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Pancreatic Nonspecific Lipase, an Enzyme Highly Specific for Micelles†

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ABSTRACT: Nonspecific lipase activity in extracts of rat pancreas has been studied using phthalate esters as selective substrates. Evidence indicates that the enzyme binds anionic micelles at a site different from the hydrolytic site. Inhibition studies implicate sulfhydryl and amino groups as active components of the binding site, hydroxyl and imidazole groups as components of the hydrolytic site. Kinetic studies suggest that apparent K_m for nonpolar substrates is determined by the micelle-forming agent used, bile salts or anionic detergents. Both K_m(app), which was the same for a variety of phthalates, and V_{max}(app), which was not, were linearly proportional to the concentration of bile salt used to solubilize the substrates. Difficulties in making "obvious" interpretations of kinetic studies with substrates as mixed micelles are discussed.

In 1966 Mattson and Volpenhein reported the presence in rat pancreatic juice of an enzyme capable of hydrolyzing esters of both primary and secondary alcohols. Later (Mattson and Volpenhein, 1968) they suggested the trivial name "nonspecific lipase" for his enzyme activity. Among the esters reportedly hydrolyzed by pancreatic "nonspecific lipase" are 2,3-dioleylbutane and isopropyl oleate (Mattson and Volpenhein, 1966), 2-monoglycerides (Mattson and Volpenhein, 1968), erythritol tetraoleate (Mattson and Volpenhein, 1972a,b), methyl oleate, ethylene glycol dioleate, triolein, pentaerythritol tetraoleate, and adonitol pentaoleate (Mattson and Volpenhein, 1972b), bis(2-ethylhexyl) phthalate, and isopropyl palmitate (Albro and Thomas, 1973).

It now seems certain that nonspecific lipase is a different enzyme (or enzymes) from pancreatic lipase (EC 3.1.1.3) (Mattson and Volpenhein, 1966, 1968; Desnuelle and Savary, 1963) and pancreatic cholesterol esterase (EC 3.1.1.13) (Hyun *et al.*, 1969; Albro and Thomas, 1973). Likewise, evidence has been presented that nonspecific lipase activity is probably not associated with trypsin, α-chymotrypsin, carboxypeptidase, or phospholipase A (Mattson and Volpenhein, 1966). Nonspecific lipase has not been obtained in any high degree of purity up to the present time, although it probably was separated from pancreatic lipase by Morgan *et al.* (1968), and a 36-fold purification that did not separate it from cholesterol esterase has been reported (Albro and Thomas, 1973).

The nonspecific lipase from rat pancreas is strongly activated by taurocholate (Mattson and Volpenhein, 1966), and, unlike cholesterol esterase (Vahouny *et al.*, 1965), by deoxycholate as well as cholate (Albro and Thomas, 1973). The acceptable substrates are apparently those that can be presented in micelles (Albro and Thomas, 1973; Morgan *et al.*, 1968). As has been pointed out by Hyun *et al.* (1969), there are great difficulties encountered in attempting to apply enzyme kinetics derived for aqueous systems to systems involving dispersed substrates. It is the purpose of the present report to discuss studies of the role of micelles in the properties of nonspecific lipase activity in extracts of rat pancreas.

Materials and Methods

Buffer Salts. Hepes[1] was either Sigma H-3375 or the equivalent product from Nutritional Biochemicals Corp. Mes and Taps were Sigma No. M-8250 and T-5130, respectively. Tris was enzyme grade from Schwarz/Mann. Phosphate buffer was prepared from mono- and dibasic sodium phosphates, Baker and Adamson reagent grade.

Surfactants. Sodium taurocholate was Sigma T-0750; sodium desoxycholate was Fisher J-285; sodium glycocholate was from Nutritional Biochemicals; sodium cholate was prepared by titrating Eastman 4952 cholic acid with sodium hydroxide to give an aqueous solution 0.232 M in cholate at pH 8. Tween 20 and Tween 80 were from Nutritional Biochemicals; Brij 35 and Cutscum were from Fisher; sodium dodecyl sulfate was Sequanal grade from Pierce Chemical Co.; cetylpyridinium chloride and cetyltrimethylammonium bromide were Eastman P-5361 and P-5650, respectively; Triton X-100 was purified grade from Packard Instrument Co. Other dispersing agents included gum acacia (gum Arabic), Fisher G-85, and bovine serum albumin (fraction V), Sigma No. A4503.

Substrates. Bis(2-ethylhexyl) phthalate, dimethyl phthalate, and isopropyl palmitate labeled with ^{14}C in the carbonyl

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid; BBOT, 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene; Egmee, ethylene glycol monoethyl ether; BEHP, bis(ethylhexyl) phthalate; cmc, critical micelle concentration; E-600, diethyl *p*-nitrophenyl phosphate.

carbons, and unlabeled di-*n*-octyl phthalate were synthesized as described previously (Albro and Thomas, 1973). Dimethyl phthalate (Eastman 318) and di-*n*-butyl phthalate (Fisher D-30) were obtained commercially.

Inhibitors. 2,4′-Dibromoacetophenone and 2,4-dinitrobenzenesulfonic acid were Eastman 2356 and 4309, respectively. Sodium *p*-hydroxymercuribenzoate, cholesterol isoamyl ether, and cholesterol oleate were Sigma H-0752, C-0504, and CH-SO, respectively. *N*-Ethylmaleimide was from Nutritional Biochemicals, *n*-hexadecane was from LaChat Chemicals, Inc., trypsin (188 units/mg) was from Worthington Biochemical Corp., and diethyl *p*-nitrophenyl phosphate (E-600) was 99+% from Pesticide Research Laboratories, Perrine, Fla. Pyridoxal 5′-phosphate was Sigma P9255.

Scintillation cocktail was made by combining 1800 ml of toluene, 1200 ml of ethylene glycol monomethyl ether, 240 g of naphthalene, and 12 g of BBOT. Counting efficiency was determined by the channels ratio method, using a Packard Tri-Carb Model 3380 scintillation counter.

All other solvents and chemicals were the best grades available from Fisher Scientific Co., Raleigh, N. C.

General Methods. Protein concentrations were determined by the Biuret method using bovine serum albumin as standard (Gornall *et al.*, 1949). The homogenate of acetone powder (see subsequently) could not be assayed with Biuret in the presence of EDTA, and protein concentration in this preparation was determined from the relationship: mg of protein/ml = 1.50(OD 280 nm) − 0.75 (OD 260 nm) when measured in a 1-cm cell. Centrifuged homogenates prepared without EDTA assayed the same protein concentration by the two methods.

Enzymatic hydrolysis of the various ^{14}C-labeled substrates was followed, after stopping the reactions with 0.2 ml of 4 N H_2SO_4, by the extraction and partition method described previously (Albro and Thomas, 1973). All incubations were carried out in 1.6 × 15 cm test tubes with Teflon-lined screw caps at 37° and 300 rpm in a New Brunswick Model G-77 shaking water bath.

