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STUDIES ON THE SUBSTRATE SPECIFICITY OF A CARBOXYL ESTER HYDROLASE FROM HUMAN PANCREATIC JUICE

I. ACTION ON CARBOXYL ESTERS, GLYCERIDES AND PHOSPHOLIPIDS

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Summary

Purified carboxyl ester hydrolase (carboxylic-ester hydrolase, EC 3.1.1.1) from human pancreatic juice was found to hydrolyze triacetin, methyl butyrate and glycerides solubilized by bile salts. It has no activity on substrate presented as emulsion or monomolecular films.

The human enzyme was found to deacylate phospholipids and lysophospholipids at different rates. The hydrolysis of short-chain phosphatidylcholines was dependent of substrate solubility and dioctanoyl phosphatidylcholine was deacylated with the highest rate. Long-chain phosphatidylcholines and lysophosphatidylcholines present in microsomal membranes were deacylated with very low rates, only lysophosphatidylcholine deacylation was faster. Evidence is presented that human carboxyl ester hydrolase is the lyophosphatidylcholine-hydrolyzing enzyme corresponding to bovine lysophospholipase.

Bile salts play an important part on the activity of human carboxyl ester hydrolase, in addition to the role of detergent that they have on insoluble substrates.

Introduction

In recent years, many lipolytic pancreatic enzymes have been reported to hydrolyze *p*-nitrophenylacetate, a water-soluble substrate described for the

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

characterization of carboxyl ester hydrolase (carboxylic-ester hydrolase, EC 3.1.1.1) activity [1]. Besides lipase whose specificity has been extensively studied [2] and whose activity on *p*-nitrophenylacetate appears only in 4% acetonitrile solution [3], other lipolytic enzymes hydrolyze water-soluble *p*-nitrophenylacetate while they display activities on other various substrates. In rat pancreas, in addition to the carboxyl ester hydrolase purified from pancreatic juice by Erlanson [4], Albro et al. [5] have isolated a nonspecific lipase. This enzyme first reported by Mattson and Volpenheim as able to hydrolyze esters of both primary and secondary alcohols [6] was later characterized by the hydrolysis of isopropyl palmitate and phtalate diesters presented in the form of anionic micelles with bile salts. A lysophospholipase hydrolyzing 1-acyl lysophosphatidylcholines was purified from beef pancreas by Van den Bosch et al. [7] and a cholesterol esterase releasing cholesterol from micellar solutions of cholesterol oleate has been recently isolated from porcine pancreas [8]. All those enzymes exhibit very broad substrate specificity and hydrolyze *p*-nitrophenylacetate.

We have recently purified a carboxyl ester hydrolase from human pancreatic juice and studied some of its molecular properties [9]. The preparation was homogeneous by ultracentrifugation and SDS-polyacrylamide gel electrophoresis. Moreover, the protein displayed one single line of precipitation when submitted to an immunoelectrophoresis against an antiserum to all proteins of human pancreatic juice. The enzyme was characterized by its activity on *p*-nitrophenylacetate which increased in the presence of biliary salts. We studied its substrate specificity in an attempt to present evidence that human carboxyl ester hydrolase covers all the activities formerly ascribed to different pancreatic enzymes. In the present paper, we describe the action of human carboxyl ester hydrolase on soluble carboxyl esters, glycerides and phospholipids. We also report the effects of biliary salts on those different activities.

