Modification of a phospholipid stabilized emulsion interface by bile salt: effect on pancreatic lipase activity

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Abstract Lipase is activated by binding to an insoluble emulsified or aggregated substrate. The extent of binding is related to the physicochemical as well as the compositional structure of the interface, the quality of the interface. 'Quality' is as yet undefined but thought to contain contributions from electrostatic interactions, orientation of substrate, and hydration forces. To investigate the electrostatic and compositional factors we have used olive oil-in-water emulsions prepared with phosphatidylcholine and four bile salts of varying hydrophobicities. By measurement of the droplet zeta potential we have monitored semi-quantitatively the incorporation of bile salts within the interface. No correlation was found between droplet surface charge as monitored by the zeta potential and lag phase. The duration of the observed lag phase was found to be inversely related to the degree of incorporation of the bile salts. Simultaneously there was evidence of lipase binding to monomeric bile salts, reducing its availability for adsorption. Calcium ions reduced the surface charge but there was no correlation with lag phase duration. The evidence presented here agrees with a more specific role for calcium ions, i.e., the formation of a new catalytically active enzyme complex, (enzyme)–(mixed micelle)–(calcium ion). — **Wickham, M., M. Garrood, J. Leney, P. D. G. Wilson, and A. Fillery-Travis.** Modification of a phospholipid stabilized emulsion interface by bile salt: effect on pancreatic lipase activity. *J. Lipid Res.* 1998. **39:** 623–632.

Supplementary key words lipase • bile salts • interfaces

The characteristic feature of pancreatic lipase is its specificity of action on insoluble emulsified substrates; the enzyme is activated when it encounters an oil/water interface. The mechanism of this catalysis is still a subject of study but there is general agreement (1) that the initial stage involves a reversible penetration of the lipase into the interface. Here irreversible denaturation can occur or, alternatively, the enzyme can undergo a conformational change (2). This change allows binding with the substrate to produce the activated complex that decomposes to give the product phase. Thus it is the interfacial binding that regulates the concentration of lipase at the interface (3).

A common kinetic feature observed during lipolysis is a lag time before the establishment of steady state hydrolysis. It has been established (4) that these lag times are not due to diffusional limitations but rather to slow interfacial penetration of the enzyme. This penetration of the interface has been related to the physicochemical nature as well as the composition of the interface thus engendering the term 'interfacial quality', but to a large extent the term 'quality' is still undefined. The interfacial activation of lipase can be explained by a substrate theory and an enzyme theory. They are not mutually exclusive but may be regarded as complimentary descriptions of the process. The enzyme theory states that upon penetration the lipases undergo a conformational change that 'activates' the lipase towards its substrate. Recent X-ray crystallographic data have shown a lid on the N-terminal domain of the lipase that peels back to reveal the active site to the interface when the enzyme is bound to mixed micelles (2). The substrate theory states that lipase binding is dependent upon the organization of substrate within the interface and the enzyme will bind only to clusters of substrate molecules of sufficient size. If the bound lipase has only limited access to substrate clusters above this critical size, then the rate of hydrolysis will be limited.

Support for this view is provided by the work of Muderhwa and Brockman (5) who concentrated on the hydrolysis of 1,3-dioleoylglycerol with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine as a relatively low-affinity lipid 'spacer' in mixed lipid monolayers. They found evidence that the inclusion of species other than the substrate could enhance lipolysis, i.e., when phospholipids are incorporated within a triglyceride monolayer

Abbreviations: PC, l-α-phosphatidylcholine; NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate; NaTCDC, sodium taurochenodeoxycholate; NaGC, sodium glycocholate; CMC, critical micellar concentration.

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to 35% of the total lipid, lipolysis occurred three times faster than in a pure triglyceride film (6). Several groups have investigated this issue using emulsified substrates with detergents as interfacial spacers, but these studies are often hindered by the use of gum arabica as the principal emulsifier (7, 8). Gum arabica is a protein/polysaccharide complex that is highly surface active, giving a robust and negatively charged interface to the emulsion droplets (9). This makes it a poor emulsifier with which to study competitive absorption and the influence of charge on lipase absorption.

In this paper we have approached the problem of interfacial quality by using a detergent of physiological origin, the bile salts. There are a number of advantages in the use of these surfactants. First, they are the natural surfactants present within the gut lumen and would be expected to competitively adsorb onto the phospholipid interface of the gastric emulsions as they enter the duodenum, the major site of lipolysis (10). Second, although bile salts share a common steroid backbone they differ not only in their head group by being either glycine or taurine conjugated but also in the number and position of the hydroxyl groups on the ring system **(Fig. 1)**. This gives a series of compounds that differ little in size or overall charge but display a range of hydrophobicities. They would thus be expected to adsorb at the interface to differing extents (11). Third, although there have been a number of studies using taurodeoxycholate/phospholipid stabilized emulsions, these have left unresolved a number of issues (12–14). Specifically, the inclusion of bile salts is thought to impart a negative charge to the interface, thereby reducing lipase penetration by increasing the electrostatic repulsion between the interface and the enzyme. The supporting evidence is the effect of added calcium ions on the lag phase of the kinetics; the addition of calcium ions reduces the lag phase by reduction in the electrostatic repulsion between the enzyme and the interface (15). No direct measurement of the surface charge of an emulsion interface prepared with phospholipid/bile salts has been undertaken until this study. In addition, the conclusions drawn from previous studies using sodium taurodeoxycholate have been assumed to hold for all bile salts.

