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# Inhibition of Lipase Adsorption at Interfaces. Role of Bile Salt Micelles and Colipase†

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ABSTRACT: The effects of bile salts and colipase on the adsorption of lipase at an interface were studied by hydrophobic affinity chromatography on phenyl- and octyl-Sepharose. In the absence of bile salts, lipase or colipase binds separately to the gel. This is unchanged in the presence of adsorbed bile salts, when one bile salt molecule is associated per hydrophobic ligand. The same data are obtained in the presence of monomeric bile salt solutions. In contrast, lipase adsorption is totally prevented in a micellar bile salt solution. These results favor the idea that the formation of a lipase–bile salt complex in solution is responsible for the lack of interfacial lipase adsorption.

Pancreatic lipase normally acts on triglyceride emulsified particles (Sarda and Desnuelle, 1958). This interfacial enzyme exerts its full activity in the presence of biliary lipids, especially bile salts (Morgan and Hofman, 1971; Borgström and Erlanson, 1973). Therefore, in the physiological concentration range, above the critical micellar concentration (cmc)[1] bile salts hinder lipase adsorption on tributyrin interfaces (Vandermeers et al., 1975; Borgström, 1975) or model interfaces such as siliconized glass beads (Chapus et al., 1975; Momsen and Brockman, 1976a). Using these interfaces, it has been demonstrated that a protein cofactor (Morgan et al., 1969), colipase (Maylié et al., 1971), reverses this inhibition and allows lipase adsorption in presence of micellar bile salts solutions (Vandermeers et al., 1975; Borgström, 1975; Chapus et al., 1975; Momsen and Brockman, 1976a,b).

The most general hypothesis is that an adsorbed bile salt monolayer counteracts the interfacial fixation of the enzyme (Borgström, 1975; Chapus et al., 1975; Morgan et al., 1969). However, the studies supporting this view do not distinguish the effect of bile salts at the interfaces from that of bile salt micelles or premicelles in solution (Small, 1971).

Moreover, it has been recently shown that bile salts may associate with lipase in the concentration range of the cmc (Borgström and Donner, 1976). This fact favors the idea that the lack of enzyme adsorption could be due to interactions between bile salts and lipase in solution (Momsen and Brockman, 1976b).

In order to contribute to the understanding of this problem, a study has been performed using hydrophobic affinity chromatography. Octyl-Sepharose and phenyl-Sepharose gels provide hydrophobic sites permitting the study of the interactions between protein and lipid structures. The advantage of the method appears by giving a clear distinction between the interactions taking place in solution or at the interface.

It was thus possible to show that the interactions in solution between bile salt micelles and pancreatic lipase hinder the enzyme adsorption on hydrophobic interfaces.

## Materials and Methods

Lipase was purified (Verger et al., 1969) from a delipidated porcine pancreas powder (Organon) by F. Ferrato and R. Verger. Traces of cofactor were removed as previously reported (Rietch et al., 1977). The specific activity of the mixture of lipases LA and LB was 3170 units/mg of protein.

The pancreatic colipase used was a mixture of porcine colipase I and II, as prepared by M. Charles and M. Astier (Maylié et al., 1973). The specific activity of the preparation was 5480 units/mg of protein.

Lipase activity was measured in presence of bile salts on triolein stabilized by arabic gum at pH 9.0 and 25 °C with the potentiometric method (Desnuelle et al., 1955). Sufficient amounts of colipase (bovine colipase, generous gift of L. Sarda, Marseille) were added to the assay system since colipase-free lipase was strongly inhibited by micellar solutions of bile salts. Advantage was taken of the activating effect of the cofactor toward purified lipase to estimate the colipase concentration under the same conditions.

Sodium deoxycholate (Merck Co.) and sodium dehydrocholate (Calbiochem, A grade) were used without further purification. Labeled [$^{14}$C]deoxycholate came from the Ra-

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[1] Abbreviation used: cmc, critical micellar concentration.

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TABLE I: Binding of Lipase to Phenyl-Sepharose Gel.[a]

| % adsorbed lipase | | | | |
|---|---|---|---|---|
| Without colipase | | | With colipase | |
| pH 9.5 | pH 7.0 | 2 M NaCl, pH 7.0 | Lipase + colipase mixed, pH 7.0 | Colipase first, lipase second, pH 7.0 |
| 39 | 36 | 97 | 81 | 77 |

[a] For experimental details, see text. Results are expressed as percentage of loaded lipase.

diochemical Centre, Amersham. 3$\alpha$-OH bile salts were quantitatively determined using an enzymatic automatic method (Domingo et al., 1972). Radioactivity determinations were done by addition of aliquots to 10 mL of Unisolve 1 (Koch light laboratories).

