Adsorption of pancreatic (pro)phospholipase A_2 to various physiological substrates

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Abstract The adsorption of pancreatic phospholipase was studied in vitro in the presence of egg yolk lipoprotein emulsion, Intralipid emulsion, and milk fat globules. When the emulsions are incubated with bile salts, the latter dissociate a considerable fraction of the phospholipids initially associated with the emulsions, leading to the coexistence of an emulsified phase and a phase of mixed micelles. After the addition of pancreatic phospholipase A_2, gel filtration shows that the enzyme was more than 90% bound to mixed micelles, regardless of the type of emulsion used. Comparable results were obtained by replacing the bile salts with human gallbladder bile. In parallel, pancreatic zymogen was never found to be bound to any of the lipid structures present (emulsion or mixed micelles). When the catalytic site of pancreatic phospholipase A_2 was blocked with 4-bromophenacylbromide, there was no fixation on mixed micelles. Fixation was restored by the presence of lysolecithins and fatty acids in the incubation mixtures. The partial transformation of all emulsified substrates to mixed micelles by bile salts in vivo would thus lead to optimum activity of pancreatic phospholipase A_2.—**Nalbone, G., M. Charbonnier-Augeire, H. Lafont, R. Grataroli, J-L. Vigne, D. Lairon, C. Chabert, J. Leonardi, J. C. Hauton, and R. Verger.** Adsorption of pancreatic (pro)phospholipase A_2 to various physiological substrates. *J. Lipid Res.* 1983; **24:** 1441–1450.

Supplementary key words mixed micelles • emulsions • phospholipid • bile salts

After emulsification in the stomach, dietary phospholipids (PL) and triglycerides (TG) pass through the pylorus into the duodenum, to be acted upon by bile salts (BS). There results a coexistence of a micellar phase and an emulsified phase (1–3) on which all pancreatic lipase (E.C. 3.1.1.3) and its cofactor are adsorbed with phospholipids (2, 4).

In a prior in vitro study using Intralipid emulsion, we showed by centrifugal flotation that pancreatic phospholipase A_2 (E.C. 3.1.1.4.) was predominantly in the phase containing bile salt-phosphatidylcholine mixed micelles (5). These micelles arise from the solubilization of phosphatidylcholines initially adsorbed at the triglyceride-water interface.

It has been shown in vitro that pancreatic phospholipase A_2 activity on synthetic mixed micelles, expressed as the bile salt-phosphatidylcholine molar ratio, was optimum for a ratio about of 2:1 (5). Other results have shown a synergistic action between pancreatic phospholipase A_2 and lipase in vitro (6–8). These authors showed that lipase action on a phospholipid-covered triglyceride emulsion was enhanced by the action of phospholipase A_2. Blackberg et al. (7) suggested that lysophosphatidylcholines are produced at the lipid globule interface by phospholipase A_2. When these products are subsequently desorbed by bile salts, they liberate the interface and enable lipase to be bound. Alternatively, Borgström (6) suggested that fatty acids generated by the hydrolysis of phospholipids at the interface would lead to lipase binding on the interface, via colipase. In these studies, however, the presence of phospholipase A_2 at the triglyceride-water interface was not investigated.

The present work was thus undertaken to study the adsorption of pancreatic phospholipase A_2 on different emulsified substrates, similar to those encountered in human nutrition. We chose egg yolk lipoproteins, Intralipid, and milk fat globules. The results show that more than 90% of the active enzyme is adsorbed on the bile salt-phospholipid mixed micelles.

MATERIALS AND METHODS

Preparation of substrates

Egg yolk lipoprotein emulsion was obtained as described by De Haas et al. (9), by mixing an egg yolk for

Abbreviations: AMPA, amidinated phospholipase A_2; AMPREC, amidinated phospholipase A_2 precursor; 4-BPB, 4-bromophenacylbromide; BS, bile salts; PL, phospholipids; PC, phosphatidylcholines; lyso-PL, lysophospholipids; lyso-PC, lysophosphatidylcholines; IRS, interfacial recognition site; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid.

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5 min with 100 ml of distilled water and 12 ml of 3.3% (w/v) $CaCl_2$. Sodium azide was added at 0.02% and the emulsion was stored at 4°C under nitrogen. It was used within 1 week of its preparation.

Intralipid emulsion (Kabi-vitrum, Paris) was used at 10% after removing phosphatidylcholines not bound to the emulsion as previously described (5) and reconstituting with standard buffer. It was stored at 4°C under nitrogen and used within 3–4 days of its preparation.

Milk fat globules were prepared from 250 ml of fresh cow's milk according to a standard procedure (10). They were taken up with 20 ml of standard buffer and experiments were performed within 24 hr of its preparation.

Human gallbladder bile was obtained during an operative procedure with sterile syringes and was stored at 4°C with 0.02% sodium azide.

Enzymes

Porcine pancreatic phospholipase A_2 was purchased from Boehringer Mannheim. It was desalted by filtration through Sephadex G-25. Specific activity in standard assay conditions (9) was 1,200 $\mu mol \cdot mg^{-1}min^{-1}$. Tritiated amidinated porcine pancreatic phospholipase A_2 and tritiated amidinated porcine prophospholipase ([^{3}H]AMPA and [^{3}H]AMPREC, respectively) were generous gifts of Dr. De Haas (Utrecht, Holland). The specific radioactivity of [^{3}H]AMPA was 13.2×10^6 dpm/mg and its specific activity towards egg yolk was 300 $\mu mol \cdot mg^{-1}min^{-1}$. The specific radioactivity of [^{3}H]AMPREC was 9.8×10^6 dpm/mg and it had no activity towards egg yolk.

