

Lipid Lateral Organization in Fluid Interfaces Controls the Rate of Colipase Association

Istvan P. Sugar,* Nancy K. Mizuno,[†] Maureen M. Momsen[†], and Howard L. Brockman[†]

*Mt. Sinai School of Medicine, Department of Biomathematical Science, New York, New York 10029 and [†]University of Minnesota, Hormel Institute, Austin, Minnesota 55912 USA

ABSTRACT Colipase, a cofactor of pancreatic triacylglycerol lipase, binds to surfaces of lipolysis reactants, like fatty acid and diacylglycerol, but not to the nonsubstrate phosphatidylcholine. The initial rate of colipase binding to fluid, single-phase lipid monolayers was used to characterize the interfacial requirements for its adsorption. Colipase adsorption rates to phosphatidylcholine/reactant mixed monolayers depended strongly on lipid composition and packing. Paradoxically, reactants lowered colipase adsorption rates only if phosphatidylcholine was present. This suggests that interactions between phosphatidylcholine and reactants create dynamic complexes that impede colipase adsorption. Complex formation was independently verified by physical measurements. Colipase binding rate depends nonlinearly on the two-dimensional concentration of phosphatidylcholine. This suggests that binding is initiated by a cluster of nonexcluded surface sites smaller than the area occupied by a bound colipase. Binding rates are mathematically consistent with this mechanism. Moreover, for each phosphatidylcholine-reactant pair, the complex area obtained from the analysis of binding rates agrees well with the independently measured collapse area of the complex. The dynamic complexes between phosphatidylcholine and lipids, like diacylglycerols, exist independently of the presence of colipase. Thus, our results suggest that lipid complexes may regulate the fluxes of other proteins to membranes during, for example, lipid-mediated signaling events in cells.

INTRODUCTION

Intestinal hydrolysis of triacylglycerols is largely complete in the proximal duodenum (Holtmann et al., 1997). This rapid and efficient process occurs in the presence of bile salts and phosphatidylcholine, major constituents of the bile needed to effectively disperse lipase substrates and facilitate the removal of hydrolysis products (Linthorst et al., 1977). In vitro, however, triacylglycerol lipolysis is inhibited by these surface-active bile constituents (Brockman, 2000a). Inhibition of triacylglycerol hydrolysis in emulsions by the nonsubstrate phosphatidylcholine is characterized by a “lag time” beyond which substrate is rapidly hydrolyzed. The explanation for the more efficient lipolysis in vivo lies in the stomach. Primary lipolysis products, like fatty acid and diacylglycerol, are produced in the stomach through the partial hydrolysis of triacylglycerols catalyzed by gastric lipase. These lipolysis products are known to shorten lag phases in lipolysis, and their presence in the surfaces of lipid droplets entering the intestine is believed to contribute to efficient intestinal lipolysis (Gargouri et al., 1986). Substantial amounts of procolipase, a protein cofactor of pancreatic lipase, but not gastric lipase, are secreted and activated to colipase in the stomach (Sörhede et al., 1996; Winzell et al., 1998) as well as in the intestine. In vitro, colipase acts synergistically with lipolysis products to shorten the lag phase of triglyceride hydrolysis by pancreatic lipase (Borgström, 1980). Thus, gastric lipolysis, coupled with colipase

addition in the stomach and at the beginning of the intestine, primes lipid droplets for attack by pancreatic lipase as they enter the intestine (Winzell et al., 1998).

Studies of the interaction of pancreatic lipase and colipase to interfaces have provided clues as to how colipase and lipolysis products help pancreatic lipase overcome the inhibition that occurs in phosphatidylcholine-rich interfaces. These studies show that colipase binds preferentially and avidly to surfaces containing lipolysis products. From such data it has been inferred that in phospholipid-rich interfaces colipase laterally concentrates substrates and products of lipolysis in its vicinity (Momsen et al., 1997). This putative lipid-colipase nanodomain of one colipase molecule and 20 to 30 reactant acyl chains facilitates the adsorption of pancreatic lipase to the interface, a prerequisite for hydrolysis of the triacylglycerols in the particle (Dahim and Brockman, 1998). Thus, a key early event in the hydrolysis of triacylglycerols is the binding of colipase to the interface, either alone or as a complex with lipase. Once this occurs, the stage is set for the initiation of lipolysis that, as a consequence of the generation of more reaction products, proceeds autocatalytically.

Structurally, colipase is a small, amphipathic wedge-shaped protein stabilized by five disulfide cross-links (Egloff et al., 1995a). Presumably as a consequence of this structure, its cofactor activity is retained after treatment with acid (Canioni et al., 1977), organic solvents (Rugani et al., 1992), and denaturants (Crandall and Lowe, 2001). Colipase binds to the gas-liquid model interface with high affinity without loss of cofactor activity (Momsen et al., 1995). These properties are in contrast to both pancreatic lipase, for which colipase serves as a cofactor, and to other lipases and lipase cofactors like apolipoprotein CII (Storjohann et al., 2000). Thus, for this study,

Received for publication 14 June 2001 and in final form 27 August 2001.

Address reprint requests to Howard L. Brockman, University of Minnesota, Hormel Institute, 801 16th Avenue, NE, Austin, MN 55912. Tel.: 507-437-9620; Fax: 507-437-9696; E-mail: hlbroc@smig.net.

© 2001 by the Biophysical Society

0006-3495/01/12/3387/11 \$2.00

we considered colipase to be a rigid, amphipathic, diffusional "projectile" for probing the availability of surface area to which it can adsorb.

Previously, we characterized the saturation level of binding of colipase to surfaces of a model diacylphosphatidylcholine, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), lipolysis reactants, like diacylglycerols and fatty acids, and their mixtures. Analysis of this data showed that colipase interacted preferentially with lipolysis reactants, implying its ability to laterally concentrate them in its vicinity (Momsen et al., 1997). In the present study we investigate the lipid species and composition dependence of the initial step of colipase adsorption using these same lipids. We have again used monomolecular films as a model interface because their composition and lipid packing density can be varied independently while they remain planar (Brockman, 1999). The results show that the initial rate of colipase binding depends strongly on the packing density of SOPC molecules in the interface. It also depends on the nonphospholipid species present but, surprisingly, only in the presence of SOPC. Analysis of the rate data is consistent with a model in which colipase adsorption occurs at small clusters of reactant molecules but is inhibited by dynamic complexes of SOPC and reactants. Because complex formation is a property of the interface and involves lipids that, in cells, are involved in lipid-mediated signaling events, the results of this study may have more general implications for the regulation of lipid-mediated protein translocation.

MATERIALS AND METHODS

Reagents

SOPC was from Avanti Polar Lipids (Alabaster, AL). 1,2-dioleoylglycerol (1,2-DO) was from Sigma (St. Louis, MO). 1,3-dioleoylglycerol (1,3-DO), 13,16-*cis,cis*-docosadienoic acid (DA), trioleoylglycerol (TO), and oleoyl methanol (OM) were from Nu-Chek Prep, Inc. (Elysian, MN). The purification of water and preparation of solvents, buffer, and lipid solutions have been previously described (Momsen et al., 1997). Colipase used in this study was prepared and converted to [14 C]colipase by reductive methylation as described previously (Schmit et al., 1996). The radiolabeled colipase had a specific radioactivity of 9.76 μ Ci/ μ mol.