In this study we have investigated interfacial quality by the use of four bile salts added to olive oil-in-water emulsions prepared with phosphatidylcholine (lecithin). The electrostatic interaction between the enzyme and the droplet interface has been investigated by characterization of the droplet zeta potential and the subsequent lipase kinetics in the presence and absence of calcium ions was determined. The long term aim of this work is to develop the appropriate physicochemical methods to characterize the role of interfacial structure in the concept of 'quality' of an interface for lipase activity.

MATERIALS AND METHODS

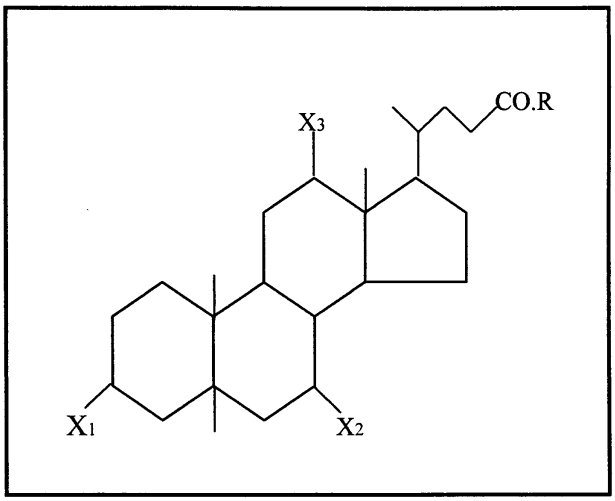
Chemicals

Olive oil (highly refined, low acidity, Sigma Chemical Company, Dorset, UK) was used without further purification following evidence that low levels of impurities have no effect on phospholipid adsorption (16). The free fatty acid content of the oil was estimated to be 0.13% (17). The bile salts were obtained from Sigma Chemical Company at 98% purity and were used without further purification. l-α-Phosphatidylcholine was obtained from Lipid Products, Surrey, UK, at 99% purity and fatty acid content 32% palmitic, 13% stearic, 31% oleic, and 15% linoleic. Porcine pancreatic colipase was obtained from Sigma Chemical Company (Dorset, UK). An SDS-page analysis of porcine pancreatic lipase (Sigma Chemical Company, Dorset, UK) showed at least 6 bands. Using protein size markers we were able to determine that all the impurities present had a lower molecular weight.

For the purification of the lipase, a Superose 6 HR10/30 column on a Pharmacia FPLC system (Pharmacia AB Biotechnology, Sweden) was used. Before loading the column, it was equilibrated with 2 mm Tris/HC1 buffer (pH 7.5, degassed overnight). The lipase was dissolved in the same buffer and injected onto the column. The flow rate through the column was set at 0.4 ml/min with a back pressure of 1 MPa. Any proteins that came off the column were detected by a 280 and 214 nm detector by the FPLC system and collected. The separation time was approximately 110 min. The sample that contained the purified lipase was collected and then desalted using a fast desalting column HR 10/10 (Pharmacia AB Biotechnology, Sweden) at a flow rate of 0.5 ml/min. The desalted lipase solution was then freeze-dried (Edwards Freeze Dryer Modulyo).

Emulsion preparation

For all studies a standard emulsion premix preparation was used of 20% w/w oil, 4.23% w/w phosphatidylcholine (PC) in 2 mm Tris/HCL buffer (pH 7.5). The phospholipid was dispersed in the buffer by constant agitation using a rotamixer (Fisons Whirlimixer, Leicester, UK), before the addition of olive oil. The emulsification was achieved by 8 passes through an orifice homogenizer (Emulsiflex, 20,000-B3, Avanti, Ottawa, Canada). Once prepared, the premix was kept con-



Compound	X_1	X_2	X_3	R
sodium taurocholate (NaTC)	α-OH	α-OH	α-OH	$NHCH_2CH_2SO_3Na$
sodium taurodeoxycholate (NaTDC)	α-OH	H	α-OH	$NHCH_2CH_2SO_3Na$
sodium taurochenodeoxycholate (NaTCDC)	α-OH	α-OH	H	$NHCH_2CH_2SO_3Na$
sodium glycocholate (NaGC)	α-OH	α-OH	α-OH	$NHCH_2CH_2CO_2Na$

Fig. 1. Molecular structure of the bile salts.

stantly agitated on an orbital shaker at room temperature under nitrogen and in the dark. The stability of the emulsion to coalescence was monitored by particle size analysis and was constant for 12 h under these conditions. Thus a premix was prepared daily and the weight mean droplet diameter was measured as 4.0(0.2)μm **(Fig. 2)**.

To prepare the final emulsion, the premix was diluted with stock solutions of bile salt, sodium chloride, and calcium chloride to give the following final compositions: 0–8 mm bile salt, 0.2% w/w olive oil, 0.74 mm phospholipid, 0–30 mm $CaCl_2$, 0.15 m NaCl, and 2 mm Tris/HCl buffer (pH 7.5).