Material and Methods

Substrates. Triacetin, tributyrin and methyl butyrate were purchased from Fluka. Triolein was a BDH product. Short-chain phosphatidylcholines, lysophosphatidylcholine, 1,2- and 1,3-diolein, 1- and 2-monoolein were obtained from Serdary Research Laboratories. Sodium taurocholate and taurodeoxycholate were A grade products from Calbiochem. 1-Palmitoylthio-2-ethylphosphatidylcholine was a generous gift of Dr. H. Van den Bosch (State University of Utrecht, Utrecht) Monomolecular films constituents: 1,3-dicaprin, 1,2-dilaurin, tricaprylin, tricaproin, β-naphthyllaurate and lauric acid butyl ester were kindly given by Dr. R. Verger (Centre de Biologie Moléculaire, CNRS, Marseille). 1-Palmitoyl[^{14}C]lysophosphatidylcholine, 2-linoleyl[^{14}C]-lysophosphatidylcholine, 1-[^{14}C]palmitoyl-2-acyl phosphatidylcholine and 1-acyl-2-[^{14}C]linoleyl phosphatidylcholine were kindly prepared by Dr. M.J. Bonnefis, (Unité 101, INSERM, Toulouse) according to the technique of Waite and Van Deenen [10] and Van den Bosch et al. [11].

Enzyme. Human carboxyl ester hydrolase was purified from pancreatic juice as previously described [9] except for the last step of purification which was a chromatography on CM-Sepharose instead of CM-Sephadex.

Enzyme assays. Hydrolysis of substrates containing short-chain fatty acids like methyl butyrate, triacetin and tributyrin were measured by a titrimetric method at constant pH 8.0 with 20 mM NaOH in 0.15 M NaCl at 25°C. Substrates were dissolved or dispersed (above saturation) by standard stirring in 10 ml of medium, for 5 min.

Hydrolysis of long-chain glycerides was measured by titration of the liberated fatty acids with 10 mM NaOH at constant pH 8.0. Titration was carried out in N_2 atmosphere at 25°C. The assay system consisted of 2 ml of 0.25 M NaCl containing 5 mM sodium taurocholate and 2 mM substrate.

Activity on olive-oil emulsion and triolein emulsion was measured according to Desnuelle et al. [12]. Activity on egg-yolk emulsion was measured by the method described by de Haas et al. [13] and modified by Figarella and Ribeiro [14].

The activities on monomolecular films were measured according to the interfacial pressure method described by Verger and de Haas [15]. In all assays, the aqueous subphase contained a 10 mM Tris/acetate buffer, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$ and 1 mM EDTA. Before each assay, remaining tensioactive impurities present in this subphase were removed by sweeping off the surface and suction with a glass capillary. Assays were performed at 25°C with 50 μg of carboxyl ester hydrolase injected under the film in the reaction compartment.

Hydrolysis of short-chain phosphatidylcholines or lysophosphatidylcholines was measured by titration at pH 7.5 and 25°C using 1 mM substrate concentration according to De Jong et al. [16]. The activity on 1-palmitoylthio-2-ethylphosphatidylcholine was measured spectrophotometrically at 412 nm in a 0.2 M sodium phosphate buffer (pH 7.4) containing 1 mM DTNB, using the molar extinction coefficient of 12 800 [17]. The hydrolysis of labelled phospholipids was carried out with 500 nmol of each substrate sonicated (twice 15 s, 0°C) in 1 ml of 0.2 M Tris-HCl buffer, pH 8.5. These preparations were diluted to 2 ml with the enzyme plus 0.3 M sucrose, then incubated at 37°C 5 min for lysophosphatidylcholine and 15 min for phosphatidylcholine. The other steps of the assay were performed as described by Bonnefis et al. [18].

Specific activities were expressed as μmol of fatty acids released/min and per mg protein. Protein concentration was measured using the absorbance coefficient of 14.5 at 280 nm [9].

Assays in presence of bile salts. Various quantities of 100 mM sodium taurocholate or taurodeoxycholate solutions were added to the assay mixtures to obtain the suitable concentrations. The values of critical micellar concentration were 2.7 mM and 0.9 mM, respectively, for sodium taurocholate and taurodeoxycholate in 0.15 M NaCl at 20°C according to Carey and Small [19].

