure was taken at (a) 16 min and (b) 44 min after reaching rotor speed of 52,000 rpm at 20°C.

Basal activity of BAL

Our previous study indicated that taurocholate represents a nonessential activator for BAL with water-soluble substrate, but an essential activator with water-insoluble long chain triacylglycerol substrate. The distinction of nonessential versus essential activation was based on the presence versus absence of basal activity of the enzyme (14).

One of the reasons for the difference of the enzyme reactivity toward these substrates could be the inaccessibility of the active site of the enzyme to the long chain triacylglycerol molecule by a steric hindrance effect. Based on this reasoning, we performed the assay by incubating each monoacid triacylglycerol of various fatty acyl-chain length with the enzyme for probing the size of the active site of the enzyme. We performed the experiment at high enzyme concentration (1 mg/ml) to exclude the possibility of failure to detect the possible presence of a low level of basal activity. As shown in **Fig. 2**, both trihexanoyl- and triheptanoylglycerol were degraded completely in the time period of incubation (10 min) while trioctanoylglycerol was partially degraded (80%). On the other hand, trinonanoyl-, tridecanoyl-, and tridodecanoylglycerol were not degraded by the enzyme even with prolonged (60 min) incubation.

Kinetic property of BAL with trioleoylglycerol as substrate

In this study, we performed kinetic analyses by maintaining the substrate at a fixed concentration while varying the activator taurocholate concentration (**Fig. 3**) or by maintaining taurocholate at several fixed levels (4, 10, and 20 mM) while varying trioleoylglycerol concentration (**Fig. 4**). In the first (Fig. 3), we observed that BAL showed a sigmoidal response in enzyme activity with increasing activator (taurocholate) concentration. Similar sigmoidal response of the enzyme has also been reported

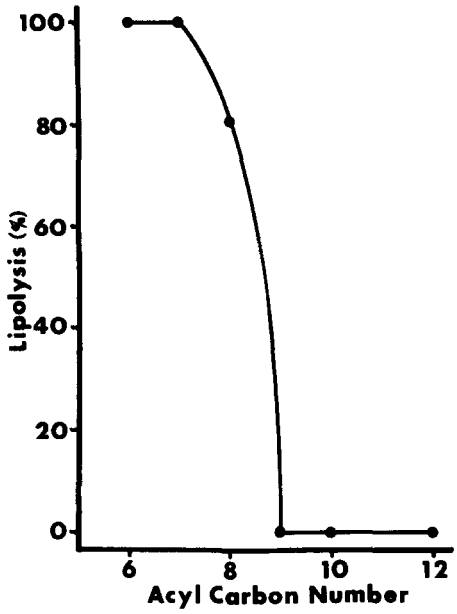

Fig. 2. The lipolysis of trihexanoyl-, triheptanoyl-, trioctanoyl-, trinonanoyl-, tridecanoyl-, and tridodecanoylglycerol with BAL (1 mg/ml) in the absence of taurocholate. Each triacylglycerol was incubated with the enzyme individually at 37°C for 10 min. Bovine serum albumin (60 mg/ml) was used as fatty acid acceptor. Acyl carbon number represents the fatty acid chain-length of each monoacid triacylglycerol. The extent of lipolysis was expressed by the extent of decrease in the intact triacylglycerol concentration as determined by gas-liquid chromatography.

previously by Hernell and Olivecrona (2). Because the enzyme is composed of a single subunit, the sigmoidal response of the enzyme to the increase of taurocholate was considered not due to the cooperativity of subunits upon binding with the ligand. Rather, we postulate that the initial lag phase for activation may be due to the interaction of taurocholate with bovine serum albumin and/or with the substrate which may reduce the effective taurocholate concentration for activation of the enzyme. This was found to be the case. As can be seen from the binding study (Table 1), the presence of bovine serum albumin could reduce the concentration of the free form of taurocholate. Emulsified trioleoylglycerol (10 mM) could also reduce the effective taurocholate concentration but to a lesser extent than bovine serum albumin (1 mM).

When taurocholate was held at several fixed levels (4, 10, and 20 mM), it was obvious that the apparent maximal catalytic rate of the enzyme (V_{max}) increased with taurocholate concentraton. Furthermore, the increase in the taurocholate concentration also resulted in a right-shift for the apparent V_{max} of the enzyme in response to the trioleoylglycerol. However, a further increase in trioleoylglycerol concentration beyond the apparent V_{max} resulted in a substrate inhibition effect (Fig. 4).

