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Mechanism of Pancreatic Lipase Action. 1. Interfacial Activation of Pancreatic Lipase†

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ABSTRACT: Hydrolysis of dissolved *p*-nitrophenyl acetate by pancreatic lipase follows the classical acyl enzyme pathway already proposed for other esterases. Kinetic parameters of the hydrolysis have been determined. The turnover rate of the reaction is many orders of magnitude slower than that for the natural emulsified substrates. Nevertheless, several arguments are in favor of the specificity of this hydrolysis: (1) triacetin, which resembles the usual substrates for the enzyme, is also hydrolyzed very slowly in solution; (2) dissolved triacetin and tripropionin are competitive inhibitors for the *p*-nitrophenyl acetate hydrolysis; (3) the same chemical structural features which are required in the case of emulsified substrates are also necessary to promote hydrolysis of dissolved *p*-nitrophenyl esters. This suggests that the same active site (or a part of the same active site) is responsible for hydrolysis of both *p*-nitrophenyl acetate and specific emulsified substrates. Since deacylation is the rate-limiting step in the catalysis of *p*-nitrophenyl acetate, the intermediate acetyl enzyme can be isolated by trapping it at pH 5.0. Kinetic competence of this intermediate has been demonstrated. Hydrolysis by pancreatic lipase of dissolved monomeric *p*-nitrophenyl acetate and triacetin is considerably enhanced (100- to 500-fold) by various interfaces. This suggests that at least the deacylation step, which is rate limiting in absence of interface, is accelerated by the presence of inert interfaces. Siliconized glass beads were directly shown to accelerate the deacylation of isolated [^{3}H]acetyl lipase by at least a hundred times. This step does not directly involve the ester substrate. Thus, it is suggested that a part of the activation of lipase at interfaces may be due to a conformational change resulting from adsorption.

Enzymes acting on insoluble lipids are of special interest for their substrates and are not molecularly dispersed in water, but form aggregates separated from water by an interface. Therefore, these enzymes provide typical examples of heterogeneous catalysis (Sémériva and Desnuelle, 1976) and they offer simple models of lipid–protein associations in membranes and other biological systems.

For example, it is noteworthy that the activity of the two best known lipolytic enzymes, pancreatic lipase (EC 3.1.1.3) and phospholipase A_2 (EC 3.1.1.4), is not impaired but, on the contrary, considerably enhanced by the insolubility of their substrates (Sarda and Desnuelle, 1958; Pieterson et al., 1974).

Several hypotheses have been proposed to explain the necessity of an interface for the full expression of lipase activity (Sémériva and Desnuelle, 1976). The simplest one is to assume that lipase preferentially reacts at the interface simply because it has no affinity for soluble molecules.

On the other hand, interfacial activation can also arise either because the substrate acquires new properties at the interface or because the enzyme itself is modified by adsorption. These two hypotheses are not exclusive and it is probably more sensible to think that the activation results from the sum of several factors.

Some of these factors have been invoked to account for the interfacial activation, such as the increase of substrate local concentration at the interface (Brockman et al., 1973), a better orientation of the strategic ester bond (Garner and Smith, 1970; Zografi et al., 1971; Wells, 1974), or the reduction of the water shell normally surrounding ester molecules dissolved in water (Brockerhoff, 1973). The alternative proposal postulating a conformational change of lipase by adsorption (Sarda and Desnuelle, 1958) has been supported by recent data (Esposito et al., 1973; Entressangles and Desnuelle, 1974). However, no conclusion can be unambiguously drawn, since, in all the results presented above, the properties of both substrate and enzyme are affected by the presence of the interface. This activating effect of interfaces will not be understood unless the mechanism of action of the enzymes on dissolved substrates is more precisely known and compared with that on emulsified substrates. Pancreatic lipase has already been shown to act at a quite measurable rate on dissolved tripropionin (Entressangles and Desnuelle, 1974; Brockman et al., 1973) and PNPA[1] (Sémériva et al., 1974) in the presence of low concentrations of water-miscible organic compounds. Data obtained with this latter substrate were consistent with an acyl enzyme mechanism (Sémériva et al., 1974).

The first purpose of the present paper is to report new results confirming that the hydrolysis of dissolved substrates by lipase

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[1] Abbreviations used are: PNPA, *p*-nitrophenyl acetate; NADH, reduced nicotinamide adenine dinucleotide; ADP, ATP, adenosine di- and triphosphates.

proceeds via an acyl enzyme intermediate and permitting calculation of the kinetic constants related to the acylation and deacylation steps. A method for the preparation of the acyl enzyme intermediate is also described.

On the other hand, various inert interfaces have been employed and their effect upon the hydrolysis of dissolved substrates has been searched for. Brockman et al. (1973) were the first to report that the hydrolysis of dissolved tripropionin by lipase was considerably enhanced by the presence of siliconized glass beads. Siliconized glass beads were shown to adsorb both enzyme and substrate so that the reaction takes place at the interface, like in the general case of lipase-insoluble substrates. A good correlation was found between enzyme adsorption and activation. This interface was thus used to evaluate its role on a discrete step of the reaction, that is the deacylation of the acetyl enzyme intermediate. It is especially noteworthy that, in this latter case, the reaction could proceed and be followed in the absence of the substrate.