Results

Activities on water-soluble carboxyl esters (triacetin and methyl butyrate)

Triacetin and methyl butyrate were hydrolyzed by human carboxyl ester hydrolase. Fig. 1 represents the enzymatic activities as a function of substrate concentration, expressed as multiples of saturation. For the hydrolysis of methyl butyrate, the activity increased to a maximal value obtained with substrate saturation. For the hydrolysis of triacetin, the maximal activity was

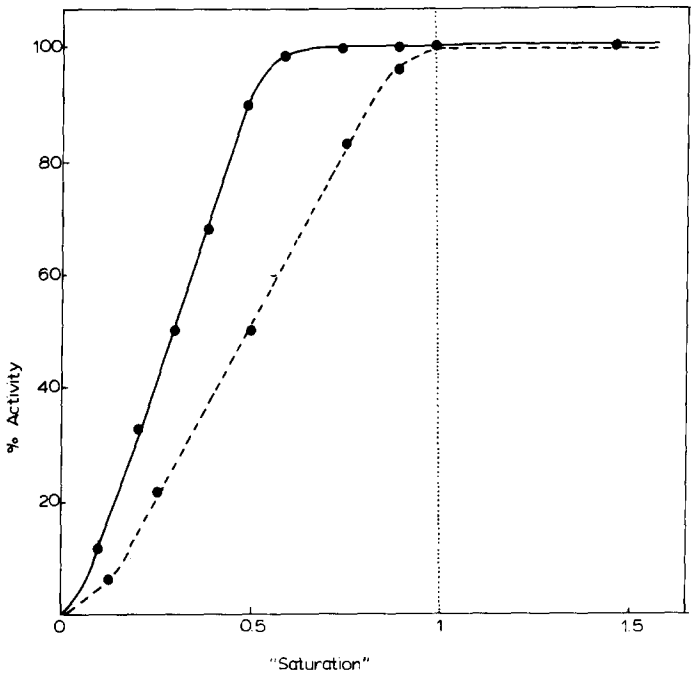

Fig. 1. Hydrolysis of triacetin and methyl butyrate by carboxyl ester hydrolase. The ordinate represents the enzyme activity expressed in percent of the maximal activity, and the abscissa the substrate concentration expressed in multiples of saturation. ●———●, triacetin hydrolysis; ●- - - - -●, methyl butyrate hydrolysis; · · · · · ·, saturation point.

reached at 65% saturation (0.65 S). This shift does not seem related to the formation of micelles since it is known that in the presence of 0.15 M NaCl, triacetin forms micelles below saturation, with a critical micellar concentration equal to 105 mM which would correspond to 0.31 S on the diagram [20]. Both substrates were hydrolyzed at low rates: maximal specific activities were 17 for the hydrolysis of methyl buryrate and 26 for the hydrolysis of triacetin.

Sodium taurocholate and taurodeoxycholate inhibit the hydrolysis of triacetin by carboxyl ester hydrolase. Fig. 2 shows the effects of each bile salt on the hydrolysis of triacetin at the concentration of 0.5 S which gives 90% of the maximal activity. No variation of the activity was observed by addition of sodium taurodeoxycholate at a concentration below 0.9 mM. The inhibition started with the bile salt critical micellar concentration and increased slowly to reach 30% at 4 mM. By contrast, the addition of sodium taurocholate immediately produced a progressive inhibition of the activity which became stable with 40% of remaining activity at 1.5 mM sodium taurocholate. This effect was independent of sodium taurocholate critical micellar concentration. Fig. 3 gives the Michaelis representation of the hydrolysis of triacetin in the absence of sodium taurocholate and in the presence of 2 mM sodium taurocholate. The two curves show that V was not modified while $K_{m(app)}$ was increased in the presence of bile salts, suggesting a competitive inhibition by sodium taurocholate at the active site. This inhibition disappeared when substrate was preincubated for 1 h with sodium taurocholate (2 or 5 mM) to form mixed

