In the present study, the stereoselectivity of *Rhizomucor miehei* lipase, lipoprotein lipase, *Candida antarctica* B lipase, and human gastric lipase towards racemic dicaprin spread as a monolayer at the air-water interface was investigated. For this purpose we have developed a method with which the enantiomeric excess of the residual substrate can be measured in monomolecular films. The stereoselectivity, which is one of the main aspects of enzymic catalysis, was found to depend on the surface pressure of the substrate. With all four lipases tested, low surface pressures enhanced the stereoselectivity while decreasing the enzymes' catalytic activity.

Lipases, which are lipolytic enzymes acting at the lipid-water interface, display stereoselectivity towards glycerides and other esters (1–3). Biological lipids, which self-organize and orientate at interfaces, are chiral molecules, and their chirality is expected to play an important role in the molecular interactions between proteins and biomembranes. Membrane-like lipid structures, such as monolayers, provide attractive model systems for investigating to what extent lipolytic activities depend upon the chirality and other physicochemical characteristics of the lipid-water interface.

The mechanism whereby an enzyme differentiates between two antipodes of a chiral substrate may be influenced by physicochemical factors such as temperature (4, 5) or solvent hydrophobicity (6), which can affect the reaction stereoselectivity. In the present study, we investigated the assumption that the stereoselectivity, which is one of the basic factors involved in enzymatic catalysis, may be pressure-dependent. When working with bulk solutions, the external pressure is not a practical variable because liquids are highly incompressible, whereas the monolayer surface pressure is easy to manipulate. To establish the effects (if any) of the surface pressure on the stereochemical course of the enzyme action, during which optical activity is generated in a racemic substrate insoluble in water, we have developed a method with which the enantiomeric excess of the residual substrate can be measured in monomolecular films.

Here we present the results of a study on the influence of the surface pressure on the enantioselectivity of lipases in lipid monolayers during kinetic resolution of racemic dicaprin. With all four lipases tested, low surface pressures enhanced the stereoselectivity while decreasing the catalytic activity.

This finding which to our knowledge is unprecedented, should help to elucidate the mode of action of water-soluble enzymes on water-insoluble substrates.

**EXPERIMENTAL PROCEDURES**

Lipases—All the lipases used were electrophoretically pure and the specific activities, which are expressed as micromole·min⁻¹·mg⁻¹ were: lipoprotein lipase 500 (measured with a pH-stat on emulsified olive oil), *Candida antarctica* B lipase 185, HGL¹ 667, and *R. miehei* lipase 5900 (measured on emulsified tributyrin (2)).

Kinetic Experiments on Monolayers—Before each utilization, the Teflon trough used for the experiments was rinsed with double-distilled water. The aqueous subphase was composed of 10 mM Tris/HCl, pH 8.0 (or pH 5.0 in case of HGL), 100 mM NaCl, 21 mM CaCl₂, and 1 mM EDTA. The buffers were prepared with double-distilled water and filtered through a 0.45-μm Millipore filter. Any residual surface-active impurities were removed before each assay by sweeping and suction of the surface. Kinetic experiments presented in Fig. 2 were performed with a KSV-2200 barostat (KSV, Helsinki) and a 29-cm long, 17.5-cm wide, 350-ml total volume single compartment Teflon trough with four 2.5-cm magnetic stirrers operating at 250 rpm. The experiments presented in Figs. 1 and 3 were performed in a two-compartment trough, as explained in the legends. The trough was equipped with a mobile Teflon barrier, which was used to compensate for the substrate molecules removed from the film by enzyme hydrolysis (monocaprin and capric acid are soluble in water), thereby keeping the surface pressure (Π) constant. The latter was measured using a Wilhelmy plate (perimeter 3.94 cm) attached to an electronic microbalance, which was connected in turn to a microprocessor controlling the movement of the mobile barrier. The reactions were performed at ambient temperature (25 °C). The enzyme solution (5–25 μl) was injected through the film with a Hamilton syringe over the 4 magnetic stirrers. Reactions were stopped when 50% of the substrate had been hydrolyzed. At this point the film of the residual, nonhydrolyzed substrate was aspirated using a water pump into a conical 50-ml flask, the cone of which had been drawn out into a tube 1.5 cm in length and 0.5 cm in diameter. Before aspiration, 2 ml of CHCl₃ and 60 μl of 0.12 M HCl (in order to reach a final pH of around 3) were placed in the flask, which were kept on ice. These steps were taken to inactivate the enzymes immediately on aspiration. A further 2 ml of cold CHCl₃ was aspirated after harvesting the film to recover the whole quantity of dicaprin from the small glass tube used as an aspirator.

