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Activity of bile-salt-stimulated human milk lipase in the presence of liposomes and mixed taurocholate-phosphatidylcholine micelles

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(1) The interaction of bile-salt-stimulated human milk lipase and liposomal membranes has been investigated in the presence or absence of sodium taurocholate. Freshly purified enzyme enhances the permeability of liposomal membranes but thermally inactivated enzyme does not. (2) The ability of the enzyme to catalyze the hydrolysis of a relatively hydrophilic substrate, 4-nitrophenyl acetate, and a more hydrophobic substrate, 4-nitrophenyl palmitate, has also been measured in media containing small unilamellar vesicles of egg phosphatidylcholine in both the absence and presence of taurocholate, and also in the presence of free taurocholate in the absence of liposomes. (3) The enzyme-catalyzed hydrolysis of 4-nitrophenyl acetate is enhanced in all of these systems, but 4-nitrophenyl palmitate is protected from enzymic attack in the phosphatidylcholine-bile salt systems. If free taurocholate be present in the system before 4-nitrophenyl palmitate is added, then, and only then, is enzymic activity observed. (4) These results have been interpreted in terms of the importance of the microenvironment around the substrate and the role played by the bile salt surfactant in stimulating the enzyme.

Introduction

It has recently been shown [1] that taurodeoxycholate and taurocholate, TC, are able to destroy the membrane of cow-, goat- and human-milk-fat globules. This membrane represents a barrier to hydrolysis of fats and esters for catalyzing enzymes, and in particular to the activity of bile-salt-stimulated human milk lipase, BSSL. The membrane in milk is composed of phospholipids, proteins and lipids [2] and although the membrane becomes thinner as lactation progresses, its gross composition remains relatively unchanged [2a]. The importance of bile-salt-stimulated lipase to the digestion of human milk is well recognized [3–5] and recently it has been shown that surfactants, other than bile salts [6], and proteins [7,8] also influence its activity.

This present study suggests that the bile salt and the enzyme play supportive roles in both

Abbreviations: BSSL, bile-salt-stimulated lipase; BTB, Bromothymol blue; CF, carboxyfluorescein; PC, phosphatidylcholine; PNPA, 4-nitrophenyl acetate; PNPP, 4-nitrophenyl palmitate; SUV, small unilamellar vesicles; TC, taurocholate; Tris, tris(hydroxymethyl)aminomethane; $R_e = [TC]_{bilayer} : [PC]$, effective molar ratio of TC to PC in the vesicles.

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hydrolyzing the contents of milk fat globules and in destroying the membrane thereof. We have found that bile-salt-stimulated lipase supports taurocholate in lysing an egg phosphatidylcholine, egg PC, liposomal membrane. The interactions of taurocholate with egg PC small unilamellar vesicles, SUV, are described in the preceding paper [9] and we now investigate the activity of bile-salt-stimulated lipase against the artificial substrates 4-nitrophenyl acetate, PNPA, and 4-nitrophenyl palmitate, PNPP, in these mixed TC-egg PC systems.

The amphiphilic nature of a protein allows it to aggregate at an interface, thereby altering the nature of the interface and, in the case of an enzyme, decreasing accessibility of the substrate to the active site. The natural substrates for lipases, the long chain triacylglycerols, are insoluble in water and in that solvent they remain associated to yield a distinct phase, separated from the water by an interface. However, bile-salt-stimulated lipase also acts as an esterase [5] and catalyzes the hydrolysis of a wide variety of water soluble esters. It is therefore useful to regard it as a special case of an esterase which has a high activity towards water insoluble substrates. Against these compounds its reactions may be regarded as interfacial and the enzymic activity will be related to the interfacial area of the insoluble substrate. Bile salts are necessary as a cofactor in order to activate these reactions. In the case of water soluble substrates, the primary hydrolytic function of the enzyme is probably to act as an acyl transfer agent and bile salts stimulate the catalysis. Surface activity of the water-soluble substrate is also an important factor in the reactivity of bile-salt-stimulated lipase and one of the roles of the bile salt is to enhance this surface activity [10]. In the light of the dual hydrolytic roles of bile-salt-stimulated lipase we deemed it necessary to carry out these present studies in the presence of both a more hydrophilic and a more hydrophobic substrate, utilizing in each case the chromophoric properties of the 4-nitrophenyl group as the probe to follow the hydrolytic reaction.