Assay for lipase activity

Pancreatic lipase activity was measured with a pH-stat titrator (Metroholm pH-stat titrino, Herisan, Switzerland) by continually titrating the released free fatty acids with 0.02 m NaOH at pH 7.5 at 37°C. The initial reaction volume for each titration was 15 ml. The emulsion prepared as above was left to equilibrate in the reaction vessel for 30 min before the addition of enzyme. The molar ratio between lipase and colipase was within physiological range at 1:5 (i.e., 20 nmol/L:100 nmol/L)

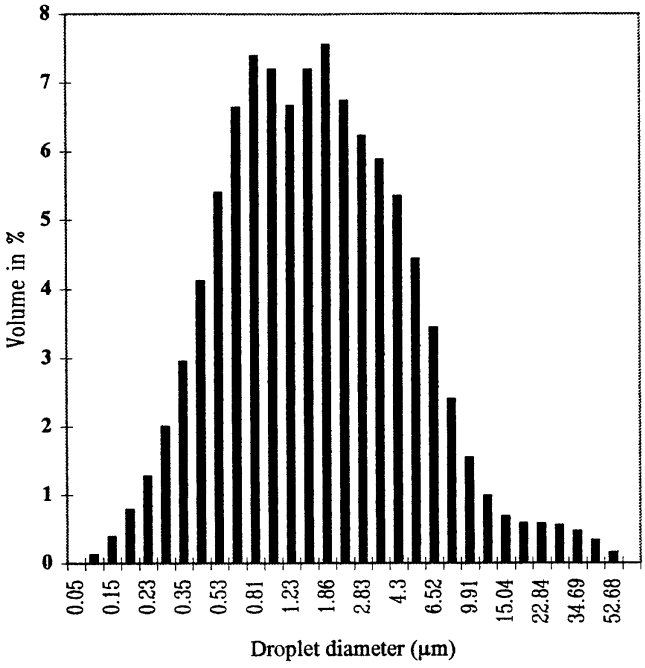


Fig. 2. Weight-mean emulsion droplet size distribution of the emulsion prepared with 0.2% w/w olive oil, 0.74 mm phosphatidylcholine in Tris/HCl buffer and 0.15 m NaCl.

and kept constant for all experiments. The reaction was started with the addition of the pancreatic lipase/colipase solution (15 mL) and followed continuously for varying lengths of time. Vigorous stirring of the reaction mixture was maintained during the kinetic run using a magnetic stirrer. The oil concentration is reported as the weight fraction of oil in the final sample before addition of the enzyme. The lipase solution was routinely prepared by dissolving the protein in buffer at a concentration of 1 mg/ml. The activity was checked against a standard emulsion system (olive oil emulsion in the presence of 10 mm calcium ions, pH 7.5, 6 mm NaTC, and 0.74 mm PC, 0.2% w/w olive oil) and found to be 5600 U/mg. Lag time was calculated from the addition of enzyme to the release of measurable fatty acid. Zero-order rate constants (initial rates) were calculated from the slopes of the product versus time dependencies after the lag phase. A degree of error was the difficulty in assessing the end of the activation phase. Nonetheless, the reproducibility was normally better than 10%. Measurements of lipase velocity were performed in the presence of increasing emulsion concentrations using the standard emulsion outlined above. The Lineweaver-Burk plot was linear but determinations of K_m and V_{max} were of limited value as shown by Benzonana and Desnuelle (18) because the interfacial area occupied by the non-substrate surface active species was unknown.

The relative distribution of the lipase between the aqueous continuous phase and the emulsion interface was determined by separation of the emulsion droplets once steady state kinetics had been reached. The emulsion system used contained 0.2% w/w olive oil, 6 mm sodium taurodeoxycholate, 0.74 mm phospholipid, 0–30 mm $CaCl_2$, 0.15 m NaCl, and 2 mm Tris/HCl buffer. The enzyme activity within the emulsion system was effectively stopped by mixing the emulsion with 2 ml of ice-cold buffer. The droplets were separated by centrifugation at 540 *g* for 15 min (no brake) at 4°C on a Beckman J2-21 rotor. The clear infranatant was collected and the lipase activity was measured potententiometrically. The emulsion system was replaced with buffer solution for the control experiment.

Particle size analysis

The droplet size distributions of the pre-mix emulsions and subsequent monitoring of oil droplet size distributions were determined using a Malvern Mastersizer (Malvern Instruments, Malvern. UK) light diffraction sizer.

Zeta potential measurement

Samples of the same emulsion premix and continuous phase prepared for the pH-stat titration were used for the measurement of droplet mobility. Emulsion droplets acquire a surface charge when brought into contact with a polar medium, in this case an aqueous solution. The presence of charged surfactants on the interface will enhance this charge which, in turn, influences the distribution of nearby ions in the polar medium, 'screening' the surface charge and producing an electric double layer. A measure of the surface charge can be gained from the measurement of the droplet velocity within an electric field, the droplet mobility. However, the surface of shear at the droplet interface does not coincide with the surface but extends a distance from the interface. As the droplet moves, it travels with the ions and solvent immediately adjacent to the surface. Thus the mobility measured is that of the droplet and its immediate environment. The electric potential at this surface of shear can be calculated from the mobility by the Henry formulation to give the zeta potential of the droplet (19).

The measurement method used, a Malvern Instrument Zeta Sizer (Malvern Instruments, Malvern, UK), requires a low volume fraction for the emulsion (0.03% w/w). The premix was diluted with a continuous phase containing 0.15 m NaCl, 0–8 mm bile salt, and 0–30 mm calcium ions. Thus the bile salt concentration was maintained but no effort was made to maintain the phospholipid concentrations due to the high turbidity of the resulting continuous phase which restricted measurement. The zeta potential of the emulsion system was monitored with time to determine whether significant desorption of surface active species occurred upon dilution; no change in the measured value was found.