**Derivatization of Diglycerides**—The flask containing the aspirated water phase, dicaprin, and CHCl₃ were gently agitated and left on ice for a few minutes. Water phase was then entirely removed with a Pasteur pipette and the CHCl₃ phase was rinsed with 2 ml of double-distilled H₂O. The water phase was discarded again and CHCl₃ was evaporated on a water pump. The flask was rinsed with 0.5 ml of CHCl₃ to ensure that all the dicaprin was collected in the tube at the base of the flask and the CHCl₃ was evaporated again. This was repeated for the second time with 0.1 ml of CHCl₃. CHCl₃ was evaporated on a water pump and the desiccator containing the flask was then connected to an oil pump to dry any residual traces of water. Teflon stirring bars (1 × 1-mm) were placed in the tubes of the flask, and 25 μl of distilled heptane and 10 μl of (+)-(-)-phenylethylisio-
cyanate were then added. All flasks were sealed with Teflon film and ground stoppers. The flasks were left under stirring for 48 h at room temperature and the excess (+)-(R)-phenylethylisocyanate was then evaporated overnight under a vacuum at 0.05 mm Hg. The diaster-
detector was used to monitor the separations. The enantiomeric
in 60 μl of distilled heptane, which sufficed to perform three or four
separations of the sample on HPLC.

HPLC Separations—The carbamates were injected on HPLC to
separate the pairs of diastereoisomers on a Beckman Ultrasphere 5-
μm column (10 × 25 cm) with 0.4% ethyl alcohol/heptane as eluent
at a flow rate of 3.3 mL/min flow rate. Both heptane and ethyl alcohol
were distilled prior to use. A light scattering Cunow DDL 11 (12)
detector was used to monitor the separations. The enantiomeric
excess percentages were calculated after automatic integration of the
separations

Chiral, optically pure (7–9), or racemic (10) compounds,
spread as monolayers at the air-water interface, have been
used previously to study enzyme kinetics under biomembrane
simulating conditions. To our knowledge, however, the prob-
lem of the steric course of enzyme action on racemic lipid
monolayers has never previously been approached experiment-
ally. The data on enzymic stereopreferences obtained with
enantiomerically pure monomolecular films (7–9) cannot be
directly extrapolated to racemates, since the physicochemical
properties of optically pure enantiomers and racemates in
monolayers may be very different (11, 12) and this in turn
may influence the enzyme behavior, which is controlled by
molecular recognition processes.

The chromatographic method for resolving enantiomeric
diglycerides developed recently at our laboratory (13) have
now been scaled down to yield insights on the interfacial
enzymic stereochemistry of monomolecular films. The ee%
(enantiomeric excess percent) measurements were adapted to
working with very small quantities (about 5 μg) of the initially
racemic substrate forming the monolayer. The substrate cho-
en here was 1,2-rac-dicaprin, since it forms stable monomo-
lar films at surface pressures as high as 40 mN·m⁻¹ and
the products of lipolysis, monopan, and capric acid, are
soluble in the water subphase, which means that they do not
disturb the monolayer during the reaction. 1,2-Dicaprin shows
a liquid-expanded phase behavior (no phase transition) within
the range of 5–40 mN·m⁻¹ at enantiomer compositions
varying between 100% 1,2-sn-dicaprin and racemate (results
not shown). The four enzymes used, R. miehei lipase, bovine
milk lipoprotein lipase (LPL), Candida antarctica B lipase,
and human gastric lipase (HGL) were chosen because they
specifically catalyze the hydrolysis of primary ester bonds in
glycerides, in positions sn-1 or sn-3.