Materials and Methods

The source of most of the materials, a description of the preparation of the SUV and the SUV loaded with carboxyfluorescein (SUV-CF) or Bromothymol blue (SUV-BTB) and the methods used to study the release of these probes are given in the preceding paper [9]. Additional experimental details are given below.

Materials. 4-Nitrophenyl acetate was obtained from Nakarai Chemicals Ltd., (Kyoto) and 4-nitrophenyl palmitate from Merck, Darmstadt. LysoPC was obtained by enzymatic digestion of egg PC using phospholipase A_2 from *Crotalus adamateus* venom [11]. Bile-salt-stimulated lipase was purified following the description in the literature [12] using heparin Sepharose CL-6B from Pharmacia Fine Chemicals, Uppsala and Affi-Gel Blue (50–100 mesh) from Bio-Rad, Richmond, CA as reported earlier [13].

The activity of the enzyme was measured, during purification, with 4-nitrophenyl acetate as substrate (initial concentration 1 mM) at pH 7.5 (0.1 M Tris-HCl) in the presence of 2 mM taurocholate at 25.0 °C [13]. The protein concentration was determined by the method of Lowry et al. [14], using bovine serum albumin as standard.

Heat inactivation of bile-salt-stimulated lipase. An aqueous solution of bile-salt-stimulated lipase (0.9 $mg \cdot ml^{-1}$) was treated at 65.0 °C for 45 min in the absence of bile salts. Under these conditions the enzyme is inactivated [15,16] against 4-nitrophenyl acetate (1 mM, pH 7.5, in the presence of 2 mM taurocholate) or 4-nitrophenyl palmitate (12 μm, pH 8.6, in the presence of 0.4 mM taurocholate). Comparison of the ultraviolet spectra of the active and heat inactivated enzyme showed that no precipitation occurred during the heating. Treatment of bile-salt-stimulated lipase at 37.0 °C for 30 min did not inactivate the enzyme.

Activity measurements of bile-salt-stimulated lipase against egg PC as a possible substrate. The activity of bile-salt-stimulated lipase against egg PC, presented in the form of SUV, has been checked qualitatively after incubation of SUV (0.11 mM PC) in the presence of taurocholate (0.8 mM) and bile-salt-stimulated lipase (5.8 or 17.7 $\mu g \cdot ml^{-1}$) at pH 8.6 (20 mM Tris-HCl, 200 mM NaCl) at room temperature for 3 or 14 h. The total volume of each incubation mixture was 1.0 ml and the incubation was stopped by freezing the samples. The lipids were then extracted by adding 90 μl of chloroform to the freeze-dried powder and

vortexing the suspension. The supernatant was analyzed by thin-layer chromatography (Silica-gel Spotfilm from Tokyo Kasei, Tokyo) with chloroform/methanol/water (70 : 30 : 4, by vol.) as eluant [17] and detected with iodine vapor.

Activity measurements of bile-salt-stimulated lipase against 4-nitrophenyl acetate (PNPA) and 4-nitrophenyl palmitate (PNPP). The following conditions prevailed in all measurements: 0.11 mM PC, $[PC]/[PNPA]_0 = 2.6$ or $[PC]/PNPP]_0 = 8.6$ (where the subscript 0 represents the initial concentration of substrate), bile-salt-stimulated lipase = 3.3 $\mu g \cdot ml^{-1}$, and $[TC]_{total} = 0$–6 mM. The substrate was added as a concentrated solution in acetonitrile to give a final acetonitrile concentration of 0.5% in a solvent containing 20 mM Tris-HCl, 200 mM NaCl (pH 8.6). We expected from results obtained in our previous study [9] that the microenvironment of the substrate within the TC-PC system would influence its availability for enzymic attack. Therefore, two methods were used to establish each set of final concentrations. In Method A, a concentrated (micellar) solution of taurocholate was added to the SUV suspension, followed (after mixing) by ester and then bile-salt-stimulated lipase. In Method B, taurocholate solutions of appropriate sub-micellar concentration were used as the base, and to these were added, in order, SUV, ester and bile-salt-stimulated lipase.

The formation of the 4-nitrophenolate ion was followed at 400 nm and 25°C using a Hitachi Spectrophotometer. The observed rate of hydrolysis was corrected for the non-enzymatic hydrolysis of ester at pH 8.6.

Results

This work deals with two main issues, namely (a) the effect of bile-salt-stimulated lipase on the lysing ability of taurocholate against PC liposomes, and (b) the effect of PC liposomes and TC-PC mixed micelles on the activity of bile-salt-stimulated lipase against 4-nitrophenyl acetate and 4-nitrophenyl palmitate. The following section presents the results according to these two issues.