Statistical analysis

All experiments were performed in triplicate and the results are expressed as means with their standard deviation (SD).

RESULTS

Stability of the emulsion systems

The stability of all prepared emulsions was monitored by measurement of their droplet size distribution. All emulsions were found to be stable up to 12 h after preparation when the oil volume fraction was kept constant, i.e., phase separation or creaming of the droplets was eliminated by constant agitation. This agrees with our previous study on phosphatidylcholine/sodium taurocholate and sodium taurocholate stabilized emulsions (20). At the lower volume fractions (0.03% w/w) re-

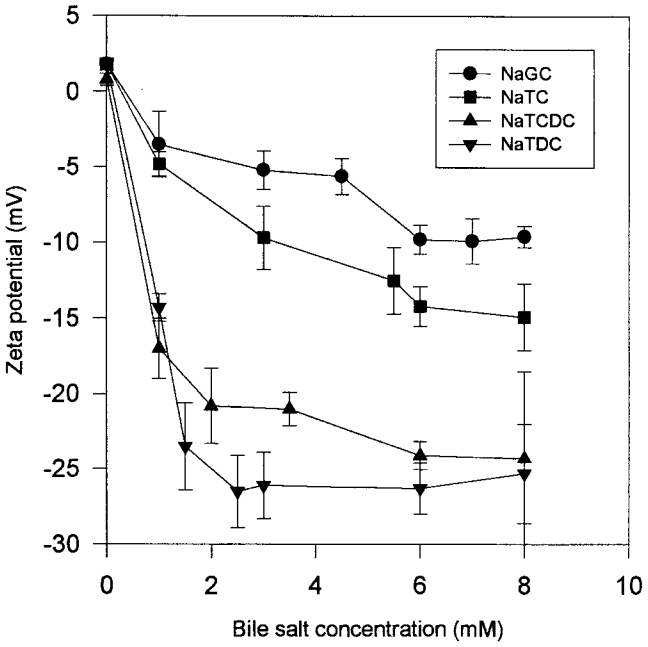


Fig. 3. Droplet zeta potential measured for each emulsion system at varying bile salt concentrations within a continuous phase of 0.15 m NaCl, 30 mm calcium, and bile salt. The phospholipid concentration was not maintained (see text for details).

quired for zeta potential measurement, the stability 'window' would be expected to increase and no evidence for coalescence of droplets was found. The size distribution of the emulsion during the initial phase of lipolysis was also monitored and found to be stable.

Effect of bile salt on zeta potential of emulsion droplets

The influence of four different bile salts on the zeta potential of the emulsion droplets is shown in **Fig. 3**. The zeta potential has been measured for emulsions prepared both in the presence and absence of phosphatidylcholine for a range of bile salt concentrations. In the absence of the bile salt, the zwitterionic headgroup of the phospholipid allows pH to be the major determinant of surface charge as reported by other workers (21). For our system, an isoelectric point at approximately pH 5 was found, but throughout the pH range of 3–8 the magnitude of the zeta potential was not more than |3| mV (22), and for pH 7.5 a value of 1.8 ± 0.7 mV was measured. Upon addition of bile salts, the zeta potential became negative and increased dramatically with increasing bile salt concentration, indicating significant absorption of the bile salt at the emulsion interface.

The general form of the relationship between bile salt concentration and zeta potential was common to all four species. The zeta potential reached a plateau at concentrations above the critical micelle concentration (cmc)of the bile salt (see Discussion). This is expected for the adsorption of an anionic surfactant at an interface (21) and mimics the behavior found in the absence of phospholipids (20). As shown in **Table 1**, the level of incorporation of the bile salt molecules, as measured by zeta potential, was proportional to their hydrophobicity, as determined by Donovan, Timofeyeva, and Carey (11), that is increasing in the order NaGC<NaTC<NaTCDC<NaTDC.

The influence of calcium ions was investigated for all bile salt species at 6 mm concentration (**Fig. 4**). The addition of calcium ions resulted in a reduction in zeta potential to the same extent for all four bile salts. This result agrees with the role of calcium ions as shielding the surface charge of the emulsion droplet (22).

Partitioning of lipase between the emulsion interface and aqueous phase during lipolysis

The fraction of pancreatic lipase bound to the the emulsion during lipolysis has been measured for the emulsion system containing; 0.2% w/w olive oil, 6 mm sodium taurodeoxycholate, 0.74 mm phospholipid, 30 mm $CaCl_2$, 0.15 m NaCl, and 2 mm Tris/HCl buffer. The lipase activity of the clear infranatant was found to be 22% of the value found for the original emulsion system. It should be noted that his value will include an uncertainty due to emulsion instability. The size distribution of the emulsion droplets within the cream layer of the centrifuged emulsion was not stable. The high local oil vol-

TABLE 1. Physicochemical properties of the bile salts in the presence and absence of the phosphatidylcholine stabilized emulsion

	NaTC	NaTDC	NaTCDC	NaGC
Droplet zeta potential above cmc in the absence of PC/mV	−23(3)	−24(2)	−30(2)	−15(1)
Hydrophobicity index[a]	0.0	+0.59	+0.46	+0.07
CMC/mm[b]	5	2	3	4
Z_a/Z_b[c]	0.6(0.1)	1.2(0.1)	0.9(0.1)	0.6(0.1)

[a]As defined in ref. 12.
[b]Ref. 25.
[c]Defined within text.

