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ACTION OF ORGANOPHOSPHATES AND SULFONYL HALIDES ON PORCINE PANCREATIC LIPASE*

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SUMMARY

Pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) is inhibited by emulsions of diethyl *p*-nitrophenyl phosphate (E_{600}) or by solutions of this organophosphate in the presence of bile salts. The inhibited enzyme contained a single phosphoryl radical per mole. This radical was shown to be bound to a serine residue in the sequence Leu–Ser–Gly–His. Therefore, lipase is a serine–histidine enzyme like ordinary carboxylesterases and a number of proteases.

However, the specific properties of lipase were confirmed when it was observed that the enzyme was not inhibited by E_{600} solutions in the absence of bile salts. Inhibition in the presence of bile salts is assumed to be caused by inclusion of E_{600} into micelles of these compounds.

Moreover, in contrast with other serine enzymes, lipase is not inhibited by diisopropylfluorophosphate (DFP) even in the presence of bile salts. It is also not inhibited by sulfonyl halides unless the nucleophilicity of the sulfur atom is enhanced by an electrophilic substituant. These results are in perfect agreement with the observations already made with the substrates of lipase.

In addition, concentrated DFP solutions were found to react with a single, non-essential tyrosine residue in native lipase. This residue is located in the sequence:

Thr–Asn–Gln–Asn–Glx–Asx(Asx, Glx)–Tyr–Glx–Leu.

INTRODUCTION

The characteristic property of lipases (glycerol-ester hydrolase, EC 3.1.1.3) of hydrolyzing carboxylic esters in an emulsified[1] or micellar[2] state may be expected to reflect a special feature of the catalytic and/or binding site of this class of enzymes.

Abbreviation: E_{600}, diethyl *p*-nitrophenyl phosphate.

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Therefore, it is of interest to investigate the residues in the enzyme molecule which are essential for activity.

The catalytic activity of pure porcine pancreatic lipase was recently shown to depend on an ionizable group with a pK of 5.8 (ref. 3). Moreover, an excellent correlation was found to exist between enzyme inactivation by photooxidation and the modification of one histidine of medium "reactivity[3]". These data strongly suggested that a histidine residue was involved in the catalytic site of lipase. The same conclusion had already been reached for a number of hydrolases including esterases and several proteases, in which the acylation site is a serine residue.

The acylation site of lipases has not yet been positively identified. It is known, however, not to be an SH group since the two SH groups of porcine pancreatic lipase can be substituted by phenylmercuric ions without extensive inactivation[4]. On the other hand, assays performed a number of years ago[5] were in favor of the possibility that it may be an OH group. Lipase was found to be insensitive to aqueous solutions of diisopropylphosphorofluoridate (DFP) and diethyl *p*-nitrophenyl phosphate (E_{600}), but to be rapidly and almost completely inhibited by emulsions of E_{600}. More recently, the enzyme was also observed to be inhibited by E_{600} solutions in the presence of bile salts[6]. In this latter system, the amount of liberated *p*-nitrophenol was approx. 1 mole per mole of inhibited enzyme.

The present report confirms that E_{600} in the presence of bile salts inhibits lipase by a stoichiometric reaction and demonstrates that the reaction involves the specific binding of a phosphoryl radical to one serine residue in the sequence Leu–Ser–Gly–His. Moreover, it shows that DFP, when employed as a concentrated solution, also binds to lipase. This binding takes place at the level of one tyrosine residue and it does not inactivate the enzyme.

MATERIALS AND METHODS

Materials

Radioactive [^{32}P]DFP and [^{32}P]E_{600} (specific radioactivities 63.1 and 11.6 mCi/mmole, respectively) were obtained from The Radiochemical Centre, Amersham, U.K. The specific radioactivity of the preparations was adjusted before use to 6.75 and 2.0 mCi/mole, respectively, by dilution with the corresponding unlabeled derivative (Boots, Nottingham, U.K. for DFP and Thomson and Joseph, Radlett, U.K. for E_{600}). Radioactive [^{35}S]pipsyl chloride (specific radioactivity 213 mCi/mmole) was obtained from The Radiochemical Centre, Amersham and the corresponding unlabeled derivative from Aldridge Company.

The following enzymes were employed for the digestion of labeled lipase and peptides:pepsin (EC 3.4.4.1) (Worthington, 2 times crystallized); chymotrypsin (EC 3.4.4.5) (Worthington, 3 times crystallized); trypsin (EC 3.4.4.4) (Worthington, 4 times crystallized); carboxypeptidase A (EC 3.4.2.1) (Worthington); pronase (Calbiochem); subtilopeptidase (EC 3.4.4.16) (Nagarse and Co.); aminopeptidase M (Rohm and Haas).

Purification of lipase and activity determination

The first experiments reported below were performed with preparations purified from fresh porcine pancreas through the "fast" form of the enzyme[7,8]. These

preparations had a high colipase content which seemed to facilitate the reaction with E_{600} (see later). But, they were heavily contaminated with lipids which interfered with the separation of peptides on paper. Consequently, further epxeriments were carried out with almost lipid-free preparations purified by the technique of Verger *et al.*[9]. Unfractionated mixtures of isolipases L_A and L_B were used throughout.