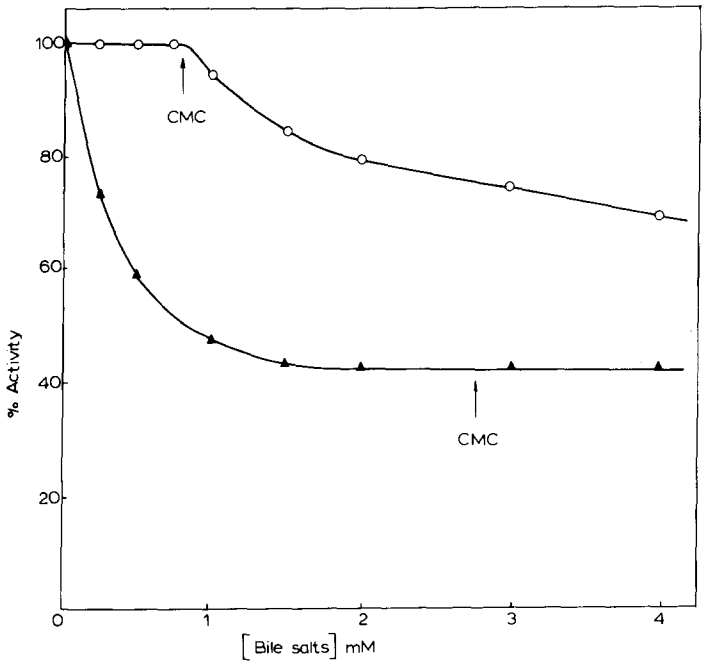

Fig. 2. Inhibition of the activity of carboxyl ester hydrolase on 0.5 S triacetin by bile salts. ○———○, effect of sodium taurodeoxycholate; ▲———▲, effect of sodium taurocholate. CMC, critical micellar concentration.

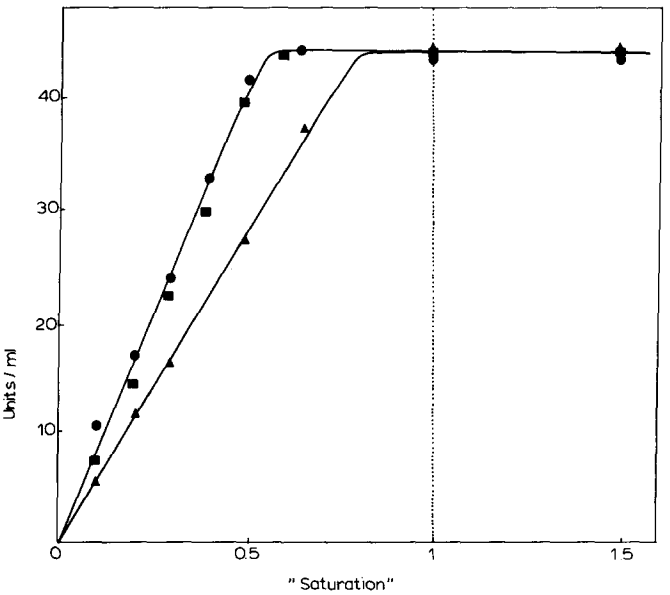

Fig. 3. Effect of bile salts on triacetin hydrolysis. The experiments have been performed in the absence of bile salts (●), in the presence of 2 mM sodium taurocholate (▲) and in the presence of 2 mM sodium taurocholate previously incubated with substrate for 1 h (■).

micelles, indicating that the steric hindrance of mixed micelles did not inhibit the enzyme activity on triacetin. The same experiments performed in the presence of 2 mM sodium taurodeoxycholate have shown a less marked competitive inhibition of triacetin hydrolysis by this bile salt.

Activities on glycerides

Long-chain glycerides solubilized by biliary salts were very poor substrates for carboxyl ester hydrolase as shown on Table I. No activity was detectable on emulsions of olive oil or triolein. The hydrolysis of short-chain triglycerides like tributyrin (100 mM) was bile salt dependent. Without bile salts the activity was very low (6—10% of the maximal activity). The addition of bile salts increased the specific activity to maximal values which were 240 and 260, respectively, in the presence of sodium taurocholate or taurodeoxycholate. However, the activator effect of each bile salt was concentration dependent. In the presence of sodium taurodeoxycholate, 90% of the activity was reached with the bile salt critical micellar concentration (0.9 mM). In the presence of sodium taurocholate, the activity increased rapidly with increasing bile salt concentration. The maximal activity was obtained at 0.5 mM, well below the bile salt critical micellar concentration and independent of the substrate concentration (between 10 mM and 100 mM) which shows that sodium taurocholate does not act only on substrate by its detergent property.