Two sets of incubation conditions were used frequently enough to be considered "standard." The first, designated "standard assay with cholate," involved combining 2.0 ml of 0.1 M Hepes (pH 8.1), 0.5 ml of 0.232 M sodium cholate (pH 8.1), 50 μl of 0.08 M ^{14}C-labeled bis(ethylhexyl) phthalate in Egmee, and 1.2 ml of water. This was preincubated for 10 min at 37°, 300 rpm, after which the hydrolysis was started by adding 0.3 ml of enzyme solution containing 0.1 mg of protein/ml. Incubation was continued for 30 min. The second standard assay, designated standard assay with deoxycholate, was the same as above but with 0.1 ml of 0.23 M sodium deoxycholate replacing the 0.5 ml of cholate and 0.04 M ^{14}C-labeled bis(ethylhexyl) phthalate in place of the 0.08 M used above.

Hydrolysis of nonradioactive substrates was followed by treating the extracted products (Albro and Thomas, 1973) with Methyl-8 or, in the case of dimethyl phthalate substrate, with Butyl-8 (Pierce Chemical Co.). The diesters were analyzed by gas–liquid chromatography (glc) using a Hewlett-Packard Model 5750 instrument coupled to an Autolab System IV computer. Injection port and hydrogen flame ionization detector temperatures were 260 and 280°, respectively. Helium flow was 35 cm^3/min. The 2 m × 2 mm (i.d.) stainless-steel columns, packed with 4.8% OV-101 on 100–120-mesh Gas Chrom Q, were linearly programmed from 150 to 200° at 10°/min. Relative molar area responses used to compute compositions of the incubation products were: dimethyl phthalate, 1.00; methyl butyl phthalate, 1.25; dibutyl phthalate, 1.58; methyl octyl phthalate, 1.60; dioctyl phthalate, 2.26.

Solubilities and Critical Micelle Concentrations. Cmc for the anionic detergents and bile salts were estimated by measuring the uptake of Oil Scarlet 6G at 485 nm. Cmc for nonionic detergents were estimated by the spectral method of Becher (1962); for cationic detergents we used *N,N*-diethylaniline (Suzuki, 1970) and Alizarin Red S (Malik and Verma, 1967). Solubilities of substrates under various conditions were estimated from turbidity measurements at 700 nm, and, for phthalates, from absorbance at 278 nm after centrifugation. When possible, concentrations were also determined from the ^{14}C label.

The accommodation of substrates in micelles was evaluated by filtration method 1 of Borgström (1967) using Millipore filters of 500-Å pore size. The results obtained by this method generally confirmed the results from absorption measurements after centrifugation as described above.

Enzyme Preparation. Acetone powder of pancreatic tissue from male CD strain rats (Albro and Thomas, 1973) was homogenized using 15 strokes of a Ten-Broeck glass–glass tissue grinder in ice-cold 0.1 M Hepes pH 8.1 buffer. The buffer contained either no additives except the NaOH used to adjust pH (type A preparation), 0.1 mg of soybean trypsin inhibitor (Sigma T9003) per ml (type B preparation), or both soybean trypsin inhibitor and 1 mM Na_2EDTA (type C preparation).

One milliliter of buffer was used per mg of acetone powder, which corresponded to approximately 3.4 mg of pancreatic tissue (fresh weight). The homogenate was centrifuged at 4°, 12,000*g*, for 10 min. The supernatant was removed and either used immediately (types A and B) or stored at 4° (type C). The type C preparation could be stored for 2 months with no decrease in nonspecific lipase activity. For most of the experiments following, the supernatant enzyme was diluted with 0.1 M Hepes (pH 8.1) to a protein concentration of 0.1 mg/ml.

Enzyme activity was expressed in units of nanomoles of ester hydrolyzed per minute (per milligram of protein for specific activity). All such values were corrected for any nonenzymatic hydrolysis detected in enzymeless controls. As indicated previously (Albro and Thomas, 1973), only one ester bond of the phthalate diesters is cleaved by the pancreatic enzymes.

Experimental Section

Stability of Nonspecific Lipase Activity. We previously reported (Albro and Thomas, 1973) observations confirming those of Mattson and Volpenhein (1967), that nonspecific lipase activity was lost when the pancreas homogenate was incubated for 1 hr at pH 9 and 40°, but retained when incubation was at pH 4. The probability that the irreversible inactivation at pH 9 was due to trypsin-like activity in the extracts led to our incorporation of soybean trypsin inhibitor in the types B and C homogenates discussed here.

Since protection against proteolysis by trihydroxy bile salts has been presented as evidence that cholate serves as a coenzyme for cholesterol esterase (Vahouny *et al.*, 1965), we ran an experiment to determine whether taurocholate could protect nonspecific lipase against trypsin digestion. One milliliter of supernatant from a type A preparation of enzyme was diluted with 9 ml of buffer to give solution 1; another milliliter of supernatant was diluted with 9 ml of buffer containing 27 mg of sodium taurocholate to give solution 2; 4-

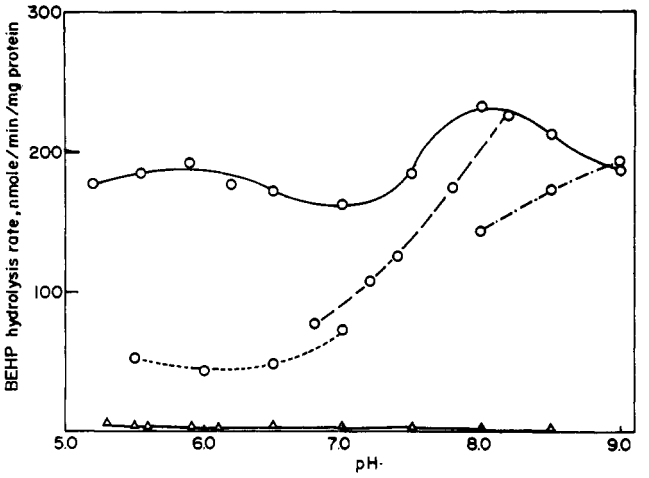

FIGURE 1: pH dependence of nonspecific lipase in several buffers with 29 mM taurocholate. The standard assay was used (see text), except that 0.1 M sodium phosphate (—), 0.1 M Taps (—·—) or 0.1 M Mes (····) were used in place of 0.1 M Hepes (— —), and taurocholate was used in place of cholate. The triangles represent the results obtained in phosphate buffer with a gum acacia emulsion of BEHP in place of micellar BEHP.