Methods

The automated, Langmuir-type film balance used for obtaining surface pressure-molecular area isotherms for pure lipids and mixtures has been recently described (Li et al., 2000). Isotherms were collected at 24°C under a humidified argon atmosphere on an aqueous subphase of phosphate-buffered saline (10 mM potassium phosphate, pH 6.6, 0.1 or 1.0 M NaCl, 0.01% NaN_3). Phase transition pressures and areas in surface pressure-molecular area isotherms were determined using FilmFit software (Creative Tension, Austin, MN). The compositions of SOPC complexes with lipolysis reactants were determined from discontinuities in plots of phase transition pressure versus the negative log of the reactant mole fraction (Smaby and Brockman, 1987).

Experimental details for measuring the adsorption of colipase have been described previously (Momsen et al., 1997; Muderhwa and Brockman, 1990; Dahim and Brockman, 1998). Briefly, a cylindrical Teflon trough

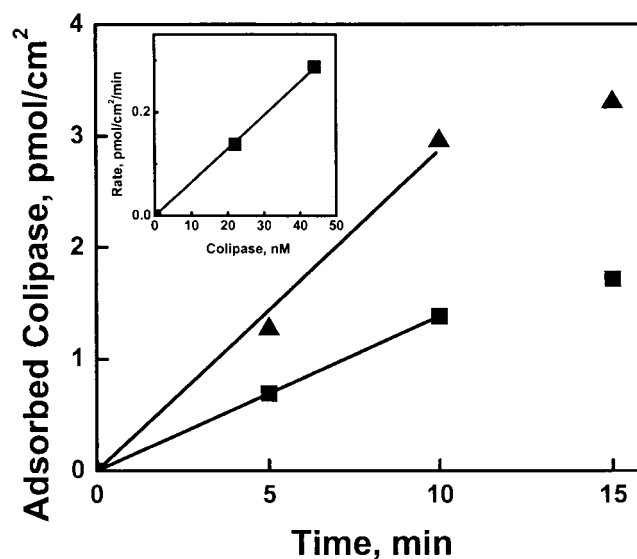


FIGURE 1 Time course of colipase adsorption to diacylglycerol monolayers. Initial surface pressure of 1,2-DO was 32 mN/m. Colipase was 22 (■) or 44 (▲) nM in the 24°C buffer subphase stirred at 50 rpm. The inset shows the slopes of the lines, determined over 10 min, as a function of subphase colipase.

(surface area = 20.4 cm², volume = 19.5 ml) was filled with phosphate-buffered saline. Temperature was held at 24°C. Lipid films were spread from a hexane/ethanol (95:5) solution until the desired surface pressure was reached. After allowing the lipid monolayer to stabilize for 5 min, stirring (50 rpm) was started, and after 2 min, colipase solution was injected to give the desired concentration in the aqueous phase. Stirring was continued for 10 min, and then the monolayer was collected on one side of a hydrophobic filter paper disk. The amount of colipase adsorbed was determined by liquid scintillation counting. Based on prior studies, 90% recovery of the monolayer and 15 μ l of carryover of subphase onto the paper was assumed (Momsen and Brockman, 1997).

RESULTS

Kinetics of colipase adsorption

Previously, we had shown that adsorption of colipase in the absence of any lipid monolayer, i.e., at the argon-buffer interface, does not cause its denaturation (Momsen et al., 1997). To establish initial rate conditions for the present study, we showed in preliminary studies that a stirring rate of 50 rpm, 44 nM colipase in the aqueous subphase, and an incubation time of 10 min resulted in colipase binding to the argon-buffer interface of 3.0 pmol/cm² (data not shown). This value is ~10% of the saturation value, i.e., complete surface coverage, for colipase adsorption (Momsen et al., 1997) and defines the upper limit measured in this study. We next measured colipase adsorption to monolayers of 1,2-DO at 32 mN/m at various times at initial colipase concentrations of 22 and 44 nM. The initial surface pressure of 32 mN/m used in these experiments is near the collapse surface pressure of the 1,2-DO monolayer. As shown in Fig. 1, adsorption

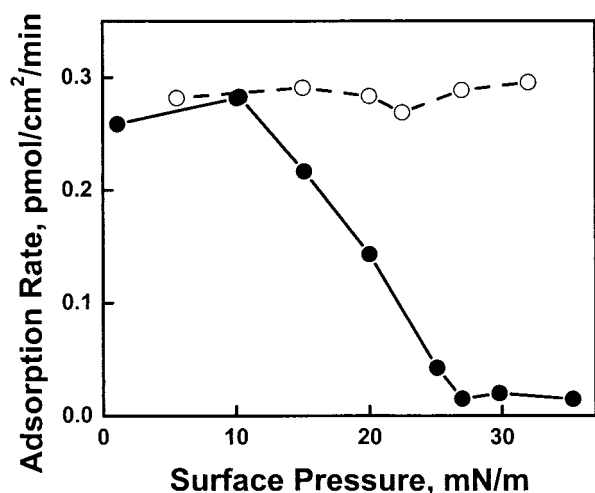


FIGURE 2 Lipid and surface pressure dependence of the colipase adsorption rate. Colipase (subphase 44 nM, 24°C) adsorption to monolayers of SOPS (●) or 1,2-DO (○).

was linear with time up to 10 min at both concentrations. Moreover, the inset shows that the rates of binding, defined as the slopes of the lines in the figure, were proportional to the concentration of colipase added to the aqueous phase. Although the absolute rate of colipase adsorption in this system still depends on the rate of subphase stirring (data not shown), these data show that when stirring is held constant at 50 rpm, the flux of colipase to the interface with a subphase concentration of 44 nM is the same as that measured in the absence of a lipid monolayer, ~ 0.3 pmol/cm²/min. Thus, colipase adsorption is proportional to its bulk concentration and independent of the presence or absence of a 1,2-DO monolayer. Based on this result, in subsequent experiments rates were calculated from a single measurement of colipase adsorption after a 10-min incubation at a bulk colipase concentration of 44 nM.

The identical adsorption rates for colipase in the presence and absence of a tightly packed 1,2-DO monolayer suggests that the diacylglycerol monolayer at the interface does not present any obstacle to colipase adsorption. To test this hypothesis more thoroughly, colipase adsorption to 1,2-DO monolayers was measured as a function of the 1,2-DO packing density in the monolayer. As shown in Fig. 2 (*open circles*), the adsorption rate is independent of surface pressure at a rate that averages 0.29 ± 0.01 pmol/cm²/min, the same rate observed in the absence of lipid. The range of surface pressures used, 5 to 32 mN/m, corresponds to essentially the entire range of existence of the liquid-expanded state and covers lipid packing densities from 211 to 292 pmol/cm². Thus, the adsorption of colipase is clearly unaffected by the presence of lipase substrate in the interface at all packing densities.

Inhibition by SOPS

Similar experiments performed with SOPS comprising the lipid monolayer gave an entirely different result. As shown in Fig. 2 (*filled circles*), up to a surface pressure of 10 mN/m, the rate of colipase adsorption is comparable with that measured with 1,2-DO lipid monolayers. With increasing surface pressure, however, the rate falls, approaching zero by 28 mN/m. This shows that tightly packed SOPS prevents colipase adsorption at surface pressures above which colipase adsorption to 1,2-DO was unimpeded. However, 28 mN/m is far below the collapse surface pressure of SOPS of ~ 47 mN/m. With respect to molecular packing density, the surface pressures of 28 and 47 mN/m correspond to SOPS concentrations of 258 and 295 pmol/cm², respectively, which are within the range of concentrations of 1,2-DO noted above. This result suggests, qualitatively, that part of the area occupied by each SOPS molecule in the interface may act as an excluded area, blocking colipase adsorption. Alternatively, the lower level of binding could reflect a marked reduction in the affinity of colipase for the interface. We earlier showed, however, that at the surface pressure at which adsorption is reduced by 50%, 20 mN/m (Fig. 2), colipase that had been spread in an SOPS monolayer remained completely bound to the monolayer for the 10-min duration of the experiment (Dahim and Brockman, 1998). Second, at a 10-fold higher concentration in the aqueous phase, colipase binding to SOPS monolayers decreased to zero at essentially the same surface pressure as observed in the present study (Momsen et al., 1997). Thus, the inhibition of binding rate observed in the present study appears to be caused by a reduction in the capacity of the surface to accommodate colipase.