Binding of BAL to trioleoylglycerol emulsion

In the absence of taurocholate, we could not demonstrate any binding of BAL to trioleoylglycerol emulsion when binding assays were performed either at 0°C or 37°C. A further increase in the incubation time from 4 min to 60 min also did not lead to any detectable binding (data not shown). However, in the presence of 40 mM taurocholate, the binding of BAL to trioleoylglycerol emulsion could be detected (**Fig. 5**). Because of the detectable lipolysis of trioleoylglycerol emulsion when taurocholate is present (40 mM) at 37°C, but not at 0°C, and the binding assay requirement for maintenance of the trioleoylglycerol at a fixed concentration, we performed the Scatchard analyses only with results obtained at 0°C. The equation for the Scatchard plot (9) was

$$\frac{[B]}{[F]} = \frac{1}{K_s}[S]_t - \frac{1}{K_s}[B] \qquad \textit{Eq. 1}$$

where [B] and [F] are the concentrations of bound and free forms of the enzyme, respectively; $[S]_t$ is the maximal binding capacity of the surface of trioleoylglycerol emulsion; K_s is the apparent dissociation constant for BAL to trioleoylglycerol emulsion when bile salt is present. From the plot, a K_s value of 5.1×10^{-8} M was obtained from Fig. 5.

DISCUSSION

Based on the sedimentation equilibrium and sedimentation velocity data, we concluded that taurocholate at 1

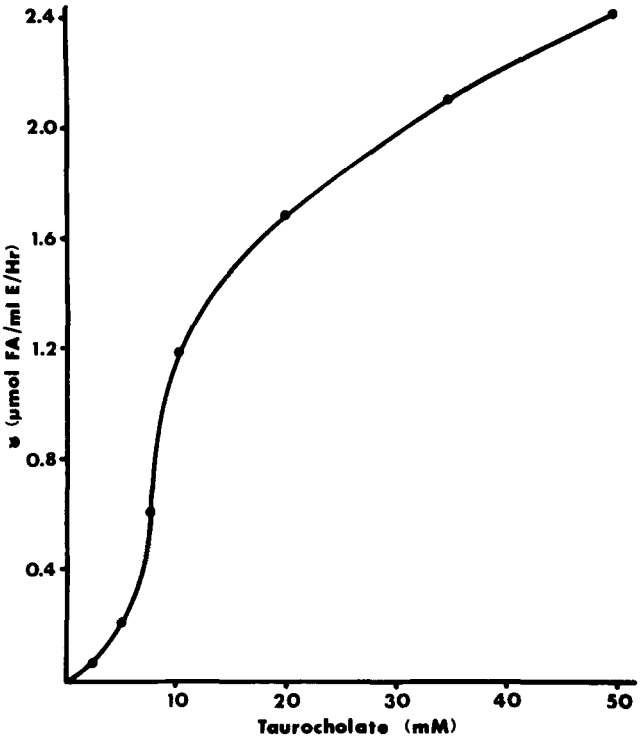

Fig. 3. Lipolytic activity of BAL as function of taurocholate concentration. The substrate trioleoylglycerol concentration was 10 mM and the enzyme concentration was 5 μg/ml. Bovine serum albumin (60 mg/ml) was used as fatty acid acceptor.

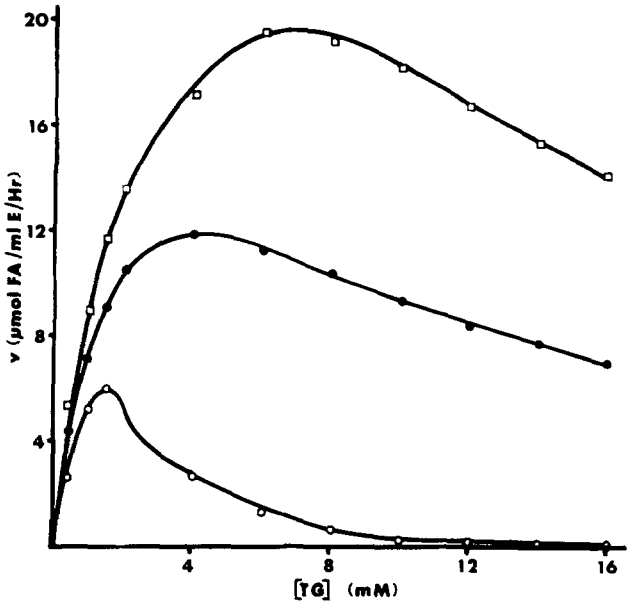

Fig. 4. Lipolytic activity of BAL as function of trioleoylglycerol (TG). Taurocholate concentrations used were: (○), 4 mM; (●), 10 mM; (□), 20 mM. The enzyme concentration was 5 μg/ml. Bovine serum albumin (60 mg/ml) was used as fatty acid acceptor.