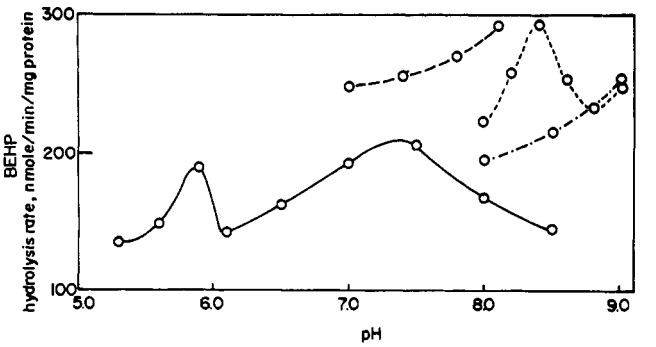

FIGURE 2: pH dependence of nonspecific lipase in several buffers with 29 mM cholate. The standard assay with cholate (see text) was used, except that 0.1 M Hepes (— —) was replaced by 0.1 M sodium phosphate (—), 0.1 M Tris-HCl (····), or 0.1 M Taps (—·—) buffers.

ml portions of each solution were incubated with and 4-ml portions without 1 mg of trypsin for 10 min at 37°.

Portions (0.3 ml) of the four variously treated enzyme samples were subjected to the standard assay with cholate. The specific activities of the four preparations were: solution 2 without trypsin, 141; solution 2 with trypsin, 112; solution 1 without trypsin, 89; solution 1 with trypsin, 1.4. Thus 5 mM taurocholate protected at least 80% against trypsin and endogenous proteolytic inactivation of nonspecific lipase.

We tested a number of antimicrobial agents and sulfhydryl compounds for their ability to protect nonspecific lipase against deterioration, with results summarized in Table I. The apparent "stimulation" of activity by antimicrobial agents seems to confirm the findings of Vahouny *et al.* (1965) concerning the sensitivity of pancreatic enzymes to bacteriolysis, and emphasizes the rapidity of the inactivation process in the absence of protecting agents. Cysteine and reduced glutathione were inhibitory if added to the enzyme before bile salt, and slightly stimulatory (cysteine) if added after the bile salt. Accordingly, we did not include sulfhydryl compounds in the homogenizing-storage medium.

TABLE I: Protection of Nonspecific Lipase Activity by Antimicrobial and Sulfhydryl Compounds.[a]

Enzyme Prep[b]	Addition to Assay Medium	Reaction Started with	Sp Act.[c]
B	None	BEHP	155
B	Sodium azide, 4 mg	BEHP	204
B	Sulfanilamide, 4 mg	BEHP	196
B	Na_2EDTA, 80 μmol	BEHP	220
C	Cysteine·HCl, 8 μmol	BEHP	172
C	Cysteine·HCl, 8 μmol[d]	Cholate + BEHP	119
C	Glutathione, 8 μmol[d]	Cholate + BEHP	135

[a] Incubation mixtures containing 0.0575 M Hepes–NaOH (pH 8.1), 0.029 M sodium cholate, and 30 μg of protein in a total volume of 4 ml received the additions indicated in the table. After a 10-min preincubation at 37°, 2 μmol of BEHP was added in 50 μl of Egmee. Incubation at 37°, 300 rpm, was carried out for 30 min. [b] Type B enzyme contains soybean trypsin inhibitor but no EDTA. Type C enzyme contains both. [c] Specific activity is given as nanomoles of substrate hydrolyzed per minute per milligram of protein. [d] Cholate was not added to these samples until after the 10-min preincubation.

Optimum Reaction Conditions. The solubility limit of BEHP in 0.5 M Hepes buffer containing 1.25% Egmee was found to be approximately 1.6×10^{-5} M. This compound and its hydrolysis products can be assayed by spectrophotometry, glc, or radioassay. Nonspecific lipase appears to be the only enzyme in rat pancreas capable of hydrolyzing this diester, and the only products formed are the monoacid and 2-ethylhexanol (Albro and Thomas, 1973). For these reasons, most of our studies of nonspecific lipase activity have utilized this substrate.

The pH optima for BEHP hydrolysis have been determined using 0.1 M buffers with taurocholate (Figure 1) or cholate (Figure 2). It is clear from the figures that there is a considerable dependence of the pH optimum on the type of buffer used, *e.g.*, anionic or cationic, different ionic strengths, and the like. It is not possible from these data to distinguish between effects of the buffer on ionizable groups of the enzyme and effects on the bile salt micelles. The conspicuously high activity with Hepes–NaOH buffer at pH 8.1, along with the convenience of Hepes relative to extraction of the reaction products (Albro and Thomas, 1973), led to our use of Hepes buffer for most of the studies reported here.

The dependence of the rate of BEHP hydrolysis on the amount of type C enzyme preparation added was tested using the standard assay with deoxycholate. Dependence was linear between 20 and 60 μg of protein; above this point the amount of available substrate decreases significantly during the 30-min incubations. Below 20 μg of protein, which corresponds to 0.2 ml of enzyme preparation in a 4-ml final volume, it appears that dilution of the EDTA below 10^{-5} M permits some degradation of the enzyme.

The time course of the hydrolysis of BEHP in the standard assay with cholate was followed with 200 μg of enzyme protein present. The rate was constant for approximately 40 min, by which time the amount of BEHP hydrolyzed corresponded to the amount found *not* to initially be in micelles by the filtration method. From this we suspected that the BEHP in excess over that accommodated in micelles was able to maintain a constant concentration of micelle-bound BEHP until all of the bulk phase was used up. Evidence confirming the contention that the true substrate is micelle-bound BEHP will be discussed later.

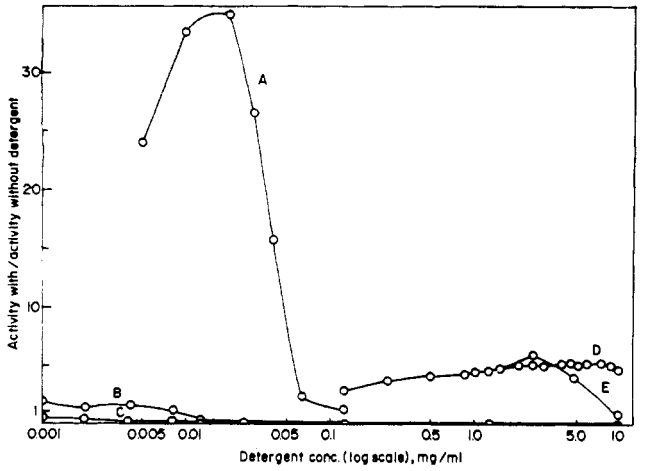

FIGURE 3: Optimum detergent concentrations for hydrolysis of 5×10^{-4} M BEHP. Hydrolysis of BEHP is expressed as the ratio of the initial rate of hydrolysis in the presence of detergent plus 1% Egmee to that in the presence of Egmee alone. Curve A applies to sodium dodecyl sulfate; curve B applies to Triton X-100 and Cutscum; curve C applies to Tween 20, Tween 80, Brij 35, cetylpyridinium chloride and cetyltrimethylammonium bromide; curve D applies to sodium cholate, taurocholate, and glycocholate; curve E applies to sodium deoxycholate.