Lipase activity was usually measured with emulsions of purified tributyrin in the absence of added emulsifier and bile salts[3]. Butyric acid released from this substrate at 25 °C and pH 8.0 was continuously titrated with the aid of a recording Radiometer pH-stat. Lipase activity was directly derived from the slope of the linear portion of the curve. In some cases, activity was also measured at 25 °C and pH 9.0 with emulsions of long-chain triglycerides (commercial olive oil). These emulsions were stabilized by 10% gum arabic[10] or 2% hydroxypropyl cellulose (Methocel, Dow Chemical Corp.) and they contained an optimal concentration of bovine bile salts (see below) or commercial sodium deoxycholate[11].

Formation of bile salts micelles

Bovine bile (1 l) was mixed with active carbon (100 g) and dried on a water bath. The solid cake was extracted with absolute ethanol (1 l). Evaporation of the extract left a white powder known to contain sodium taurocholate[12] (approx. 40%), glycocholate (25%), taurodeoxycholate (20%), glycodeoxycholate (15%) and traces of cholate. Fig. 1 illustrates the spectral changes at 540 nm occuring in mixtures of Rhodamine 6 G with varying proportions of these salts. Similar changes are generally assumed[13] to indicate the inclusion of the dye into micelles formed at the expense of the amphipath present in the mixture. They can, therefore, be used for the detection and quantitation of these micelles. The curve in Fig. 1 is seen to rise at once for very low concentrations of bile salts. An almost imperceptible breakpoint may be discerned for a concentration of approx. 0.08%. This value is reduced to 0.06% in the presence of E_{600}.

Colorimetric determination of p-nitrophenol

The hydrolysis of *p*-nitrophenol esters can be followed in alkaline medium through the strong absorption of the released nitrophenolate ion at 400 nm. Accordingly, 0.5 ml samples of the mixture resulting from incubation of lipase with E_{600} were added to 2 ml of a 0.1 M Tris–HCl buffer (pH 9.0) and the absorbance was read immediately at 400 nm in a Zeiss Spectrophotometer Model PMQ II. The molar absorbance coefficient of pure *p*-nitrophenol was found to be $18 \cdot 10^3$ $l \cdot mole^{-1} \cdot cm^{-1}$ under these conditions. Spontaneous hydrolysis of E_{600} was negligible within the few min required for the assays.

Radioactivity determination and evaluation of the number of labeled radicals attached to the derivatives

The radioactivity of ^{32}P or ^{35}S-labeled derivatives of proteins or peptides was measured with the aid of a Packard Tricarb Scintillation Spectrometer after dissolution of the samples in Bray's mixture. The number of phosphoryl radicals attached to lipase during incubation with organophosphates was estimated by comparison with a α-chymotrypsin sample treated in exactly the same way. α-Chymotrypsin is known to bind a single radical of either the diisopropylphosphoryl derivative or the

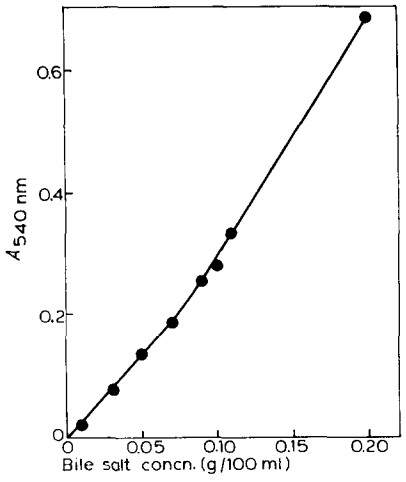


Fig. 1. Micelle formation in bile salt solutions of varying concentration. Rhodamine 6 G (20 mg) was dissolved in 1 l of a 0.1 M acetate buffer, pH 6.0, containing 0.1 M NaCl and 50 mM $CaCl_2$. Bile salts were added to an aliquot to a final concentration of 0.5%. Samples containing varying concentrations of bile salts for a constant dye concentration were obtained by mixing different volumes of the two solutions. The absorbance at 540 nm was measured in each mixture with the aid of a Zeiss Spectrophotometer Model PMQ II.

diethylphosphoryl derivative per mole. The specific radioactivity of commercial [^{32}P]E_{600} could also be calculated after total hydrolysis of an aliquot in 1 M NaOH at room temperature for 24 h and estimation of the released *p*-nitrophenol by spectrophotometry. Results obtained by either technique were in good agreement.

Techniques related to protein structure determination

Reduction of disulfide bridges. The proteins were treated by 0.13 M mercaptoethanol for 4 h in the presence of 8 M urea and then converted into their *S*-carboxymethyl derivatives by iodoacetic acid[14]. At the end of the reaction, the excess of reagent was removed by dialysis against 0.1% NH_4HCO_3 and desalting through Sephadex G-25 coarse equilibrated with the same solution. Peptides were reduced by 0.14 M dithiothreitol in the absence of urea. Subsequent alkylation of SH groups was carried out by treatment with iodoacetamide[15].

Enzymatic hydrolysis. The diethyl[^{32}P]phosphoryl derivative of lipase (350 mg) in 35 ml water was incubated for 18 h at 30 °C with pepsin (13 mg). The pH was maintained at 3.0 by addition of 10 mM HCl. At the end of the incubation, the pH was raised to 6.5 by 0.1 M ammonia and 13 mg of trypsin were added. After 8 h, incubation was pursued at the same pH for 10 h in the presence of 13 mg of chymotrypsin. The hydrolyzate was finally acidified and centrifuged.