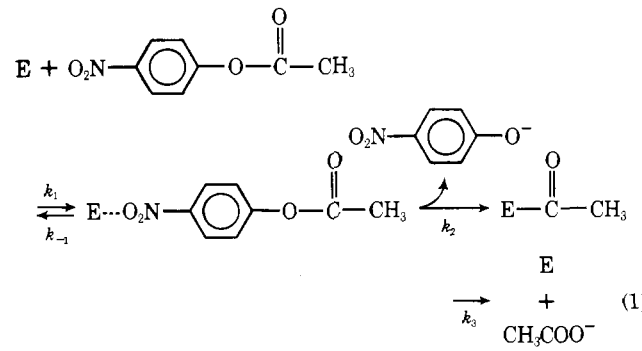
Materials and Methods

Substrates. *p*-Nitrophenyl acetate (PNPA) was purchased from Aldrich and recrystallized twice from absolute ethanol. Tritium-labeled PNPA was synthesized (Sémériva et al., 1974) by esterification of *p*-nitrophenol (2 mmol) with an equimolar amount of [^{3}H]acetic acid (CEA, France; specific radioactivity, 25 mCi/mmol) in the presence of dicyclohexylcarbodiimide (3.2 mmol) in dry ethyl acetate (4 ml). After two crystallizations in absolute ethanol, the compound was judged pure by thin-layer chromatography on silica gel G in a hexane–diethyl ether–methanol (90:10:1, vol/vol) system. Approximately 1 mmol of ester with a specific activity of 25 mCi/mmol was obtained. This latter value was derived from absorbance determinations at 400 nm after total hydrolysis in 0.1 M NaOH. *p*-Nitrophenyl butyrate was prepared by reaction of butyryl chloride with *p*-nitrophenol in chloroform–pyridine and purified by chromatography on silicic acid in the solvent hexane–diethyl ether (90:10, vol/vol). This product yielded a single band by thin-layer chromatography. The yield was 70%. Other *p*-nitrophenyl esters were purchased from Sigma and used without further purification. Triacetin, tripropionin, and tributyrin were purified as already described (Dufour and Sémériva, 1972).

Enzymes. Pancreatic lipase was purified according to an already published method (Verger et al., 1969). Lipase concentrations were calculated assuming a molecular weight of 48 000 and a $E_{1cm}^{1\%}$ of 13.3 at 280 nm. Pyruvate kinase, lactate dehydrogenase, and acetate kinase were of the best available grade and purchased from Sigma.

Hydrolysis of p-Nitrophenyl Esters. This hydrolysis was followed at 25 °C and pH 7.5 with the aid of a Zeiss recording spectrophotometer, Model PMQ II, adjusted at 400 nm. The molar extinction coefficient of the mixture of *p*-nitrophenolate ions and *p*-nitrophenol released by the enzyme, in a 0.1 M Tris-HCl buffer at pH 7.5 containing 4% acetonitrile, was found to be 14 000. Care was taken to maintain a constant pH in all assays because of the strong variability of the ionization of *p*-nitrophenol in this range. Lipase (~0.01 μmol in 40 μl) was added to one of the cuvettes containing 1 ml of the above buffered substrate solution. Spontaneous substrate hydrolysis was automatically compensated by placing the solution without lipase in the other cuvette.

The well known acyl enzyme mechanism has been extensively analyzed by Kezdy and Bender (1962) in terms of the following equation:



(1)

The first order rate constant b of the presteady state is given by:

$$b = \frac{k_2[S_0]}{K_S + [S_0]}$$

and

$$1/b = 1/k_2 + \frac{K_S}{k_2 1 s_0]} \quad (2)$$

This constant is experimentally determined by extrapolating to zero time the straight line of the steady state and measuring the differences ΔA between the extrapolated line and the presteady-state curve (see Figure 1). The amount of *p*-nitrophenol liberated at the end of the burst (π) (see Figure 1) is given by:

$$\frac{1}{\sqrt{\pi}} = \frac{1}{\sqrt{E_0}} + \frac{K_m(app)}{[S_0]\sqrt{[E_0]}} \quad (3)$$

The reaction rate at the steady state (v) is given by:

$$1/v = 1/k_3[E_0] + \frac{K_m(app)}{k_3[E_0][S_0]} \quad (4)$$

Microtitration of Acetate. A new microtechnique involving three coupled enzymatic reactions has been worked out for determining the very low amount of acetate released by lipase from dissolved triacetin. The ADP generated from ATP (6.2 mM) during the phosphorylation of acetate to acetyl phosphate by acetate kinase (20 μg/ml) was measured through the oxidation of NADH (2.6 mM) in the presence of phosphoenol pyruvate (11 mM), pyruvate kinase (20 μg/ml), and lactate dehydrogenase (20 μg/ml) in a 0.1 M triethanolamine-HCl buffer at pH 7.4 and containing 7 mM $MgCl_2$. For acetate concentrations far below the K_m of acetate kinase, the reaction did not run to completion, but its rate was strictly proportional to the acetate concentration. Kinetics were followed during 20 min. It was checked that the reaction catalyzed by acetate kinase was rate limiting under the conditions of the assays. The rate was independent from the concentration of the other reagents, including those of the two enzymes, pyruvate kinase and lactate dehydrogenase.

Preparation of [^{3}H]Acetyl Lipase. This preparation was essentially carried out as described in a previous publication (Sémériva et al., 1974). Unspecific acylation sites have been designated as the sites present in the enzyme which cannot be deacylated after 1 h of incubation at pH 7.5 and room temperature. These unspecific acylation sites were first saturated by incubation of the enzyme (44 μM) for 90 min at 20 °C with 2.5 mM unlabeled PNPA in the 0.1 M Tris-HCl buffer, pH 7.5, containing 4% acetonitrile. The losses due to hydrolysis of PNPA during the treatment were compensated by four additions of the reagent (2.5 μmol each). Then, the reaction was stopped by filtration of the solution through a Sephadex G-25 column (0.9 × 21 cm) equilibrated and eluted with the