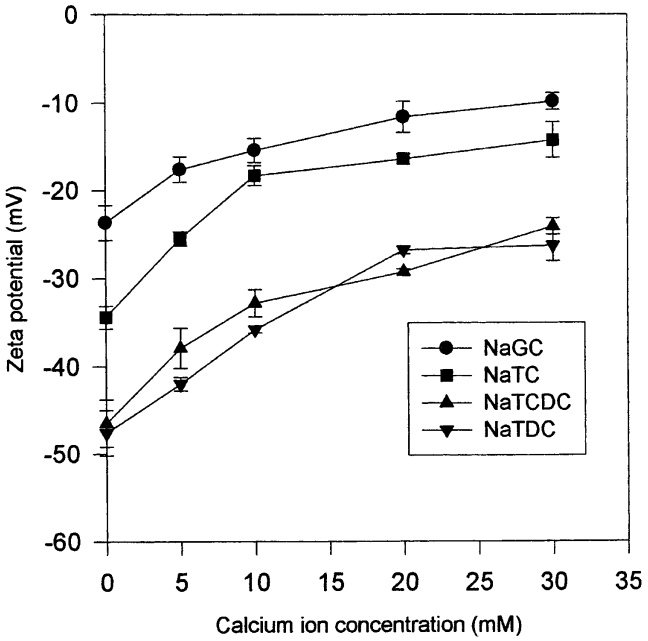


Fig. 4. Droplet zeta potential measured at varying calcium ions concentration at 6 mm bile salt and 0.15 m NaCl. The phospholipid concentration was not maintained.

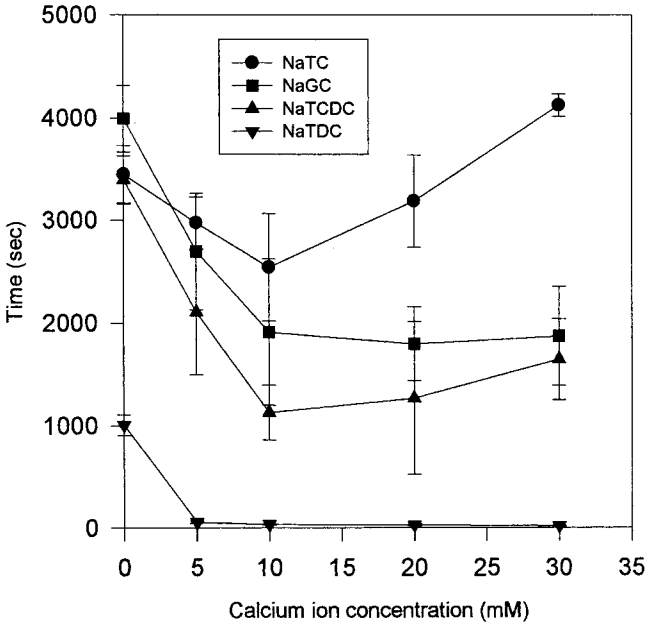


Fig. 5. The lag phase duration measured for each emulsion system at 0.2% w/w olive oil, 6 mm bile salt, 0.15 m NaCl, 2 mm Tris/HCl buffer (pH 7.5) and varying calcium ion concentrations.

ume fraction of the droplets promoted aggregation and coalescence of the emulsion system and thereby decreased the effective surface area of the emulsion.

Effect of bile salt on lag phase of lipolysis in the presence of calcium ions

Above the cmc of the bile salts. The lag time was measured for all PC/bile salts stabilized emulsions at 6 mm bile salt and varying calcium ions concentrations. The results are given in **Fig. 5**. The bile salt concentration was chosen as being well above each bile salt cmc as shown in Table 1. There was no correlation between the measured lag phase and the zeta potential, thereby ruling out a purely electrostatic determinant of ease of lipase penetration. Furthermore, a consistent decrease in lag time with calcium ion concentration was only observed for the system containing the bile salt NaTDC. A reduction of 54 ± 9 sec to 19 ± 5 sec was found upon increasing the calcium ion concentration from 5 mm to 30 mm. In contrast, the emulsion containing NaTC showed a significant increase in lag phase over the same range in calcium concentration.

Below the cmc of the bile salts. The lag phase of the emulsions containing the bile salts NaTDC and NaTC was measured at bile salt concentrations below the cmc, **Fig. 6**. For each system the lag time reached a maximum at concentrations approaching the cmc of the bile salt, confirming the generality of the study by Alvarez and Stella (12) with NaTDC. At higher bile salt concentrations the lag time decreased dramatically. The rate of lipolysis of emulsion systems containing NaTDC was found to increase with increasing concentrations of bile salt. In the presence of NaTC, however, a minimum in activity was observed a bile salt concentrations of 2–4 mm.

Influence of bile salt on velocity of lipolysis in the presence of calcium ions

Again the concentration of bile salt was chosen as 6 mm and the calcium ion concentration varied between 0 and 30 mm. The rate of lipolysis increased for all emulsions that contained NaTDC and NaTC as the calcium ion concentration increased (**Fig. 7**). The extent of increase varied with bile salt species in agreement with previous workers (4). Comparison with the data obtained from mobility measurements revealed no correlation with zeta potential and enzyme velocity. These results suggest that the observed variations in enzyme activity are not a consequence of the surface charge of the emulsion droplet or of the degree of incorporation of the bile salt into the emulsion interface.