Role of lipase substrates

The data in Fig. 2 show that some portion of the area occupied by SOPS, but not 1,2-DO, impedes the initial rate of colipase adsorption to the interface. In those experiments the surface pressure necessarily varied as a consequence of changing the surface concentration of SOPS. Based on the data in Fig. 2 it should also be possible to vary the area of SOPS by mixing it with 1,2-DO at constant surface pressure. Accordingly, monolayers of 1,2-DO/SOPS at various mole fractions were spread to a surface pressure of 32 mN/m, and the initial rate of colipase adsorption was determined. The surface pressure of 32 mN/m was chosen because it is a value above which adsorption to SOPS alone was negligible (Fig. 2). As shown in Fig. 3 A (*filled circles*), the initial rate of colipase adsorption shows an increase from near zero to ~ 0.3 pmol/cm²/min in the range of 0.3- to 0.6-mole fraction of 1,2-DO. This is qualitatively consistent with the idea that the initial rate of colipase adsorption increases as the excluding SOPS molecules are spread farther apart by an increasing mole fraction of 1,2-DO. Another variation of this experiment was to set the initial

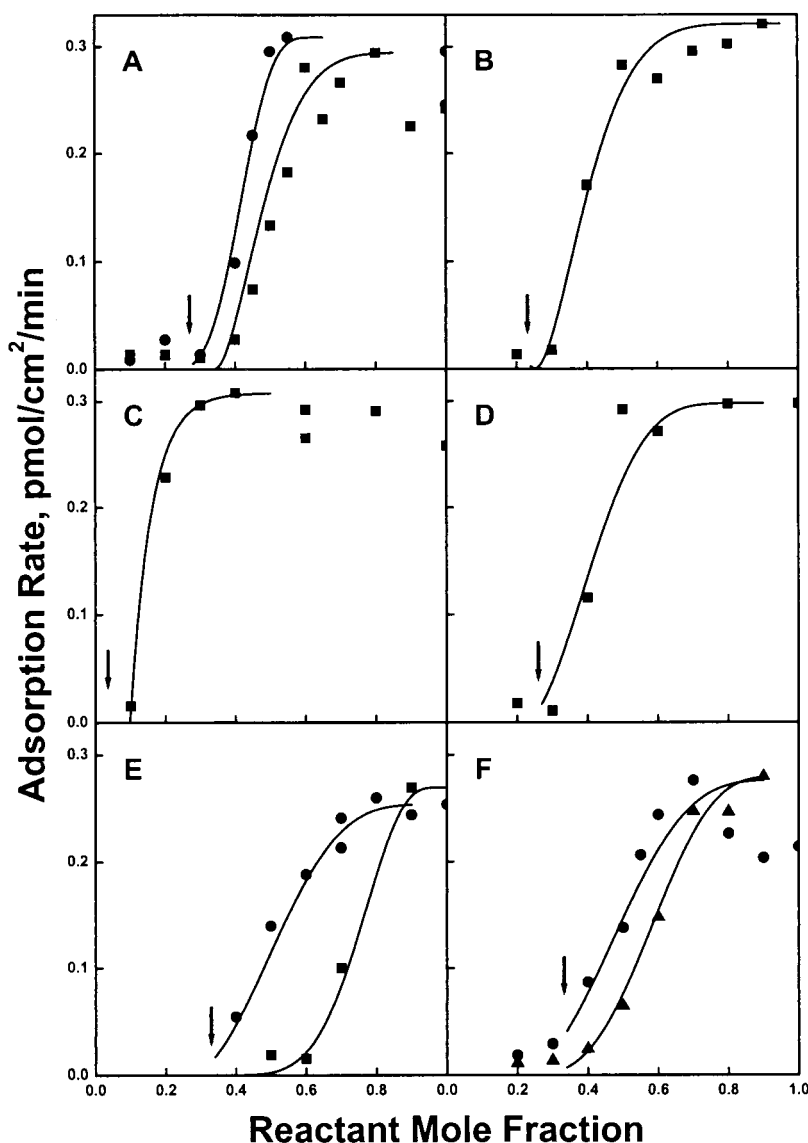


FIGURE 3 Dependence of colipase adsorption rate on interfacial lipid composition. Colipase (subphase 44 nM, 24°C) adsorption to monolayers of SOPC and 1,2-DO (A), 1,3-DO (B), TO (C), OM (D), DA on 0.1 M NaCl (E), and DA on 1.0 M NaCl (F). Initial surface pressure was 32 mN/m (●), 38 mN/m (▲), or >90% of monolayer collapse surface pressure (■). For reference, arrows show complex compositions as determined from data in Fig. 5. Theoretical curves (solid lines) were generated from the parameters in Table 1 as described in the text.

surface pressure at a value $\geq 90\%$ of the collapse surface pressure of the monolayer at a given composition. This allowed the initial surface pressure to follow more closely the limit of solubility of 1,2-DO in the interface as the composition was changed, as was done in earlier enzymatic studies (Tsujita et al., 1989). With this protocol, molecular packing was tighter at all but the highest mole fractions of 1,2-DO, resulting in a shift of the curve to the right (Fig. 3, filled squares).

The specificity of the observed inhibition of colipase adsorption rate by lipolysis reactants was examined using 1,3-DO, TO, OM, and DA (Fig. 3, B–F). This group of compounds is comprised of both substrates and products of lipolysis, and these can be collectively referred to as lipolysis “reactants.” In the case of the fatty acid, DA, the “hydrolysis” reaction measured in earlier studies was the lipase-catalyzed exchange of carboxyl oxygen atoms (Muderhwa and Brockman, 1992a,b).

For some experiments with DA, the subphase salt concentration was increased from 0.1 (Fig. 3 E) to 1.0 M (Fig. 3 F) to increase the stability of monolayers of DA at high surface pressures. The figures show that, with each reactant, there is an abrupt increase in the initial rate of colipase adsorption as reactant mole fraction is increased. For all SOPC/reactant mixtures studied, the maximal rate observed in each set is 0.29 ± 0.02 pmol/cm²/min. This is consistent with the idea that the reactants alone do not provide a significant barrier to colipase adsorption. The data for DA obtained at >90% of collapse on buffered 0.1 M NaCl are shifted to the right relative to data obtained on that buffer at 32 mN/m (Fig. 3 E) or on buffered 1.0 M NaCl at either 32 or 38 mN/m (Fig. 3 F). It is not clear if this reflects instability of the monolayers at these relatively high pressures or, alternatively, if DA presents some obstacle to adsorption of colipase under these conditions.