The gels used for hydrophobic affinity chromatography were phenyl-Sepharose CL-4B and octyl-Sepharose CL-4B purchased from Pharmacia (Sweden). The hydrophobic substituents in such gels are known as "arms" with the following structure as given by the manufacturer:

$$\text{(Sepharose CL-4B)-O-CH}_2\text{-}\underset{\text{OH}}{\overset{\text{CH}}{}}\text{-CH}_2\text{-O-R}$$

where R is an octyl or a phenyl group in octyl-Sepharose and phenyl-Sepharose, respectively. The ligand (phenyl or octyl group) concentration was approximately 40 $\mu$mol/mL gel bed.

Gels were packed in 1-mL columns (0.5 × 5.9 cm) consisting of disposable syringes and the columns were equilibrated by running 10 mL of the buffer solution. Gel filtrations were carried out at 4 °C at a mean flow rate of 2 mL/h. All solutions were made in 20 mM Tris, 1 mM $CaCl_2$ buffer. Twenty-five micrograms of lipase and/or 25 $\mu$g of colipase in 0.2 mL of solution were loaded on each column. Ten fractions of 1.5 mL (40 void volume) were collected. Lipase and colipase activities were determined in the fractions as described above.

To determine the amount of adsorbed bile salts on the gels, 60 to 90 $\mu$mol of sodium deoxycholate and trace amounts of [$^{14}$C]deoxycholate were loaded in 0.2 mL of buffer. Bile salts and radioactivity were measured in the eluted fraction. In our experimental conditions, the cmc of sodium deoxycholate and sodium dehydrocholate were respectively 2 mM and 140 mM (Small, 1971).

## Results

I. *Binding of Lipase and Colipase to Hydrophobic Gels in Absence of Bile Salts.* As shown in Table I, 39% of the lipase was bound when loaded on a phenyl-Sepharose column equilibrated in buffer at pH 9.5. Lowering the pH to 7.0 does not change significantly the amount of adsorbed enzyme (36%). However, when the ionic strength of the buffer was raised to 2 M (NaCl), 97% of the enzyme adsorbed to the gel. This result indicates that hydrophobic forces are probably involved in the binding of the enzyme to the gel since it is known that raising the ionic strength enhances hydrophobic interactions (Tanford, 1973). Octyl-Sepharose gel retained more tightly the lipase since all the loaded enzyme remained adsorbed to the gel column in all sets of conditions tested: pH 7.0 or pH 9.5, low or high (2 M NaCl) ionic strength.

Colipase alone was found to bind strongly both types of hydrophobic gels. More than 90% of the colipase remained attached to the columns equilibrated in the buffers described above.

Lipase binding was enhanced more than two times, when a mixture of lipase and colipase (1 to 4 molar ratio) was loaded on a column equilibrated with buffer at pH 7.0. The same type of results were obtained by loading first colipase and, after rinsing the column with 15 void volumes, percolating lipase through the column. Under these conditions, 77% of the lipase was retained on the gel (see Table I). These results clearly indicate the existence of lipase–colipase interactions on the hydrophobic gels surfaces.

When lipase alone was adsorbed to the gel under the various conditions indicated in Table I, nearly all the enzyme was eluted by a micellar solution (12 mM) of sodium deoxycholate. These results clearly indicate the effect of deoxycholate on the reversibility of lipase adsorption to hydrophobic gels.

II. *Binding of Bile Salts to Hydrophobic Gels.* As indicated by the manufacturer, detergents can be tightly bound to hydrophobic gels. The octyl-Sepharose gel has a ligand concentration of about 40 $\mu$mol/mL of gel bed. Three columns each containing 1 mL of octyl-Sepharose gel were equilibrated with buffer, and then 60, 70, and 90 $\mu$mol of sodium deoxycholate in a volume of 0.2 mL were respectively loaded on each column. Then 60 void volumes of buffer were filtered through the gel of each column; 39, 42, and 43 $\mu$mol, respectively, of deoxycholate remained bound to the three columns. In another experiment, 80 $\mu$mol of sodium deoxycholate plus trace amounts of [$^{14}$C]deoxycholate were submitted to the same chromatography. By radioactivity measurements, 46 $\mu$mol of adsorbed bile salts without change of the specific radioactivity was found in the eluted fraction.

These results indicate that bile salts can adsorb tightly to the hydrophobic gel in a ratio of one detergent molecule per octyl chain. Thus one can assume that the hydrophobic sites are "covered" with bile salts. Furthermore, adsorbed bile salts molecules cannot be appreciably washed out by rinsing the columns with buffer.