TABLE II: Critical Micelle Concentrations of Various Detergents.[a]

Detergent–Surfactant	Critical Micelle Concn	
	mg/ml	mM
Triton X-100	0.14	
Cutscum	0.12	
Brij 35	0.095	
Tween 20	0.022	
Tween 80	0.010	
Cetylpyridinium chloride	0.010	0.03
Cetyltrimethylammonium bromide	0.10	0.28
Sodium dodecyl sulfate[b]	0.46	1.6
Sodium cholate[c]	2.07	4.8
Sodium deoxycholate[d]	0.46	1.1
Sodium taurocholate	2.21	4.1
Sodium glycocholate	2.05	4.2

[a] In 0.0575 M Hepes–NaOH (pH 8.1); best estimates. [b] No detectable cmc with 1% Egmee present. [c] Cmc with 1% Egmee present increases to 6.8 mg/ml. [d] Cmc with 1% Egmee present increases to 2.25 mg/ml.

Egmee alone will disperse BEHP sufficiently to permit a minimal level of hydrolysis in the type C enzyme system. The optimum amount of Egmee for 2.5×10^{-5} M BEHP was found to be 1.0%; larger amounts gave rapidly decreasing rates of hydrolysis. With 1% Egmee and 2.5×10^{-5} M BEHP the initial rate of hydrolysis was 1.25 nmol/min mg of protein. Under the same conditions but with 2.9×10^{-3} M cholate present the rate was 20.5 nmol/min per mg of protein.

A variety of detergents was tested for ability to support BEHP hydrolysis by the type C enzyme system at pH 8.1 in 0.0575 M Hepes–NaOH. BEHP was added in sufficient Egmee to give a final concentration of 1% Egmee, 5×10^{-4} M BEHP. Some of the results are shown in Figure 3. Only the anionic detergent sodium dodecyl sulfate and the bile salts supported active BEHP hydrolysis. The nonionic detergents Triton X-100 and Cutscum were inhibitory above 0.01 mg/ml (although not below), while both of the cationic detergents as well as the nonionics Tween 20, Tween 80, and Brij 35 were inhibitory at all concentrations above 0.001 mg/ml. Critical micelle concentrations for the various detergents in 0.0575 M Hepes buffer at pH 8.1 and 37° are listed in Table II.

All of the trihydroxy bile salts gave essentially identical support of the BEHP hydrolysis reaction over the range of concentrations tested. Deoxycholate was similar in effectiveness to the trihydroxy bile salts up to about 4 mg/ml, above which its apparent activation decreased.

With 1% Egmee present, sodium dodecyl sulfate at 0.02 mg/ml was much more effective at activating the BEHP hydrolysis than were the bile salts. However, cholate was much more effective in the absence than in the presence of Egmee. Without Egmee, cholate gave a very sharp peak of activation having "activity with/activity without cholate" equal to 4.2 at the optimum cholate concentration of 2.0 mg/ml. The omission of Egmee had the effect of eliminating the near independence of the reaction rate from the cholate concentration, however, and therefore the inclusion of Egmee has an advantage in the performance of a generally applicable assay for nonspecific lipase activity. As will be discussed later, nonspecific lipase assays in the absence of Egmee require that an optimum bile salt: substrate ratio be determined for each concentration of substrate used.

In the presence of Egmee, the optimum deoxycholate concentration was 2.5 mg/ml. This was the optimum whether 30 or 90 μg of protein was used in the assay, indicating that the optimum detergent concentration does not derive primarily from a direct interaction of the detergent with the enzyme. As will be discussed later, there is a relationship between optimum detergent concentration and amount of substrate.

We next compared the ability of the type C pancreas extract to hydrolyze BEHP in four different types of dispersions; 2.5×10^{-5} M BEHP (final concentration) was supplied to an assay system consisting of 0.0575 M Hepes (pH 8.1), 7.5 μg of protein/ml, and various dispersing agents. Preincubation, incubation, and analysis of products were as described for the standard assay.

BEHP was provided in a minimal volume (10 μl) of ethanol, yielding a cloudy suspension, in a sonicated dispersion with concentrations of gum acacia varying from 1 to 10% (yielding a fine emulsion), and in a mixture of bovine serum albumin and calf serum as described previously (Albro and Thomas, 1973) for assay of lipoprotein lipase (yielding a clear solution). The fourth preparation was that portion of BEHP in 2.0% aqueous sodium cholate capable of passing through a 500-Å pore size Millipore filter, *i.e.*, micelles. This last gave the only incubation mixture containing bile salt. The results are summarized in Table III. Clearly micelles, and judging from the results of the previous experiment only micelles of anionic surfactants, are the preferred substrate form for hydrolysis of BEHP by rat pancreas enzymes.

Micellar Accommodation of BEHP. Oil Scarlet 6G, a fat-soluble dye, is held in the hydrophobic interior of cholate micelles as evidenced by the shift of its absorption maximum from 494 nm in 95% ethanol to 485 nm in aqueous cholate above its cmc (λ_{max} 483 nm in ethyl acetate) (*e.g.*, Suzuki, 1970). A "saturated" micellar solution of dye was shaken with various amounts of BEHP, giving the results shown in Figure 4. BEHP uptake by the dye-loaded micelles resulted in dis-

TABLE III: Influence of Dispersing Agent on Hydrolysis of BEHP.[a]

Dispersing Agent, Final Concn	App Sp Act.
Ethanol, 0.25–0.5%	4.5
Acacia, 0.025–0.25%	1.7
Serum proteins, various	3.9
Cholate, 0.25%	41.0

[a] Final concentration of BEHP = 2.5×10^{-5} M, of pancreas protein = 7.5 μg/ml, of Hepes–NaOH (pH 8.1) = 0.0575 M (in Hepes). Conditions described in the text.

placement of the dye, suggesting that BEHP also locates inside the cholate micelles.

Indirect evidence that BEHP is normally solubilized inside the cholate micelles was also obtained using two "competitors." The relative abilities of dimethyl phthalate and *n*-hexadecane to compete with ^{14}C-labeled BEHP for micellar uptake and their effects on BEHP hydrolysis were examined as shown in Table IV.

Although the effects were not great, it appears from Table IV that *n*-hexadecane, equimolar with BEHP, both reduced its uptake into micelles and its rate of hydrolysis by the lipase. Dimethyl phthalate had little or no effect on uptake or hydrolysis of BEHP. The amount of dimethyl phthalate used here was greater than its solubility in 0.05 M Hepes (3.8 mg/ml), but less than its "solubility" in the presence of deoxycholate at this level (5.8 mg/ml). Dimethyl phthalate is presumed to be associated with the outside of micelles by adsorption (Suzuki, 1970), while hexadecane is presumed to enter the micelles.