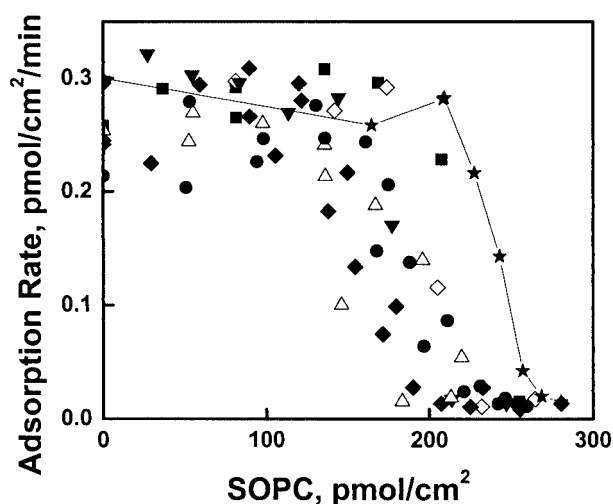


FIGURE 4 Dependence of colipase adsorption rate on the surface concentration of phospholipid. Data from Figs. 2 and 3 are shown for SOPC (★) alone and SOPC mixed with 1,2-DO (▲), 1,3-DO (▼), TO (■), OM (◇), DA on 0.1 M NaCl (△), and DA on 1.0 M NaCl (●).

Mechanism of inhibition by SOPC

The data presented show that the rate of adsorption of colipase to interfaces is inhibited by monolayers of SOPC but not, with the possible exception noted, by monolayers of lipolysis reactants. Inhibition of colipase adsorption by SOPC is complete at surface pressures ≥ 28 mN/m and is relieved by lowering the surface pressure (Fig. 2). At surface pressures above 28 mN/m inhibition can also be relieved by progressive replacement of SOPC by lipolysis reactants (Fig. 3, *A–F*). Substitution of reactant for SOPC increases the spacing of SOPC molecules as does lowering the surface pressure. These observations indicate that it is not simply the surface pressure but the presence of SOPC that inhibits colipase adsorption. If the presence of SOPC is the sole cause of the inhibition of colipase adsorption, then inhibition should depend only on the two-dimensional concentration of SOPC in the interface. However, plotting the data for colipase adsorption to SOPC alone and to the mixed monolayers as a function of SOPC concentration reveals that this is not the case (Fig. 4). With all mixed monolayers, adsorption is inhibited at SOPC concentrations lower than observed with SOPC alone. This indicates that in the mixed monolayers at least part of the reactant molecules contribute to the inhibition of colipase adsorption by SOPC.

How can lipolysis reactants contribute to the inhibition of colipase adsorption in the presence of SOPC but not in its absence? One way is through the formation of exclusionary complexes with SOPC, in the sense of preferred packing arrays (Dervichian, 1958). As recently reviewed, there is increasing evidence for the existence of dynamic lipid complexes or “superlattices” in fluid interfaces (Somerharju et al., 1999). Much earlier, physical studies of lipid-lipid interactions in

monolayers also suggested complex formation between either SOPC or its homolog, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and all of the lipolysis reactants used in this study (Smaby and Brockman, 1985; Cunningham et al., 1989; Smaby et al., 1994). Because phase diagrams for only two of the reactants used in this study have been determined with SOPC (Cunningham et al., 1989; Smaby et al., 1994), experimental surface pressure-area isotherms were measured for SOPC and each of the reactants. The phase diagrams derived from them as described in Materials and Methods are shown in Fig. 5, *A–F*. For all reactants, the phase diagrams are similar to those determined earlier using the SOPC homolog and indicate complex formation. This is evident from the discontinuity in each phase diagram at which the collapse surface pressure deviates downward from that of SOPC (Dervichian, 1958) and is indicated by a down arrow in each panel of Fig. 5. Although for DA there clearly appears to be a discontinuity at or near the previously identified complex mole fraction of 0.67 (Smaby et al., 1994), the collapse surface pressure at 0.67 DA is ~ 1 to 2 mN/m lower at that composition than that for SOPC alone. However, the collapse pressures do appear to be relatively constant in the vicinity of 0.0 to 0.3 DA (Fig. 5 *E*) as revealed by dotted lines showing the average of those values ± 1 SD. The data obtained with 1.0 M salt show a relatively continuous decrease between the collapse surface pressure of SOPC and 0.67 DA. It is relevant here that in addition to the well-known complex between saturated fatty acids and phosphatidylcholines in the bilayer gel state at 0.67 (Lohner, 1991), studies with mixtures of oleic acid and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine show complex formation at 0.33 oleic acid in the gel state (Ortiz and Gómez-Fernández, 1987). Thus, the data are suggestive of, but certainly do not prove, the existence of a second complex composition in the vicinity of 0.33 DA in fluid monolayers. This is indicated by up arrows in Fig. 5, *E* and *F*. If the complex composition for DA is considered to be 0.33, the complex stoichiometry for all reactants is at or below the reactant mole fraction at which colipase adsorption begins (Fig. 3, *arrows*). This suggests that colipase adsorption to reactants in the presence of SOPC (Fig. 3) depends on the availability of surface that is not part of the complex with SOPC.

Requirements for colipase adsorption

One feature of the inhibition of colipase adsorption by SOPC and its complexes with lipase reactants is the abruptness of changes in adsorption rate with changes in surface composition (Fig. 3) or packing (Fig. 2). This suggests that colipase adsorption does not depend simply on the fraction of surface not occupied by excluding lipid. Indeed, it is readily shown that if the excluded area of SOPC or any of its complexes is a constant, the rate of adsorption should vary linearly with SOPC concentration and attain its maximal value at zero SOPC. As shown in Fig. 4, this is clearly not the case for either SOPC alone or for colipase adsorp-