Phospholipase A_2 and its precursor were labeled with ^{125}I (C.E.A. France) as described by Slotboom et al. (11). The specific radioactivities of phospholipase A_2 and the precursor were 1.8×10^5 cpm/mg and 2×10^6 cpm/mg. The specific activity of the iodinated enzyme towards egg yolk was 1,300 $\mu mol \cdot mg^{-1}min^{-1}$, while the precursor had no activity.

In certain experiments, ^{125}I-labeled pancreatic phospholipase A_2 was inactivated by incubating with 4-bromophenacylbromide (Fluka, 99% pure) as described by Volwerk, Pieterson, and De Haas (12). As was also found by these authors, the enzyme was completely inactivated resulting from blocking histidine 48, in 5 hr with an inhibitor-enzyme molar ratio of 50. Excess inhibitor was removed by dialysis against distilled water. The inhibited enzyme was further purified by using HPLC (Spectra-physics) on a μBonda-pack column C18 (Waters). The column was equilibrated with H_2O containing 0.05% TFA. Elution was performed in 0.05% TFA with a linear acetonitrile gradient (0 to 60% in 30 min). Protein detection was at 220 nm.

Porcine pancreatic lipase was purified from the organ as described (13). The specific activity of the lipase preparation measured with tributyrin was 3200 $\mu mol \cdot mg^{-1}min^{-1}$. The mixture of porcine pancreatic colipase I and II was prepared as described by Maylie et al. (14).

Products

The standard buffer used in all experiments was composed of 0.01 M Tris-HCl (pH 7.0), 0.1 M NaCl, 1 mM $CaCl_2$, and 0.02% NaN_2.

All gel filtrations were performed with standard buffer containing a mixture of four bile salts at the total final concentration of 1.2 mM, pH 7.0. The molar proportions of the four salts were 0.29 glycocholate, 0.40 glycodeoxycholate, 0.08 taurocholate, and 0.23 taurodeoxycholate. All were purchased from Calbiochem (A grade). In certain experiments, triple mixed micelles were prepared, containing bile salts, lysophosphatidylcholines (Sigma, egg, 99% pure), and oleic acid (Merck, 99% pure) in 6:1:1 molar proportions. Lipids evaporated to dryness were taken up by the mixture of the four bile salts until a perfectly clear solution was obtained.

Incubation of substrates with bile salts and pancreatic phospholipase A_2

Egg yolk lipoprotein emulsion, Intralipid emulsion, and milk fat globules were incubated with variable quantities of the BS mixture in solution at pH 7.0 in such a way as to vary the BS-PL molar ratios. The desired PL concentrations were obtained by diluting the incubation mixture with standard buffer; total volume was 4 ml. Incubation was at ambient temperature, pH 7.0, and with slight agitation for 30 min. An aliquot (0.85 ml) was then removed for gel filtration, except for the experiments with milk fat globules, which were studied by centrifugation. In other experiments and after the 30-min incubation, 4 μg of pancreatic phospholipase A_2 or its precursor labeled with ^{3}H or 53 μg of PA_2 labeled with ^{125}I were added. After a 30–60-sec incubation, a 0.85-ml aliquot was immediately deposited on the gel, or subjected to centrifugation in the case of milk globules. In certain experiments, the solution of four BS was replaced by human gallbladder bile in such a way as to obtain the same BS concentration.

In other experiments, the solution of four BS was replaced by mixed BS-lysophosphatidylcholine-oleic acid micelles (see above). In some experiments, 10 μg of lipase and 90 μg of colipase were added to the Intralipid emulsion incubated with BS (BS-PL 2:1). After 1 min, 4 μg of ^{3}H-labeled AMPA was added and incubation was continued for an additional minute, whereupon a 0.85-ml aliquot was removed for gel filtration.

Gel filtration

All filtrations were performed in a 50 × 1.1 cm column filled with agarose A4 (IBF, France). Elution was with standard buffer containing the mixture of four BS at 1.2 mM, pH 7.0, at ambient temperature and at a rate of 11–12 ml/hr. One-ml fractions were collected and elution volumes were determined gravimetrically.

Centrifugations

Intralipid emulsions were purified with an SW 40 rotor in a Beckman L5-75 B ultracentrifuge. Milk globules were studied with the same rotor. Centrifugations were at 20°C at 5,000 rpm for 10 min. Cream and the underlying phase were recovered separately by sectioning the tube just under the cream.

Assays

Lipid phosphorus was assayed semi-automatically (15). In experiments with egg yolk emulsion and milk lipid globules, it was necessary to first extract lipids with the method of Folch, Lees, and Sloane Stanley (16). Bile salts were enzymatically assayed with an automatic method (17). Tritiated molecules were assayed for radioactivity in a liquid scintillation spectrometer (Beckman model LS 9000) in 10 ml of Readysolve MP (Beckman) scintillation fluid. Calculations were performed with a Texas Instruments Silent 700 calculator connected to the counter. ^{125}I radioactivity was determined with an Intertechnique CG 4000 gamma counter.