Kinetic Study. When cholate concentration was held constant at 29 mM, Egmee at 5%, and enzyme at 30 μg of protein in a final volume of 4 ml, varying the amount of ^{14}C-labeled BEHP with a 10-min preincubation before adding enzyme gave a linear double-reciprocal plot up to a concentration of 2 mM BEHP as shown in Figure 5A. Substrate inhibition was apparent at concentrations of BEHP greater than 2 mM. This linear plot, which gave an apparent K_m of 1.3×10^{-3} M and apparent V_{max} of 393 nmol/min per mg of protein, was not surprising in view of the independence of the hydrolysis rate from the ratio of cholate to substrate in the presence of Egmee. When the values for 1/[S] were corrected such that [S] referred only to that fraction of the BEHP accommodated

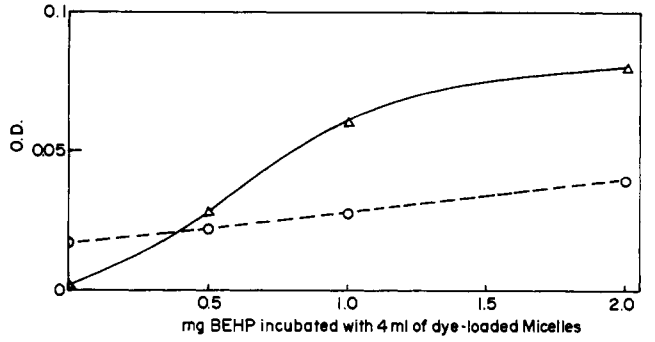

FIGURE 4: Exclusion of Oil Scarlet 6G from cholate micelles by BEHP. 4 ml of 29 mM cholate in 57.5 mM Hepes (pH 8.1) saturated with Oil Scarlet 6G was incubated with BEHP for 1 hr at 37° and centrifuged. The precipitated dye was dissolved in ethyl acetate and its absorbance at 485 nm measured (Δ—Δ). Light scattering associated with uptake of BEHP was measured by turbidity at 700 nm in the supernatant fraction (- - -).

TABLE IV: Effects of Hexadecane and Dimethyl Phthalate on Support of Nonspecific Lipase Activity by Deoxycholate.[a]

Competitor	μmol of BEHP Solubilized/ 3.7 ml	BEHP Hydrolyzed (nmol/ min)	Hydrolysis (nmol/min per μmol of BEHP)
None	1.80	14.6	8.21
n-Hexadecane	1.51	11.2	7.44
Dimethyl phthalate	1.89	16.0	8.46

[a] 2.4 μmol of ^{14}C-labeled BEHP with or without an equimolar amount of "competitor" was preincubated for 1 hr at 37° with 4.7-ml aliquots of 0.05 M Hepes buffer (pH 8.1) and 2.5-mg portions of sodium deoxycholate. After centrifugation, 0.2-ml aliquots were assayed for ^{14}C and 3.7-ml aliquots were treated with type C nonspecific lipase as usual.

in micelles at the end of the preincubation period, the apparent K_m was lowered to 1.48×10^{-4} M.

In the absence of Egmee, the data previously discussed imply that changes in the ratio of cholate to substrate would significantly alter the reaction rate at any given substrate concentration. Accordingly Figure 5 also shows double-reciprocal plots for experiments in which the molar ratio of cholate to BEHP was held constant at approximately 12:1. Two slightly different experiments were performed. For the first, the mixture of cholate and BEHP was sonicated and various amounts were added to the incubation medium at 37° with enzyme already present. For the second, the sonicated

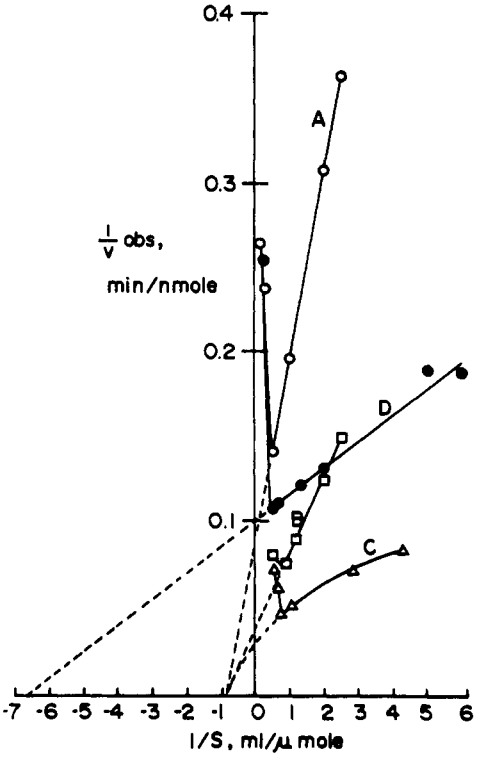

FIGURE 5: Dependence of initial rate of hydrolysis of BEHP on substrate concentration. Curve A: cholate constant at 29 mM, Egmee 5%; curve B: [cholate]/[BEHP] = 12:1, micelles added to dilute enzyme; curve C: as B, but enzyme added to diluted micelles; curve D: ([cholate] minus 5 mM)/[BEHP] = 12:1, enzyme added to diluted micelles. In all cases, reactions were run in 57.5 mM Hepes buffer (pH 8.1).

mixture of cholate and BEHP was preincubated with the buffer at 37° for 15 min before adding enzyme.

As Figure 5B shows, the addition of micellar BEHP directly to the enzyme-containing incubation medium again gave a linear double-reciprocal plot until substrate inhibition appeared at about 1.5 mM BEHP. The apparent K_m, 1.1×10^{-3} M, did not differ significantly from the uncorrected one observed at a constant cholate concentration, but the apparent V_{max} was increased to 830 nmol/min per mg of protein.

When the micelles were preincubated after dilution with buffer prior to adding enzyme, the double-reciprocal plot (Figure 5C) was nonlinear. Substrate inhibition was again apparent. Intuitive extension of that portion of the curve preceding substrate inhibition suggested that an apparent K_m would be similar to those above, but apparent V_{max} would be close to 1 μmol/min per mg of protein.

Cholate micelles once formed would probably dissociate slowly upon dilution below the cmc (Hofmann and Small, 1967). This phenomenon may account for the nonlinear plot resulting from the preincubation experiment. In addition, the concentrations of cholate used here are sufficiently close to the rather large cmc of 2.07 mg/ml that the amount of nonmicellar, dissolved cholate is a significant fraction of the total. Accordingly, we ran a fourth experiment in which the molar ratio of presumably *micellar* cholate (only that above 5 mM after dilution in the assay) to BEHP was held constant at 12:1, and these data are also plotted in Figure 5D. We again obtained a straight line showing substrate inhibition starting at 2 mM BEHP. Apparent V_{max} under these conditions was only 333 nmol/min per mg of protein, but the apparent K_m was reduced to 1.5×10^{-4} M.