III. *Binding of Lipase and Colipase to Hydrophobic Gels Coated with Bile Salts.* For these studies we used 1-mL columns loaded with 80 $\mu$mol of sodium deoxycholate and washed with 60 void volumes of buffer to remove the bile salt excess as described previously. Only trace amounts of bile salts could be detected in the washing solution.

Using phenyl-Sepharose coated with bile salts, 40% of the lipase activity remained attached to the gel columns after elution with the buffer. Under the same conditions, all the lipase was bound to octyl-Sepharose.

In a second step, the columns of the two kinds of gels were eluted with a buffer containing 12 mM sodium deoxycholate. Almost all the adsorbed enzyme was recovered in the eluted fractions: 98% and 95% with phenyl-Sepharose and octyl-Sepharose, respectively.

All colipase was bound to both kinds of gel coated with deoxycholate molecules. When a mixture of lipase and colipase was loaded on such an octyl-Sepharose column, no lipase emerged from the column and elution with 12 mmol of sodium deoxycholate removed only 6% of the adsorbed enzyme.

IV. *Binding of Lipase and Colipase in Presence of Monomeric Bile Salts Solutions.* Two kinds of experiments were performed on octyl-Sepharose columns equilibrated and eluted with monomeric bile salt solutions. In the first one, a 0.24 mM sodium deoxycholate solution, which is well below the cmc, was used. In the other experiments, we equilibrated the columns with a 12 mM sodium dehydrocholate solution, which does not form micelles under these conditions (Small, 1971). In both cases, 93% and 100%, respectively, of the lipase remained adsorbed to the gels. In a subsequent elution step, 90% of the lipase activity was eluted from the column by percolating a micellar solution of 12 mM sodium deoxycholate.

TABLE II: Binding of Lipase to Hydrophobic Gels in a Micellar Deoxycholate Solution.[a]

| Na deoxycholate concn (mM) | % adsorbed lipase | |
|---|---|---|
| | Phenyl-Sepharose | Octyl-Sepharose |
| 2.4 | 0 | 7 |
| 12 | 0 | 3 |
| | Lipase + colipase mixed | |
| 12 | 59 | 96 |
| | Colipase first, lipase second | |
| 12 | 65 | 93 |

[a] For experimental details, see text. Results are expressed as percentage of loaded lipase.

V. *Binding of Lipase and Colipase in Presence of Micellar Deoxycholate Solutions.* As shown in Table II, there was a negligible adsorption of pancreatic lipase to octyl-Sepharose or phenyl-Sepharose in the presence of a 2.4 mM or 12 mM sodium deoxycholate micellar solution.

In contrast, all the colipase loaded was found to be adsorbed on columns equilibrated with 12 mM sodium deoxycholate. Colipase has a higher affinity for a hydrophobic gel coated with deoxycholate than for a deoxycholate micelle. Respectively 59% and 96% of the lipase activity was retained on phenyl-Sepharose and octyl-Sepharose (see Table II) when a mixture of colipase and lipase (4 to 1 molar ratio) was percolated through a hydrophobic gel column equilibrated in 12 mM sodium deoxycholate. The amounts of enzyme adsorbed to the same gels were respectively 65% and 93% when colipase was first loaded on the columns. It appears clear that colipase permits lipase fixation on a hydrophobic surface in the presence of micellar deoxycholate solutions.

## Discussion

"Hydrophobic affinity chromatography" had been applied recently to some lipolytic enzyme purification such as human (Verine et al., 1974) and pig (Nieuwenhuizen et al., 1976) adipose tissues lipases and *Pseudomonas mephitica* var. *lipolytica* lipase (Kosugi and Suzuki, 1976). In these reports, desorption of the protein from the gel was achieved by increasing the salt concentration. This behavior indicates the involvement of electrostatic forces between the proteins and the gels. In contrast, during the present work the presence of 2 M NaCl increased significantly the affinity of the lipase for the phenyl-Sepharose column. Such an effect is in agreement with the role of salt on hydrophobic interactions (Tanford, 1973). Thus the present study shows for the first time the involvement of hydrophobic forces between the lipase and the hydrophobic agarose derivatives. This result can be understood if one compares the chemical structure of the hydrophobic gels used (see Materials and Methods) and the lipase substrates. However, lipase binds more strongly to the octyl-Sepharose than to the phenyl-Sepharose. This can be explained by the higher hydrophobic character of the octyl chain compared with the phenyl group (Hofstee, 1973). Colipase adsorbs completely to both types of gel and enhances the lipase binding.