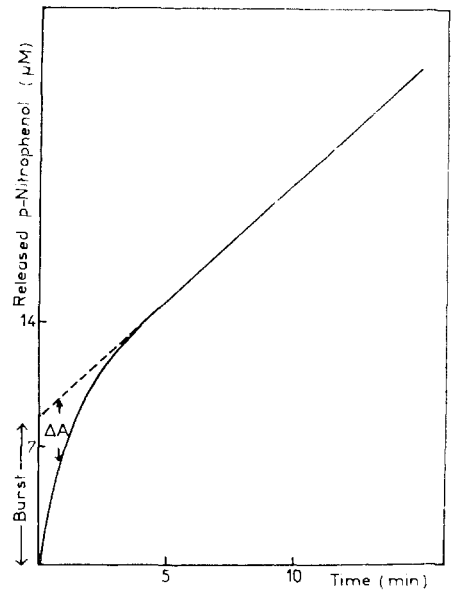


FIGURE 1: Time course of the hydrolysis of dissolved PNPA (3 mM) by pancreatic lipase (8.6 μM) in a 0.1 M Tris-HCl buffer, pH 7.5, containing 4% acetonitrile.

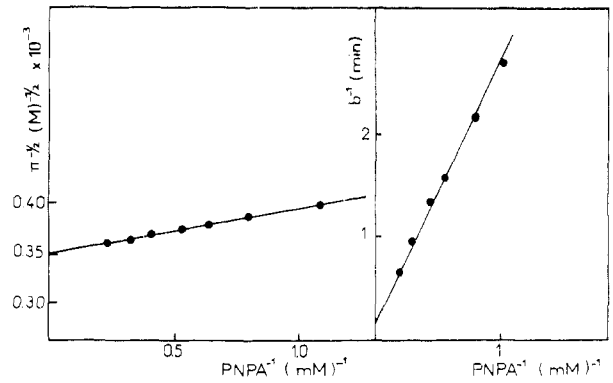


FIGURE 2: Variation of the burst, π (left), and of the first-order rate constant of the presteady state, b (right), with substrate concentration. Lipase concentration, 8.5 μM in all assays.

Tris-HCl buffer, pH 7.5. In a second step, after a further incubation of the enzyme at pH 7.5 for 1 h, the excluded material (1 mg/ml in the Tris-HCl buffer) was again incubated with 2.5 mM [^{3}H]PNPA. After exactly 3 min, the reaction was stopped by addition for each ml of 15 μl of a 1 M sodium acetate buffer at pH 4.5.

Determination of Lipase Activity in the Presence of Interfaces. Lipase activity was routinely measured at pH 8.0 and 25 °C on emulsified tributyrin (Sémériva et al., 1971). Activity on emulsified triolein was determined by titrimetry at pH 8.5 and 25 °C in an assay containing 20 mg of olive oil, 3.6 mM sodium deoxycholate, 0.1 M NaCl, and 5 mM $CaCl_2$, in a total volume of 15 ml. In the presence of inert interfaces, lipase activity was measured, at pH 7.5 and 25 °C, either spectrophotometrically, as indicated above, or titrimetrically in a recording Radiometer pH-stat Model TTT 60. When applied to the same enzyme solution, both techniques yielded concordant results. The titrimetric technique required higher reaction rates to give significant data. Thus, it was only used in the presence of added interfaces (see below).

Inert Interfaces. These interfaces were brought about either by the walls of the vessels containing the substrate solution and the accessories of the pH-stat, or by added siliconized glass beads, emulsified dodecane particles, and lipid micelles. The beads were purchased from Serva (Fein Biochimica, Heidelberg). Their mean diameter, measured with a Nikon microcomparator Model 6 CT 2, was 160 ± 16 μm. They were added (0.31 g/ml) in a 1 mM Tris-HCl buffer containing substrate and enzyme in the pH-stat beaker. The mixture was agitated by a glass-coated magnetic stirrer set at low speed in order to avoid air bubbles and the NaOH outflow was constituted by an extremely thin glass tubing. In this manner, the beads represented, by far, the main source of interfaces. Dodecane (0.7 ml) was sonicated in 4 ml of 3.2 mM sodium taurocholate. A 1-ml aliquot of the resulting emulsion was added to 14 ml of a 80 mM Tris-HCl buffer containing, besides enzyme and substrate, 1.5 mM taurocholate, 5 mM $CaCl_2$, and 0.1 M NaCl.

The lipoprotein complex formerly designated "fast lipase" was reconstituted free of esterolytic activities by mixing in 0.75 M NaCl, 0.25 M $CaCl_2$, pure lipase (2.5 mg), pure colipase (1 mg), and the lipids extracted from 60 g of fresh porcine pancreas. The homogenate (in 600 ml of water) was centrifuged at 85 000g for 24 h, the supernatant was lyophilized, and the resulting powder (500 mg) was extracted by chloroform–methanol (2:1, v/v). The dry residue was taken up in a small volume of chloroform–methanol (98:2, v/v) to remove the last traces of proteins. The mixture of enzyme, cofactor, and lipid was stirred for a few min and the clear solution was passed through a (0.8 × 150 cm) Sephadex G-200 column in 0.75 M NaCl, 0.25 M $CaCl_2$. The excluded peak, containing the complex, was collected separately.

Miscellaneous. Radioactivities were measured in a Packard Tricarb scintillation spectrometer after dissolution of the sample in 15 ml of Bray's mixture.

The absence of micelles in all solutions was checked spectrophotometrically in the presence of Rhodamine 6 G (Corrin and Harkins, 1947).

Results

Hydrolysis of Monomeric PNPA by Lipase. The typical curve reproduced in Figure 1 confirms an early observation (Sémériva et al., 1974) that the lipase-catalyzed hydrolysis of dissolved PNPA proceeds via a two-step reaction: an exponential burst attributable to a presteady-state phase and liberating about 1 mol of product/mol of enzyme, and a linear phase corresponding to the steady-state reaction. Moreover, the burst, π, and the steady-state rate, v, are both proportional to the enzyme concentration. These data are consistent with an acyl enzyme mechanism.