The reduced and S-carboxymethylated diisopropyl[^{32}P]phosphoryl derivative of lipase (200 mg in 35 ml water) was incubated for 24 h with 25 mg chymotrypsin. The pH was maintained at 7.9 by 0.1 M NaOH. The hydrolyzate was acidified to pH 3.0 and centrifuged.

Terminal residues and sequences. The N-terminal residues in peptides were identified by dansylation[16]. The dansylated amino acids were separated by thin

layer chromatography on Micro polyamide sheets coated on both sides without bonding agent (F. 1700 Carl Schleicher and Schüll). The solvents used were: benzene–pyridine·acetic acid (80:20:5, by vol.); toluene–glycol monochlorhydrine–25% aqueous ammonia (60:100:40, by vol.); *n*-butanol saturated with 0.2 M NaOH.

The N-terminal sequences were determined, either by Edman degradation, or with the aid of an aminopeptidase preparation purified from hog duodenal mucosa by Drs S. Maroux and J. Baratti in this laboratory. In the first case, the phenyl thiohydantoins were identified by thin layer chromatography on microscope slides coated with silicagel G. The solvents were: *o*-xylene[19]; water saturated with formamide[19]; heptane–*n*-butanol–75% formic acid (50:30:9, by vol.)[20]. In the second case, the peptides (0.05 μmole) dissolved in a 0.1 M NH_4HCO_3 buffer (pH 8.0) were incubated at 30 °C with varying amounts of aminopeptidase. The released amino acids were identified by thin layer chromatography on silica gel-coated microscope slides using *n*-butanol–acetic acid–water (4:1:1, by vol.) and phenol–water (75:25, by vol). as solvents[21].

For C-terminal sequence determinations, the peptides (0.05 μmole) were incubated in a 0.1 M ammonium bicarbonate buffer (pH 8.0) at 30 °C with varying amounts of DFP-treated carboxypeptidase A (ref. 22). The released amino acids were identified by thin-layer chromatography as described before.

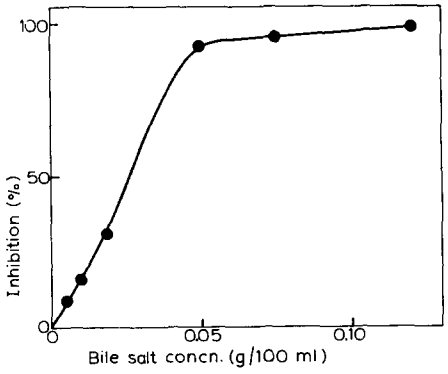
Identification of phosphoserine. [^{32}P]Phosphoserine was identified after total acid or enzymatic hydrolysis of labeled lipase. In a typical experiment, radioactive diethylphosphoryl derivative of lipase (0.5 μmole) or any ^{32}P-labeled peptide was treated for 18 h at 100 °C in 2 M HCl. The hydrolyzate was taken to dryness and the residue was dissolved in 2 ml of 10 mM HCl. Unlabeled phosphoserine (0.2 μmole) was added as a tracer and the solution was chromatographed in a 1 cm × 70 cm Dowex 50 — X8 column equilibrated and eluted with 10 mM HCl.

Because of the known lability of phosphoserine during acid hydrolysis, enzymatic digestion was used in another experiment. The labeled material (0.1 μmole) dissolved in 0.5 ml of a 0.1 M NH_4HCO_3 buffer (pH 8.0) was treated successively by 0.01 μmole of carboxypeptidase A, aminopeptidase M and pronase. Each incubation lasted 8 h. After acidification at pH 6.5, the digest was submitted to electrophoresis–chromatography on paper. Electrophoresis was conducted for 45 min at 44 V/cm in a pyridine–acetic acid–water (25:1:475, by vol.) buffer at pH 6.5 and was followed by chromatography with the solvent butanol–acetic acid–pyridine–water (15:3:10:2, by vol.). An aliquot of the radioactive spot was introduced into an amino acid analyzer for the direct detection of phosphoserine. Another aliquot was hydrolyzed in 5.6 M HCl for 18 h for the detection of free serine.

RESULTS

Action of E_{600} on pancreatic lipase

General aspects. It has already been pointed out earlier that lipase is not inactivated by aqueous solutions of E_{600}, but that it is readily inhibited, either by an emulsion of this compound[5], or by a solution in the presence of bile salts[6]. The rate and yield of inhibition has now been found to be controlled by a number of parameters including pH and the concentration of bile salts, organophosphate and enzyme. Fig. 2 illustrates the dependence on the concentration of bile salts. The



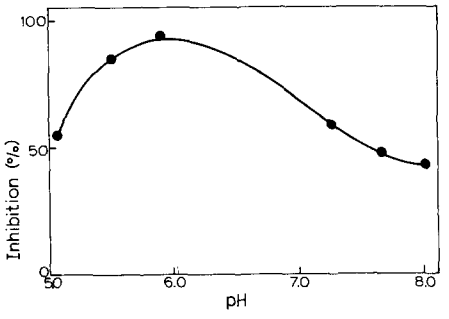


Fig. 2. Dependence of lipase inhibition on bile salt concentration. Lipase solution (1 μM) in a 0.1 M acetate buffer at pH 6.0 were incubated for 180 min at room temperature with 4.3 mM E_{600} (0.46 saturation) in the presence of varying bile salt concentrations.