Activities on phospholipids

Table II summarizes the extent of hydrolysis of various synthetic phospholipids by carboxyl ester hydrolase. Only short-chain phosphatidylcholines were hydrolyzed. Dioctanoyl phosphatidylcholine was the best substrate and the constant ratios between specific activities towards *p*-nitrophenylacetate and dioctanoyl phosphatidylcholine during purification steps (Table III) indicate that both substrates were hydrolyzed by the same enzyme. The hydrolysis of dioctanoyl phosphatidylcholine was inhibited by the addition of sodium taurocholate (Fig. 4) and the inhibition was almost complete (95%) at the bile salt critical micellar concentration. A similar inhibitor effect was observed with taurodeoxycholate but the effect started somewhat below the critical micellar concentration.

Table IV gives the percentage of hydrolysis of long-chain phosphatidylcholines and lysophosphatidylcholines isolated from microsomal membranes

TABLE I

HYDROLYSIS OF LONG-CHAIN GLYCERIDES BY CARBOXYL ESTER HYDROLASE

Specific activities are expressed in μmol/min per mg protein. All assays were performed in the presence of 5 mM sodium taurocholate.

Substrate	Specific activity
1-Monoolein	7.2
2-Monoolein	4.7
1,2-Diolein	19.7
1,3-Diolein	14.0
Triolein	16.8

TABLE II

HYDROLYSIS OF SYNTHETIC PHOSPHOLIPIDS BY CARBOXYL ESTER HYDROLASE

Specific activities are expressed in μmol/min per mg protein.

Phospholipid	Specific activity
Dihexanoyl phosphatidylcholine	30
Dioctanoyl phosphatidylcholine	197
Didecanoyl phosphatidylcholine	5
Dilauroyl phosphatidylcholine	0
1-Decanoyl lysophosphatidylcholine	0
1-Palmitoylthio-2-ethyl phosphatidylcholine	270

TABLE III

SPECIFIC ACTIVITIES TOWARDS *p*-NITROPHENYLACETATE AND DIOCTANOYL PHOSPHATIDYLCHOLINE DURING PURIFICATION OF CARBOXYL ESTER HYDROLASE

The activities on dioctanoyl phosphatidylcholine were performed at 37°C. Specific activities are expressed in μmol/min per mg protein.

Purification step	Specific activity on *p*-nitrophenylacetate	Specific activity on dioctanoyl phosphatidylcholine	Ratio
Pancreatic juice	2	12.8	0.156
Fraction from Sephadex G-100	26	175.0	0.149
Carboxyl ester hydrolase eluted from CM-sepharose	40	250.0	0.160

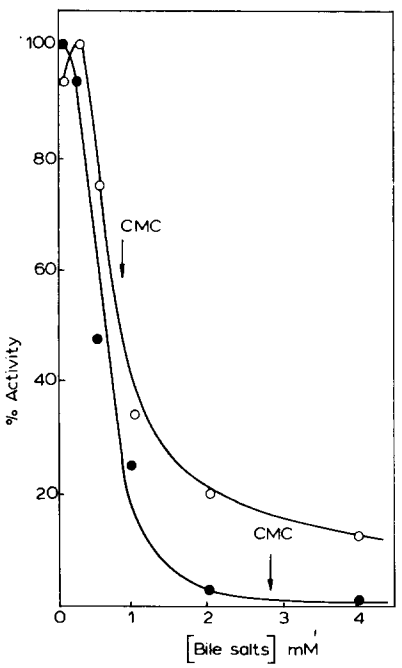

Fig. 4. Inhibition of the activity of carboxyl ester hydrolase on dioctanoyl phosphatidylcholine by sodium taurocholate (●) and taurodeoxycholate (○). CMC, critical micellar concentration.