mM, i.e., at 200-fold concentration of taurocholate over BAL, did not lead to alteration in molecular size of the enzyme. Analytical ultracentrifugation of BAL in the presence of high concentration of taurocholate (50 mM) was also performed; however, the high concentration of taurocholate interfered with the molecular weight determination due to micelle formation. Based on the dissociation constant of BAL-taurocholate of 0.37 mM as determined by equilibrium dialysis (1), the interaction of the enzyme with taurocholate (1 mM) could be considered to essentially reach completion. Because of the glycoprotein nature of BAL and probably with its abnormal secondary structure due to its rich proline content, the molecular weight could not be accurately determined by the empirical method of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). This could account for the wide range of subunit molecular weights reported for the enzyme—from 90,000 (16), 107,000 (5), to 125,000 (17). The more reliable measurement of molecular weight of BAL by sedimentation equilibrium indicated that it has a molecular weight of 105,000–107,000. Using this value, and assuming the presence of 15 moles of methionine per polypeptide chain, the best-fitted molecular weight of the enzyme gave a calculated molecular weight of 107,000. Based on a similar magnitude of molecular weight as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (mol wt = 125,000) and by sedimentation equilibrium (mol wt = 105,000–107,000), the results indicated that the enzyme is composed of a single subunit. Thus, we have further concluded that the sigmoidal response of BAL to an increase in taurocholate concentration (Fig. 3) is not due to the cooperativity of subunits, as seen for hemoglobin binding with oxygen (18).

From probing the accessibility of the active site of BAL with various monoacid triacylglycerol substrates, we have concluded that, in the absence of the activator, the enzyme could interact directly only with trioctanoylglycerol and shorter chain monoacid triacylglycerols. For longer chain monoacid triacylglycerols, the binding of BAL to these substrates requires the presence of bile salt. The lack of basal activity of BAL with these substrates is correlated with the lack of binding of BAL to a trioleoylglycerol emulsion when bile salt activator is absent. Based on this study, we also suggest that the binding of BAL with the substrate involves direct interaction of the active site of the enzyme with the acyl-chain in the trioleoylglycerol emulsion rather than through nonspecific hydrophobic interactions. Thus, the BAL-catalyzed lipolysis of long chain triacylglycerols can be described to follow a compulsory, sequential mechanism as described below:

$$E + A \overset{K_A}{\rightleftharpoons} EA + S \overset{K_S}{\rightleftharpoons} EAS \xrightarrow{k_p} EA + P + Q \quad \textit{Eq. 2}$$

where E is the enzyme, BAL; A is the activator (taurocholate); S is the long chain triacylglycerol substrate; P and Q represent the alcoholic and the fatty acid lipolysis

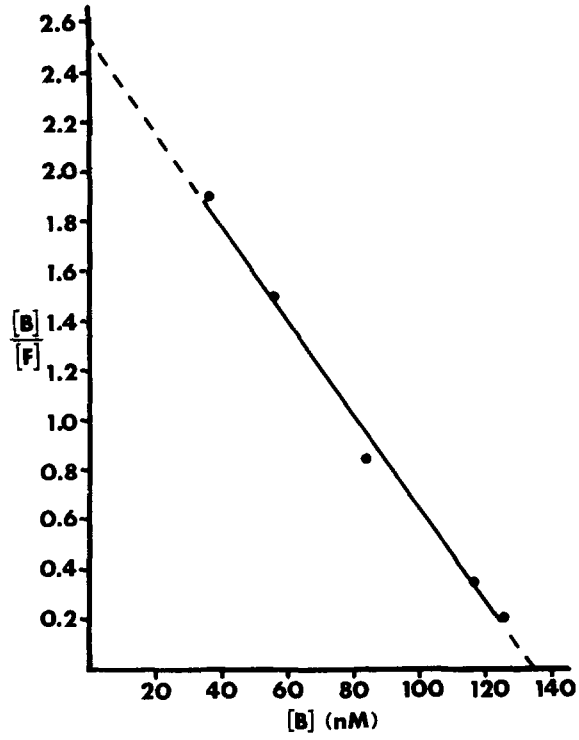

Fig. 5. Binding of BAL to the trioleoylglycerol emulsion. [B] and [F] represent the concentration of bound form and free form of BAL. Details are described in Experimental Procedures.

products; K_A represents the dissociation constant for E:A binary complex, while K_S represents the dissociation constant of EA:S ternary complex; k_p is the rate constant for the breakdown of EAS to EA + P + Q. Previously, we have demonstrated that the interaction of BAL with taurocholate leads to a conformational change of the enzyme (4). Obviously, the conformational change of the enzyme could further facilitate the interaction with the bulky triacylglycerol molecule. This reaction scheme (equation 2) is different from the reaction kinetics observed with *p*-nitrophenyl acetate as substrate (1). In contrast to the sigmoidal response of BAL with trioleoylglycerol as substrate, the enzyme exhibited a saturable hyperbolic response to the increase in taurocholate concentration with respect to the intrinsic fluorescence change (4) and with the activity of the enzyme with *p*-nitrophenyl acetate as substrate. With *p*-nitrophenyl acetate as substrate, it is possible to form E:S binary complex and the enzyme reaction was found to occur through a random pathway with respect to the sequence of the activator and substrate binding to the enzyme (1). It is likely that the lipolysis of trioctanoyl- and shorter chain triacylglycerol as catalyzed by BAL undergoes a reaction pathway similar to that described for *p*-nitrophenyl acetate due to the presence of basal activity of the enzyme with these substrates.