The most widely accepted hypothesis by which bile salts prevent lipase adsorption is the existence at the interface of a bile salt monolayer counteracting the enzyme fixation (Borgström, 1975; Chapus et al., 1975; Morgan et al., 1969; Brockerhoff, 1973). The use of hydrophobic gel columns allows a quantitative estimation of the tightly adsorbed bile salts under conditions where really no bile salts are present in the column eluent. We found a stoichiometric adsorption of one bile salt molecule per hydrophobic ligand. This system could be schematically compared with an immobilized mixed micelle in which one of the micellar constituents is the immobilized hydrophobic ligand, the other component being the bile salt. From these results it appears likely that the presence of a bile salts coat on the hydrophobic gels does not hinder significantly the lipase binding. Furthermore the equilibration of the column with a monomeric bile salt solution does not affect the binding.

In contrast, when the hydrophobic column is washed with a micellar bile salt solution, the lipase is completely eluted from the column. Similarly no lipase binding was observed when the same column was initially equilibrated with a micellar bile salt solution. These results support the idea of the formation of lipase–bile salt micelle complex which has a negligible affinity for the interface. Such an hypothesis has been previously formulated by Momsen and Brockman (1976b) and is reinforced by the recent finding of an association between lipase and bile salt micelles (Borgström and Donner, 1976).

Our results indicate that colipase binds tightly to hydrophobic gels in absence or presence of a micellar bile salt solution. This observation raises the question about the differences in interfacial qualities between a bile salt micelle and a hydrophobic surface covered with bile salt. Due to an unknown molecular mechanism, the binding of colipase is more efficient to a mixed interface containing bile salt than to a pure bile salt micelle. The situation is exactly the opposite in the case of lipase. Its affinity for the micellar solution probably prevents its interfacial fixation to the gel by a competition process (Momsen and Brockman, 1976b).

Similar lipase adsorptions were observed when colipase and lipase were mixed before loading them on the column or when colipase was adsorbed in a first step and lipase in a second. Recently Donner et al. (1976) reported that pancreatic lipase binds to colipase, to form a 1:1 molar complex in the absence of any lipid interface. Formation of such a complex is certainly not necessary for the positive effect of colipase on lipase fixation to hydrophobic gels in absence or presence of bile salts.

Specificity for the interface is clear from the work of Borgström: colipase does not favor the binding of lipase to a bile salt covered hydrocarbon-water interface (Borgström, 1976). The presence of bile salt is not per se required to observe an influence of colipase on the interfacial fixation and consequently lipase activity. It has been previously shown that, without bile salt, rat pancreatic lipase can adsorb on a lipid mixture in the presence of colipase (Lairon et al., 1976). Studies using monomolecular lipid films indicate a clear interaction between the enzyme and its cofactor in the interface for a given range of the quality of the lipid interface (Verger et al., 1977). Such specific effects of colipase on lipase adsorption explain the activity enhancement toward triglycerides observed in the absence of bile salts (Borgström and Erlanson, 1973; Maylié et al., 1973; Nano and Savary, 1976; Julien et al., 1972).

Thus hydrophobic gels could be used as immobilized model interfaces which can mimic the quality of a lipase–substrate interface.

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## References

Borgström, B. (1975), *J. Lipid. Res. 16*, 411.
Borgström, B. (1976), *FEBS Lett. 71*, 201.
Borgström, B., and Donner, J. (1976), *Biochim. Biophys. Acta*