Fig. 3. Effect of pH on inhibition. A series of assays were carried out with the same concentration of enzyme (2 μM), E_{600} (4.3 mM) and bile salts (0.12%). The buffer was 0.1 M sodium acetate for assays at pH 6.0 and lower. 0.1 M Tris–HCl was used at higher pH values. Both buffers contained NaCl 0.1 M and $CaCl_2$ 50 mM.

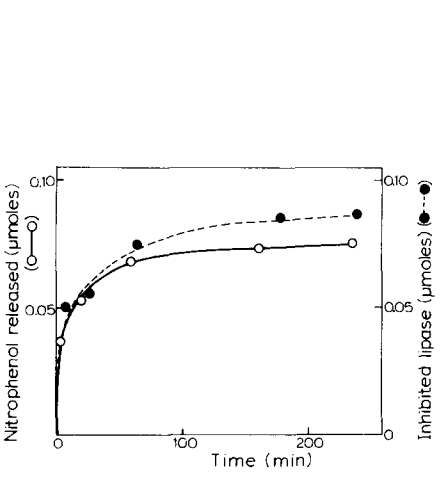
curve confirms that dissolved E_{600} is not an inhibitor for lipase in the absence of bile salts. It further shows that inhibition is already measurable for very low concentrations of the salts and that it is complete within 180 min when their concentration is 0.05% or higher. In these assays, the concentration of inhibitor and enzyme was, respectively, 4.3 mM (0.46 saturation) and 1 μM (inhibitor–enzyme molar ratio, $4.3 \cdot 10^3$).

The pH dependence of the inhibition reaction is reproduced in Fig. 3. The rate is seen to be maximal for a pH value of approx. 6.0.

Stoechiometry of the reaction. The stoechiometry of the inhibition was evaluated by a determination of the released *p*-nitrophenol and of the number of phosphoryl radicals bound to the enzyme.

A solution of 0.1 μmole of lipase in 2 ml of the 0.1 M acetate buffer (pH 6.0) containing NaCl 0.1 M and $CaCl_2$ 50 mM was mixed with a solution of E_{600} in bile salts (final concentration 4.3 mM and 0.1%, respectively). Spontaneous E_{600} hydrolysis and lipase inactivation during the assays were evaluated by suitable blanks. Fig. 4 indicates that inhibition runs parallel with the release of 1 mole of *p*-nitrophenol per mole of inhibited enzyme.

In another series of assays, ^{32}P-labeled E_{600} was used for the evaluation of the number of phosphoryl radicals attached to lipase during inhibition. Lipase (15 μmole) was dissolved in 1250 ml of a solution of labeled E_{600} and bile salts (final concentration, 4.6 mM and 0.06%) in the pH 6.0 acetate buffer. After 72 h, the mixture was extensively dialyzed against 0.1% NH_4HCO_3 and the non-dialyzable compounds were filtered through a Sephadex G-100 column equilibrated with 0.12 M NH_4HCO_3. All fractions under the resulting peak (Fig. 5) possessed approximately the same specific radioactivity from which it was calculated that about 1 phosphoryl radical was attached to each inhibited lipase molecule. Data depicted in Figs 4 and 5 demon-



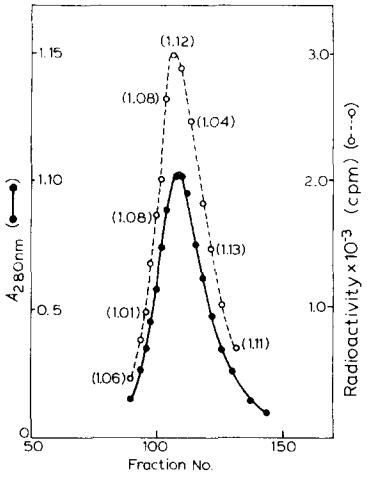


Fig. 4. Comparative time course of *p*-nitrophenol release and lipase inhibition. The experimental conditions of the assays are given in text.

Fig. 5. Filtration of labeled diethylphosphoryl derivative of lipase through Sephadex G-100. The derivative (10 μmoles) was passed through a column (5 cm × 200 cm) equilibrated and eluted with 0.12 M NH_4HCO_3. Volume of fractions: 12 ml. The figures in parentheses indicate the number of phosphoryl radicals per mole of inhibited lipase.

strate that the reaction leading to lipase inactivation by E_{600} can be formulated as follows:

Active lipase (1 mole) + E_{600} → inactive diethylphosphoryl derivative of lipase (1 mole) + *p*-nitrophenol (1 mole)

Identification of the essential serine and related sequence. Preliminary assays indicated that small but significant amounts of phosphoserine could be detected in total hydrolyzates of diethylphosphoryl derivative of lipase. However, the isolation of the corresponding serine peptides proved to be difficult because of the unusual lability of the phosphoryl bond, especially in the acidic pH range.