Substrate inhibition was clearly due to BEHP, since it occurred even at constant cholate concentration. The results of the last experiment indicate that the inhibition is brought about by excess *solubilized* BEHP (since in the last three experiments bulk phase was removed by centrifugation before adding the micelles to the assay system, and in the final system the concentration of micelles remained proportional to the concentration of BEHP).

Apparent K_m and V_{max} values were determined at several different but constant cholate concentrations. Under these conditions both K_m for BEHP and V_{max} were linearly proportional to the cholate concentration. Extrapolation to zero cholate gave a K_m of 8×10^{-5} M BEHP and a V_{max}(app) of 153 nmol/min per mg of protein, the latter being about 13 times the maximum velocity actually observed in the absence of cholate.

When data for the rates of hydrolysis of 1×10^{-3} and 2.5×10^{-5} M BEHP in the presence of varying amounts of cholate in excess of the optimum [cholate]/[BEHP] ratios were plotted by the method of Dixon (1953) (not shown), it could be seen that cholate was behaving as a competitive inhibitor with $K_i = 4.48 \times 10^{-3}$ M. When data from the experiment at 1 mM BEHP but from the regions of cholate concentrations below the optimum were plotted as 1/velocity *vs.* 1/[cholate], a straight line resulted with an apparent K_m for cholate of 2.25×10^{-3} M. These experiments were interpreted as indicating that the enzyme has the same affinity for "empty" cholate micelles as for micelles containing BEHP, and therefore for each BEHP concentration there is only one suitable bile salt concentration for a valid measurement of the enzyme activity. Further, these experiments imply that the enzyme binds the micelle, not the "substrate" (*i.e.*, the true "substrate" is the mixed micelle). This is supported by the observation that, in the system containing 5% Egmee and 29 mM cholate, apparent K_m for dibutyl phthalate, dioctyl phthalate, and BEHP was the same, $1.3 \times 10^{-3} \pm 0.1 \times 10^{-3}$ M. However, the apparent V_{max} for the three were 660, 775, and 393 nmol per min of mg of protein, respectively, suggesting that the hydrolysis process does distinguish between different micelle "contents." When dimethyl phthalate was used as substrate in this system, strong substrate inhibition became conspicuous at concentrations below the solubility limit of dimethyl phthalate in the buffer, suggesting that it may not be a substrate for nonspecific lipase. Further, the hydrolysis of dimethyl phthalate by the pancreas extract was maximal at cholate concentrations below the cmc.

Inhibitor Studies. METAL IONS. The "stimulatory" effect of EDTA suggested that nonspecific lipase might be susceptible to inhibition by heavy metal ions. We tested a variety of salts in the standard assay with cholate, using the type C enzyme preparation. The EDTA concentration during preincubation and assay is approximately 7.5×10^{-6} M, which presumably would not interfere when the various salts were tested at concentrations of 1 mM.

Amost all salts had a slightly inhibitory effect, possibly due to reducing the cmc of cholate. In that case the "excess" cholate micelles would somewhat inhibit. When the inhibition was expressed relative to that shown by 1 mM NaCl set equal to zero, it was seen that fluoride, strontium, magnesium, calcium, and bismuth subnitrate were slightly stimulatory (7, 24, 16, 11, and 22% stimulation, respectively). This suggests that ionization of a carboxyl group on the enzyme may not be critical for optimum activity at pH 8.1, or that removal of acid products may be advantageous. Chloride, nitrate, acetate, and sulfate anions showed no inhibition as sodium or potassium salts. Manganous, ferrous and aluminum salts were slightly inhibitory (19–20% inhibition relative to NaCl). Nickelous (51%), cupric (78%), cadmic (83%), zinc (97%), mercuric (99%), and silver (100%) ions inhibited strongly, generally in the order of their affinity for sulfhydryl groups.

Dixon plots were prepared for several of the metal ions. In all cases tested (Ag^+, Hg^{2+}, Zn^{2+}, and Cd^{2+}), inhibition was noncompetitive. K_i values for these cations were estimated from the plots to be 3.5×10^{-7}, 4.5×10^{-7}, 7.8×10^{-7}, and 1×10^{-6} M, respectively. It made no difference whether cholate was present while the enzyme was preincubated with inhibitor or added later. We did observe, however, that while preincubation of the enzyme with 0.1 mM zinc acetate for 5 min at pH 8.1 resulted in 81% inactivation, similar preincubation at pH 7.2 gave only 41% inactivation. For 10^{-5} M silver nitrate, the values were 49% at pH 8.1 and only 9% at pH 7.2. This suggests that the cations react with some group whose degree of ionization changes significantly between pH 7.2 and 8.1.

We tested several sulfhydryl reagents, as summarized in Table V, and found that cholate could protect the enzyme against them. This was also the case with cholesterol esterase (Hyun *et al.*, 1969). Bile salt protection was also seen in another series of experiments in which we treated undiluted type C enzyme with 0.1 mM sodium dinitrobenzenesulfonate, 0.1 mM pyridoxal 5′-phosphate, or 1 mM maleic anhydride for 20 min at 37° in the presence or absence of 6 mM sodium deoxycholate. The treated enzyme was diluted with four volumes of 0.1 M Hepes (pH 8.1), and 0.3-ml portions were tested for nonspecific lipase activity in the standard assay with cholate (the deoxycholate being thus diluted to ≃1/300 of the cholate concentration in the assay). The results were quite clear-cut; there was no inhibition when deoxycholate was present during the exposure of the enzyme to either reagent.

TABLE V: Effect of Sulfhydryl Reagents on Nonspecific Lipase Activity.[a]

Reagent	Final Concn (mM)	Bile Salt[b]	BEHP Hydrolyses (nmol/min)	% Inhibn
None			6.71	
N-Ethylmaleimide	0.50	Before	5.31	20.9
N-Ethylmaleimide	0.50	After	2.40	64.3
Sodium arsenite	0.10	Before	5.28	21.3
Sodium arsenite	0.10	After	2.61	61.1
Sodium *p*-hydroxymercuribenzoate	1.13	Before	6.60	1.6
Sodium *p*-hydroxymercuribenzoate[c]	1.13	After	0.21	97.0
2-Iodoacetamide	1.00	Before	6.58	2.3
2-Iodoacetamide	1.00	After	3.39	50.5

[a] The inhibitor, in water, was added to 0.3 ml of type C enzyme (0.1 mg/ml), 2.0 ml of 0.1 M Hepes (pH 8.1), and 0.5 ml of 0.232 M sodium cholate, giving a final volume of 4 ml. After a 20-min preincubation at 37°, the reaction was started either by adding 4 μmol of [^{14}C]BEHP in 50 μl of ethylene glycol monoethyl ether, or by adding 116 μmol of sodium cholate and then the substrate. [b] Indicates whether the cholate was added before the inhibitor in which case it was preincubated with the enzyme for 5 min, or added after the enzyme had been incubated with the inhibitor. [c] This inhibition was completely reversed by 2 mM cysteine or glutathione.