*450,* 352.
Borgström, B., and Erlanson, C. (1973), *Eur. J. Bichem. 37,* 60.
Brokerhoff, H. (1973), *Chem. Phys. Lipids 10,* 215.
Chapus, C., Sari, M., Semeriva, M., and Desnuelle, P. (1975), *FEBS Lett. 58,* 155.
Desnuelle, P., Constantin, M. J., and Baldy, J. (1955), *Bull. Soc. Clin. Biol. 37,* 285.
Domingo, N., Amic, J., and Hauton, J. C. (1972), *Clin. Chim. Acta 37,* 399.
Donner, J., Spink, C. M., Borgström, B., and Sjöholm I. (1976), *Biochemistry 15,* 5413.
Hofstee, B. H. J. (1973), *Biochem. Biophys. Res. Commun. 53,* 1173.
Julien, R., Canioni, P., Rathelot, J., Sarda, L., and Plummer, J. M. (1972), *Biochim. Biophys. Acta 280,* 215.
Kosugi, Y., and Suzuki, H. (1976), *J. Lipid Res. 17,* 307.
Lairon, D., Nalbone, G., Domingo, N., Lafont, H., Hauton, J. C., Julien, R., Rathelot, J., Canioni, P., and Sarda, L. (1975), *Lipids 10,* 262.
Maylié, M. F., Charles, M., Gache, C., and Desnuelle, P. (1971), *Biochim. Biophys. Acta 229,* 286.
Maylié, M. F., Charles, M., Astier, M., and Desnuelle, P. (1973), *Biochem. Biophys. Res. Commun. 52,* 291.
Momsen, W. E., and Brockman, H. L. (1976a), *J. Biol. Chem. 251,* 378.
Momsen, W. E., and Brockman, H. L. (1976b), *J. Biol. Chem. 251,* 384.
Morgan, R. G. H., and Hofman, N. E. (1971), *Biochim. Biophys. Acta 248,* 143.
Morgan, R. G. H., Barrowman, J., and Borgström, B. (1969), *Biochim. Biophys. Acta 175,* 65.
Nano, L., and Savary, P. (1976), *Biochimie 58,* 917.
Nieuwenhuizen, W., Reman, F. C., Vermeer, I. A. M., and Vermond, T. (1976), *Biochim. Biophys. Acta 431,* 288.
Rietch, J., Pattus, F., Desnuelle, P., and Verger, R. (1977), *J. Biol. Chem. 252,* 4313.
Sarda, L., and Desnuelle, P. (1958), *Biochim. Biophys. Acta 30,* 513.
Small, D. M. (1971), in The Bile Acids, Vol. I, Nair, P., and Kritchevsky, D., Ed., New York, N.Y., Plenum Press, p 249.
Tanford, C., Ed. (1973), in The Hydrophobic Effect, New York, N.Y., Wiley.
Vandermeers, A., Vandermeers-Piret, M. C., Rathé, J., and Christophe, J. (1975), *FEBS Lett. 49,* 334.
Verger, R., De Haas, G. M., Sarda, L., and Desnuelle, P. (1969), *Biochim. Biophys. Acta 188,* 272.
Verger, R., Rietch, J., and Desnuelle, P. (1977), *J. Biol. Chem. 252,* 4319.
Verine, A., Guidicelli, H., and Boyer, J. (1974), *Biochim. Biophys. Acta 369,* 125.

# In Vitro Metabolism of 4-Chlorobiphenyl by Control and Induced Rat Liver Microsomes†

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ABSTRACT: The in vitro metabolism, mechanism of metabolism, and macromolecular binding of a monochlorobiphenyl component of commercial polychlorinated biphenyls (PCB) have been investigated. 4-Chlorobiphenyl was metabolized by rat liver microsomes in the presence of NADPH to yield a major metabolite, 4′-chloro-4-biphenylol, and a number of minor metabolites. The metabolism of deuterium-labeled 4-chlorobiphenyl proceeded with the NIH shift of the isotope and no observed isotope effect thus indicating the intermediacy of an arene oxide. Noninduced rat liver microsomes mediated the covalent binding between the 4-chlorobiphenyl and 4′-chloro-4-biphenylol substrates and endogenous microsomal protein. Prior in vivo administration of a commercial PCB preparation, Aroclor 1248 (Monsanto Chemical Co., containing 48% by weight of chlorine), resulted in an induced microsomal preparation which significantly increased the substrate–protein binding. The effect of various inhibitors on protein binding was investigated. Aroclor 1248 induced microsomes mediated binding of 4-chlorobiphenyl to endogenous and exogenous nucleic acids, indicating a possible mechanism for the previously reported mutagenic action of this chlorobiphenyl. The spectral properties of Aroclor 1248 induced cytochrome P-450 were investigated and compared with the pentobarbital-induced cytochrome fraction.

Polychlorinated biphenyls (PCB)[1] are a class of chemically unreactive and thermally stable compounds that have found widespread industrial application as fire retardants, heat transfer fluids, plasticizers, and dielectric fluids. PCB were first identified in the environment during the late 1960's (Widmark, 1967) and since that time have been found to be among the most ubiquitous and persistent chemical pollutants in the global ecosystem (Hutzinger et al., 1974). The lipophilic nature of PCB results in their accumulation in tissues of species at higher trophic levels within terrestrial communities, and in aquatic species generally.

The biological effects of PCB are diverse and dependent on both the test animal and the dose levels; however, their hepatoxicity is probably the most widely recognized effect (Kimbrough, 1974). Recently it has been shown that female Sher-

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[1] Abbreviations used: PCB, polychlorinated biphenyls; TLC, thin-layer chromatography; TCPO, 1,2-epoxy-3,3,3-trichloropropane; BF, benzoflavone; PPO, 2,5-diphenyloxazole; PAH, polynuclear aromatic hydrocarbons.

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