Bile-salt-stimulated lipase-SUV interactions

The effect of bile-salt-stimulated lipase on the lysing effect of various concentrations of

TABLE I

EFFECT OF BILE-SALT-STIMULATED LIPASE (BSSL) ON THE CARBOXYFLUORESCEIN (CF) OR BROMOTHYMOL BLUE (BTB) RELEASE FROM SUV IN THE PRESENCE OF TAUROCHOLATE

Run	$[TC]_{tot}$ (mM)	R_e [a]	BSSL ($\mu g \cdot ml^{-1}$)			Released after 15 min (%)	
			active	heat inactivated [b]	heat treated	CF [c]	BTB [d]
1 [c,d]	0	0				1.0	50
2 [c,d]	0	0	5.8			1.0	50
3 [c,d]	0.4	0.09				1.0	64
4 [c,d]	0.4	0.09	5.8			1.0	65
5 [c,d]	0.8	0.17				4.0	69
6 [c,d]	0.8	0.17	5.8			5.0	76
7 [d]	0.8	0.17		5.8			69
8 [d]	0.8	0.17			58		76
9 [c,d]	1.2	0.26				22.0	78
10 [c,d]	1.2	0.26	2.9			29.5	83
11 [c,d]	1.2	0.26	5.8			35.5	85
12 [c]	1.2	0.26		5.8		22.0	

[a] $R_e = [TC]_{bilayer} : [PC]$. For calculation of values see legend to Fig. 1, preceding paper [9].

[b] BSSL treated at 37°C for 30 min prior to assay.

[c] 20 mM Tris-HCl, 200 mM NaCl (pH 8.6), 25°C; 0.092 mM PC; carboxyfluorescein concentration in the interior water phase at time zero 200 mM.

[d] Internal pH 5.0 (20 mM NaOAC, 200 mM NaCl); external pH 8.6 (20 mM Tris-HCl, 200 mM NaCl), 25°C; 0.11 mM PC, $[BTB]_{total}$ 5.5 μM.

taurocholate is given, in Table I, as values of percent release, after 15 min reaction time, of either a hydrophilic (carboxyfluorescein) or a hydrophobic (Bromothymol blue) probe from the SUV, at pH 8.6. Under conditions used, bile-salt-stimulated lipase had no effect on the release of either carboxyfluorescein or Bromothymol blue from the liposomes in the absence of taurocholate (runs 1 and 2). At a total taurocholate concentration of 0.4 mm, there was still no effect of bile-salt-stimulated lipase on the carboxyfluorescein release (runs 3^c and 4^c), whereas in the presence of 0.8 mM taurocholate the effect was measurable, but comparatively small (runs 5^c and 6^c). However, at 1.2 mM taurocholate (or 1.6 mM taurocholate, data not shown) the release of carboxyfluorescein was markedly increased in the presence of bile-salt-stimulated lipase. This increase was dependent on the enzyme concentration (runs 9^c, 10^c and 11^c) but was not observed in the presence of heat inactivated lipase (run 12^c). On the other hand, the effect on the Bromothymol blue release was small at a total taurocholate concentration of 0.4 mM (runs 3^d and 4^d), moderate at 0.6 mM (data not shown) and relatively high at 0.8 (runs 5^d and 6^d), 1.2 (runs 9^d, 10^d and 11^d) and 1.6 (data not shown) mM taurocholate. Although heat inactivated enzyme had no effect on the Bromothymol blue release in the presence of 0.8 mM taurocholate (run 7^d), bile-salt-stimulated lipase which had been incubated for 30 min at 37.0 °C prior to the assay remained fully active (run 8^d). These data indicate that active enzyme increases the release of carboxyfluorescein or Bromothymol blue from the liposomes most noticeably in solutions of taurocholate more concentrated than 0.6–0.8 mM. Quantitative estimation of the effect of the enzyme is difficult, but based on these observations, there is evidence that active bile-salt-stimulated lipase interacts with SUV in the presence of a critical concentration of taurocholate. Preliminary data showed that the effect of the enzyme on the release of carboxyfluorescein or Bromothymol blue is not a consequence of the hydrolysis of a substantial amount of PC. Even after an incubation period of 14 h at room temperature, hydrolysis of egg PC could not be detected, under the conditions used. (See Materials and Methods in the preceding paper [9]: the PC spot, R_f 0.29, did not disappear and no new spot appeared on the silica-gel plate. LysoPC would have appeared at R_f 0.12.)