TABLE IV

HYDROLYSIS OF LONG-CHAIN PHOSPHATIDYLCHOLINES AND LYSOPHOSPHATIDYLCHOLINES ISOLATED FROM LIVER MICROSOMAL MEMBRANES

Specific activities are expressed in nmol/min per mg protein.

Phospholipid	Specific activity
1-[^{14}C]Palmitoyl-2-acyl phosphatidylcholine	9
1-Acyl-2-[^{14}C]linoleyl phosphatidylcholine	19
1-Palmitoyl [^{14}C]lysophosphatidylcholine	53
2-Linoleyl [^{14}C]lysophosphatidylcholine	49

of rat liver. Phospholipid hydrolysis was extremely low like previously observed with synthetic long-chain phosphatidylcholines. Only the conversion of lysophosphatidylcholine into glycerylphosphorylcholine was slightly higher whatever the fatty acid position. This lysophospholipase activity was more obvious on the synthetic lysophosphatidylcholine, 1-palmitoylthio-2-ethyl phosphorylcholine whose rate of deacylation was high (Table II). However, it is worthwhile noting that 1-decanoyl lysophosphatidylcholine hydrolysis was undetectable.

Activities on monomolecular films of lipids

Table V gives the list of the different substrates used in the form of monomolecular films and the results of the assays. Human carboxyl ester hydrolase did not hydrolyze any of these lipids except triglycerides like tricaprylin and tricaproin whose rates of hydrolysis were very low. In the case of tricaprylin the specific activity of human carboxyl ester hydrolase represents about 1% of the activity of porcine pancreatic lipase. Diglycerides, phospholipids and carboxyl esters were not hydrolyzed and it is particularly important to notice that dioctanoyl phosphatidylcholine which was a good substrate in monomeric form, was not hydrolyzed in this physical state.

TABLE V

ACTIVITIES ON MONOMOLECULAR FILMS OF VARIOUS LIPIDS

Pressure is given in dynes/cm. Specific activities are expressed in μmol/min per mg protein. n.d., not determined.

Substrate	Collapse pressure	Pressure used	Specific activity
1,3-Dicaprin	36	8, 15, 20	0
1,2-Dilaurin	40	20, 30, 40	0
Tricaprylin	20	15	0.17
Tricaprylin	20	19.5	0.5
Dioctanoyl phosphatidylcholine	n.d.	10	0
Didecanoyl phosphatidylcholine	n.d.	8, 12, 20	0
Dilauroyl phosphatidylcholine	n.d.	8, 12, 20	0
β-Naphthyllaurate	8	7	0
Lauric acid butyl ester	4	3.5	0

Discussion

The experiments using different classes of lipids show that human carboxyl ester hydrolase hydrolyzes water-soluble carboxyl esters as well as substrates solubilized by biliary salts and has no activity on insoluble substrates when emulsified or presented as monomolecular films.

The hydrolysis of soluble carboxyl esters such as methyl butyrate and also *p*-nitrophenylacetate, as previously reported [9], demonstrates that the enzyme is a true esterase like the enzyme of rat pancreatic juice [4,21]. Human esterase hydrolyzes also ester bonds of water-soluble phospholipids such as dioctanoyl phosphatidylcholine and 1-palmitoylthio-2-ethyl phosphatidylcholine. The decreased activity on the other phospholipids can be explained by differences in solubility or physicochemical properties of these phosphatidylcholines. At 1 mM, didecanoyl phosphatidylcholine and dilauroyl phosphatidylcholine form liposomes which were not degraded by the enzyme as shown for bovine lysophospholipase [16]. The low activity on dihexanoyl phosphatidylcholine whose critical micellar concentration is relatively high, 9.5 mM (compared to dioctanoyl phosphatidylcholine critical micellar concentration, 0.19 mM) could be due to the low substrate concentration used in the assay.