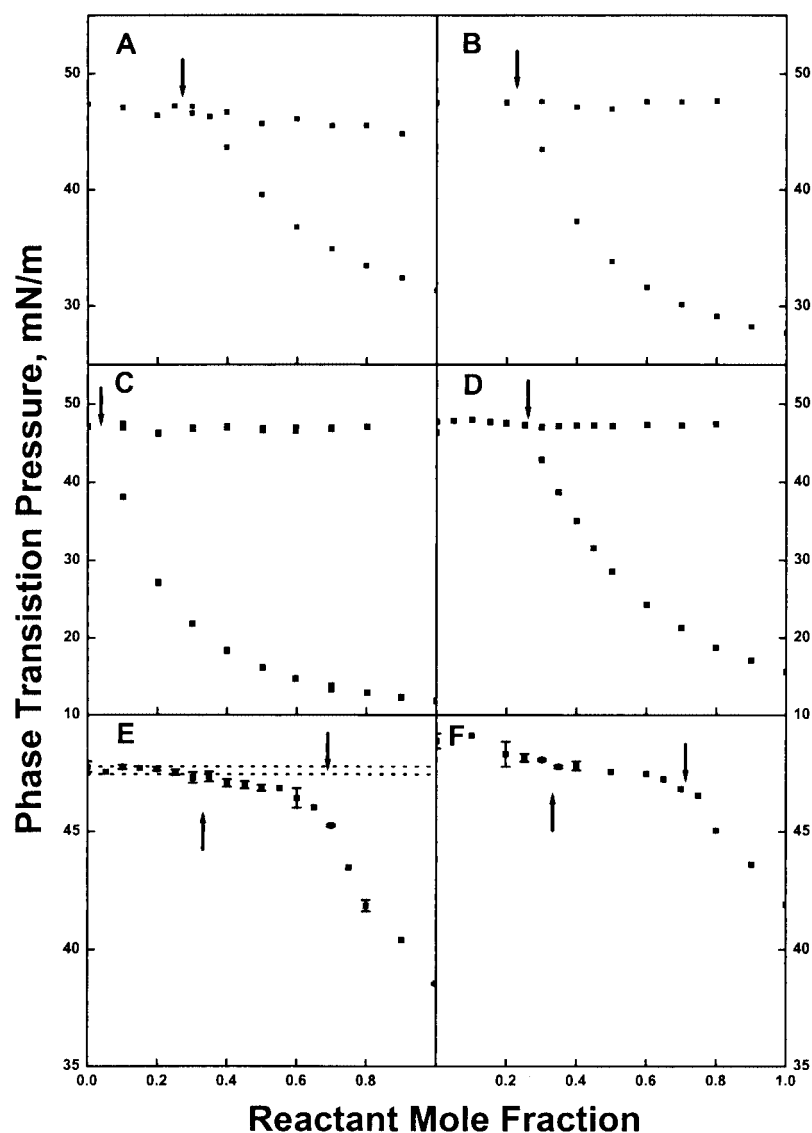


FIGURE 5 Monolayer phase diagrams for SOPC reactant mixtures. Phase transition pressures were determined from the surface pressure-molecular area isotherm at each composition as described in Materials and Methods for SOPC mixtures with 1,2-DO (A), 1,3-DO (B), TO (C), OM (D), DA on 0.1 M NaCl (E), and DA (F) on 1.0 M NaCl. Downward arrows indicate the SOPC-reactant complex composition determined as described in Materials and Methods. The upward arrows denote possible complex compositions for SOPC/DA as described in the text. The dotted lines in E represent the average of the transition surface pressures from 0.0 to 0.3 DA \pm 1 SD.

tion to the mixed monolayers. For the example of SOPC alone, the colipase adsorption rate is zero at 256 pmol/cm² but reaches its maximal value by 206 pmol/cm². This suggests that only a relatively small fraction of the surface must be free of excluding complex for colipase adsorption rate to be maximal.

One way that only a small fraction of free surface would be required is if only part of the colipase molecule needs to interact with the free surface to effect adsorption of the entire molecule. In this model the surface is viewed as a matrix in which a surface-bound colipase molecule replaces lipid in n matrix sites. However, if a cluster of only m ($\leq n$) sites are

required to trigger colipase binding from the aqueous phase, then the fraction of total surface area available for colipase binding, F_a , can be expressed (see Appendix 1) as,

$$F_a = \sum_{j=m}^n X^j (1-X)^{(n-j)} n! / [j!(n-j)!] \quad (1)$$

As described in Appendix 1, X was defined in terms of the excluded area of an SOPC molecule and its complexed reactant, A_{ex} , and n was defined as $500/A_{ex}$. With these substitutions, Eq. 1 was used to analyze the colipase adsorption rate data in the range over which it varied with composition by

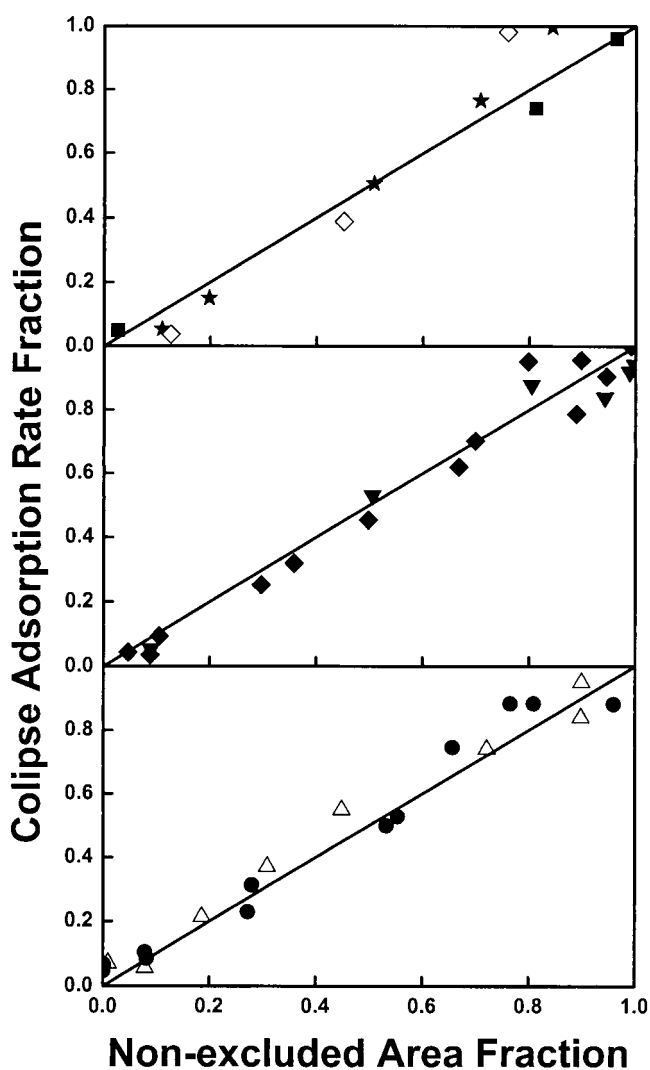


FIGURE 6 Relationship of the relative rate of colipase adsorption to the fraction of nonexcluded area. Nonexcluded areas were calculated using the fitting parameters in Table 1. Lipids were SPC alone (★) and SPC mixed with 1,2-DO (◆), 1,3-DO (▼), TO (■), OM (◇), DA on 0.1 M NaCl (△), and DA on 1.0 M NaCl (●). The solid lines show the theoretical slope of 1.0.

testing the hypothesis that the fractional rate of colipase adsorption, defined as each rate divided by the maximal rate measured in that set of data ($= F_{\text{exp}}$), equals the fraction of uncomplexed reactant surface area, F_a . These best fits of the data to this model are shown in Fig. 6 and indicate reasonable agreement for each data set. The values of m , n , A_{ex} and the coefficient of correlation (r) for the best fits are shown in Table 1. Values of A_{ex} varied between 55 and 76 Å² and, hence, n ranged from 6 to 8. Values of m ranged from 1 to 4, showing that generally one-half or less of a group of sites need to be unoccupied by SOPC for colipase adsorption to occur. Also given in the table is the product of $m \times A_{\text{ex}}$. This represents the size of an interaction site and has an average value of 145 ± 59 Å² or approximately one-third the area occupied by a colipase molecule after becoming bound to the interface. The variability in these values is due in part to scatter in the data and to the requirement that m and n assume only integer values. It may also reflect nonideality of mixing of the complexes with the various lipase reactants. More rigorous testing of this model will require that measurements be made with wider variation of packing for each mixture mole fraction studied. It is interesting to note, however, that the parameters obtained by varying the surface pressure of SOPC monolayers (Fig. 2) and by adding TO to SOPC monolayers at high surface pressures (Fig. 3) gave almost identical results (Table 1). This is expected because the mole fraction of TO in the complex with SOPC is very small, only 0.034. Using the values of m , n , and A_{ex} from the table and the maximal adsorption rate from each data set, theoretical curves were calculated, and these are shown in Fig. 3 (solid lines).