When deoxycholate was not present, the sulfonate inhibited 79%, pyridoxal phosphate 60%, and maleic anhydride 82%. These results suggest that the micelle binding site may have not only an essential sulfhydryl group, but an essential (lysine?) amino group as well. This may also explain bile salt protection against trypsin. Although sulfonates may react both with –SH and –NH_2 groups, only the derivative with amino groups is thought to be stable at pH 8.1 (Hollenberg *et al.*, 1971).

In a similar experiment, type C enzyme experienced 12% inactivation by 0.1% Methylene Blue in 1 hr in dim incandescent light, but 53% inactivation by 0.1% Methylene Blue in 1 hr in direct sunlight, compared with enzyme exposed to sunlight without the dye. This suggests the presence of an essential histidine in nonspecific lipase, as is typical of many esterases.

That the hydrolytic site is different from the micelle binding site was suggested by an experiment using diethyl *p*-nitrophenyl phosphate (E-600). The standard assay with cholate was used, but the reaction was started with 4 μmol of BEHP and the dilute enzyme was exposed to various concentrations of E-600 in the incubation medium, for 5 min at 37°, either with cholate present or before the cholate was added. In addition, E-600 was added to some samples after the complete assay mixture including substrate and enzyme had been incubating for 10 min. It was found that preincubation of the enzyme with 10^{-6} M E-600 inhibited the subsequent hydrolysis of BEHP by 93–95% whether 29 mM cholate was present during the preincubation or not. The inhibitory process was accompanied by the visible release of *p*-nitrophenol.

Adding E-600 after the reaction with substrate was under way resulted in considerably less inhibition than seen above. Without inhibitor, 29.1 nmol of BEHP was hydrolyzed during each 10 min of the 30-min reaction. If E-600 had produced 95% inhibition of the last 20-min hydrolysis, only a total of 32 nmol of BEHP should have been hydrolyzed. However, if E-600 required 5 min to *reach* 95% inhibition, a maximum of 32 + 7.3 or 39.3 nmol of BEHP should have been hydrolyzed. The actual amount hydrolyzed when E-600 was added after 10 min was 52.5 nmol, indicating that substrate was able to protect to a considerable extent.

These observations suggested that the hydrolytic site may contain an essential hydroxyl group (serine?), and that this site may not be blocked by the cholate micelles in the absence of ester substrate. We made no attempt to "titrate" with E-600 as can often be done with esterases (*e.g.*, Runnegar *et al.*, 1969), since the enzyme system available to us thus far is highly impure.

We found no inhibition of the nonspecific lipase activity by 1 mM *p*-bromophenacyl bromide or 0.012% Safranin O, compounds which react with or bind to ionized carboxyl groups. Cholesterol isoamyl ether showed no inhibition of BEHP hydrolysis, possibly because it has no carbonyl group. Cholesterol oleate, however, showed a rather peculiar type of competitive inhibition. At very low concentrations cholesterol oleate acted as a competitive inhibitor of the hydrolysis of BEHP with K_i approximately 9×10^{-6} M. However, at a concentration of 10.6 nmol/ml, the inhibition by cholesterol oleate began to decrease and at any concentration above 1.6 μmol/ml there was no inhibition.

The limit of solubility of cholesterol oleate in the incubation medium was found to be about 10.6 nmol/ml. Possibly the appearance of a bulk phase resulted in the extraction of the otherwise solubilizable cholesterol oleate from the micelles. This phenomenon will require further study at some future date.

In passing, we observed that isooctyl 2,4,5-T and isopropyl 2,4-D, common herbicides, were effective competitive inhibitors of BEHP hydrolysis, having similar K_i values of approximately 1.8×10^{-5} M in the standard assay with deoxycholate. Monoolein was competitive with BEHP, but the apparent V_{max} for BEHP hydrolysis was increased by monoolein, as well as the apparent K_m. The effects of mixed bile salt–monoglyceride micelles on nonspecifie lipase also need extensive future study.

Discussion

The results obtained to date suggest a visualization of the action of nonspecific lipase as follows. Anionic micelles are bound to a site on the enzyme that involves a sulfhydryl group or groups and possibly an amino group. At pH 8.1 the sulfhydryl group would likely be at least partially ionized, as would the amino group (Edsall, 1943). Once the micelle is bound, its contents are apparently brought within range of a separate catalytic site, which may include an imidazole moiety and a serine hydroxyl group. If the micelle contents include an ester, the usual mechanism appears to follow, *i.e.*, formation of an acyl-enzyme intermediate plus free alcohol, then release of the acid.

The distinguishing feature of this suggested mechanism is the presence on the enzyme of a separate binding-recognition site for the anionic micelle which accounts for the relative nonspecificity (relative to the ester substrate). Otherwise, the hydrolytic mechanism is apparently similar to that proposed for chymotrypsin (Westheimer, 1962) and liver esterases (Wynne *et al.*, 1973; Stoops *et al.*, 1969). Since there is some

hydrolysis of substrate in the absence of bile salt, it appears that access to the catalytic site is not denied, merely greatly reduced in the absence of bound micelles.

When substrates are soluble, kinetic studies may be made relative to total molar concentrations of substrate. When the appropriate substrates are insoluble and an enzyme acts at the substrate particle–water interface, kinetic studies may be advantageously made relative to the surface area of that interface rather than to the absolute "concentration" of substrate (Desnuelle and Savary, 1963). When substrates form micelles directly, for example, lysolecithin (Shiloah *et al.*, 1973) or fatty acyl coenzyme A (Zahler *et al.*, 1968), then if the micellar solutions are truly monodisperse, good kinetic plots can be obtained using either the concentration of substrate molecules or concentration of micelles as relevant parameter.

When the true substrate is the micelle form, quantitatively misleading results will be obtained when absolute molar concentrations of monomer are assumed in the kinetic calculations. This would seem to be the case, for example, with phospholipases (*e.g.*, Shiloah *et al.*, 1973). But if transfer of monomer from micelle to enzyme occurs, as would explain some of the results obtained with palmitoyl coenzyme A: carnitine palmityltransferase (EC 2.3.1.-) (Bremer and Norum, 1967), absolute molar concentrations would be the more useful parameters.