Study of the Presteady State. The left diagram of Figure 2 shows that, as predicted by eq 3, $1/\sqrt{\pi}$ is proportional to $1/[S_0]$ for a constant enzyme concentration. Extrapolation of the straight line to infinite substrate concentration leads to a value of $E_0 = 8.4 \times 10^{-6}$ M in excellent agreement with that yielded by direct spectrophotometric determination of the enzyme concentration (8.5×10^{-6} M). The K_m(app), derived from the curve in Figure 2, is 0.2 ± 0.02 mM. The first-order rate constant b of the presteady state could be evaluated with a reasonable accuracy without the aid of special equipment for fast kinetics. The right diagram of Figure 2 shows that the plot of $1/b$ vs. $1/[S_0]$ is linear. Using eq 2, the two kinetic constants, k_2 and K_S, of the acylation step were estimated to 7 min^{-1} and 14 mM, respectively.

Study of the Steady State. A nonlinear relationship was obtained when $1/v$ was plotted against $1/[S_0]$, in disagreement with eq 4 (Figure 3). At high substrate concentration, the plot

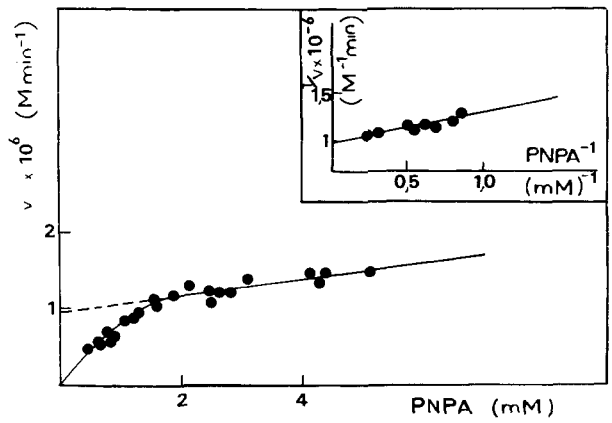


FIGURE 3: Substrate concentration dependence of the steady-state rate. Upper diagram, reciprocal plot (Lineweaver-Burk) $1/v$ vs. $1/[S_0]$ after correction for unspecific hydrolysis (see text).

of v vs. $[S_0]$ gave a straight line, whose slope and intercept on the v axis are both proportional to $[E_0]$. This process, called nonspecific hydrolysis, might be attributed to the high reactivity of *p*-nitrophenyl esters and has already been observed with other esterases (Kezdy and Bender, 1962). According to these authors, the steady state may best be described by an equation of the form:

$$v = \frac{k_3[E_0][S_0]}{K_m(\text{app}) + [S_0]} + k_{ns}[E_0][S_0]$$

in which k_{ns} is the second-order rate constant of the nonspecific hydrolysis.

For each substrate concentration, v is the sum of two terms. One, related to specific hydrolysis, varies hyperbolically with $[S_0]$, while the other, corresponding to nonspecific hydrolysis, varies linearly. The value of k_{ns}, derived from the slope of the linear portion of the curve in Figure 3, is 13 min^{-1} M^{-1}.

By subtracting to each value of v, at a given substrate concentration, the corresponding value of the nonspecific rate, the steady-state rate of the specific reaction can be calculated. A linear Lineweaver-Burk representation was then obtained in the range of concentrations used from which the values of 0.12 min^{-1} and 0.24 ± 0.02 mM could be derived, respectively, for the steady-state kinetic constants k_3 and K_m(app) of the specific process. The agreement between the measured K_m(app) and that calculated above from the presteady-state plot $1/\sqrt{\pi}$ vs. $1/[S_0]$, even though it might be fortuitous, provides a check for internal consistency of the model. Moreover, the values found for k_2, k_3, K_S, and K_m are consistent with the condition $k_2 \gg k_3$ necessary for establishing eq 2, 3, and 4, and also with the relation $k_3/K_m(\text{app}) = k_2/K_S$, which is a direct consequence of the acyl enzyme mechanism.

Hydrolysis of Other p-Nitrophenyl Esters. A detailed kinetic analysis with the higher homologues, *p*-nitrophenyl butyrate and octanoate, could not be performed because of their low solubility. However, Table I shows that the specificity constant (Bender and Kezdy, 1965) k_{cat}/K_m of lipase is much larger for these medium-chain substrates than for short-chain PNPA. This observation suggests that substrate hydrophobicity favors lipase action in solution as it does in emulsions (Brockerhoff, 1968). By contrast, *p*-nitrophenyl trimethylacetate as well as other substituted esters (*p*-nitrophenyl diazoacetate and *p*-nitrophenyl *N,N'*-diethylcarbamate) are not attacked at all, probably because of the steric hindrance around the β carbon of the acyl chain. The same chemical requirements have been observed in the case of emulsified esters

TABLE I: Specificity Constant k_{cat}/K_m of Lipase for Several *p*-Nitrophenyl Esters.