RESULTS

Gel filtration study of the adsorption of pancreatic phospholipase A_2 on egg yolk lipoprotein emulsion

Column equilibration and elution were performed with a 1.2 mM BS solution. This BS concentration caused no change in the elution profile of the PLs in the emulsion compared to the elution obtained from the same emulsion eluted with standard buffer without BS. At elution concentrations of BS higher than 1.2 mM, however, the elution profile of PLs changed. An important part of PLs was solubilized and consequently eluted in retarded fractions as micellar structure (3, 18). Incubations were performed with BS-PL molar ratios in the range of 0.5 to 4.

The egg yolk lipoprotein emulsions profile obtained after an incubation with a BS-PL molar ratio of 0.5 and a PL concentration of 7.0 mM is shown in **Fig. 1A.** Almost all the phospholipids were eluted with the void volume of the column in the highly opalescent fractions. When phospholipase A_2 (^{3}H-labeled AMPA or ^{125}I-labeled phospholipase A_2) was added to the incubation (Fig. 1B), all the enzyme eluted in the retarded fractions (K_{av} = 0.85). When the enzyme was filtered alone, it eluted at K_{av} = 0.97. The presence of minor quantities of PLs eluted in the retarded fractions may explain this difference in observed K_{av} values. When the lipoprotein emulsion was incubated with BS at a BS-PL ratio of 2, a considerable proportion of PL (40–45%) eluted in the clear fractions as a peak whose K_{av} was 0.70 (**Fig. 2A**). The estimated molecular weight after calibration is $8–10 \cdot 10^4$ indicative of a micellar organization (3, 18). The BS-PL molar ratio in the fractions corresponding to the peak was about 3, with a PL concentration of 0.37 mM. Excess BS in the load was eluted at total column volume. When identical incubations were performed with other BS-PL molar ratios, the proportion of PL eluting as a peak (K_{av} = 0.7) increased with increasing molar ratio (**Fig. 3**).

Comparable curves were obtained when the PL concentration was 3.5 mM in the incubations (Fig. 3). At this former PL level, its solubilization was about 10% lower than when the PL concentration was 7.0 mM.

When phospholipase A_2 (^{3}H-labeled AMPA) was added to an incubation with a BS-PL molar ratio of 2 (Fig. 2B), the elution profile of the PLs was comparable to that obtained in the absence of enzyme. Fifty to 55% of total PLs were eluted as a single retarded peak. Nearly all of the enzyme was also eluted with the phospholipid peak (K_{av} = 0.73). Only 2–3% of the enzyme was eluted with the emulsified fraction in the void volume.

In an attempt to determine the extent of hydrolysis of PLs at column output, powdered EDTA was added to each collection tube in order to stop the enzymatic reaction. The phospholipid peak (K_{av} = 0.73) and the excluded peak were subjected to lipid extraction (16) and PLs and lyso-PLs were assayed after separation by thin-layer chromatography on silica gel. It was found that the micellar phospholipid peak (K_{av} = 0.73) contained about 30% lyso-PL, while the peak eluting with the void volume contained about 3% of lyso-PL in relation to total PL. Using a radiolabeled mixture of oleic acid and lyso-PL (30% in relation to total PL), added to egg yolk lipoprotein emulsion prepared with a BS-PL molar ratio of 2, we observed a comparable distribution ratio of the added lipolytic products between the emulsion and micellar peaks (data not shown).

When the mixture of four BS was replaced by human gallbladder bile, almost 100% of phospholipase A_2 was eluted with the retarded phospholipid peak, which in this case had a K_{av} of 0.75.

Gel filtration study of the adsorption of pancreatic phospholipase A_2 on Intralipid emulsion

This series of experiments was similar to that conducted with egg yolk emulsion. The elution profiles during gel

