Carboxylic ester hydrolases of rat pancreatic juice

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ABSTRACT An attempt was made to establish the number and characteristics of the enzymes in pancreatic juice that hydrolyze nitrogen- and phosphorus-free esters of fatty acids. For this purpose model compounds were hydrolyzed by lyophilized rat pancreatic juice under conditions that accelerated or inhibited the reactions. Although it is not established with certainty, it is suggested that three enzymes are responsible for the hydrolysis of fatty acid esters.

The first enzyme is glycerol-ester hydrolase (EC 3.1.1.3) or lipase. This enzyme hydrolyzes water-insoluble esters of primary alcohols. The reaction occurs at an oil/water interface and is inhibited by bile salts at pH 8. The enzyme is relatively stable at pH 9, but unstable at pH 4. It has a broad pH optimum between 7.5 and 9.5.

The second enzyme hydrolyzes esters of secondary alcohols and of other alcohols as well. It has an absolute requirement for bile salts and has a pH optimum at about 8. The enzyme is unstable in pancreatic juice when maintained at pH 9, probably due to the action of trypsin. It may be identical with sterol-ester hydrolase (EC 3.1.1.13).

The third enzyme hydrolyzes water-soluble esters. It too has an absolute requirement for bile salts, although a smaller amount is necessary for maximum activity. This enzyme also is unstable at pH 9, but can be differentiated from the preceding enzyme by its stability at pH 4 and its pH optimum of 9.0.

Carboxylic-ester hydrolase (EC 3.1.1.1) is not found in pancreatic juice, although it is present in pancreatic tissue.

KEY WORDS digestion · pancreatic juice · pancreatic tissue · lipase · esterase · cholesterol esterase · esters · primary alcohols · secondary alcohols · water-soluble esters · water-insoluble esters · bile

In 1952 we (1) reported that the hydrolysis of triglycerides in the lumen of the intestinal tract yields predominantly 2-monoglycerides. On the basis of this observation, it was suggested that the hydrolysis is a series of directed stepwise reactions from triglyceride, via 1,2-diglyceride, to 2-monoglyceride. Subsequently, Borgström (2) demonstrated the formation of 1,2-diglyceride. This sequence of reactions was confirmed by studies in vitro (3–5). Sarda and Desnuelle (6) demonstrated that the enzyme functions only at an oil/water interface and cannot hydrolyze water-soluble esters. From these and other reports it thus appeared that pancreatic lipase could be described as an enzyme that hydrolyzes water-insoluble esters of a primary alcohol. It was proposed that the word esterase be restricted to enzymes that hydrolyze water-soluble substrates.

The question whether pancreatic lipase can hydrolyze esters of secondary alcohols, e.g. at the 2-position of a triglyceride, has never been answered unequivocally. Studies in vitro (7) always show a small amount of the fatty acid that was esterified at the 2-position of a triglyceride to be present as free fatty acid at the end of the digestion period. However, this could arise as the result of the isomerization of 2-monoglyceride to 1-monoglyceride (8) and the subsequent hydrolysis of this primary ester. Borgström (9) has proposed that 2-monoglycerides can be hydrolyzed by pancreatic lipase without first being isomerized. Our studies (10) on the absorption of triglycerides showed that a portion of the fatty acid esterified at the 2-position is converted in vivo to free fatty acid prior to absorption. In spite of these observations there has never been a demonstration of the direct hydrolysis of long-chain fatty acid esters of secondary alcohols, with the exception of those of cholesterol, by the enzymes of pancreatic juice.

The hydrolysis of water-soluble esters also presents a conflicting picture. Marchis-Mouren, Sarda, and Desnuelle (11) have described the separation of an esterase from a lipase in pancreatic tissue. On the other hand, Borgström (12) could find no esterase in pancreatic juice. An extensive discussion of the distribution and properties of esterases is given in the articles by Myers (13) and Hofstee (14).

In the studies reported here four model compounds were used as substrates. Methyl butyrate at a concentration of 0.2% was used as the water-soluble ester. The hydrolysis of esters of secondary alcohols was followed

with the dioleate ester of butane-2,3-diol, hereafter referred to as 2,3-dioleoyl butane, as the substrate. Triolein was used as a substrate for the study of esters of primary alcohols. Since cholesterol esterase is known to occur in pancreatic juice (15), cholesteryl linolenate was the fourth substrate.

MATERIALS

Cholesteryl linolenate and 2,3-dioleoyl butane were prepared from the appropriate fatty acid anhydrides and alcohols (16). Triolein was synthesized by base-catalyzed transesterification of methyl oleate with glycerol (17). Methyl butyrate was purchased and purified by distillation. These substrates were examined by gas–liquid and thin-layer chromatography; all were of better than 98% purity.