DISCUSSION

The aim of this study was to investigate the influence of interfacial structure and composition on lipase binding and activity at the oil/water interface. The interface

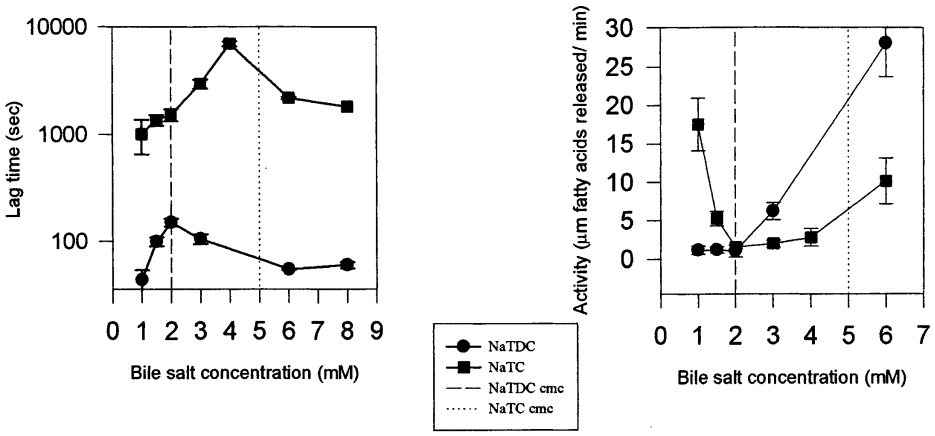


Fig. 6. The lag phase duration for NaTC/PC and NaTDC/PC stabilized emulsions prepared with 1–8 mm bile salt, 0.2% w/w olive oil, 0.15 m NaCl, 2 mm Tris/HCl buffer (pH 7.5) and 10 mm calcium.

is in equilibrium with the continuous phase surrounding the emulsion; changes in the composition of the continuous phase will result in changes in the interfacial composition. Before discussion of the observed lipase kinetics, we will discuss the physicochemical properties of each bile salt/PC system.

The physical characteristics of bile salt/phosphatidylcholine systems have been studied for a number of years and a range of phase diagrams has been constructed (23–25). These confirm that at low phospholipid/bile salt ratios the phospholipid is incorporated into minimum sized mixed micelles in conjunction with bile salt. Excess bile salts are present either as monomers at concentrations below their cmc or as a mixture of monomers and simple micelles at concentrations above their cmc. The concentration of bile salt/phospholipid adsorbed onto the interface is small (20) compared to the total lipid and can be neglected when considering the phase composition of the continuous phase. For NaTC/PC at 6 mm bile salt, the continuous phase consists of mixed micelles and monomeric bile salt. At higher concentrations of bile salt, simple micelles are also present. For the more hydrophilic bile salts, the coexistence boundary between the mixed micelle and mixed+simple micelle phases occurs at lower PC/bile salt ratios and hence, at 6 mm bile salt, the continuous phase will consist of simple and mixed micelles (23).

To investigate semiquantitatively the variation in phospholipid/bile salt composition of the interface, we have measured the zeta potential of the mixed lipid stabilized emulsions. The measurement of microelectrophoretic mobility allows the electric potential to be measured only at a certain distance from a surface. By application of the appropriate model, the zeta potential can be calculated and, for relatively simple systems, has commonly been taken as a measure of the surface charge at an emulsion droplet. The phosphatidylcholine used in these studies is zwitterionic and imparts a low surface charge to the droplets at the pH used. Upon addition of the bile salts to the emulsion, the negative zeta potential was found to increase with increasing concentration of bile salt, reaching a maximum value around the cmc of the bile salt. Such behav-

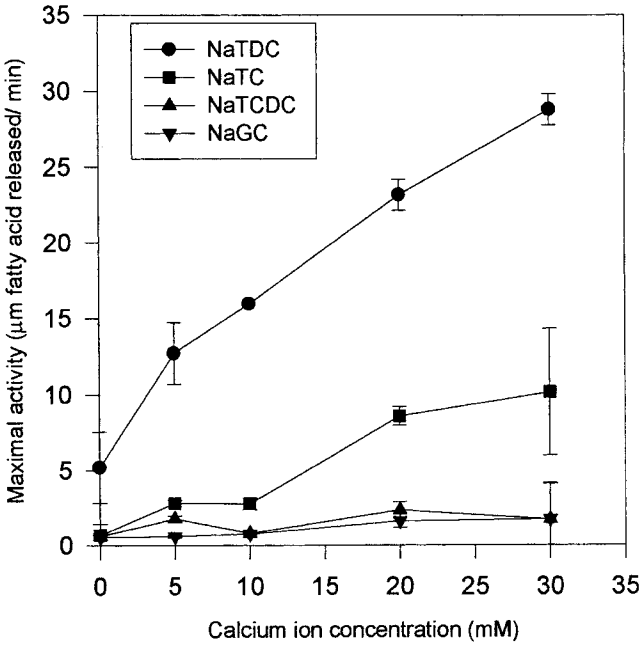


Fig. 7. Lipase activity for each emulsion system at 0.2% w/w olive oil, 6 mm bile salt, 0.15 m NaCl, 2 mm Tris/HCL buffer (pH 7.5) for varying calcium ion concentrations.

ior is as expected for the adsorption of an anionic surfactant at an interface. This indicates that the change in zeta potential is attributable to bile salt adsorption.