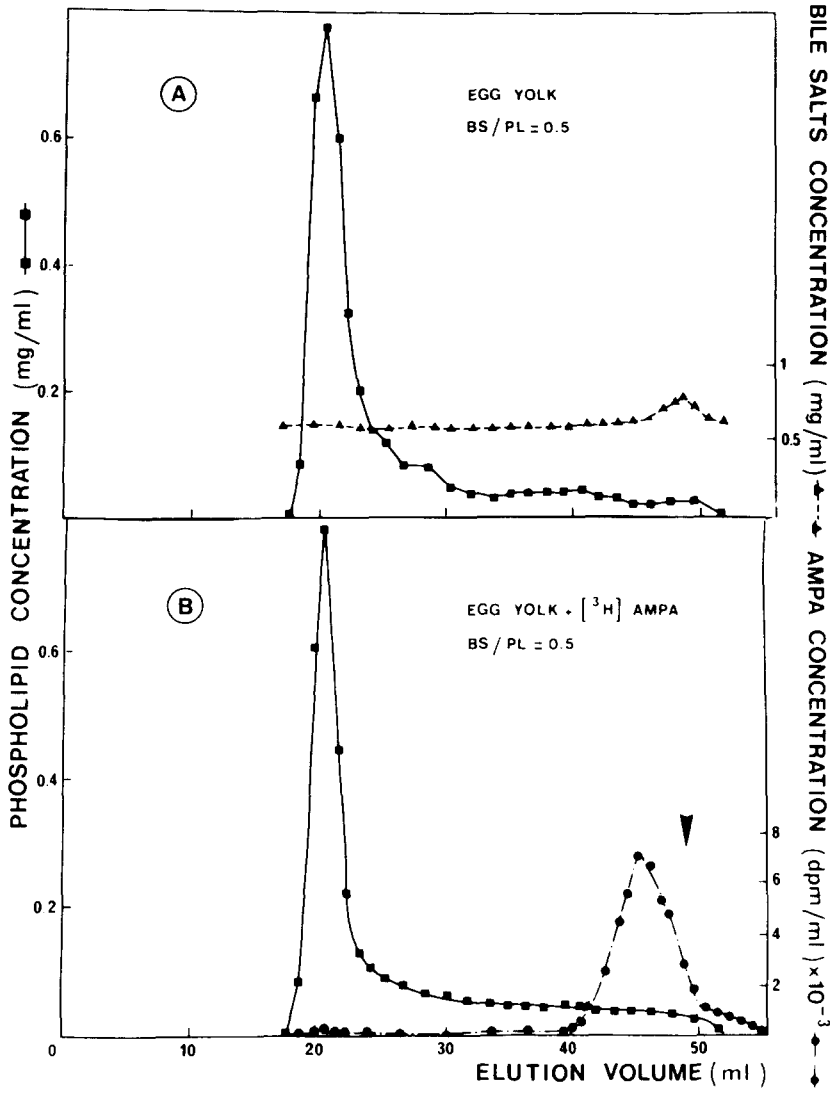

Fig. 1. Agarose A4 gel chromatography of incubations. Columns equilibrated and eluted with standard buffer containing 1.2 mM bile salts. A) Egg yolk lipoprotein emulsion and BS. The 4-ml incubation mixture contained 28 μmol of PL and 14 μmol of BS (BS-PL = 0.5); 0.85 ml of this incubation was chromatographed. B) Same conditions as in Fig. 1A + 5 μg of [^{3}H] AMPA; 0.85 ml was chromatographed. The arrow indicates the elution volume of the enzyme when chromatographed alone.

filtration (data not shown) were comparable, and the extents of PC desorption are shown (Fig. 3) for 3.9 and 7 mM phosphatidylcholines. When phospholipase A_2 was added (^{3}H-labeled AMPA or ^{125}I-labeled) with a BS-PC molar ratio of 0.5, almost all the enzyme was eluted with its elution volume (K_{av} = 0.97) (data not shown), regardless of whether the PC concentration was 3.9 or 7 mM. In the case of a molar ratio of 2, 90–93% of the enzyme was eluted with the retarded PL peak (K_{av} = 0.7). The triglyceride emulsion fraction contained 2–3% bound ^{3}H-labeled AMPA and 6–8% ^{125}I-labeled phospholipase. Intralipid emulsion is also a substrate for pancreatic lipase (6), which is why we examined the effect of this enzyme on the phase distribution of phospholipase A_2 (see Materials and Methods). The resulting elution profile showed that the phase partition of the latter enzyme was not affected by the presence of lipase and colipase (data not shown).

Centrifugation study of the adsorption of pancreatic phospholipase A_2 on milk fat globules

Preparations of milk fat globules cannot be subjected to gel filtration because of their high viscosity. Separation was thus performed with a low speed centrifugation. For the same reasons of viscosity, the maximum globule concentration in the experiments corresponded to 2.5 mM PL. The level of desorption was calculated by assaying the PLs in the aqueous phase. As in the case of incubations with egg yolk and Intralipid emulsions, the level of PL desorption was a function of the BS-PL