Ester	k_{cat}/K_m (min^{-1} M^{-1})
p-Nitrophenyl acetate	50
p-Nitrophenyl butyrate	$4.45\ 10^3$
p-Nitrophenyl octanoate	$2.3\ 10^4$
p-Nitrophenyl trimethylacetate	0

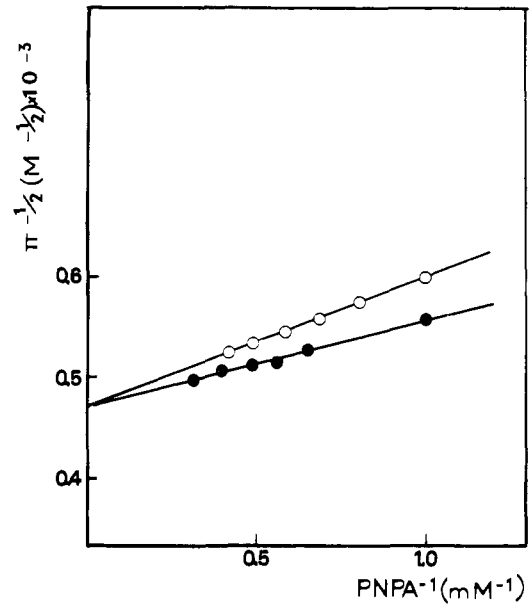


FIGURE 4: Competitive inhibition of the lipase-catalyzed hydrolysis of PNPA by monomeric tripropionin. π designates the initial burst of *p*-nitrophenol. Open and filled circles are related, respectively to assays performed at pH 7.5 and 25 °C in the presence of tripropionin (3.8 mM) and in its absence. Lipase concentration, 7.4 μM.

(Brockerhoff, 1968).

Competitive Inhibition by Monomeric Short-Chain Triglycerides. The purpose of these assays was to see whether the same site in lipase was responsible for the hydrolysis of dissolved PNPA and more usual lipase substrates, such as short-chain triglycerides. The fact that dissolved triacetin, likewise dissolved tripropionin (Entressangles and Desnuelle, 1974), is a substrate for lipase was first checked by incubating, at pH 7.5 and 25 °C, a 20 mM solution of the glyceride in 4% acetonitrile with 6.7 μM lipase. The enzyme was inactivated by acidification to pH 2, the pH was raised back to 7.4, the released acetate was titrated, as previously described, and the results were corrected for spontaneous hydrolysis of triacetin. Under these conditions, hydrolysis was found to be linear for at least 15 min. The k_{cat} of the reaction was 2.5 min^{-1}.

The effect of dissolved tripropionin and triacetin on the hydrolysis of PNPA was next investigated. Figure 4 shows that the *p*-nitrophenol burst, π, was significantly reduced by the presence of tripropionin, except for an infinite substrate concentration as expected from competitive inhibition. The differences were relatively small due to the limited tripropionin concentration range that could be explored before micelle formation and, also, to the high K_m of the hydrolysis of dissolved substrates by lipase (Entressangles and Desnuelle, 1974). Using the value already reported for this K_m under the same conditions (Entressangles and Desnuelle, 1974), the K_m(app) for PNPA could not be expected to vary by more than

TABLE II: Catalytic Properties of Lipase Pretreated with Unlabeled PNPA at pH 7.5.

	Burst (mol of PNP/mol of lipase)	k_3 (min^{-1})	K_m (mM)
Native lipase	1	0.11	0.24
Lipase pretreated with unlabeled PNPA	0.98	0.11	0.28

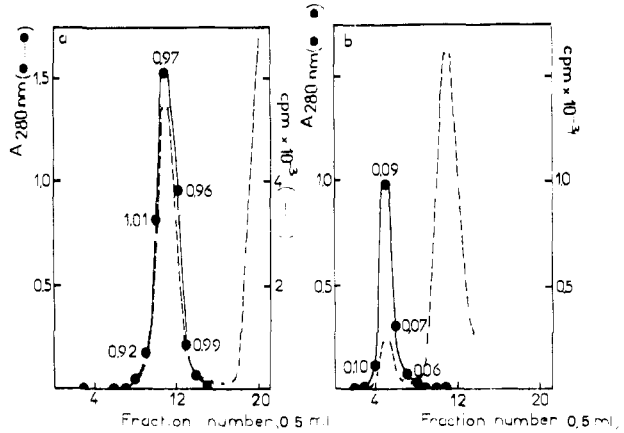


FIGURE 5: Acylation and deacylation of lipase as materialized with the aid of tritium-labeled PNPA. The number of labeled acetyl groups per mol of enzyme is given for each fraction of the protein peaks. Left, pretreated [^{3}H]acetyl lipase (see text) is filtered at 4 °C through a Sephadex G-25 column (0.9 × 21 cm) in a 1 M sodium acetate buffer, pH 5.0. The first peak is [^{3}H]acetyl lipase, whereas the excess of PNPA emerges later. Right, filtration in a column (0.9 × 13 cm) of the above derivative after a 1-h exposure at pH 7.5 and 25 °C. The second peak of this diagram is formed by the radioactive acetate released by deacylation.

20%, which is in good agreement with the experimental data. Triacetin yielded comparable results.

An accurate determination of the kinetic parameters of monomeric triacetin or tripropionin hydrolysis was hampered by the lack of a simple activity assay in these cases. It must be recalled that the lipase activity on these dissolved substrates is very low and cannot be easily measured by titrimetry. Work is in progress to devise a routine assay to measure these activities.

Isolation of [^{3}H]Acetyl Lipase and Characterization as an Intermediate. Considering that $k_2 \gg k_3$ (7 and 0.11 min^{-1}, respectively (see above)), it should be possible to isolate the acyl enzyme intermediate if suitable conditions can be found for its stabilization. Data reported in a previous publication (Séméria et al., 1974) have already suggested that the intermediate deacylates fast at pH 7.5 and 25 °C but fairly slowly at pH 5.0 and 0 °C.