Obviously there are difficulties in comparing zeta potentials obtained for different bile salts that may differ in size and orientation at the interface. This may be overcome by comparison of zeta potentials obtained for a bile salt/phospholipid stabilized emulsion (Z_A) with those obtained for emulsions stabilized only by the relevant bile salt (Z_B). Thus the influence of the phospholipid at the interface can be inferred. The ratio Z_A/Z_B for each system is given in Table 1. For PC/NaTC and PC/NaGC, the partitioning of bile salt into the interface is significantly lower than found for the bile salt-only system whereas for NaTCDC and NaTDC the level approaches and seems to exceed that found for the bile salt-only case. The relative degree of incorporation of each bile salt into the interface is in agreement with their relative hydrophobicities.

The question then arises whether adsorption of the bile salts is additive leading to a mixed lipid interface or competitive resulting in the complete desorption of one component. We have investigated the relative stability of NaTC/PC-stabilized emulsion droplets to coalescence in a previous study and have found clear evidence of additive adsorption resulting in an improved stability over that observed for emulsion systems stabilized by either surfactant alone (20). Similarly, the zeta potential measurements for the NaTC/PC and NaGC/PC-stabilized emulsions indicate that the surface charge of the emulsion droplets is significantly lower that for the corresponding bile salt-only stabilized system. Obviously a full characterization of the interfacial tension of these mixed surfactant species will be necessary to confirm additive behavior and we are currently undertaking such a study.

For the NaTCDC/PC and NaTDC/PC stabilized emulsions, the measured zeta potential is comparable to that measured for the bile salt-only stabilized system. In this case the absorption of bile salt may be competitive, resulting in the complete desorption of the phospholipid from the interface. The desorbed phospholipid would be solubilized within the aqueous phase by the bile salt within mixed micelles. If the adsorption process is additive the high level of bile salt incorporation for NaTCDC and NaTDC would suggest a condensation of the interface by the bile salt. This agrees with past workers who have suggested that fully ionized bile salts associate to form dimers or larger aggregates within the hydrophobic portion of membranes and are thus orientated with the steroid nucleus perpendicular to the interface (26). But recent work by Fahey, Carey, and Donovan (27), who investigated the molecular orientation of non-ionized bile salt in monolayers mixed with POPC (1-palmitoyl-2-oleoyl-3-*sn*-glyceroposphatidylcholine), suggests that no change in orientation of the bile salt occurs within the mixed lipid system. In comparing our results with this most recent study, we must consider two points. First, the authors used non-ionized bile salts to eliminate problems arising from the aqueous solubility of the bile salt. Second, their measurements were made at the air–water interface, whereas in this study we are concerned with the oil–water interface where the contribution of hydrophobic interactions of the acyl chains of the phospholipid and the oil phase have to be considered.

Thus, in summary, the four bile salt/phospholipid systems have been constructed that, to the first approximation, contain a relatively invariant concentration of mixed micelles but increasing concentrations of monomeric and micellar bile salt. The bile salt content of the interface has also been found to increase in line with the increasing hydrophobicity of the bile salt. The effect on lipase function is now discussed.

Of immediate interest is the duration of the lag phase in these four systems. The lag phase is accepted as a measure of the ease of penetration of the lipase into the interface. Verger's model (1) assumes that binding to the interface is fast and reversible. Our results on the partitioning of lipase between the emulsion interface and the aqueous continuous phase suggest that the lipase was not all bound to the interface but a proportion remained free within the aqueous phase. After binding, the enzyme may either desorb from the interface, denature irreversibly, or change conformation to the active form. Obviously the quality of the interface will determine the relative rate of these processes. In this study there was no correlation between surface charge (as measured by zeta potential) and lag phase duration, thereby ruling out a major contribution from electrostatic repulsion between the emulsion interface and the enzymic complex.

There is a large difference between the lag times observed for the NaTC/PC and the NaTDC/PC systems. At the concentrations investigated there is a comparable range of concentrations of monomers and aggregates but at no point do the lag times become comparable. The significant difference between these two systems is the degree of incorporation of the bile salt into the oil/water interface. The lag phase durations measured for bile salt/PC stabilized emulsions at bile salt concentrations well above their cmc (6 mm for NaGC, NaTC, NaTCDC, and NaTDC and 8 mm for NaTC and NaTDC) are shown in **Fig. 8**. For comparison, the lag time for a purely phosphatidylcholine interface is included. As the degree of bile salt incorporation increases (as ranked by Z_A/Z_B) the lag time is seen to reduce from ~30 min to seconds. Lipase cannot