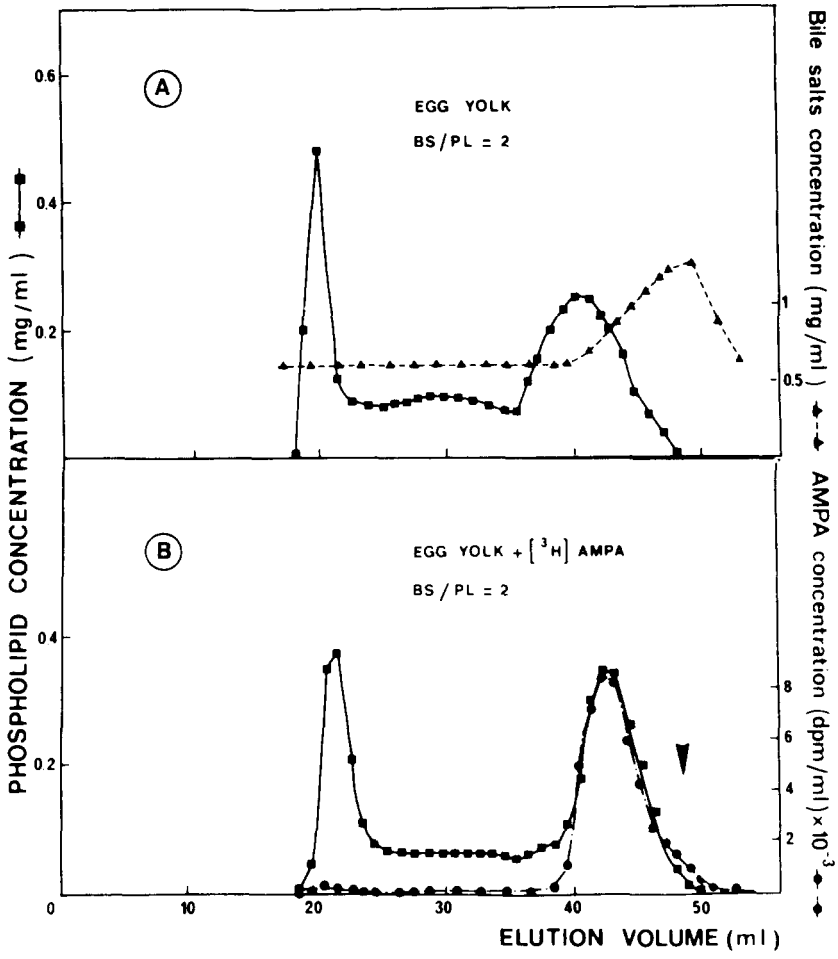

Fig. 2. Agarose A4 gel chromatography of incubations. Columns equilibrated and eluted with standard buffer containing 1.2 mM bile salts. A) Egg yolk lipoprotein emulsion and BS. The 4-ml incubation mixture contained 28 μmol of PL and 56 μmol of BS (BS-PL = 2); 0.85 ml was chromatographed. B) Same conditions as in Fig. 2A + 4 μg of [^{3}H]AMPA; 0.85 ml was chromatographed. The arrow indicates the elution volume of the enzyme when chromatographed alone.

molar ratio, reaching 50% for a molar ratio of 5. When ^{3}H-labeled AMPA was present in the incubation, 80% of the enzyme was detected in the post-centrifugation micellar phase.

During centrifugation, a proportion of the aqueous phase was carried with the cream formed during coalescence of the globules. A fraction of the enzyme thus may have been nonspecifically trapped in the cream. The contribution of this type of artifact was estimated in similar incubations with ^{3}H-labeled inulin, a polymer of D-fructose, and was found to be about 10%. The quantity of phospholipase A_2 really adsorbed to milk globules could thus be estimated at about 10%.

Adsorption of pancreatic (pro)phospholipase A_2 on egg yolk and Intralipid emulsions

Experiments identical to the above with phospholipase A_2 were repeated with zymogen (^{3}H-labeled AMPREC or ^{125}I-labeled prophospholipase). It was found that the latter was never bound to the lipid structures in the incubation (emulsion or mixed micelles) (**Fig. 4**). Zymogen was consistently eluted in fractions with a K_{av} of 0.98.

Gel filtration study of the adsorption of pancreatic phospholipase A_2 inactivated with 4-BPB in the presence of egg yolk or intralipid emulsions

This series of experiments was performed to determine whether enzyme binding to the PL peak (K_{av} = 0.7) was partially due to the presence of hydrolysis products in this peak (fatty acids and lyso-PLs), generated by enzyme activity. The experimental protocol was the same as in the preceding experiments, with ^{125}I-labeled enzyme previously inactivated by blocking the active site with 4-BPB (12).

The behavior of the active ^{125}I-labeled enzyme in the presence of egg yolk emulsion is shown for a BS-PL

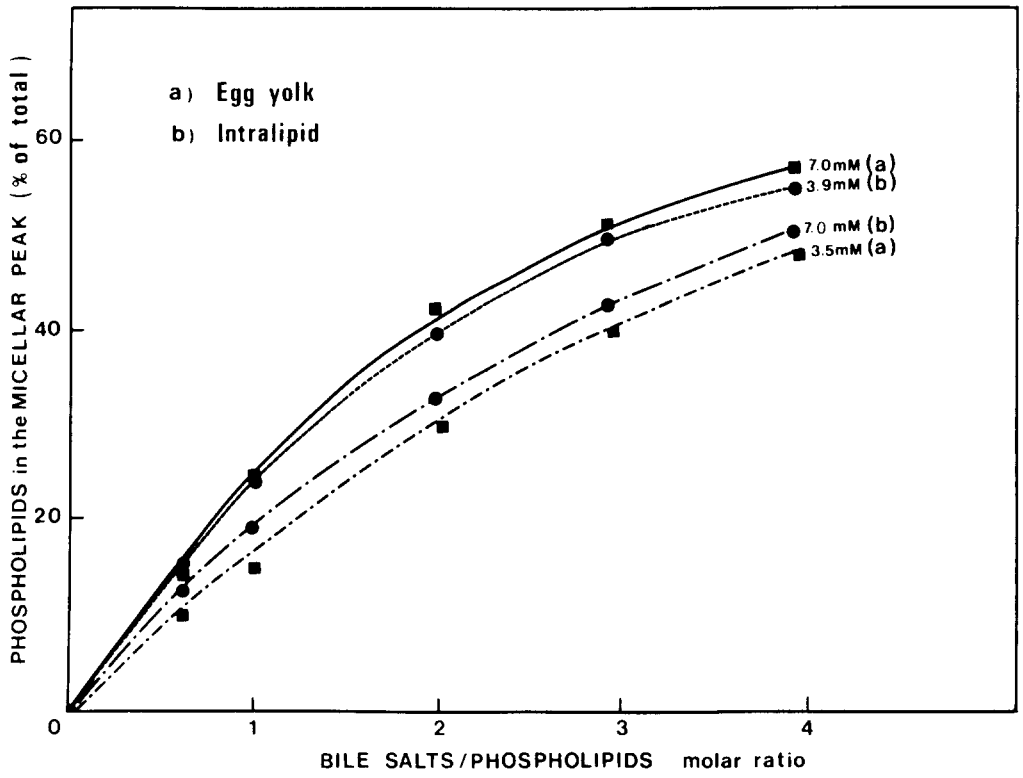

Fig. 3. Micellar solubilization of PLs as a function of the BS-PL molar ratio in the incubation. a) Egg yolk lipoprotein emulsion for PL concentrations of 3.5 and 7.0 mM. b) Intralipid emulsion for PL concentrations of 3.9 and 7.0 mM.