Lipase (8 μmoles) was kept for 72 h at room temperature in 800 ml of a solution containing E_{600} (4.6 mM) and bile salts (0.06%). The proteins were precipitated by $(NH_4)_2SO_4$ saturated and centrifuged. The sediment was taken up in 50 ml of 0.12 M NH_4HCO_3 and dialyzed against large volumes of this solution. The last traces of low molecular weight radioactive contaminants were removed by filtration through Sephadex G-25 coarse and the labeled lipase derivative was digested by pepsin, trypsin and chymotrypsin. The digest was filtered through Sephadex G-25 superfine with the results shown by Fig. 6. A smaller and a larger radioactive peak (Peaks I and II) are seen to separate on this type of column.

The heterogeneous Peak I was not investigated. Material under Peak II was further purified on Sephadex G-50 superfine in 1 mM HCl and then on Dowex 50X2. This latter chromatography separated two radioactive peaks designated II_a and II_b in Fig. 7, which contained, respectively, 20% and 80% of the total radioactivity introduced into the column.

Final purification of the radioactive peptide was achieved by submitting the

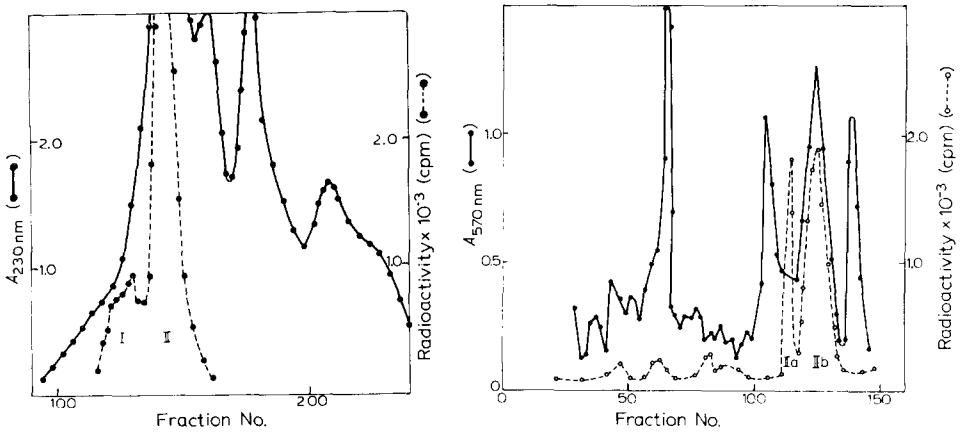


Fig. 6. Filtration through Sephadex G-25 superfine of the peptides resulting from digestion of diethylphosphoryl derivative of lipase with pepsin, trypsin and chymotrypsin. The column (2.5 cm × 200 cm) was equilibrated and eluted with 1 mM HCl. Volume of fractions: 4 ml.

Fig. 7. Chromatography on Dowex 50-X2 (200–400 mesh) of the material under Peak II in Fig. 6. The column (1.5 cm × 70 cm) was equilibrated with a 0.2 M pyridine–formic acid buffer at pH 2.9 and eluted by a linear increase of the pyridine concentration from 0.2 to 1.0 M. The resulting pH change was 2.9 to 6.1. After alkaline hydrolysis, aliquots of the fractions were mixed with ninhydrin and heated in a boiling water bath prior to spectrophotometric reading at 570 nm.

material under Peak II_a to electrophoresis at pH 6.5 and chromatography (solvent, *n*-butanol–acetic acid–pyridine–water (15:3:10:2, by vol.) on paper. The single radioactive spot thus obtained was eluted with water and the eluate was hydrolyzed in 6 M HCl for 24 h. Hydrolysis liberated leucine (1.04), serine (0.75), glycine (1.05) and histidine (0.99). The attachment of the phosphoryl radical to serine was demonstrated by submitting the peptide to total hydrolysis by carboxypeptidase A, aminopeptidase M and pronase. An electrophoresis on paper at pH 6.5 of the digest followed by a chromatography with the same solvent as before revealed the existence of one radioactive spot which, after elution and hydrolysis in 6 M HCl, gave in the automatic analyzer a single peak corresponding to serine.

In addition, leucine was found to be N-terminal. Carboxypeptidase A released histidine and then glycine. It could, therefore, be concluded that the sequence of the tetrapeptide was:

Leu–Ser–Gly–His
|
P

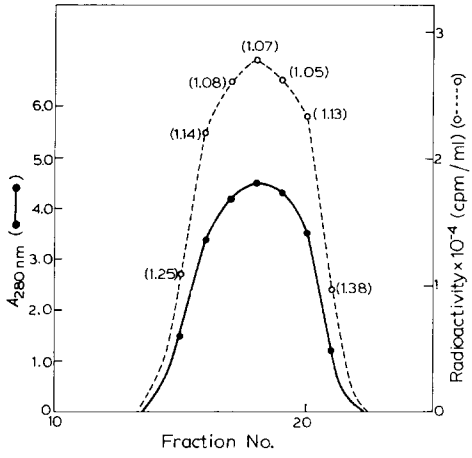
Action of DFP

DFP is generally considered to be a more potent inhibitor for esterases than E_{600} and to cause a more permanent inhibition because of the higher stability of the diisopropylphosphoryl–serine bond when compared to its diethylphosphoryl analog. Preliminary experiments[6] performed with a commercial sample of DFP and another sample synthesized in the laboratory led some years ago to the assumption that concentrated solutions of the organophosphate inhibited pancreatic lipase in the absence

of added bile salts. Data were also consistent with the view that DFP reacted in the enzyme molecule with a single tyrosine residue in the sequence:

Thr–Asn–Gln–Asn–Gln–(Asx$_{0-1}$, Glx$_{0-1}$, Tyr)–Leu

It was discovered later that other DFP samples were not inhibitory. However, these samples were still able to react in a highly specific manner with a tyrosine residue in the enzyme. Lipase (4.5 μmoles) was incubated for 24 h in 50 ml of a 50 mM (0.66 saturated) DFP solution at pH 8.2. After a prolonged dialysis against 0.1% NH_4HCO_3 and a gel filtration, the number of radicals of diisopropylphosphoryl derivatives per mole of enzyme was evaluated, as indicated earlier, by comparison with an authentic sample of mono diisopropylphosphoryl derivatives of α-chymotrypsin prepared under the same conditions. The diagram of the chromatography of diisopropylphosphoryl derivatives of lipase on Sephadex G-25 at pH 8.0 is reproduced in Fig. 8. Most of the fractions composing the single, almost symetrical peak are seen to contain approx. 1 phosphoryl radical per mole of enzyme.



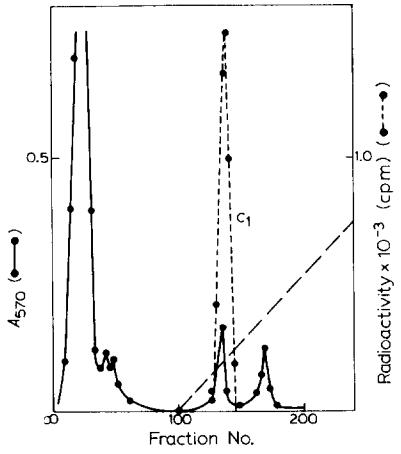


Fig. 8. Chromatography of diisopropylphosphoryl derivative of lipase (4.5 μmoles) on Sephadex G-25. The column (4 cm × 40 cm) was equilibrated with a 0.1% NH_4HCO_3 buffer (pH 8.3). Elution was performed with the same buffer. The figures in parentheses indicate the number of radioactive phosphoryl radicals bound to one mole of lipase during the reaction with labeled DFP. Volume of fractions: 15.2 ml.

Fig. 9. Chromatography on DEAE–Sephadex A-50 of the chymotryptic digest of labeled diisopropylphosphoryl derivative of lipase. The column (2 cm × 53 cm) was equilibrated with 0.1 M acetic acid, washed with 500 ml of the same acid and finally eluted by an acetic acid concentration gradient from 0.10 to 0.37 M. Volume of fractions 5 ml.

Another diisopropylphosphoryl derivative of lipase preparation (6.45 μmoles) was reduced, S-carboxymethylated and digested for 24 h at pH 7.9 with 10% chymotrypsin. The resulting peptides were passed through a 2 cm × 200 cm Sephadex G-25 column equilibrated and eluted with 1 mM HCl. The fractions under the radioactive peak emerging from the column at the end of the second breakthrough volume were pooled, evaporated, taken up in a small volume of 0.1 M acetic acid and again chromatographed on DEAE-Sephadex A-50 equilibrated with 0.1 M acetic acid. As

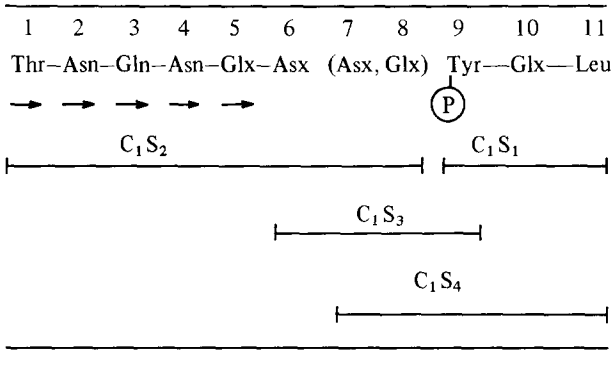
TABLE I

AMINO ACID COMPOSITION OF THE CHYMOTRYPTIC PEPTIDE C_1

Residue	*μmoles in analyzed sample*	*Number of residues per mole*	
		Calculated	*Nearest integer*
Asx	0.190	3.7	4
Glx	0.209	4.0	4
Leu	0.058	1.1	1
Thr	0.038	0.7	1
Tyr	0.041	0.8	1

TABLE II

SEQUENCE OF THE CHYMOTRYPTIC PEPTIDE C_1



shown by Fig. 9, a single radioactive peak (C_1) emerged from the column for an approximate acetic acid concentration of 0.18 M.

Peptide C_1 was further purified by electrophoresis at pH 3.5 and chromatography (solvent, *n*-butanol–pyridine–acetic acid–water) on paper. Table I shows that it is an undecapeptide containing 1 residue each of leucine, threonine and tyrosine, 4 residues of aspartic acid (or asparagine) and 4 residues of glutamic acid (or glutamine).