The remaining materials were obtained from the following suppliers: sodium taurocholate and carboxypeptidase (3× cryst.), Nutritional Biochemicals Corp., Chagrin Falls, Ohio; ovomucoid and α-chymotrypsin (3× cryst.), Worthington Biochemical Corp., Freehold, N.J.; trypsin 1× cryst. (bovine pancreas), Calbiochem, Los Angeles, Calif.; diethyl *p*-nitrophenyl phosphate (E-600), K & K Laboratories Inc., Plainview, N.Y. Thin-layer chromatography of the sodium taurocholate showed that although this bile salt was the predominant constituent, four other minor components were present as well, at least two of which appeared to be other bile salts. The critical micelle concentration in 1 M NaCl at pH 8, as determined by surface tension measurement, was 0.5 mg/ml (0.93 mM). This value is one-tenth to one-third that reported by others and suggests the presence of a water-insoluble, surface-active contaminant. In spite of this not being a pure material, it is referred to here as sodium taurocholate.

Pancreatic juice was obtained from young adult rats. A cannula was inserted into the bile duct as near to the liver as possible. A second cannula was inserted into the common bile–pancreatic duct near its entrance into the duodenum. The common bile–pancreatic duct was severed between the two cannulas. In this way pancreatic juice was obtained free from bile. The two cannulas were led from the animal and the bile and pancreatic juice were collected separately. After the incision had been closed, the animal was placed in a restraining cage and offered water and Purina Laboratory Chow. The pancreatic juice was collected in a flask that was immersed in iced water; if collected at room temperature, the juice could hydrolyze only triolein. The first day's collection was discarded. The juice obtained from each succeeding 24-hr period was freeze-dried. These conditions of collection and processing resulted in no loss of any enzymatic activities. The dried powder when stored at 0°C was stable for several months. The lyophilized juice was reconstituted by dissolving it (0.6 mg/ml) in 0.01 M, pH 7.0 histidine. This solution was prepared on the day it was used. All the enzymes that were being studied were stable in this solution for at least 6 hr.

Some of the animals that had been used as donors of pancreatic juice were killed and their pancreatic tissue obtained by dissection. Since the bile duct of these animals was cannulated, the possiblity of bile juice contaminating the pancreatic tissue was minimized by using tissue from these animals. The tissue was homogenized at 0°C in 50% glycerol. The homogenate was strained through a few layers of cheese cloth and the filtrate used immediately.

METHODS

The course of hydrolysis of methyl butyrate was studied with the aid of a pH Stat and the use of 0.02 N KOH. The rate of addition of KOH, which was linear over the first several minutes, was used for determining the rate of the reaction. The digestion mixture consisted of 100 mg (0.98 mmole) of methyl butyrate, 0.44 mg/ml (0.82 mM) sodium taurocholate, 0.0018 M Tris, 1 M NaCl, and 3 mg of lyophilized pancreatic juice in a total volume of 55 ml at pH 8.0 and at 25°C. Agitation was by stirring.

For the digestion of triolein, the mixture contained 200 mg (0.226 mmole) of triolein, 0.1 M Tris, 1 M NaCl, 0.33 mmole of $CaCl_2$, and 0.3 mg of lyophilized pancreatic juice in a total volume of 55 ml at a pH of 8.0 and at 25°C. With even the most active of enzyme preparations, only 15% of the total ester groups was hydrolyzed in 10 min under these conditions. Analysis of the digestion products showed that 75% of the resulting free fatty acid arose by the conversion of triglyceride to 1,2-diglyceride. The other digestion product was almost exclusively 2-monoglyceride.

For the digestion of 2,3-dioleoyl butane, the mixture consisted of 240 mg (0.388 mmole) of substrate, 0.73 mg/ml (1.35 mM) sodium taurocholate, 6 mg of lyophilized pancreatic juice, 0.1 M Tris, 1 M NaCl, and 0.33 mmole of $CaCl_2$ in a total volume of 55 ml at a pH of 8.0 and at 25°C.

Cholesteryl linolenate was digested in a mixture consisting of 240 mg (0.371 mmole) of cholesteryl linolenate, 40 mg of mineral oil, 0.73 mg/ml (1.35 mM) sodium taurocholate, 6 mg of lyophilized pancreatic juice, 0.1 M Tris, 1 M NaCl, and 0.33 mmole of $CaCl_2$ in a total volume of 55 ml at a pH of 8.0 and at 25°C. Mineral oil was added to this digest because cholesteryl linolenate is a solid at 25°C. Separate studies established that mineral oil does not inhibit the enzymatic hydrolysis of this substrate.