The R. miehei lipase ee% measurements are given in Fig. 1
as a function of the reaction yield. With this lipase, the
residual substrate recovered from the surface was found to
have an optical purity as high as 80% ee at 50% yield and
90% ee at 75% yield. The results of this experiment, performed
at the arbitrarily chosen surface pressure of 30 mN·m⁻¹
confirmed the accuracy and reproducibility of the method.

The main advantage of the monolayer technique, however,
is that by changing the surface pressure it becomes possible
to modulate the conformation and interactions of the film-
forming molecules (12). We expected the enzyme-substrate
chiral recognition to be affected by these changes and to result
in surface pressure-dependent ee% variations.

The experimental results confirmed our expectations (Fig.
2). We noted that in all the lipases studied, the reaction stereoselectivity was governed by the surface pressure and

had the highest values at low surface pressures. In the most
spectacular case, that of lipoprotein lipase, the initially con-
stant stereoselectivity of about 85% ee, observed in the 10 to
30 mN·m⁻¹ surface pressure range, dropped sharply to about
30% ee at 40 mN·m⁻¹. With C. antarctica B lipase, HGL, and
R. miehei lipase the difference in the stereoselectivity mea-
ured at the two extreme surface pressures was about 25, 20,
and 10%, respectively. Unlike the stereoselectivity, the enzyme activity showed the highest values at high surface pressures (Fig. 3).

Any interpretations of the observed phenomenon can only be speculative in the absence of analytical methods with which to determine the changes occurring at the molecular level in the lipid film and in the associated water layer and enzyme as a function of the surface pressure. Enzyme-substrate molecular recognition is a critical step in enzyme-catalyzed reactions. Formation or cleavage of covalent bonds in the substrate is possible only if a sufficient number of non-bonding enzyme-substrate interactions have occurred for the steric control of the reaction to be possible (14). Lipases are usually capable of catalyzing the hydrolysis of monolayers of both of the optical antipodes of their substrates, but at different rates (7, 8). This means that the efficiency of the molecular recognition is defined by the absolute configuration at the chiral center of the substrate.

On the basis of crystallographic data (15, 16) we speculate that the aliphatic chain of the fatty acid to be cleaved from glyceride position 1 (or alternatively, position 3) occupies a hydrophobic channel within the enzyme interior. Furthermore, we hypothesize that the ester group and its aliphatic chain in position 2 (chiral carbon) in both enantiomers may fit a specific non-polar pocket within the molecular recognition site (the fact that the ester group in position 2 is rarely hydrolyzed supports this idea). In the better fitting enantiomer, the carbinol group of the chiral center may also be stabilized by some specific interactions lacking in the other enantiomer.

The results of the crystallographic studies cited above (15), as well as NMR, UV, and fluorescence spectroscopy data (17–19), show the ability of lipolytic enzymes to undergo conformational changes upon lipid binding. Assuming that the first step in interfacial catalysis, i.e., lipid binding, is not stereoselective, then the anticorrelation observed between the catalytic activity and the ee% depending on the surface pressure can be explained as follows. The presumed surface pressure-dependent enzyme conformational changes (20) may result in a deterioration of the molecular recognition of both enantiomers due to the progressive loss of the residue-specific interactions and the concomitant decrease in catalytic activity at low surface pressures. The deteriorating molecular recognition will, however, have a relatively stronger destabilizing effect on the already less well fitted enantiomer, which may then lose the catalytically efficient orientation before its antipode does so, thus enhancing the reaction stereoselectivity.

The differences in the catalytic activity and ee% patterns between the four enzymes probably reflect their individual susceptibility to undergo different conformational changes due to their flexibility and/or interfacial binding efficiency, i.e., a flexible enzyme sticking strongly to the interface would undergo changes more easily than a rigid enzyme with a limited capacity to interact with the interface.

The preliminary results on several other lipases tested at our laboratory (21) suggest that the dependence of lipase stereoselectivity on surface pressure is a general phenomenon and may therefore be applicable to enantioselective biocatalysis as well as being relevant in general to the enzyme reactions associated with biological membranes.

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