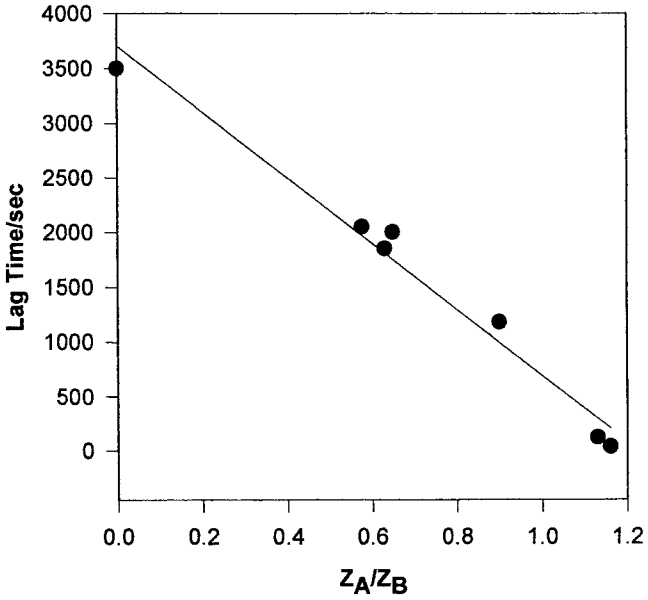


Fig. 8. The measured lag time for emulsion systems containing 0.2% w/w olive oil, 0.15 m NaCl, Tris/HCl buffer (pH 7.5), 10 mm calcium ions, 0.74 mm PC with: 0 mm bile salt, 6 mm NaTCDC, NaTDC, NaTC, and NaGC, 8 mm NaTC and NaTDC as a function of Z_A/Z_B.

bind effectively to a phosphatidylcholine interface presumably as the structure of the close-packed interface does not allow sufficient penetration by the substrate for cluster formation. It can be suggested that the degree of incorporation of the bile salts mirrors the breakup of the tightly packed phospholipid interface. We would speculate that this would allow greater access to the interface for the substrate molecules allowing cluster formation to occur. Our results indicate that this breakup of the phospholipid interface is partial for the bile salts of lower hydrophobicity, i.e., NaTC and NaGC, but may be complete for the more highly hydrophobic bile salts, NaTDC and NaTCDC. When phospholipid is lost to the continuous phase, the concentration of substrate (in conjunction with bile salt) at the interface will increase relative to that of phospholipid and lead to activation of lipase as described by substrate theory. Below the cmc of the bile salt the displacement of the phospholipid from the interface is incomplete and a decrease in lipase activation is observed.

Direct measurement of the partitioning of phospholipid between the emulsion interface and the aqueous phase by separation of the droplets is not possible for this relatively unstable system. Centrifugation and filtration methods induce aggregation and coalescence of the emulsion droplets thereby changing the surface area of the emulsion interface significantly. The equilibrium distribution of the phospholipid and bile salt between the interface and the aqueous phase is highly dependent upon the interfacial area. Again this issue cannot be fully addressed without a more complete investigation of the interfacial tension of this system and the mixing of the lipid at the interface.

The relatively smaller variations in lag time observed for each bile salt at concentrations around the cmc indicate that the continuous phase contributes to the availability of the enzyme to bind to the interface. There is evidence that lipase can bind to monomeric bile salts (26) and mixed micelles (2) within the continuous phase. The increase in lag phase duration with bile salt concentration below the cmc (at constant phospholipid concentration) indicates that the enzyme is bound to monomeric bile salt, thus reducing the availability of lipase for binding to the emulsion interface. The reduction in lag time that occurs above the cmc suggests that binding of the lipase by mixed and simple micelles does not reduce penetration of the enzyme and may indeed facilitate it. No literature is available concerning the variation in binding strength of the lipase/bile salt complex but it would be expected to be affected both by bile salt structure and calcium ion concentration.

Once established, the rate of lipolysis increases until zero-order kinetics are observed, that is the enzyme affinity for its 'supersubstrate' increases with time. It is now accepted that this reflects an increase in the product concentration which changes the 'quality' of the interface allowing greater penetration of the enzyme. We have found a non-linear increase in lipase activity with bile salt concentration in agreement with Alvarez and Stella (12) for NaTDC but for NaTC a minimum at 2–4 mm was found. There are two possible mechanistic interpretations of this data. A parallel pathway for lipolysis may be opened by the binding of calcium ions, bile salt, and enzyme as suggested by Alvarez and Stella (12). Alternatively, the greater concentration of micelles within the continuous phase may allow the removal of the products of lipolysis before an inhibitory concentration is achieved. We are presently investigating the fatty acid carrying capacity of mixed micelles of varying composition to address this issue, but the results presented here do not show a simple relationship between concentration of micelles and enzyme activity.

CONCLUSIONS

Our results confirm that the addition of bile salts to a phospholipid stabilized emulsion destroys the well-packed phospholipid interface resulting in a mixed phospholipid/bile salt interface. By measurement of the droplet zeta potential we have monitored semi-quan-

titatively the incorporation of bile salts within the interface. There was found to be no correlation between droplet surface charge, as monitored by the zeta potential, and lag phase. The duration of the observed lag phase was found to inversely related to the degree of incorporation of the bile salts. It can be postulated that this breakup of the phospholipid interface allows greater binding of the enzyme to the interface by facilitating the formation of substrate clusters. Simultaneously there was evidence that monomeric bile salts present within the continuous phase bound lipase molecules and reduced their availability for adsorption onto the emulsion interface. Calcium ions were found to 'screen' the surface charge of the emulsion droplets but no correlation was found between the resulting surface charge and lag phase duration, thereby ruling out a significant contribution from an electrostatic repulsion between the emulsion interface and the enzyme. The evidence presented here agrees with a more specific role for calcium ions as recently formulated by Alvarez and Stella (12), i.e., the formation of a new catalytically active enzyme complex, (enzyme)–(mixed micelle)–(calcium ion).

We would like to acknowledge funding (a studentship) for one author (M. Wickham) from the Institute of Food Research.

Manuscript received 19 May 1997 and in revised form 3 November 1997.

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