In a first series of assays, lipase directly treated with tritium-labeled PNPA was found to lose not more than 30% of its radioactivity at pH 7.5 and 25 °C for 25 h. This unexpected result is probably related to the already reported unspecific hydrolysis (see Figure 3), which should induce the fixation of acetyl groups to the enzyme by relatively stable bonds. The corresponding site(s) in lipase must be saturated by nonradioactive PNPA before proceeding to the specific labeling. As shown by Table II, the resulting compound possessed a fully functional active site which could be labeled by a 3-min incubation at pH 7.5 with radioactive PNPA, immediately followed by acidification to pH 5.0. The left diagram of Figure 5 shows

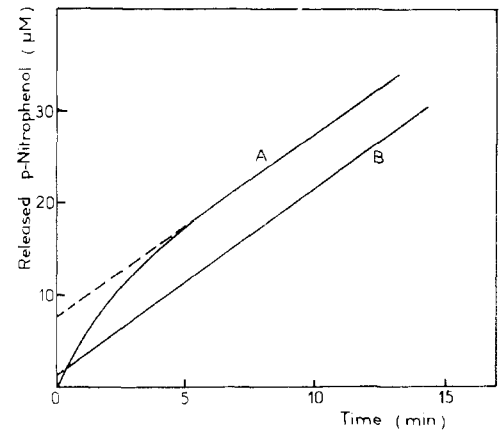


FIGURE 6: Kinetic competence of [^{3}H]acetyl lipase. PNPA (2.5 mM in the usual buffer at pH 7.5) was incubated at 25 °C with 7.5 μM pretreated lipase (curve A) and with the same concentration of the acetyl derivative (curve B). The nitrophenol release was monitored as indicated in text.

that a [^{3}H]acetyl lipase derivative is formed in good yield under these conditions. Rechromatography at pH 5.0 was possible without loss of radioactivity but, as indicated in the right diagram of the figure, deacylation of the derivative was now almost complete after standing for 1 h at pH 7.5 and 25 °C in an ordinary vessel.

The fact that the above acetyl derivative is an intermediate in catalysis was definitely confirmed by comparing its action on PNPA to that exerted by the enzyme itself. Figure 6 shows that the release of *p*-nitrophenol is no longer biphasic, but linear in accordance with the expected suppression of the initial burst, since the enzyme is already acylated. Moreover, the steady-state rate maintained throughout by the derivative is equal to that induced by an equivalent amount of enzyme under comparable conditions, and this agrees with the already reported finding that deacylation is the rate-limiting step. The same kinetics of hydrolysis of emulsified tributyrin and triolein were also observed with native lipase and its monoacetyl derivative.

Stimulation of Lipase Action by Inert Interfaces. The early observations made with siliconized glass beads and tripropionin (Brockman et al., 1973) have been extended herein to other substrates, including PNPA and triacetin, and to other types of interfaces. To obtain quantitative results, the stimulations were calculated by reference to the activity displayed by lipase ($\simeq 10\mu M$) towards a 2.5 mM PNPA or 20 mM triacetin solution in 1 ml of the buffer placed in a spectrophotometer quartz cuvette (1 × 0.4 × 2 cm). The small area of the interface thus created its nature, and the relatively high lipase concentration in the solution induced an especially weak stimulation taken as unity. This stimulation was enhanced by a factor ranging from 100- up to 500-fold by realizing the assays in a pH-stat beaker (internal diameter, 4 cm) with 15 ml of a solution containing the substrate (2.5 mM), the enzyme (0.2 mg), and added substances providing interfaces of various nature. The highest effect was observed with siliconized beads (500-fold). The lowest was induced by the dodecane particles (100-fold), probably because of the presence of bile salts in the emulsion impairing the interfacial adsorption of lipase when a suitable complement of colipase is not present (Chapus et al., 1975). The stimulation induced by previous binding of lipase to lipid micelles ("fast lipase") was as high as 300-fold. Addition of extraneous interfaces is not required for a high stimulation to occur. The glass walls of the beaker, the polyethylene stirrer, the ordinary polyvinyl tubing for NaOH

TABLE III: Deacylation of [^{3}H]Acetyl Lipase in the Presence of Siliconized Glass Beads.[a]

	Siliconized Glass Beads (g)	% Enzyme in Supernatant	% Adsorbed Enzyme	% Total Radioactivity in Supernatant	% Deacylation of Adsorbed Material in 30 s
Acetyl lipase (30 μg)	0.25	58	42	96	82
	0.50	29	71	87	78
	0.75	14	86	77	70
	1.00	13	87	76	68
Acetyl lipase (30 μg)	0.25	45	55	99	99
+ colipase (12 μg)	0.50	24	76	76	82
	0.75	21	79	99	99
	1.00	7	93	83	71

[a] The percentage of adsorbed enzyme was determined by measuring lipase activity in the supernatant. The radioactivity found in the supernatant was compared to the total radioactivity in the assay brought about by [^{3}H]acetyl lipase. Percentage of rapid deacylation was calculated from the specific radioactivity of [^{3}H]acetyl lipase, the amount of adsorbed and nonadsorbed enzyme, and the amount of radioactivity in the supernatant, taking account of [^{3}H]acetyl irreversibly bound to lipase (see Figure 1).

outflow used in the pH-stat assays, and the air bubbles produced by a purposedly violent agitation of the reaction mixture suffice to multiply lipase activity by a factor of 350. This latter result shows that the action of the enzyme on dissolved substrates may vary within very large limits according to the experimental conditions. Very similar activation factors were obtained with both triacetin and PNPA as the substrates.