According to the simple statistical model used to analyze the colipase adsorption rates, A_{ex} is the excluded area of one SOPC molecule and its complexed lipase reactant or of SOPC alone if no reactant is present. If this is the case, A_{ex} should be related to the surface pressure-area properties of the lipid monolayer measured at, or interpolated to, the composition of the complex. It is not clear what defines A_{ex} ,

TABLE 1 Cluster analysis of colipase adsorption to SOPC-reactant monolayers

Lipids	X_c	A_c , Å ²	m	n	A_{ex} , Å ²	mA_{ex} , Å ²	r
SOPC*	0.00	54.9	1	8	64	64	1.00
SOPC + 1,2-DO†	0.27	76.2	2	7	76	152	0.99
SOPC + 1,2-DO‡	0.27	76.2	2	6	79	158	0.96
SOPC + 1,3-DO‡	0.23	69.5	2	7	72	144	0.98
SOPC + TO‡	0.03	58.1	1	8	65	65	1.00
SOPC + OM‡	0.26	64.1	2	8	66	132	0.92
SOPC + DA†	0.69	111 (65.4)‡	2	7	67	134	0.98
SOPC + DA‡	0.69	111 (65.4)‡	4	7	67	268	0.96
SOPC + DA§	0.71	125 (69.2)‡	2	7	67	134	0.97
SOPC + DA¶	0.71	125 (69.2)‡	3	8	65	195	0.99

All adsorption rates were measured at 24°C on 10 mM phosphate buffer, pH 6.6 containing at 0.1 M*–‡ or 1.0 M§¶ NaCl. Initial monolayer surface pressure was various*, 32 mN/m†, 90% of collapse‡, 32 mN/m§, and 38 mN/m¶. Values of the reactant mole fraction at the complex composition, X_c , were determined from data in Fig. 5 as described in Materials and Methods. Values of the apparent area of SOPC at the collapse of the complex, A_c , were evaluated at X_c by linear interpolation between values obtained at nearby compositions. Values of m , n , and A_{ex} were determined from the data shown in Fig. 3 using Eq. 1 as described in the text and Appendix I. ‡, Values in parentheses are calculated assuming $X_c = 0.33$ for DA and were used in Fig. 7.

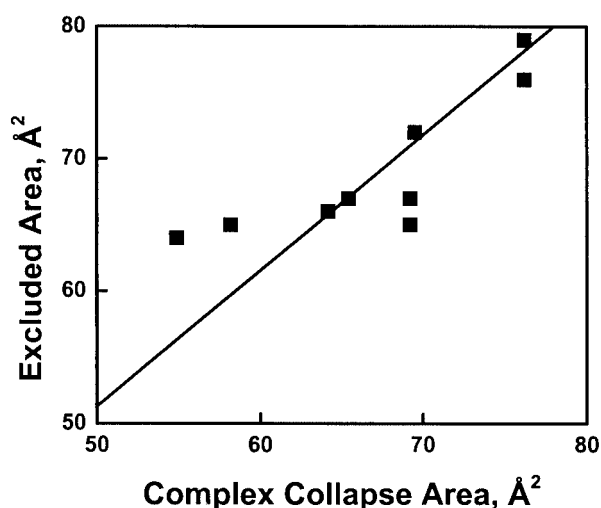


FIGURE 7 Identity of collapse areas of SOPC-reactant complexes with excluded areas for colipase adsorption. Excluded areas are from Table 1 and complex areas at monolayer collapse were determined from surface pressure-molecular area isotherms as described in Materials and Methods. The solid line is a least squares fit of the data forced through the origin.

but as a first approximation the collapse area of the monolayer at the complex composition can be used. This is the area at which further compression of the monolayer causes it to buckle into the third dimension as a consequence of steric repulsion between lipids (Fenwick et al., 2001). A plot of A_{ex} as a function of the interpolated collapse area of each reactant pair at the complex composition, A_c , shows reasonable proportionality with a coefficient of correlation of 0.83 (Fig. 7). A complex composition of 0.33 was assumed for SOPC/DA for reasons described above. The slope of 1.03 is close to the theoretical value of 1.0, supporting the hypothesis that the binding rate of colipase is determined by the fraction of surface area not occupied by SOPC-reactant complex.

DISCUSSION

Phosphatidylcholine is known to prevent the adsorption of proteins to surfaces (e.g., Brash, 1996; Orban et al., 2000). The data presented here show, not surprisingly, that SOPC decreases colipase adsorption rates in a concentration-dependent manner (Fig. 2). Paradoxically, lipolysis reactants were also found to contribute to the lowering of colipase adsorption rate in the presence of SOPC (Fig. 3) but not in its absence (Figs. 2 and 3). A qualitative explanation for this observation is that lipid-lipid interactions between SOPC and lipolysis reactants create inhibiting complexes with excluded areas greater than or equal to that of SOPC alone. The existence of such complexes is shown by physical measurements using SOPC/reactant monolayers (Fig. 5). However, colipase adsorption rate depends on the concentration of SOPC in a nonlinear way (Fig. 4). This suggests

that colipase binding initially requires less nonexcluded surface sites than the total number of sites a colipase molecule occupies after it is bound. Application of a simple statistical model to the binding data is consistent with the model (Fig. 6, Table 1) and indicates approximate identity between the excluded area for colipase binding and the collapse area of SOPC/reactant complexes (Fig. 7). Whereas more extensive data will be required to unequivocally prove or disprove this hypothesis, the present data are supportive and will guide future research. Not addressed in this study was the specificity for phospholipid of either complex formation or inhibition of colipase adsorption. However, phase diagrams do show complex formation for DA with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine and for 1,3-DO with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, or bovine phosphatidylinositol (M. M. Momsen, J. M. Smaby, H. L. Brockman, unpublished data). Future experiments will address the ability of these phospholipid-reactant complexes to inhibit adsorption of colipase and other proteins.

Implications for lipase regulation

In a phosphatidylcholine-rich interface, such as the surface monolayer of an emulsion particle, the challenge for pancreatic lipase is to bind in a catalytically productive manner. This means it must bind in the open conformation of the N-terminal domain in which it has a rather large, flat hydrophobic footprint of $\sim 900 \text{ Å}^2$ (Egloff et al., 1995b). Because of the interaction of reactants and phosphatidylcholine to form dynamic complexes, as indicated from physical measurements (Fig. 5), clusters of reactant molecules sufficiently large to support catalytically efficient lipase binding via the opened lid should be nonexistent up to the complex composition. As reactant mole fraction is further increased, the complex is miscible with uncomplexed reactant (Fig. 5). Unless miscibility is highly nonideal, the existence of clusters containing more than a few uncomplexed reactant molecules should be statistically rare until the percolation threshold of uncomplexed reactant is reached (Stauffer, 1985). Thus, between the complex composition and the percolation threshold, lipase binding should be inhibited. Experimentally, some lipase binding is observed in this compositional region with DA/SOPC monolayers (Dahim and Brockman, 1998). However, the apparent mechanism of the oxygen exchange is coupled or concerted in this compositional regime compared with the random sequential mechanism observed in DA-rich interfaces (Muderhwa and Brockman, 1992b). Also, the extent of the reaction is limited. This suggests that lipase binding in the range of compositions between the complex composition and the percolation threshold occurs in the closed or catalytically inefficient conformation. How this could occur is suggested by recent studies of the C-terminal domain of

lipase. These have shown the potential contribution to lipid binding of the $\beta 5'$ hydrophobic loop in the C-terminal, noncatalytic domain of pancreatic and related lipases (Bezine et al., 1998; van Tilbeurgh et al., 1999). However, this hypothesis remains to be tested. As the percolation threshold for reactant is crossed, lipase binding should increase and should occur in the catalytically efficient conformation as a consequence of uncomplexed reactant becoming the continuum. For pancreatic and other lipases, a lipid composition exists at which binding begins to increase and catalysis increases discontinuously (Tsuji et al., 1989; Brockman, 2000a; Muderhwa and Brockman, 1992b). The reactant mole fraction at which this occurs is approximately twice the complex composition.