Activity of bile-salt-stimulated lipase against 4-nitrophenyl acetate and 4-nitrophenyl palmitate

The activity of bile-salt-stimulated lipase was measured by using two synthetic 4-nitrophenyl esters as substrates. 4-Nitrophenyl acetate is water soluble and it is not expected that this hydrophilic compound will interact with the hydrophobic domain of a phosphatidylcholine bilayer. In contrast to 4-nitrophenyl acetate, the 4-nitrophenyl ester of palmitic acid, PNPP, has low water-solubility and therefore it is expected that it will be intercalated into the liposomal membranes. In the case of the hydrophobic 4-nitrophenyl palmitate, the order of addition of the different components in the system studied is of great importance (see Materials and Methods and below).

Activity of bile-salt-stimulated lipase against 4-nitrophenyl acetate (PNPA). It has been shown previously that bile-salt-stimulated lipase catalyzes the hydrolysis of water soluble PNPA [14,18,19]. The activity of bile-salt-stimulated lipase against PNPA (initial concentration 12.9 μM), measured as percent PNPA hydrolyzed after 3 min at pH 8.6 in the absence and presence of 0.11 mM egg PC at a total taurocholate concentration of 0.4 mM or 6.0 mM, is given in Table II. Although the order of addition of the substrate and the bile salt to the vesicles was not important (indicating that PNPA is not significantly intercalated into the TC-SUV under the conditions used), the order of addition of bile salt to vesicles, or vice versa, was important. Consider the data obtained in the presence of 0.4 mM taurocholate. If a micellar solution of taurocholate were added to the SUV, the activity of bile-salt-stimulated lipase against PNPA was greater than it was under conditions when SUV were added to submicellar taurocholate (compare methods A and B). In the latter case, however, (runs 3, 5 and 7) the activity was decreased compared with that obtained in the absence of PC (run 1). In the case of 6.0 mM taurocholate the order of addition of SUV and taurocholate was not important (runs 9 and 10). At this higher concentration of taurocholate the SUV are transformed relatively rapidly into mixed

TABLE II

ACTIVITY OF BILE-SALT-STIMULATED LIPASE (BSSL) AGAINST WATER SOLUBLE 4-NITROPHENYL ACETATE (PNPA) IN THE PRESENCE OF EGG PC AND TAUROCHOLATE (TC)

20 mM Tris-HCl, 200 mM NaCl (pH 8.6), 25°C; $[PNPA]_o$ 12.9 μM, 0.5% (v/v) acetonitrile, BSSL 3.2 $\mu g \cdot ml^{-1}$.

Run	$[TC]_{tot}$ (mM)	[PC] (mM)	Method [a]	% PNPA hydrolyzed in 3 min (%)
1	0.4	0		42
2	0.4	0.02	A	42
3	0.4	0.02	B	32
4	0.4	0.04	A	48
5	0.4	0.04	B	28
6	0.4	0.11	A	64
7	0.4	0.11	B	23
8	6.0	0		74
9	6.0	0.11	A	100
10	6.0	0.11	B	100

[a] See Materials and Methods: Method A, TC added to SUV; Method B, SUV added to TC.

bile salt-PC micelles [9]. These mixed micelles seem to be better activators of the enzyme than is taurocholate alone (run 8). Some of these mixed micelles are also produced [9] when micellar taurocholate is added to SUV, $[TC]_{total} = 0.4$ mM (for example, run 6) and they enhance the enzyme-activity. This enhancement is dependent on the PC concentration, the effect being larger with a high initial SUV concentration (runs 2, 4 and 6).

Addition of SUV to 'submicellar' taurocholate leads to the formation of mixed vesicles [9], and consequently there is a reduction in the concentration of free taurocholate and a decrease in the activity of the enzyme (runs 0, 3, 5, and 7). It has previously been shown that the activity of bile-salt-stimulated lipase against PNPA is strongly dependent on concentrations of bile salt less than 1 mM taurocholate [3,5]. Moreover, the actual concentration of enzyme in the bulk water phase may be decreased due to interactions between bile-salt-stimulated lipase and SUV (see above). However, the decrease in the activity is dependent on the PC-concentration (runs 3, 5, and 7).