Role of Interfaces in the Deacylation of [^{3}H]Acetyl Lipase. The effect of inert interfaces on the overall hydrolysis reaction may be expected to result, at least in part, from an acceleration of the rate-limiting step, i.e., the deacylation of the covalent acyl enzyme intermediate. To check this point, the time course of the deacylation of [^{3}H]acetyl lipase, prepared as indicated above, was first followed in the absence of added interfaces. Solutions of the derivative were maintained at pH 7.5 and 25 °C in the presence of 4% acetonitrile. At given time intervals, the reaction was quenched by reducing the temperature to 0 °C and the pH to 5 with a suitable volume of a 1 M sodium acetate buffer at pH 4.5. The resulting mixtures were immediately filtered through Sephadex G-25 and the number of [^{3}H]acetyl groups remaining attached to the enzyme was derived from the specific radioactivity (radioactivity over enzymatic activity) of the material under the excluded protein peak. Deacylation was shown to be first order at least up to 80% completion with a half-life time for the derivative of 12 ± 2.5 min. This value is in the same range as that (7 ± 1.5 min) deduced from purely kinetic considerations and in good agreement, taking account of the experimental difficulties. Both show that, in the absence of interfaces other than that of the vessel containing the solution, the deacylation of acetyl lipase at pH 7.5 and 25 °C is relatively slow.

The acceleration induced by hydrophobic interfaces was next investigated. Siliconized glass beads were selected because their decantation takes less than 30 s. This permits quick separation of the interface where the enzyme is adsorbed from the aqueous phase (Chapus et al., 1975) and then evaluation of the progress of deacylation in the clear supernatant, after a very short time.

The amount of released acetate was derived from the radioactivity in supernatant minus that brought about by the unadsorbed enzyme fraction. This latter was directly deduced from the enzymatic activity in the supernatant. It was checked that acetate was not adsorbed by the beads. In a preliminary experiment, 30 μg of freshly isolated [^{3}H]acetyl lipase was incubated with 1 g of beads in 3.5 ml of buffer. After exactly 30 s, an aliquot of the supernatant was removed and 0.5 ml was dissolved in 15 ml of Bray's mixture for radioactivity counting. Lipase activity in the supernatant was very low in this assay, showing that nearly all the enzyme was adsorbed to the beads. Therefore, all the radioactivity was attributed to the released acetate. Deacylation attained 80% in the imparted time of 30 s and, therefore, was considerably faster than in the absence of beads.

Then, an attempt was made to better correlate enzyme adsorption and amount of rapidly removable acetate for varying weights of beads. The upper half of Table III shows that enzyme adsorption increased as expected when the amount of beads was increased, whereas the percent of radioactivity in the supernatant remained roughly constant. This result cannot be explained without invoking a rapid release of acetate in the supernatant from the adsorbed lipase. The percent of deacylation of the adsorbed material is reported in the last column of Table III. The quasi-totality of the adsorbed molecules are deacylated within 30 s. When compared to the half-life time of acetyl lipase in the absence of glass beads (12 min), this finding proves that deacylation is considerably accelerated by adsorption of the derivative to the inert interface of the beads.

It is, however, surprising that a value of 100% of rapid deacylation could not be obtained especially at high beads concentration. Yet, an incubation of 1 h with glass beads did not lead to a further increase of the percentage of deacylation. Thus, it seems that all the deacylable enzyme has been deacylated and that the remaining cannot be at all deacylated, probably because a fraction of the enzyme was rapidly denatured by adsorption to glass beads. A similar fall of interfacial stimulation at high beads concentrations has also been observed in our conditions for lipase acting on the substrate tripropionin. When the enzyme activity on this glyceride was plotted vs. the weight of beads, a considerable rise was noted at first, followed by a steady decline.

To test the possibility that this decline was due to increased interfacial denaturation of acetyl lipase prior to deacylation, a last series of assays was performed with 2 mol/mol of colipase, known for its stabilizing effect on lipase (Rietsch and Verger, personal communication). Indeed, as shown by the lower half of Table III, colipase increased slightly, but significantly, the yield of deacylation in 30 s. This yield now attained on the average 90%, thus definitely attesting the effect of interfaces on the deacylation step.

Since inert interfaces, such as glass beads, enhance the deacylation rate of [^{3}H]acetyl lipase, it seems important to investigate the behavior of this intermediate in the presence of substrate interfaces. Because of obvious experimental difficulties, it is impossible with emulsified substrates to measure the rapid release of [^{3}H]acetate resulting from adsorption of [^{3}H]acetyl lipase. The only possibility is to examine the behavior of this intermediate during the hydrolysis of the substrate emulsions. Exactly the same rates and shapes of the kinetics of hydrolysis of tributyrin or triolein emulsions were observed either with native lipase or with [^{3}H]acetyl lipase. This result strongly suggests that the [^{3}H]acetyl group is, again, rapidly released in the presence of substrate interfaces. Indeed, if it had been slowly released with a half-life of 12 min, as it is the case in solution, a lag of the same duration would have been observed, since k_{cat} with emulsified substrates is much larger than with soluble ones.

Discussion

The characteristics of the hydrolysis of dissolved PNPA by lipase are consistent with the model proposed a number of years ago for chymotrypsin by Hartley and Kilby (1954) and Kezdy and Bender (1962). The enzyme first binds PNPA to form a Michaelis–Menten adsorption complex. Then, a covalent acyl enzyme is produced which later deacylates according to eq 1. Because deacylation is rate limiting under the conditions of the assays, the acyl enzyme accumulates with a concomitant rapid release of *p*-nitrophenol. Using the equation developed for a similar purpose by Kezdy and Bender (1962), it was possible to calculate the kinetic constants of the acylation and deacylation steps of the reaction at pH 7.5 and 25 °C. These constants ($k_3 = k_{cat} = 0.11 \pm 0.01$ min^{-1}; $k_2 = 7 \pm 0.7$ min^{-1}; K_m(app) $= 0.22 \pm 0.02$ mM; $K_s = 15$ mM) agree with the relation $k_2/K_s = k_{cat}/K_m$ required by the acyl enzyme mechanism.