Table II indicates how the sequence of this peptide was determined. Five successive Edman degradations were consistent with the N-terminal sequence: Thr–Asn–Gln–Asn–Glx. Results of the 5th degradation did not permit us to discriminate between Gly and Gln. Leucine was found to be C-terminal by carboxypeptidase digestion. The remaining 5 residues (2 Asx, 2 Glx, 1 Tyr) were partly positioned after identification of 4 peptides (C_1S_1–C_1S_4) set free by subtilisin. The leucine-containing tripeptide C_1S_1 showed that Tyr and one out of the two Glx were near the end of the chain. The threonine-containing octapeptide C_1S_2 confirmed that the two Asx and the second Glx residues should be placed before tyrosine. Finally, the position of Asx_6 and the sequence Tyr_9–Glx_{10}–Leu_{11} were ascertained by comparative examination of peptides C_1S_3 and C_1S_4. Lack of material prevented the identification of the N-terminal residue in peptide C_1S_4 and, consequently, the determination of the positions of the last Asx and Glx residues 7 and 8.

The binding of the phosphoryl radical to tyrosine rather than to the N-terminal

threonine in the undecapeptide was demonstrated by the following observations: (a) the peptide was still fully radioactive after one or several Edman degradations; (b) its spectrum was similar to that of synthetic *O*-diisopropylphosphoryl derivative of tyrosine in the 260–290 nm range[23] and it was not modified by alkalinization to pH 12 where free phenols would normally be ionized; (c) chymotrypsin cleaved the chain after leucine, not after tyrosine; (d) the 3 tyrosine-containing fragments C_1S_1, C_1S_3 and C_1S_4 were found to be radioactive whereas the others were not radioactive; (e) the bond linking the phosphoryl radical to the peptide was unstable in acid but relatively stable in alkali. This is a known property of phenol phosphate derivatives.

In early experiments inducing partial inhibition of lipase by DFP, a second labeled peak including about 30% of the total radioactivity and migrating unretarded was consistently observed in diagrams similar to that presented in Fig. 8. The corresponding serine-containing peptide has not been fully identified.

Action of sulfonyl halides

Sulfonyl halides such as methane-, phenylmethane-, benzene- and toluenesulfonyl fluoride are known to combine with the essential serine in chymotrypsin, trypsin, cholinesterase and other serine esterases[24–28]. The action on lipase of this class of compounds has been investigated in the hope of confirming the results obtained with E_{600}.

In the course of preliminary assays, methanesulfonyl fluoride and the more hydrophobic butanesulfonyl fluoride, toluenesulfonyl chloride and fluoride proved to be not inhibitory for lipase in the presence or absence of bile salts. For these assays, the water-insoluble compounds were dissolved in methylcellosolve and the solution was added dropwise to the enzyme solution in a 0.1 M Tris buffer (pH 8.0). The final concentration of methylcellosolve was 10% (v/v) and the molar excess of halide over enzyme was 100 fold. Incubation of the resulting turbid mixtures was conducted under conditions similar to those employed for E_{600}.

While these assays were in progress, R. Verger[29] announced that lipase was easily inhibited by aromatic sulfonyl halides provided that the sulfur atom was made more nucleophilic by an electronegative substituent in the ring. An interesting point, therefore, was to see whether these substituted derivatives were able to react in a specific way with the essential serine identified above in lipase.

A 15 min incubation at pH 8.0 and room temperature of the enzyme with a 27 molar excess of *p*-iodobenzene[^{35}S]sulfonyl chloride (pipsyl chloride) resulted in a 65% inhibition. This value was not increased by a longer incubation, but it reached 84 and 94% after two additions at 15 min intervals of the same amount of the halide (total molar excess, 81-fold). After dialysis and desalting of the labeled protein over Sephadex G-25, 12 sulfonyl groups were found to be attached per mole of inhibited lipase. This number could be reduced to 5 by limiting the reagent excess to 45, but at the expense of the inhibition yield which did not exceed 77% under these conditions.

In another series of assays performed with 1-dimethylaminonaphtalene-5-sulfonyl chloride (dansyl chloride), the number of bound dansyl groups was evaluated by spectrophotometry at 340 nm (ref. 30). The value $4.3 \cdot 10^6$ l·mole^{-1}·cm^{-1} was used for the extinction coefficient of the dansyl group. Approx. 10 dansyl groups were

found to be bound per mole of inhibited lipase after a 4 h incubation at pH 8.0 with a 50 molar excess of the reagent. The inhibition was about 75%.

An indication that one of the sulfonyl groups was attached to a serine was obtained in the following way: The pipsyl chloride-treated lipase (0.5 μmole) containing 5 pipsyl groups per mole (see above) was incubated at 40 °C for 5 h at pH 8.0 in a solution 8 M in urea and 2 M in thioethylamine[31]. After acidification at pH 3, dialysis against 1 mM HCl and hydrolysis in 5.6 M HCl at 40 °C for 22 h, the protein was observed to contain 1.03 moles/mole of *S*-aminoethylcysteine. An assay carried out simultaneously with the monopipsyl derivative of α-chymotrypsin resulted in the identification of 1.07 mole/mole of *S*-aminoethylcysteine.

In conclusion, these assays were consistent with the view that pipsyl and probably also dansyl chloride inhibited lipase by a reaction with the essential serine in the enzyme molecule. However, both halides also reacted with several other groups with the result that the overall process was by far less specific than with E_{600}. Experimentation with these derivatives, therefore, was not pursued.

DISCUSSION

The experiments described in the preceding section indicate that pancreatic lipase is a serine enzyme. Since an histidine has already been shown to be involved in the catalytic site of the enzyme[3], it is now apparent that the splitting of carboxylic ester bonds by lipase requires the joint participation of a serine and a histidine. In this respect, lipase does not differ from ordinary carboxyl esterases and a number of proteases. But, the specificity of lipase is demonstrated by the conditions that must be used for the characterization of the essential serine.