The 2,3-dioleoyl butane and cholesteryl linolenate digestion mixtures containing all of the constituents,

except the enzyme, were shaken on a wrist-action shaker (330 cycles/min, amplitude 25°) for 2 hr. The enzyme was then added and the shaking continued for 10 min. The triolein was similarly digested for 10 min with agitation by the wrist-action shaker. However, this digest was not shaken for 2 hr prior to the addition of the enzyme.

The conditions under which the water-insoluble substrates were digested were such that the hydrolyses followed an essentially linear course for at least 15 min.

The agitation conditions were selected on the basis that any further increase in the rate of shaking did not result in an increase in the rate of hydrolysis. Thus dispersion and diffusion should not be rate-limiting steps. The shaking of the cholesteryl linolenate and 2,3-dioleoyl butane with the taurocholate prior to the addition of the enzyme results in a dispersion, possibly in micellar form. If the digestion mixture minus the enzyme was shaken for less than 1.5 hr, the rate of hydrolysis on adding the enzyme was lower than the maximum rate. Agitation for longer than 1.5 hr did not influence further the rate of hydrolysis.

At the end of the 10 min digestion period, sufficient (1 ml of 25%) HCl was added to lower the pH to 2. This stops enzymatic activity. Chloroform, 50 ml, was added and the flask shaken for a further 20 min. The contents of the flask were transferred to a separatory funnel and the chloroform phase was drawn off. This was dried with sodium sulfate and a 40.0 ml sample was taken. The chloroform was removed in a 40°C water bath with the aid of a stream of nitrogen. The residue in the flask was dissolved in ethanol–chloroform 3:1, to which was added 1 drop of 1 M NaCl, and was titrated potentiometrically using 0.02 N KOH. Preliminary experiments with free oleic acid established the instrument settings that were necessary for this titration. The rates of hydrolysis of all substrates are expressed as microequivalents of fatty acid released per minute per milligram of enzyme (lyophilized pancreatic juice). On the basis of a large number of experiments, the values for the rates of hydrolysis under these conditions of all four substrates had a relative error no greater than ±5%.

RESULTS

The effect of the concentration of NaCl on the rates of hydrolysis of these various substrates is shown in Fig. 1. Whereas the addition of salt changes only slightly the speed with which triolein is hydrolyzed, the rates at which the other substrates are cleaved is markedly altered. Among these three substrates, differences are apparent in the concentration which gives maximum activity and in the response to higher or lower NaCl concentrations. The effect of NaCl can be duplicated by other electrolytes such as calcium chloride or sodium sulfate, and could be due to a number of factors known to be influenced by electrolyte concentration. Such factors include configurational changes in proteins, which could alter the activity of the enzyme; changes in potential at the oil/water interface, which could affect the enzyme–substrate association as well as the rate of diffusion of the product (free fatty acids); and alterations in the free fatty acid–soap equilibrium, which could inhibit or speed up the reaction. The dioleoyl butane, cholesteryl linolenate, and methyl butyrate digestion mixtures contained added sodium taurocholate. The activities observed when NaCl was not added to these digests could be due to the sodium taurocholate supplying sufficient electrolyte to permit this rate of hydrolysis. If this is the case, the cleavage of these substrates requires the presence of electrolyte. Since the rates of hydrolysis of all substrates were at or near their maxima in 1 M NaCl, this concentration was selected for the subsequent experiments.

The rates at which the substrates are hydrolyzed are influenced also by the level of taurocholate in the digest, as is shown in Fig. 2. The hydrolysis of triolein is markedly inhibited by taurocholate. This effect at pH 8.0 has been reported by Borgström (18), although he did not describe it as an inhibition. The hydrolysis of methyl butyrate, dioleoyl butane, and cholesteryl linolenate requires the presence of taurocholate. However, the level of taurocholate (0.7 mM) required for the maximum rate of methyl butyrate hydrolysis is approximately one-half that required by the other two substrates, that is, 1.5 mM. At higher levels of taurocholate, the hydrolysis of methyl butyrate is markedly inhibited, but not that of dioleoyl butane and cholesteryl linolenate. The levels of taurocholate selected for use in the standard digestions were those that gave the maximum activity with each particular substrate.

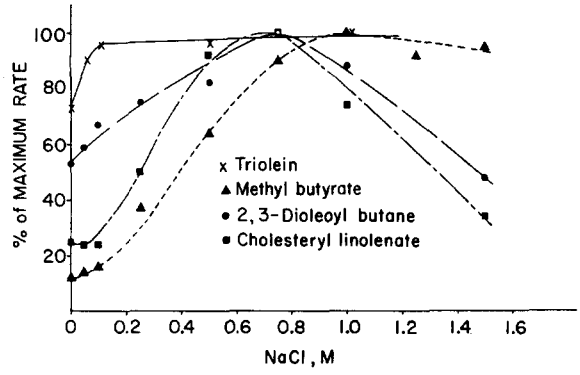

FIG. 1. Effect of NaCl concentration on the rate of hydrolysis of various substrates by pancreatic juice. Digestion conditions were as given under Methods except for the level of NaCl. Rate, μeq/min per mg of enzyme, equivalent to 100% was: methyl butyrate, 1.8; 2,3-dioleoyl butane, 0.80; cholesteryl linolenate, 0.66; triolein, 47.