Activity of bile-salt-stimulated lipase against 4-nitrophenyl palmitate (PNPP). The activity of bile-salt-stimulated lipase against PNPP at pH 8.6 was measured using an initial substrate concentration of 12–15 μM and in the presence of varying amounts of taurocholate. The ultraviolet spectrum of PNPP (10 μM) in water containing 0.5% acetonitrile (by vol.) has a maximal absorbance at 276 nm, whereas λ_{max} of PNPA is 271 nm. This difference indicates self-aggregation of PNPP in the aqueous system, under the conditions employed. It was found that the enzyme is active against PNPP and that the activity is dependent on the concentration of taurocholate. This dependency is complex, reflecting different concentration-dependent interactions among the three species – substrate, bile salt and enzyme. Depending on the taurocholate concentration, three different activity patterns were observed.

(a) In the absence of taurocholate, about 25% of PNPP was hydrolyzed within 1 min under the conditions used (Fig. 1, curve 1) and then no further hydrolysis took place. (The same result was obtained in the presence of twice as much enzyme.) Further addition of substrate did not lead to continued hydrolysis (data not shown). This result indicates that, under these conditions, the activity of bile-salt-stimulated lipase is inhibited during the initial hydrolysis of PNPP.

(b) In the presence of as little as 20 μM taurocholate no inhibition of bile-salt-stimulated lipase activity was observed. All the substrate was hydrolyzed within 1 min under the conditions used. The same observation has been made in the presence of taurocholate in the concentration range 0.02–0.80 mM and the example of 0.4 mM taurocholate is shown in Fig. 1, curve 2.

(c) In the presence of 4 mM or 6 mM taurocholate, PNPP was initially hydrolyzed very slowly for one or two minutes under the conditions used, the rate of hydrolysis then increased and finally all the substrate was hydrolyzed (data not shown). Induction periods in lipolysis are very often observed in the hydrolysis in emulsions, micelles or monolayers and different explanations have been discussed [20]. The origin of the 'induction-period' in our case was not further investigated and we generally limited our further studies to a total taurocholate concentration of 0.4 mM.

We determined that, under the conditions used, bile-salt-stimulated lipase was not active against PNPP if the latter were incorporated into the lipid bilayer of egg PC-SUV. The observations leading

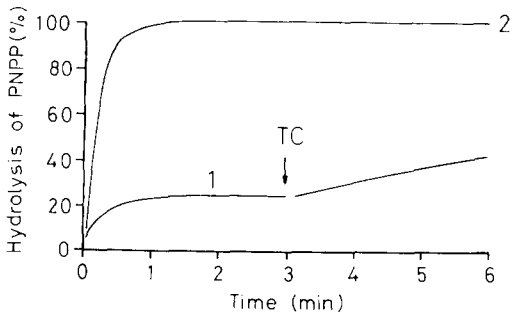

Fig. 1. Activity of bile-salt-stimulated lipase (BSSL) against 4-nitrophenyl palmitate (PNPP) in the absence of taurocholate (TC) (1) and presence of 0.4 mM TC (2). 20 mM Tris-HCl, 200 mM NaCl (pH 8.6), 25°C; $[PNPP]_0 = 12.8$ μM; 0.5% acetonitrile (by vol.); and $[BSSL] = 1.6$ μg·ml^{-1}. The arrow indicates the time of addition of taurocholate to give a final concentration of 0.4 mM.

to this conclusion follow. If PNPP were added to a SUV-solution, followed by taurocholate and finally bile-salt-stimulated lipase to give a total taurocholate concentration of either 0.4 or 0.2 mM, PNPP was not hydrolyzed. The same result was obtained when first bile-salt-stimulated lipase was added, then PNPP, and finally taurocholate (or first PNPP, then bile-salt-stimulated lipase, and finally taurocholate). On the other hand, when taurocholate was first added to the SUV solution, followed by PNPP and finally BSSL (or first taurocholate, then BSSL and finally PNPP; or first BSSL, then taurocholate and finally PNPP) 39% of the substrate was hydrolyzed in 3 min. However, the rate of hydrolysis of PNPP was decreased compared with a similar measurement carried out in the absence of PC (91% of the substrate hydrolyzed in 3 min).