The lipolysis scenario described in the preceding paragraph implies that the surface of a triacylglycerol emulsion stabilized by phosphatidylcholine will resist attack by pancreatic lipase. In fact, this is observed, and the inhibition can be relieved by increasing the proportion of fatty acids in the emulsion surface (Borgström, 1980). As recently reviewed (Brockman, 2000b), colipase acts synergistically with reactants like fatty acid to more efficiently overcome the inhibition. This it accomplishes by laterally concentrating lipolysis reactants in its vicinity, helping lipase to adsorb to phosphatidylcholine-rich interfaces (Dahim and Brockman, 1998), presumably in the catalytically efficient conformation. This study shows that the initial step of colipase adsorption to an interface is, like that for lipase, inhibited by phosphatidylcholine-reactant complexes. However, once the reactant mole fraction has exceeded the complex composition, colipase adsorption can occur (Fig. 3). Although not determined with high accuracy from the present data, colipase appears to require only $\sim 145 \text{ \AA}^2$ of uncomplexed reactant (Table 1) for binding to occur even though it occupies $\sim 500 \text{ \AA}^2$ when completely bound. Thus, colipase binding is initiated at reactant mole fractions below the putative percolation threshold. Moreover, because of its ability to act as a nucleating center for reactant molecules after it binds, bound colipase creates relatively complex-free sites in phospholipid-rich interfaces for lipase to adsorb in the catalytically efficient conformation. Whether this lipase comes from the solution, is already bound in the catalytically inefficient conformation, or binds simultaneously with colipase is not known with certainty or addressed in this study. In any case, the observed result is that colipase shifts the apparent percolation threshold for lipolysis to lower reactant mole fractions (Brockman, 2000a).

Once lipolysis is initiated on emulsion particles with a substrate core, more reactants are generated. This increases reactant mole fraction in the interface and lipolysis becomes autocatalytic. Thus, the production in the stomach of sufficient fatty acid and diacylglycerol by the action of gastric lipase allows colipase binding either in the stomach or intestine. This, in turn, primes the particle for attack by pancreatic lipase in the intestine (Gargouri et al., 1986;

Winzell et al., 1998). The results of this work suggest that for the priming mechanism to be effective, sufficient gastric lipolysis may need to occur to exceed the mole fraction of these reactants in their complexes with phospholipid. Hence, these results emphasize the importance of gastric lipolysis for efficient fat digestion in the intestine. Unanswered by this or prior studies is whether bound colipase laterally concentrates all reactant present or only that not complexed to phosphatidylcholine.

Implications for lipid-mediated signal transduction

This work clearly demonstrates that interactions between a typical membrane phospholipid and nonphospholipids, like diacylglycerols and fatty acids, can regulate the flux of a peripheral protein, colipase, to an interface. The translocation of colipase appears to be regulated by the size of clusters of the nonphospholipid molecules in the phospholipid matrix. Diacylglycerols and free fatty acids are generated in membranes in response to stimuli that activate enzymes like phospholipases A, C, and D (Khan et al., 1995; Cornell and Arnold, 1996). Once released, these signaling molecules trigger subsequent events often involving the translocation of proteins from the aqueous compartment to the membrane surface (e.g., Cornell and Northwood, 2000). The present study suggests that the same lipid-lipid interactions that regulate colipase adsorption may influence rates of binding of other peripheral proteins to cellular membranes.

APPENDIX 1

The surface area can be defined on the basis of the apparent area per molecule of phospholipid, A_{pc} , which is the total surface area divided by the number of phospholipid molecules present. At reactant mole fractions above the complex composition, where all phospholipids are complexed, each molecule of phospholipid alone or its complex with reactant exhibits an excluded area, A_{ex} . A_{ex} is independent of lipid packing density, surface pressure, or the presence of uncomplexed reactant.

The surface can be viewed as a lattice in which A_{ex} defines one lattice site and a surface-bound colipase occupies n lattice sites. The probability that a lattice site is free from complex is $X = 1 - A_{ex}/A_{pc}$. Let us consider a cluster of n lattice sites. Colipase binding can be initiated if at least m of the n lattice sites are free from complex. The probability that given j sites, in which $j \leq n$, are free of complex, whereas the remaining $n - j$ sites are occupied by complex is $X^j(1 - X)^{n-j}$. The probability that any j of the n sites are free from complex, while the remaining $n - j$ sites are occupied by complex is $X^j(1 - X)^{n-j} n!/[j!(n - j)!]$. Finally, the probability that at least m of the n sites are free from complex is

$$\sum_{j=m}^n X^j(1 - X)^{n-j} n!/[j!(n - j)!].$$

This probability is equal to the fraction of the total surface area that is available initially for colipase binding, F_n , as expressed in Eq. 1.

Based on earlier measurements of colipase binding to lipid monolayers at equilibrium, we fixed the molecular area of bound colipase to that measured in monolayers, 500 \AA^2 (Momsen et al., 1997), and equated n with

the integer nearest $500/A_{\text{ex}}$. With the substitution for X in terms of A_{ex} and the experimentally determined A_{pc} , the number of unknown parameters is reduced to two, A_{ex} and m . For the analysis of each data set, m was progressively varied by integers, 1, 2, 3, . . . With each value of m the value of A_{ex} was progressively varied in increments of 1 Å² to determine the value A_{ex} that best minimized the absolute differences between a plot of F_a versus F_{exp} and a theoretical line through the origin with a slope of unity. This process was then repeated with the next value of m . The overall best fit was the A_{ex} , m pair that gave the minimal least squares deviation from the theoretical line. A best fit was obtained with each data set.

We gratefully acknowledge the critical comments of P. Mattjus and R.E. Brown.

This work was supported by U.S. Public Health Service grant HL-49180 (to H.L.B.), the Hormel Foundation (to H.L.B.), a grant from Pfizer, Inc. (to I.P.S.), and by Mrs. Larwence Garner (to I.P.S.).