An important remark is that the k_{cat} of lipase for monomeric PNPA is very much lower than that for emulsified substrates. But, it is of the same order of magnitude as the k_{cat} of other esterases such as chymotrypsin, trypsin, and elastase (Bender et al., 1966).

The reaction of lipase with dissolved PNPA was also of special relevance for the isolation of the acyl enzyme intermediate. To prove that the isolated material was really the transient covalent intermediate suggested by the kinetic analysis, the first criterion was fast and complete deacylation at pH 7.5. The second was that the derivative hydrolyzed dissolved PNPA at exactly the same rate as the native enzyme, but no longer displayed biphasic kinetics because of the suppression of the burst.

The hydrolysis of dissolved substrates was not affected by colipase. This agrees with the view that the stimulating effect of the cofactor is linked to a problem of interfacial recognition (Chapus et al., 1975) or stabilization (Rietsch and Verger, unpublished results). No effect of colipase can be expected in the absence of an interface.

The possibility that nonspecific hydrolysis, due to the high reactivity of PNPA, was responsible for the *p*-nitrophenol release was excluded by the hyperbolic substrate concentration dependence of the reaction rate in the presence of the native enzyme and, also, by the very weak activity of enzyme preparations previously denatured by 0.6% sodium dodecyl sulfate. However, a small proportion of the liberated total *p*-nitrophenol was attributable to unspecific hydrolysis, and this must be taken into account when calculating the kinetic constants of the steady state and preparing the acyl enzyme. SH groups, known to readily react with PNPA, are involved neither in specific nor unspecific hydrolysis for diphenylmercuric lipase, in which the two thiols of lipase are blocked (Verger et al., 1971), which has exactly the same kinetic behavior as the native enzyme.

It was of interest to check that the same site in lipase was responsible for the specific hydrolysis of PNPA and that of more usual carboxylic esters. A preliminary indication comes from the fact that monomeric tripropionin or triacetin are competitive inhibitors for PNPA. On the other hand, substitution at the β carbon of the acyl chain abolished lipase activity on dissolved *p*-nitrophenyl esters, as it does with emulsified esters (Brockerhoff, 1968).

It must, however, be pointed out that the k_{cat} calculated for dissolved triacetin was 20 times higher than that obtained with dissolved PNPA, although the deacylation of the same acetyl intermediate should have been rate limiting in both cases. More work on the kinetics of soluble substrates is in progress to completely elucidate this apparent discrepancy.

A last indication for the validity of the acyl enzyme mechanism was that various interfaces can considerably enhance both the hydrolysis of dissolved substrates and deacylation of the acetyl enzyme intermediate. If lipase action at interfaces did not comply with the acyl enzyme mechanism demonstrated for the enzyme in aqueous solution, the effect of these interfaces on the deacylation of acetyl lipase could hardly be understood. It seems preferable to believe that the same mechanism is followed in both situations. Therefore, the existence on the enzyme of two sites, one moderately active in solution and the other acting more energically at interfaces, is unlikely. A more plausible hypothesis is that the enzyme possesses a normal esteratic site which becomes much more efficient after adsorption of the enzyme to an interface.

To explain the necessity of an interface for the full expression of lipase activity, one could simply assume that the enzyme has no affinity for soluble molecules. The saturation of the enzyme by monomeric substrates dissolved in water shows that the effect of interface is not to facilitate the formation of the primary Michaelis–Menten complex, but rather to increase the maximal rate of the reaction and, consequently, the catalytic efficiency of the enzyme. The same is true for pancreatic phospholipase A_2, an enzyme sharing many properties with lipase (Pieterson et al., 1974).

Thus, the origin of the stimulation has still to be ascertained, for the interface adsorbs, not only the enzyme, but also the substrate (Brockman et al., 1973). Consequently, in a conventional system containing enzyme and substrate, they may be assumed to activate anyone of the two components, or both. A better approach to the problem consists in following, in the absence of the substrate, the simulation exerted by glass beads on the deacylation of preformed acetyl lipase. We have, indeed, the opportunity to study, in this case, a particular step which does not involve the substrate. Thus, the contribution of the substrate adsorption on this particular step can be eliminated. The real extent of this stimulation is not easy to evaluate with our present technique, which requires a minimum of 30 s for a measure to be made. Anyhow, most of adsorbed acetyl lipase molecules were shown to deacylate in less than 30 s in the presence of beads. When a solution of the derivative was simply placed in a glass tube, under exactly the same conditions of concentration, pH and temperature, a comparable deacylation required at least 50 min (half-life time of the derivative, 12 min). Therefore, the stimulation of acetyl lipase deacylation by adsorption to the beads is probably higher than 100-fold and thus accounts for a substantial part of the overall stimulation

of 500-fold observed under the same conditions for the hydrolysis of PNPA.

The above findings do not exclude the possibility for the acylation step to be also stimulated by the beads and/or for the substrate to be activated. In the same way, it is likely that the activation also depends on the nature of the interface, as shown in this work by different activation factors brought about by different interfaces and, also, by previous observations, especially with monolayer substrates (Verger et al., 1973; Esposito et al., 1973). The main interest of these findings resides in the fact that they unambiguously prove for the first time an enhancement of the catalytic properties of lipase consecutive to adsorption to an interface. Adsorption of proteins may be expected to alter their hydrophilic–hydrophobic balance and, consequently, to modify their conformation. In other words, the data presented here suggest that a part of the activation of lipase at interfaces may be due to a conformational change resulting from adsorption.

On the other hand, as discussed above, the behavior of acetyl lipase upon emulsified substrates hydrolysis is also consistent with an acceleration of the deacylation brought about by substrate interfaces. Lipase may possibly serve in this respect as a model for other enzymes known to be activated by addition of lipids (see, for example, Metcalfe et al., 1975).

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