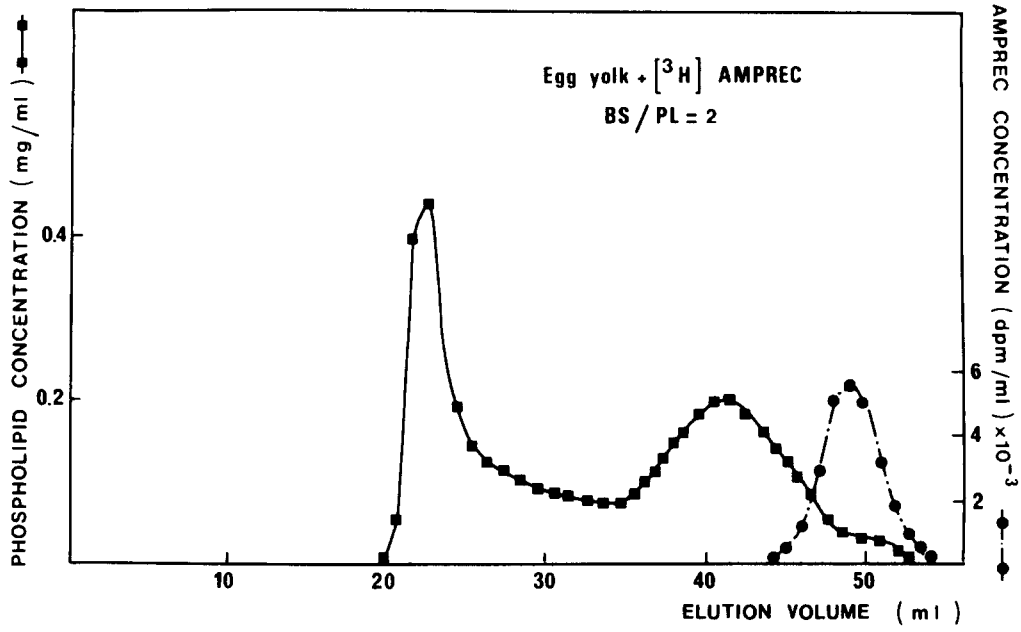

Fig. 4. Agarose gel chromatography of an incubation of [^{3}H] AMPREC and egg yolk lipoprotein emulsion in the presence of BS (BS-PL = 2). Column equilibrated and eluted with standard buffer containing 1.2 mM bile salts. Experimental conditions identical to Fig. 2B. Ten μg of precursor was added and 0.85 ml of the incubation was chromatographed.

molar ratio of 2 (**Fig. 5A**). More than 90% of the enzyme was bound to the micellar phospholipid peak (K_{av} = 0.7) and about 8% was bound to the emulsified phase. When inactive phospholipase was filtered alone, it eluted as a sharp peak with a K_{av} of 0.98. The elution profile of the inactive enzyme incubated with egg yolk emulsion and at a BS-PL molar ratio of 2 is shown in Fig. 5B. In comparison with Fig. 5A, it is seen that the