REFERENCES

- Bezzine, S., F. Carrière, J. De Caro, R. Verger, and A. De Caro. 1998. Human pancreatic lipase: an exposed hydrophobic loop from the C-terminal domain may contribute to interfacial binding. *Biochemistry*. 37:11846–11855.
- Borgström, B. 1980. Importance of phospholipids, pancreatic phospholipase A₂, and fatty acid for the digestion of dietary fat: in vitro experiments with the porcine enzymes. *Gastroenterology*. 78:954–962.
- Brash, J. L. 1996. Behavior of proteins at interfaces. *Curr. Opin. Colloid Interface Sci.* 1:682–688.
- Brockman, H. L. 1999. Lipid monolayers: why use half a membrane to characterize protein-membrane interactions? *Curr. Opin. Struct. Biol.* 9:438–443.
- Brockman, H. L. 2000a. Kinetic behavior of the pancreatic lipase-colipase-lipid system. *Biochimie*. 82:987–995.
- Brockman, H. L. 2000b. Pancreatic lipase. Physiological studies. In *Intestinal Lipid Metabolism*. C.M. Mansbach, II, P. Tso, and A. Kuksis, editors. Plenum Press, New York. 61–79.
- Canioni, P., R. Julien, J. Rathelot, H. Rochat, and L. Sarda. 1977. Characterization of Triton X 100 extracted colipase from porcine pancreas. *Biochimie*. 59:919–925.
- Cornell, R. B. and R. S. Arnold. 1996. Modulation of the activities of enzymes of membrane lipid metabolism by non-bilayer-forming lipids. *Chem. Phys. Lipids*. 81:215–227.
- Cornell, R. B. and I. C. Northwood. 2000. Regulation of CTP: phosphocholine cytidyltransferase by amphitropism and relocalization. *Trends Biochem. Sci.* 25:441–447.
- Crandall, W. V. and M. E. Lowe. 2001. Colipase residues Glu⁶⁴ and Arg⁶⁵ are essential for normal lipase-mediated fat digestion in the presence of bile salt micelles. *J. Biol. Chem.* 276:12505–12512.
- Cunningham, B. A., T. Tsujita, and H. L. Brockman. 1989. Enzymatic and physical characterization of diacylglycerol-phosphatidylcholine interactions in bilayers and monolayers. *Biochemistry*. 28:32–40.
- Dahim, M. and H. L. Brockman. 1998. How colipase-fatty acid interactions mediate adsorption of pancreatic lipase to interfaces. *Biochemistry*. 37:8369–8377.
- Dervichian, D. G. 1958. The existence and significance of molecular associations in monolayers. In *Surface Phenomena in Chemistry and Biology*. J. F. Danielli, K. G. A. Pankhurst, and A. C. Riddiford, editors. Pergamon Press, London. 70–87.
- Egloff, M.-P., F. Marguet, G. Buono, R. Verger, C. Cambillau, and H. van Tilbeurgh. 1995b. The 2.46 Å resolution structure of the pancreatic lipase-colipase complex inhibited by a C11 alkyl phosphonate. *Biochemistry*. 34:2751–2762.
- Egloff, M.-P., L. Sarda, R. Verger, C. Cambillau, and H. van Tilbeurgh. 1995a. Crystallographic study of the structure of colipase and of the interaction with pancreatic lipase. *Protein Sci.* 4:44–57.
- Fenwick, N. I. D., F. Bresme, and N. Quirke. 2001. Computer simulation of a Langmuir trough experiment carried out on a nanoparticulate array. *J. Chem. Phys.* 114:7274–7282.
- Gargouri, Y., G. Pieroni, C. Rivière, P. A. Lowe, J.-F. Saunière, L. Sarda, and R. Verger. 1986. Importance of human gastric lipase for intestinal lipolysis: an in vitro study. *Biochim. Biophys. Acta*. 879:419–423.
- Holtmann, G., D. G. Kelly, B. Sternby, and E. P. DiMagno. 1997. Survival of human pancreatic enzymes during small bowel transit: effect of nutrients, bile acids, and enzymes. *Am. J. Physiol.* 36:G553–G558.
- Khan, W. A., G. C. Blobe, and Y. A. Hannun. 1995. Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. *Cell. Signal.* 7:171–184.
- Li, X.-M., J. M. Smaby, M. M. Momsen, H. L. Brockman, and R. E. Brown. 2000. Sphingomyelin interfacial behavior: the impact of changing acyl chain composition. *Biophys. J.* 78:1921–1931.
- Linthorst, J. M., S. B. Clark, and P. R. Holt. 1977. Triglyceride emulsification by amphipaths present in the intestinal lumen during digestion of fat. *J. Colloid Interface Sci.* 60:1–10.
- Lohner, K. 1991. Effects of small organic molecules on phospholipid phase transitions. *Chem. Phys. Lipids*. 57:341–362.
- Momsen, M. M., M. Dahim, and H. L. Brockman. 1997. Lateral packing of the pancreatic lipase cofactor, colipase, with phosphatidylcholine and substrates. *Biochemistry*. 36:10073–10081.
- Momsen, W. E. and H. L. Brockman. 1997. Recovery of monomolecular films in studies of lipolysis. *Methods Enzymol.* 286:292–305.
- Momsen, W. E., M. M. Momsen, and H. L. Brockman. 1995. Lipid structural reorganization induced by the pancreatic lipase cofactor, procolipase. *Biochemistry*. 34:7271–7281.
- Muderhwa, J. M. and H. L. Brockman. 1990. Binding of pancreatic carboxylester lipase to mixed-lipid films: implications for surface organization. *J. Biol. Chem.* 265:19644–19651.
- Muderhwa, J. M. and H. L. Brockman. 1992b. Lateral lipid distribution is a major regulator of lipase activity: implications for lipid-mediated signal transduction. *J. Biol. Chem.* 267:24184–24192.
- Muderhwa, J. M. and H. L. Brockman. 1992a. Regulation of fatty acid ¹⁸O exchange catalyzed by pancreatic carboxylester lipase: II. Effects of lateral lipid distribution in mixtures with phosphatidylcholine. *Biochemistry*. 31:149–155.
- Orban, J. M., K. M. Faucher, R. A. Dluhy, and E. L. Chaikof. 2000. Cytomimetic biomaterials: IV. In-situ photopolymerization of phospholipids on an alkylated surface. *Macromolecules*. 33:4205–4212.
- Ortiz, A. and J. C. Gómez-Fernández. 1987. A differential scanning calorimetry study of the interaction of free fatty acids with phospholipid membranes. *Chem. Phys. Lipids*. 45:75–91.
- Rugani, N., C. Dezan, L. de La Fourmière, P. J. Cozzone, B. Bellon, and L. Sarda. 1992. Separation and characterization of the precursor and activated forms of porcine and human pancreatic colipase by reversed-phase liquid chromatography. *J. Chromatogr.* 583:246–253.
- Schmit, G. D., M. M. Momsen, W. G. Owen, S. Naylor, A. Tomlinson, G. Wu, R. E. Stark, and H. L. Brockman. 1996. The affinities of procolipase and colipase for interfaces are regulated by lipids. *Biophys. J.* 71:3421–3429.
- Smaby, J. M. and H. L. Brockman. 1985. Miscibility, chain packing, and hydration of 1-palmitoyl-2-oleoyl phosphatidylcholines and other lipids in surface phases. *Biophys. J.* 48:701–708.
- Smaby, J. M. and H. L. Brockman. 1987. Acyl unsaturation and cholesteryl ester miscibility in surfaces: formation of lecithin-cholesteryl ester complexes. *J. Lipid Res.* 28:1078–1087.
- Smaby, J. M., J. M. Muderhwa, and H. L. Brockman. 1994. Is lateral phase separation required for fatty acid to stimulate lipases in a phosphatidylcholine interface? *Biochemistry*. 33:1915–1922.
- Somerharju, P., J. A. Virtanen, and K. H. Cheng. 1999. Lateral organization of membrane lipids: the superlattice view. *Biochim. Biophys. Acta*. 1440:32–48.

- Sörhede, M., H. Mulder, J. Mei, F. Sundler, and C. Erlanson-Albertsson. 1996. Procolipase is produced in the rat stomach: a novel source of enterostatin. *Biochim. Biophys. Acta.* 1301:207–212.
- Stauffer, D. 1985. Introduction to Percolation Theory. Taylor and Francis, London.
- Storjohann, R., A. Rozek, J. T. Sparrow, and R. J. Cushley. 2000. Structure of a biologically active fragment of human serum apolipoprotein C-II in the presence of sodium dodecyl sulfate and dodecylphosphocholine. *Biochim. Biophys. Acta.* 1486:253–264.
- Tsujita, T., J. M. Muderhwa, and H. L. Brockman. 1989. Lipid-lipid interactions as regulators of carboxylester lipase activity. *J. Biol. Chem.* 264:8612–8618.
- van Tilbeurgh, H., S. Bezzine, C. Cambillau, R. Verger, and F. Carrière. 1999. Colipase: structure and interaction with pancreatic lipase. *Biochim. Biophys. Acta.* 1441:173–184.
- Winzell, M. S., M. E. Lowe, and C. Erlanson-Albertsson. 1998. Rat gastric procolipase: sequence, expression, and secretion during high-fat feeding. *Gastroenterology.* 115:1179–1185.