DFP is generally preferred to E_{600} for the inhibition of serine enzymes, because of the higher stability of the diisopropylphosphoryl–serine bond. It was soon realized, however, that aqueous solutions of DFP were not inhibitory for lipase. Aqueous solutions of E_{600} had also no effect. By contrast, emulsions of E_{600} readily inhibited lipase in aqueous solutions containing bile salts.

These observations are fully consistent with the well documented view[32,33] according to which lipase is exclusively active on substrates in an emulsified[1] or micellar state[2]. Explicit results concerning micelle formation were not expected with the complex mixture of bovine bile salts used for the assays depicted in Fig. 1. This figure, however, strongly suggests that under our conditions, micelles are already present in dilute solutions of bile salts. Hence, a plausible assumption is to suppose that the inhibitory effect of E_{600} in the presence of bile salts is caused by inclusion of the compound into micelles. It is not yet known whether this inclusion determines an activation of the inhibitor or of the enzyme itself, for instance by inducing the appearence of a functional active site as a result of the adsorption of the molecule at a micelle or emulsified particle.

A further argument in favor of the inclusion of E_{600} into bile salt micelles is given by the pH-dependence curve reproduced in Fig. 3. The fact that the optimal pH value observed for lipase inhibition is 3 pH units lower than that normally observed for the reaction of the enzyme with a substrate can be readily explained by a local accumulation of alkaline ions around negatively charged micelles. Two optimal pH values, one at 6 and the other at 9, have been observed under certain conditions

also with lipase substrates in the presence of bile salts[34]. Experiments are presently in progress to give additional experimental support to this assumption. Inhibition of lipase by E_{600} has now been shown to be induced also by pure sodium glycocholate and taurocholate (M. Sémériva, personal communication).

With the system consisting of E_{600} and bile salts, it was possible not only to show that lipase inhibition resulted from the binding of a single organophosphoryl radical, but also to identify the essential serine and related sequence. To our knowledge, it is the first time that E_{600} has been employed for the identification of an essential serine peptide. Great difficulties were encountered in this respect, due to the intrinsic instability of the diethylphosphoryl-serine bond and also to the proximity of an histidine which undoubtedly increased the instability of the bond by enhancing the nucleophilicity of the serine oxygen. Finally, suitable conditions could be set up for the purification from diethylphosphoryl derivatives of lipase of a labeled serine peptide in a yield permitting full identification.

It was also noteworthy that the sequence around the essential serine in lipase differed appreciably from those already identified in ordinary carboxylesterases and proteases. The residue just before the serine in the 3 carboxylesterases so far investigated (acetyl and butyrylcholine esterase[35,36]; liver carboxylesterase[37]) and in a number of proteases is aspartic or glutamic acid. In lipase, it is a bulky and strongly hydrophobic leucine residue. Moreover, no other known sequence appears to contain an histidine so close to the serine. No indication is available so far that this histidine is the one shown to be involved in the catalytic site of the enzyme.

Assays performed some years ago had suggested that concentrated aqueous solutions of DFP inhibited lipase even in the absence of bile salts[6]. Using other DFP samples and other enzyme preparations, these earlier results could not be confirmed. DFP does not appear to inactivate lipase even in the presence of bile salts. This new observation may be explained, either by a lack of fit between the organophosphate and the geometry of the enzyme active site, or by the inability of DFP to be included into bile salts micelles. The first explanation is corroborated by the fact that esters of isopropyl alcohol are poor substrates for lipase[38]. However, the origin of the discrepancy between our first and more recent results has not yet been discovered.

In spite of their inability to inhibit lipase, concentrated DFP solutions still reacted in a highly specific manner with a non-essential tyrosine residue in lipase. No interpretation can be offered at the present time for this reaction which has previously been found to occur also with ficin and bromelain[23,39].

Finally, the action of sulfonyl halides on lipase is worthy of some comment. The physical state of these compounds during their incubation with lipase has not been precisely ascertained. However, the water-insoluble compounds were dissolved in methylcellosolve. Addition of the solution to the enzyme resulted in turbid mixtures indicating heterogeneity. In spite of this heterogeneity, aliphatic or aromatic sulfonyl halides were not inhibitory for lipase and, consequently, did not react with the essential serine. By contrast, an inhibitory effect and probably also a reaction with the essential serine were observed when the nucleophilicity of the sulfur atom in the sulfonyl group was increased by an electrophilic substitution in the ring. It is interesting to note that monoester substrates of lipase must also be activated by an electrophilic substituant near the ester bond[38]. Sulfonyl halides, however, were much less specific for the essential serine than E_{600} and their study was not pursued.

In conclusion, an essential serine has been detected in pancreatic lipase as it has long been in ordinary carboxylesterases. However, in contrast with these latter enzymes, the serine does not appear to be especially "reactive" in native lipase. DFP is unable to react. E_{600} is an inhibitor, but only in an emulsified state or in the presence of bile salts acting as adjuvants. Sulfonyl halides must be activated and then their action is not specific. In the same connection, it may be recalled that the histidine residue involved in lipase catalytic site is not among the most "reactive" ones towards photooxidation[3].

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