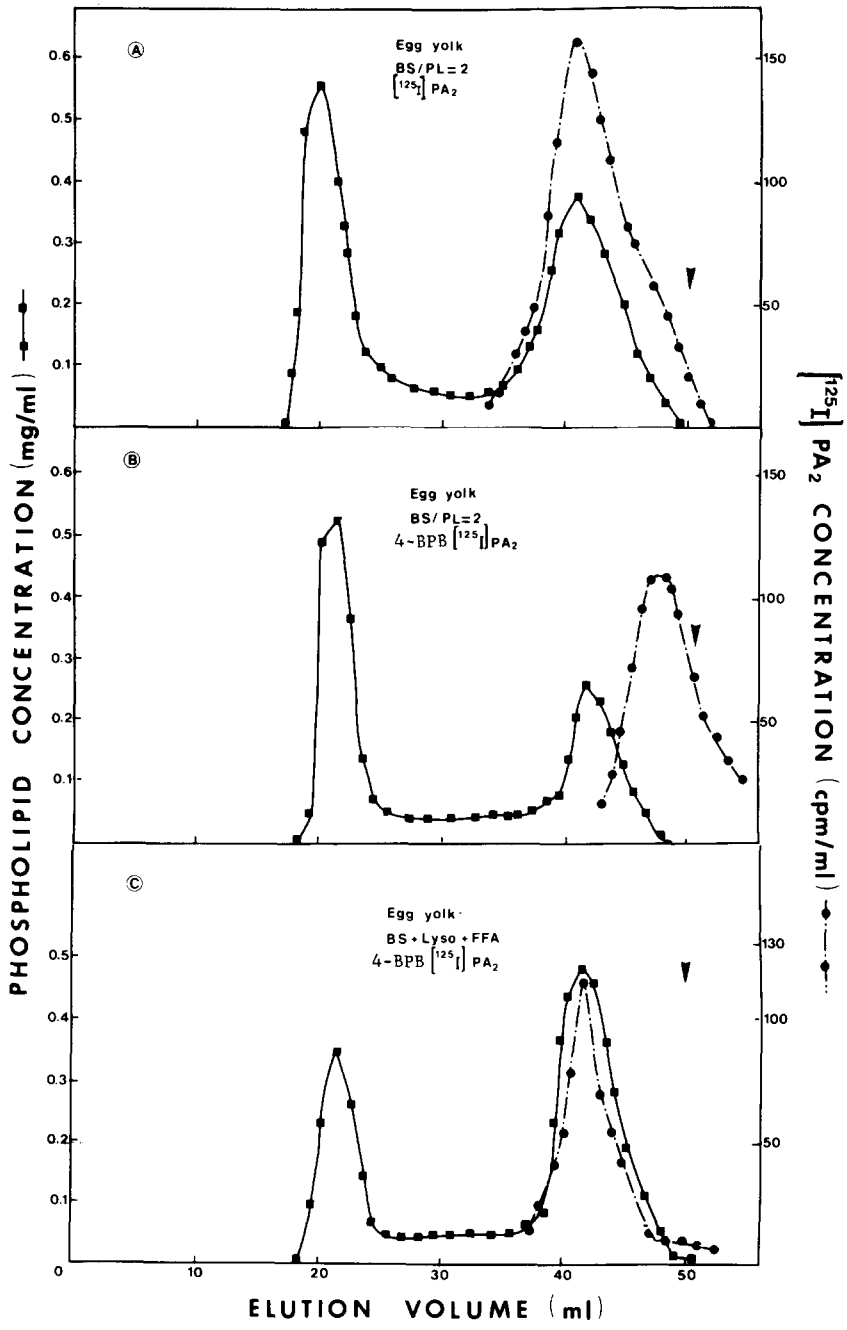

Fig. 5. Agarose A4 gel chromatography of incubations. Elutions with standard buffer containing 1.2 mM bile salts. A) Egg yolk lipoprotein emulsion (BS-PL = 2) + 53 μg of ^{125}I-labeled phospholipase A_2. Experimental conditions identical to Fig. 2B. B) Egg yolk lipoprotein emulsion (BS-PL = 2) + 57 μg of ^{125}I-labeled phospholipase inactivated with 4-BPB. Experimental conditions identical to Fig. 2B. C) Egg yolk lipoprotein emulsion incubated with mixed BS-lyso-PC-oleic acid micelles + 57 μg of ^{125}I-labeled phospholipase A_2 inactivated with 4-BPB. The 4-ml incubation contained 22 μmol of lyso-PC, 9.4 μmol of oleic acid, and 62.8 μmol of BS; 0.85 ml was chromatographed. The arrows indicate the elution volumes of the active and inactivated enzyme when chromatographed alone.

inactive enzyme was not eluted with the phospholipid peaks but rather emerged from the column as a single peak, with K_{av} 0.93. Similar results were obtained with the Intralipid emulsion (data not shown).

In order to determine if the lipolytic products were responsible for the association of PA_2 with the mixed micellar peak, incubations were performed by using an egg yolk emulsion and a solution of mixed BS-lyso-PC-oleic acid micelles. The molar proportions of lyso-PC and oleic acid were each 0.30 in relation to PL. The elution profile of this incubation (Fig. 5C) shows that the presence of lyso-PC and oleic acid led to a significant change of the K_{av} of inactive phospholipase A_2; in this case, all the inactive enzyme was co-eluted with the micellar PC peak with a K_{av} of 0.75.

DISCUSSION

The present research was undertaken in an attempt to determine the adsorption of pancreatic phospholipase A_2 to different physiological substrates in the presence of BS. The substrates were chosen as a function of their similarity to those encountered in normal human nutrition. Thus, we selected egg yolk lipoprotein emulsion, Intralipid emulsion, and milk fat globules. The bile salts used were a mixture of four BS in molar proportions consistent with the glycoconjugate-tauroconjugate and dihydroxyl-trihydroxyl ratios in human gallbladder bile.

The bile salts acted upon these substrates to produce a solubilization of the PLs. A considerable proportion of the latter eluted as a complex of 8–10 $\times$ 10^4 daltons, with a BS-PL molar ratio of 3, consistent with the characteristics of a mixed BS-PL micellar structure (18). These results are in agreement with our own previous findings (5), as well as with other work (6, 8), showing a partition of PL between the emulsified and micellar phases when an emulsion is incubated with BS.

The coexistence of emulsified and micellar phases, both containing PLs, raises the question of the phase partition of pancreatic phospholipase A_2. The present results clearly show that 90–95% of the enzyme was bound to mixed BS-PL micelles, regardless of the substrate. Only 3–8% of the enzyme was found in the emulsified phase. This preferential partition in the micellar phase could be due to the fact that the available surface of PLs in the micellar phase would be greater than that in the emulsified phase. However, in the case where only the emulsified phase was present (BS-PL = 0.5, Fig. 1B), the enzyme was not adsorbed to this phase. Furthermore, where both phases coexisted (Fig. 2B), the PLs were about equally distributed between the emulsified and micellar phases. It may thus be reasonably admitted that the surface occupied by the PLs at the interface of emulsified particles is about the same as that occupied at the interface of mixed micelles. It may be concluded that the distribution of the enzyme in favor of the micellar phase is not related to a difference in available surface.