Discussion

Although the importance of the bile-salt-stimulated lipase in human milk to the nutrition of the neonate has been recognized [3,21,22] a detailed biochemical characterization has not yet been made. Let us first discuss the results obtained from investigation of the effect of active or inactive bile-salt-stimulated lipase on SUV (Table I). These measurements were made before we studied the activity of bile-salt-stimulated lipase against the esters in the presence of the liposomes. The carboxyfluorescein-release technique has been used previously to monitor the lipoprotein lipase and phospholipase A_2 catalyzed hydrolysis of egg PC [17]. We found that active bile-salt-stimulated lipase influences the permeability of liposomal membranes, suggesting that the active enzyme interacts with the phospholipid bilayer in the presence of taurocholate. However, since such interactions do not correlate with a substantial hydrolysis of the PC molecules, the effect appears to be purely physicochemical and not chemical. It seems that, although active bile-salt-stimulated lipase binds to taurocholate and the BSSL-TC complex increases the permeability of the lipid membrane, heat-inactivated bile-salt-stimulated lipase does not bind to the bile salt and therefore has no effect on the permeability of membranes. It could also be that the increased permeability is due to an increased binding of bile-salt-stimulated lipase to taurocholate-containing SUV. It has been reported earlier that bile-salt-stimulated lipase is not active against PC [23] (a finding in agreement with our result), and neither was the closely related [24,25] human pancreatic carboxylic ester hydrolase when this was tested against an egg yolk suspension [2,6]. However, in the case of lipolytic enzymes, the physicochemical state of the substrate plays an important role in controlling the activity [20], and therefore it is unwise to jump to unjustified conclusions.

Let us now discuss the results obtained from the studies of the activity of bile-salt-stimulated lipase against the two synthetic substrates, 4-nitrophenyl acetate and 4-nitrophenyl palmitate, in the presence of liposomes. It has been reported [14,24,27] recently that the mechanism of bile-salt-stimulated lipase-catalyzed reactions in a bile salt-substrate-enzyme system is dependent on the substrate used (4-nitrophenyl acetate or trioleylglycerol). These findings are not surprising when we consider the different physical properties of these two substrates: namely, 4-nitrophenyl acetate is water soluble but trioleylglycerol is insoluble. In the present work, we studied the activity of bile-salt-stimulated lipase against 4-nitrophenyl acetate and 4-nitrophenyl palmitate. The latter substrate was chosen in order to utilize a property generally considered to be a disadvantage in enzyme studies [28], i.e. its ability to self-aggregate in an aqueous medium [29]. The estimated critical micelle con-

centration of PNPP in water at 25°C is 0.1 μM [28] and therefore at the concentrations we have used (10–15 μM) the substrate is presented to the enzyme as an aggregate. Differences in the physicochemical state of PNPP and PNPA in an aqueous medium are also indicated by differences in the ultraviolet spectra. PNPP has a maximal absorbance at 276 nm (identical with λ_{max} of 4-nitrophenyl laurate (10 μM) [30]), whereas λ_{max} of PNPA is 271 nm. The physicochemical state of PNPP in an aqueous medium can be changed by the presence of taurocholate. In fact, bile salts may strongly perturb the non-enzymatic hydrolysis rates of long chain 4-nitrophenyl carboxylates [31]. It was found that bile-salt-stimulated lipase seems to be most effective against PNPP, under the conditions used, in the presence of 0.02–0.40 mM taurocholate, being much more rapidly hydrolyzed than is PNPA. This result may be a reflection of differences in the affinity of bile-salt-stimulated lipase for the two substrates. It is known, for example, that K_m for 4-nitrophenyl acetate or 4-nitrophenyl propionate is much larger than the K_m for a more hydrophobic myristic acid ester [10]. The marked decrease in bile-salt-stimulated lipase activity against 4-nitrophenyl palmitate in the absence of bile salts (Fig. 1) may be the result of inhibition by one of the products of hydrolysis, namely palmitate. The subsequent addition of taurocholate to the reaction mixture (see arrow, Fig. 1), to give a final total concentration of 0.4 mM, only increased the rate of hydrolysis of 4-nitrophenyl palmitate very slightly, indicating a strong association of the fatty acid with the enzyme. If taurocholate were present initially (at a concentration, for example, of 0.4 mM) bile-salt-stimulated lipase is protected from this product-inhibition. It could be that taurocholate changes the aggregation state of the substrate (4-nitrophenyl palmitate) and that in this state the substrate is more rapidly hydrolyzed by the enzyme. However, we know that oleate is a competitive inhibitor of bile-salt-stimulated lipase, whereas acetate (or phenolate) is not [18], results which support our suggestion. In the present study, we selected 4-nitrophenyl palmitate as substrate for bile-salt-stimulated lipase because the hydrophobic nature of 4-nitrophenyl palmitate allows it to be intercalated into the phospholipid bilayer. Furthermore, palmitic acid is the most abundant saturated fatty acid in egg PC [32,33] and it is also known that bile-salt-stimulated lipase catalyzes the hydrolysis of emulsified retinol palmitate [18].