When the substrate for an enzyme does not form micelles itself but must be incorporated into mixed micelles, as is the case for the present study, there are many opportunities for misleading results. If each micelle incorporates only a single molecule of substrate, the effective concentration unit is the molar concentration of that fraction of the total substrate that is present in mixed micelle form. Since distribution between mixed micelles and bulk phase at equilibrium can be described by a partition coefficient (Borgström, 1967), using total concentration of substrate may give linear kinetic plots but the results will be quantitatively misleading. This consideration applies, for example, to Figure 5 in the present paper. The apparent K_m calculated from plot A may give the amount of BEHP that must be added to the system to obtain one-half of the maximum velocity, but it does not actually indicate the *substrate* concentration (micellar BEHP concentration) giving $(1/2)V_{max}$.

Even when the substrate is initially all in mixed micelles, dilution in the assay system may release bulk substrate when the cmc of the surfactant is relatively high, which would tend to invalidate the apparent K_m determined from curves B and C in Figure 5. Thus a valid K_m(app) would only be obtained when the substrate is retained in the micelles while setting up the assay (curve D, Figure 5), or when the values for concentration are corrected for that portion of the substrate present in bulk phase.

We found that the optimum ratio of deoxycholate to BEHP was approximately 13, and of cholate to BEHP approximately 5, when the amount of BEHP was sufficiently high for the majority of the bile salt to be in micelles. In experiments not presented here we found that the optimum ratio of bile salt to BEHP was quite high, up to 400 in some cases, at very low ($<5 \times 10^{-5}$ M) BEHP concentrations. This was apparently related to the fact that it is the ratio of micelles to BEHP that is relevant.

The optimum ratios of deoxycholate and of cholate to BEHP, 13 and 5, at 1 mM BEHP, suggest that each micelle of deoxycholate (aggregation 9–13 at 37°, 0.05 M salt; Hofmann and Small, 1967) and of cholate (aggregation 5–6 at 37°, 0.05 M salt; Hofmann and Small, 1967) is associated with one molecule of BEHP, although this remains to be directly demonstrated. However, the optimum levels of sodium dodecyl sulfate were extremely low, and we were unable to detect a cmc for sodium dodecyl sulfate under conditions of 57.5 mM Hepes–1% Egmee. The effective sodium dodecyl sulfate produced form may be "pre-micellar aggregates," or possibly mixed micelles of sodium dodecyl sulfate–BEHP can form in this medium with some apparent critical "mixed micelle" concentration.

When bile salt micelles intercalate a nonpolar lipid, the aggregation number is not ordinarily expected to change and typically only (at most) one molecule of lipid enters each micelle (Hofmann and Small, 1967). Although it is tempting to associate the minimum apparent K_m on extrapolating the bile salt concentration to zero with the "affinity" of the enzyme for its ester substrate (*e.g.*, Kaplan and Teng, 1971), our observation that the "recognition site" of the enzyme seems to bind the bile salt rather than the ester casts doubts on the validity of this approach.

A different type of problem relates to the dependence of apparent V_{max} on bile salt concentration. The difference between the maximum velocity observed in the absence of bile salt and the minimum value of V_{max} when the plot of bile salt concentration against apparent V_{max} is extrapolated to zero bile salt probably reflects the greater access of substrate bound in the "micelle site" than of free substrate to the catalytic site on the enzyme. However, any attempt to measure the true rate constant for the hydrolysis step is thwarted by the fact that no *maximum* value of apparent V_{max} with bile salt present can readily be determined. However, a practical maximum rate for the overall hydrolysis process would be that given by approximately 2 mM BEHP and the optimum [micelle]/[BEHP] ratio. This follows from the substrate inhibition above 2 mM.

We have routinely supplied BEHP in Egmee for assays of nonspecific lipase activity in tissues (Albro and Thomas, 1973). This procedure has the advantages of eliminating the need for a specific, critical ratio of bile salt to BEHP, and of greatly shortening the time required for equilibration of the BEHP with the micelles (although sonication is even more effective); but as the results shown here demonstrate, the effect of Egmee on cmc of the detergents and the probable formation of mixed Egmee–bile salt micelles complicate kinetic studies considerably.

Many of the suggestions and speculations presented in this paper, especially those concerning functional groups on the enzyme, will require confirmation after purified nonspecific lipase (perhaps better, micelle lipase) becomes available.

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Interaction of Phospholipase A_2 and Its Zymogen with Divalent Metal Ions†

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ABSTRACT: Porcine pancreatic phospholipase A_2 as well as its zymogen bind calcium ions in a 1:1 molar ratio at calcium concentrations ranging from 5×10^{-3} to 5×10^{-5} M. Binding of calcium to both the zymogen and the active enzyme changes the protein absorption spectrum. The ultraviolet difference spectrum is characterized by a strong band at 242 nm and two smaller bands at 282 and 288 nm. The origin of the difference spectrum can be understood as a partial shielding of a tyrosine residue from the aqueous solvent and in addition, most likely, a charge effect on a histidine residue. Calcium does not influence the tryptophan fluorescence of either protein. However, addition of calcium enhances the 8-anilino-1-naphthalenesulfonate fluorescence induced by phospholipase A and its precursor. Besides the spectral changes binding of calcium protects the enzyme against inactivation by *p*-bromophenacyl bromide and diminishes its susceptibility to trypsin attack. These criteria suggest that the enzyme and the zymogen undergo a conformational change upon calcium binding. Substitution of calcium by barium or strontium results in a similar but somewhat smaller conformational change, in agreement with the behavior of these ions as competitive inhibitors. Magnesium, on the contrary, does not bind to the enzyme according to both kinetic and direct binding experiments. The dissociation constants of the various enzyme and zymogen metal complexes have been determined. Values were found ranging from 10^{-1} M at pH 4.0 to 2×10^{-4} M at pH 10.0. The data show a good agreement on using the various techniques and suggest that the metal ion binding site contains one or more carboxylates with an additional contribution of a residue with a pK of 6–7, presumably histidine.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of fatty acid ester bonds at the 2 position of 1,2-diacyl *sn*-phosphoglycerides (van Deenen and de Haas, 1964). The stimulating effect of calcium ions on the hydrolysis of phospholipids by phospholipase A_2 has long been recognized (Hayashi and Kornberg, 1954). The requirement of Ca^{2+} seems to be a general property of many phospholipases A isolated from such diverse sources as snake (Habermann, 1957) and bee venom (Shipolini *et al.*, 1971) and mammalian pancreatic tissue or juice (Figarella *et al.*, 1971). In those cases where calcium has been found to be an obligatory requirement for proper enzymatic action, the possibility of substitution by other divalent ions has been investigated (Long and Penny, 1957; Roholt and Schlamowitz, 1961; de Haas *et al.*, 1968; Wu and Tinker, 1969). From these studies it became clear that several phospholipases from snake and bee venom could be activated by a number of other divalent cations.

The high specificity of the porcine pancreatic enzyme for calcium suggests a specific function of the metal ion in catalysis. Investigations toward elucidation of the function of the

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