The direct effect of taurocholate on BAL to facilitate its further binding with bulky triacylglycerol substrate is clearly demonstrated by the binding studies (Fig. 5). However, the amphiphilic nature of the bile salt could lead to additional interaction with other constituents in the lipase assay mixture, and this in turn will affect the observed lipolytic activity. For these reasons, the influence of such interaction on lipolytic activity of BAL deserves a more detailed discussion. Both bovine serum albumin and trioleoylglycerol substrate in the lipase mixture could interact directly with the activator, taurocholate, as shown in Table 1. In addition, these various components in the lipase assay mixture could also possibly alter the equilibrium of the interconversion of monomer-micellar forms of taurocholate. For these reasons, the taurocholate concentration in the lipase mixture can only be considered to be nominal. The effective taurocholate concentration is obviously much smaller, as evidenced from the results shown in Table 1. Thus, the presence of a lag phase of activation of BAL in response to the increase in taurocholate concentration could be attributed to the above described interactions. Similarly, the substrate inhibition effect of trioleoylglycerol at high [trioleoylglycerol]/[taurocholate] ratio could be attributed to the resulting decreased "effective taurocholate concentration" in the lipase assay mixture due to interaction of trioleoylglycerol emulsion and taurocholate. The results also suggest that the enzyme could not recognize substrate-bound taurocholate as activator. Conversely, the increase in the taurocholate concentration could lead to a better emulsification of the substrate and thereby increase the "effective substrate concentration" even if the quantity of the substrate in the assay mixture was not varied. The increase in "effective substrate concentration" probably contributed to the apparent nonsaturable behavior of taurocholate in the activation of BAL (Fig. 3). Because of the aforementioned interactions, the Michaelis-Menten kinetic treatment cannot be applied to this reaction system.

In this kinetic study, we have performed the binding assays by taking advantage of the fact that concentrations of the free form and substrate-bound form of BAL can be readily determined due to the water-insolubility of the substrate. We expressed the substrate concentration as the number of the enzyme binding sites, so that the problem of determining the "effective substrate concentration" can be avoided. We performed the binding study at 0°C, which was found to be useful for observing the binding effect of BAL when taurocholate was present (Fig. 5), without any detectable degradation of the trioleoylglycerol. Under the experimental conditions, it is interesting to note that taurocholate transforms BAL from no affinity to a high binding affinity with K_S value of 5.1×10^{-8} M trioleoylglycerol emulsion at 0°C.

The optimum reactivity of BAL occurs when the [bile salt]/[trioleoylglycerol] ratio is high. This is in contrast to pancreatic lipase, which does not require bile salt as "true cofactor" (19) by enzyme-bile salt interaction. In fact, pancreatic lipase is inactivated by bile salt unless colipase is present (20). Based on this kinetic property of pancreatic lipase, we suggest that human milk BAL may be functional at its optimum catalytic capacity after the initial partial digestion of milk triacylglycerol by pancreatic lipase. The lipolysis of bile salt-emulsified milk triacylglycerol by pancreatic lipase in the intestinal lumen will lead to a gradually decreasing efficiency of pancreatic lipase. On the other hand, the resulting increase in [bile salt]/[triacylglycerol] ratio will increase the catalytic efficiency of BAL due to the high affinity of the EA complex to long chain triacylglycerols. Consequently, the milk fat may be quantitatively digested. This dual lipolytic system serves to minimize the amount of fat escaping from lipolysis by the lipolytic enzymes in the upper intestine. An incomplete lipolysis of milk fat could result in steatorrhea in the infant. We have also shown recently (21) that BAL has a special potential for a rapid release of short chain and polyunsaturated fatty acids from mixed-acid triacylglycerols. Thus, the presence of BAL in human milk ensures a maximal digestion of milk fat and optimal absorption of essential fatty acids by the infant.

We would like to express our gratitude to Dr. P. Alaupovic for his continued interest in this study and to Drs. Walter J. McConathy and H. U. Kloer for their criticism and suggestions. The technical assistance of H. B. Bass, D. Downs, and R. Whitmer is also appreciated. We also wish to thank Ms. D. C.

Link, Director of Mothers Milk Bank, Shawnee Mission Medical Center, Shawnee Mission, Kansas, for providing human milk for this study. This study was supported by Grants HL 23181 and HD 14104 from the U.S. Public Health Service and by the resources of the Oklahoma Medical Research Foundation.

Manuscript received 22 October 1984.

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