It has been suggested that stimulation of the esterase activity arises from two effects. First, there is a hydrophobic interaction between the enzyme and the bile salt, which essentially involves only the steroidal portion of the bile salt amphiphile [10]. Only one bile salt molecule is required per binding site in order to stimulate the enzyme but many bile salt monomers or small oligomers are adsorbed onto the surface of the enzyme [10]. Secondly, there is an electrostatic interaction. The bile salts will give a negative charge to the substrate surface, and the substrate-bile salt complex is more effective in stimulating the enzyme and generally presents a more attractive surface to the enzyme than does the substrate alone [10].

The hydrophobicity of the substrate is important in consideration of the activity of bile-salt-stimulated lipase against 4-nitrophenyl palmitate and 4-nitrophenyl acetate in the presence of egg PC. 4-Nitrophenyl acetate is water soluble and therefore it is unlikely to be intercalated into the hydrophobic domain of the lipid membranes. The base-catalyzed, non-enzymatic hydrolysis of 4-nitrophenyl acetate (12.8 μM) at pH 8.6 is not affected by liposomes (0.1 mM PC) (results not shown), thereby indicating that there is also no interaction between 4-nitrophenyl acetate and the liposomal membranes. Therefore, it seems that 4-nitrophenyl acetate remains in the bulk water phase. The observed increase in the activity of bile-salt-stimulated lipase against 4-nitrophenyl acetate in the presence of mixed TC-PC micelles (Table II) is in agreement with earlier observations, made using 4-nitrophenyl propionate as the substrate and a MLV dispersion of egg PC, under slightly different conditions [8]. An explanation for this observation cannot be made at present. Although it is uncertain whether these observations may be of physiological significance, it is probable that mixed micelles of bile salt-PC (or other lipophilic substances) are present in the small intestine of the breast fed neonate. In contrast to PNPA, PNPP is only sparingly soluble in water and can easily be incorporated into the

liposomal membranes at 25 °C, a temperature above the phase transition temperature of egg PC liposomes (approximately −15 °C [34]). The palmitate moiety has a relatively high affinity for the membranes [35], and polysaccharides, such as pullulan or amylopectin, which bear a hydrophobic palmitate anchor, have been used for coating the surface of black lipid membranes. This present study which compares the behavior of bile-salt-stimulated lipase against PNPP in the presence and absence of egg PC suggests that PNPP associates, under the conditions used, with lipid membranes and that this partition into the vesicles is perturbed by taurocholate. The different activity-behavior patterns may arise from the different distribution environments of the substrate between the phospholipid bilayer and the bulk water phase. PNPP is rapidly incorporated into the PC bilayer when it is added to the SUV before taurocholate is added, and in this location it cannot be attacked by the enzyme. However, when PNPP is added to the SUV after taurocholate has been added, the substrate remains (probably associated with taurocholate) in the bulk water phase and can be attacked by bile-salt-stimulated lipase. The presence of bile salt-PC mixed vesicles and free taurocholate do not favor incorporation of the fatty acid ester into the bilayers.

In the system we have studied there is a number of allowed and identified interactions: taurocholate [37] and PNPP [29] aggregate in an aqueous medium to form micellar structures whereas PNPA [28] does not; taurocholate binds strongly to bile-salt-stimulated lipase [18,38]; taurocholate weakly influences the non-enzymatic hydrolysis of 4-nitrophenyl esters [31,39] but strongly influences their bile-salt-stimulated lipase-catalyzed hydrolysis [5,38,40]; interactions between taurocholate and SUV lead to the formation of mixed bile salt-PC vesicles or mixed micelles [9,41], and bile-salt-stimulated lipase assists taurocholate to penetrate the liposomal bilayer [this work].