The hydrolysis of micellar triacetin and the hydrolysis of di- and triglycerides solubilized by bile salts demonstrate that the enzyme also hydrolyzes micellar substrates. By contrast, emulsified tributyrin in the absence of bile salts, and diglycerides or phospholipids in monomolecular film are not hydrolyzed, which is in agreement with the lack of activity of carboxyl ester hydrolase on olive oil or triolein as well as on egg-yolk emulsions. The very slow hydrolysis of triglycerides in monomolecular film is surprising but cannot be due to a lipase contamination since in the same conditions, diglycerides are not hydrolyzed by the enzyme that we have purified. Consequently, human carboxyl ester hydrolase would be complementary to pancreatic lipase and phospholipase that hydrolyze glycerides and phospholipids in emulsion or monomolecular film.

We verified that carboxyl ester hydrolase is completely devoid of proteolytic activity. No hydrolysis of casein or ester bonds on amino acids such as acetyl-L-tyrosine ethyl ester, benzoyl-L-arginine ethyl ester of tosyl-L-arginine methyl ester was obtained. This property differentiates carboxyl ester hydrolases of pancreatic or hepatic origin [22].

Human carboxyl ester hydrolase behaves like bovine lysophospholipase towards various substrates. Both enzymes hydrolyze triacetin, tributyrin, *p*-nitrophenylacetate and dioctanoyl phosphatidylcholine. The two enzymes display a high and similar specific activity on dioctanoyl phosphatidylcholine and this activity is inhibited by bile salts. No cofactor able to remove this inhibition was found in human pancreatic juice. The degradation of long-chain phospholipids present in biological membranes is extremely slow, only lysophosphatidylcholines deacylation being slightly faster. This lysophospholipase activity is confirmed by the high rate of hydrolysis of 1-palmitoylthio-2-ethyl phosphatidylcholine, synthetic thioester substrate described to reflect the specificity of lysophospholipase [23]. This activity is also inhibited by the presence of bile salts. From all these results, it is clear that in human pancreatic

juice carboxyl ester hydrolase is the lysophosphatidylcholine hydrolyzing enzyme corresponding to the enzyme characterized in beef pancreatic juice and we can postulate that pancreatic carboxyl ester hydrolase possessed the property of hydrolyzing lysophosphatidylcholines, fact questionned by de Jong et al. [16].

If the identity between carboxyl ester hydrolase and nonspecific lipase is more difficult to assert, it is interesting to note that human carboxyl ester hydrolase hydrolyzes the same substrate as rat nonspecific lipase [5]. A sample of the purified human enzyme has been kindly assayed on diethylhexyl phtalate by Dr. Albro. The specific activity has been found to be 3.25 μequiv./min per mg protein. This value is virtually identical to that obtained for the rat nonspecific lipase.

It is worthwhile noting the effects of bile salts on carboxyl ester hydrolase activity towards the different substrates. In addition to the role of detergent played on insoluble substrates, bile salts seems to act directly on the enzyme activity. On one side sodium taurocholate and taurodeoxycholate increase the activity of human carboxyl ester hydrolase on tributyrin and *p*-nitrophenylacetate [9] and on the other they inhibit the activity on dioctanoyl phosphatidylcholine and triacetin and we have demonstrated that the latter substrate is competitively inhibited. The two opposite effects show that the enzyme recognizes bile salts as well as its specific substrates. However, if we try to relate the activator or inhibitor effect of each bile salt with the critical micellar concentration, it is obvious that the effect of sodium taurocholate appears well below the critical micellar concentration, whereas the effect of sodium taurodeoxycholate appears for a concentration close to the critical micellar concentration. Those results suggest that carboxyl ester hydrolase recognizes the monomers of sodium taurocholate and the micelles of sodium taurodeoxycholate. The different extent of inhibition or activation observed with those two bile salts can be explain by an additional effect of trihydroxylated bile salts which will be discussed in the following paper.

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