It is known that the hydrolysis step of pancreatic phospholipase A_2 is preceded by its binding to the substrate (19–21) via the participation of a precise region termed the interfacial recognition site (IRS) (20, 22, 33). The capacity of the IRS to penetrate the lipid layer depends on the physico-chemical nature of the interface, e.g., surface potential, fluidity, packing, and compressibility of fatty acid chains (19, 21–26). Demel et al. (26) concluded that the lack of pancreatic phospholipase A_2 activity towards erythrocyte membranes was due to an elevated lateral compression of the membrane phospholipid fatty acid chains. It is thus consistent to suggest that the quality of the interfaces (20) of lipoprotein and triglyceride emulsions are not favorable for the fixation of the enzyme, as shown particularly by incubations with a BS-PL molar ratio of 0.5. With higher BS-PL ratios, mixed BS-PL micelles appear, enabling the enzyme to be fixed on these micelles. This agrees with our prior findings (5), which showed that phospholipase A_2 activity toward Intralipid or egg yolk emulsions was optimum for a BS-PL molar ratio of 1.4 and 1.8, respectively. Enzyme activity was null for a molar ratio of 0.5. Enzyme activity is thus apparently a function of the presence of mixed micelles in the incubation medium. These results support the idea that micelle interface quality favors enzyme binding. The annular zone of the mixed micelle could be the site of penetration of the IRS, since the fatty acid chains are the most accessible in this zone (19, 27, 28).

We showed that hydrolysis products of PLs (fatty acids and lyso-PL) enhance the enzyme-mixed micelle binding. This phenomenon has been observed for pancreatic lipase (6, 29) and colipase (30). The presence of fatty acids in mixed micelles favors the formation of a stable complex among lipase, colipase, and the micelle (29). In order to study this phenomenon with phospholipase A_2, it is necessary to dissociate the fixation and hydrolysis steps. This was rendered possible by the use of ^{125}I-labeled enzyme whose catalytic site had been blocked with 4-BPB (12). The results (Fig. 5B) show that the peak of the inactivated enzyme did not coincide with that of the mixed micelles, although the elution profile of the inactivated enzyme was modified in comparison with that of the same enzyme eluted from an incubation in the absence of substrate. These results indicate a weak association between the inactivated enzyme and mixed micelles. We observed, however, that when the incubation containing the egg yolk emulsion

as substrate also received mixed lyso-PC-BS-oleic acid micelles, the binding of inactive enzyme to PLs was restored (Fig. 5C). It is thus possible that the presence of PL hydrolysis products generated in the molecular environment close to the IRS participates in the stability of the enzyme-micelle binding by modifying the mixed micelle interfacial quality. This observation is consistent with previous data showing a high affinity between PA_2 and negatively charged lipids (31, 32). Recently, Jain et al. (32) showed convincingly that pancreatic phospholipase A_2 binds instantaneously only to ternary codispersions of dialkyl (or diacyl) phosphatidylcholine, lysophosphatidylcholine, and fatty acid. Binding of the pig pancreatic enzyme to vesicles of 1,2-di(tetradecyl)-rac-glycero-3-phosphocholine could not be detected even after long incubation below, at, or above the phase transition temperature.

In the case of pancreatic (pro)phospholipase A_2, the results clearly show that the precursor is bound neither to the emulsified nor to the mixed micelle phase. This is consistent with prior studies (33) showing that the precursor lacks a functional IRS. This implies that zymogen activation in vivo by trypsin probably occurs in aqueous medium.

Our results demonstrating the presence of pancreatic phospholipase A_2 in the micellar phase are to be compared with the conclusions from previous work (6, 7). These authors indeed showed that under certain circumstances there exists in vitro a synergy between lipase and pancreatic phospholipase A_2 activities towards triglyceride emulsions, whose oil-water interface is covered with PLs. According to these authors, PL hydrolysis by phospholipase A_2 would lead to the subsequent adsorption of lipase to the lipid globule interface. Nevertheless, in order to demonstrate the influence of this phenomenon, these authors used an excess of phospholipase A_2 in relation to the pancreatic lipase. This excess is probably necessary, since we showed that only a small percent of phospholipase is present in the emulsified phase.

Another interpretation of the stimulation of lipase activity by phospholipase A_2 could be the release of trapped colipase/lipase on phospholipid hydrolysis of these mixed micelles which behave as competitive lipase-colipase inhibitors. This interpretation is in line with data presented by Patton and Carey (8) who demonstrated the inhibition of human pancreatic lipase-colipase activity by mixed taurodeoxycholate-phospholipid micelles.

In addition, based on certain results (34, 35), it may be estimated that human pancreatic juice contains a lipase-phospholipase molar ratio close to unity. It is thus possible that under conditions of intestinal physiology, the synergy between the two enzymes is less significant than that observed in vitro with an excess of phospholipase A_2. On the other hand, it appears that bile salts are also effective for the removal of PL from the interface (5–8). In conclusion, these results show the requirement for a sufficient biliary secretion in order to obtain an efficient micellar solubilization of phospholipid substrates, leading to the subsequent hydrolysis by pancreatic phospholipase A_2.

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