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References

1 Patton, S., Borgström, B., Stemberger, B.H. and Welsch, U. (1986) J. Pediatr. Gastroenterol. Nutr. 5, 262–267
2 (a) Anderson, M. and Cheeseman, G.C. (1970) in 18th Int. Dairy Congress (Flowers, G.W. and Loftus-Hills, G., eds.), Vol. IE, p. 72, 18th Dairy Congress Committee, Sydney; (b) Steward, P.S. and Irvine, D.M. (1970) in 18th Int. Dairy Congress (Flowers, G.W. and Loftus-Hills, G., eds.), Vol. IE, p. 71, 18th Dairy Congress Committee, Sydney
3 Hernell, O. and Bläckberg, L. (1982) Pediatr. Res. 16, 882–885
4 Bläckberg, L. and Hernell, O. (1983) FEBS Lett. 157, 337–341
5 O'Connor, C.J. and Wallace, R.G. (1985) Adv. Colloid Interface Sci. 22, 1–111
6 O'Connor, C.J. and Walde, P. (1986) Langmuir 2, 139–146
7 Erlanson-Albertsson, C., Sternby, B. and Johannesson, U. (1985) Biochim. Biophys. Acta 829, 282–287
8 O'Connor, C.J. and Walde, P. (1986) J. Colloid interface Sci. 112, 488–496
9 Walde, P., Sunamoto, J. and O'Connor, C.J. (1987) Biochim. Biophys. Acta 905, 30–38
10 O'Connor, C.J., Walde, P. and Wallace, R.G. (1986) J. Pediatr. Gastroenterol. Nutr. 5, 622–629
11 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314–326
12 Bläckberg, L. and Hernell, O. (1981) Eur. J. Biochem. 116, 221–225
13 O'Connor, C.J., Mitha, A.S.H. and Walde, P. (1986) Aust. J. Chem. 39, 245–257
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
15 Hernell, O. (1975) Eur. J. Clin. Invest. 5, 267–272
16 O'Connor, C.J., Longbottom, J.R. and Walde, P. (1986) J. Pediatr. Gastroeneterol. Nutr. 5, 630–637
17 Fugman, D.A., Shirai, K., Jackson, R.L. and Johnson, J.D. (1984) Biochim. Biophys. Acta 795, 191–195
18 Wang, C.-S. (1981) J. Biol. Chem. 256, 10198–10202
19 Hernell, O. and Olivecrona, T. (1974) Biochim. Biophys. Acta 369, 234–244
20 Verger, R. (1980) Methods Enzymol. 64B, 340–392
21 Fredrikzon, B., Hernell, O., Bläckberg, L. and Olivecrona, L. (1978) Pediatr. Res. 12, 1048–1052
22 Hernell, O. and Bläckberg, L. (1983) J. Pediatr. Gastroenterol. Nutr. 2 (Suppl. 1), 242–247

23 Freudenberg, E. (1966) Experientia 22, 317
24 Wang, C.-S. and Kloer, H.-U. (1983) Biochim. Biophys. Acta 754, 142–149
25 Bläckberg, L., Lombardo, D., Hernell, O., Guy, O. and Olivecrona, T. (1981) FEBS Lett. 136, 284–288
26 Lombardo, D., Fauvel, J. and Guy, O. (1980) Biochim. Biophys. Acta 611, 136–146
27 Wang, C.-S. and Lee, S.M. (1985) J. Lipid Res. 26, 824–830
28 Guthrie, J.P. (1973) Can. J. Chem. 51, 3494–3498
29 Murakami, Y., Aoyama, Y. and Kida, M. (1977) J. Chem. Soc. Perkin II, 1947–1952
30 Menger, F.M. and Portnoy, C.E. (1968) J. Am. Chem. Soc. 90, 1875–1878
31 Menger, F.M. and McCreery, M.J. (1974) J. Am. Chem. Soc. 96, 121–126
32 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53–56
33 Sunamoto, J., Baba, Y., Iwamoto, K. and Kondo, H. (1985) Biochim. Biophys. Acta 833, 144–150
34 Bach, D., Miller, I.R. and Sela, B.-A. (1982) Biochim. Biophys. Acta 686, 233–239
35 Shaw, I.H., Knight, C.G. and Dingle, J.T. (1976) Biochem. J. 158, 473–476
36 Moellerfeld, J., Prass, W., Ringsdorf, H., Hamasak, H. and Sunamoto, J. (1986) Biochim. Biophys. Acta 857, 265–270
37 Carey, M.S. and Small, D.M. (1972) Arch. Intern. Med. 130, 506–527
38 O'Connor, C.J. and Wallace, R.G. (1984) J. Colloid Interface Sci. 102, 539–547
39 O'Connor, C.J. and Wallace, R.G. (1984) Aust. J. Chem. 37, 1881–1893
40 O'Connor, C.J. and Wallace, R.G. (1984) Eur. J. Biochem. 141, 379–383
41 Mazer, N.A., Benedek, G.B. and Carey, M.C. (1980) Biochemistry 19, 601–615