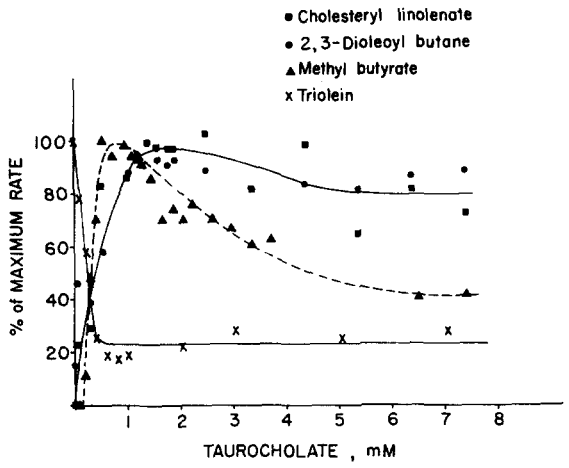

FIG. 2. Effect of sodium taurocholate concentration on the rate of hydrolysis of various substrates by pancreatic juice. Digestion conditions were as described under Methods except for the level of taurocholate. Rate, μeq/min per mg of enzyme, equivalent to 100% was: methyl butyrate, 1.9; 2,3-dioleoyl butane, 0.81; cholesteryl linolenate, 0.59; triolein, 36.

The retention or loss of ability by pancreatic juice after various treatments to hydrolyze the four substrates was determined. A stock solution of lyophilized pancreatic juice powder in 0.01 M histidine, pH 7.0, was prepared. A portion of this, the control sample, was diluted to 0.6 mg/ml and used immediately to determine the rates at which the four substrates were hydrolyzed. Other portions of the stock solution were made more acid or alkaline by the addition of HCl or KOH. These solutions were then diluted so that the final concentration of the pancreatic juice powder was 0.6 mg/ml. The solutions were placed in a water bath at 40°C for 1 hr. At the end of this time, the rates at which the various substrates were hydrolyzed by these preparations were determined. The assays of the control and experimental samples were by the standard procedure at pH 8.0. The results, expressed as the percentage of the rate at which the control sample effected hydrolysis, are given in Table 1.

The relative stabilities at pH 4.0 were investigated further by maintaining a solution of the pancreatic juice powder at this pH and 40°C for various periods of time. The procedure described in the preceding paragraph was used. The results are given in Table 2. The rapid loss of hydrolytic activity toward cholesteryl linolenate and 2,3-dioleoyl butane was observed in other experiments when the solution of pancreatic juice powder was maintained at pH 4 for only 3 min. Such exposure resulted in a loss of 10–15% of the initial hydrolytic activity toward these two substrates.

Since only the enzyme that splits water-insoluble esters of primary alcohols is stable at pH 9, this affords a convenient method of treating pancreatic juice so that it will contain this single lipid hydrolase. It seems likely that the inactivation of the other enzymes at pH 9.0 is due to the action of trypsin, for the addition of 1 mg of ovomucoid, a trypsin inhibitor, to a solution containing 20 mg of pancreatic juice powder prior to adjusting the solution to pH 9.0 prevented any loss in activity.

The ability of ovomucoid to protect the enzymes from trypsin inactivation made it possible to determine the pH optima for the various substrates. The rates of hydrolysis of the substrates at different pH values, when the digest contained 1 mg of ovomucoid per 20 mg of pancreatic juice powder, are shown in Fig. 3. Since the substrates are nonionic, these curves have been drawn analogously to titration curves to demonstrate better the changes in the enzymes. The enzyme that splits triolein has a broad pH optimum between 7.5 and 9.5. The maximum rates of hydrolysis for the other substrates were attained at 7.8 for cholesteryl linolenate, 8.3 for dioleoyl butane, and 9.0 for methyl butyrate.

The inhibitory action of diethyl *p*-nitrophenyl phosphate (E-600) was determined by adding various amounts of this material to a pH 7.0, 0.01 M histidine solution of 0.6 mg/ml of pancreatic juice powder. After 30 min at 25°C, the residual enzymatic activity toward the four

TABLE 1 RETENTION OF HYDROLYTIC ACTIVITY TOWARD VARIOUS SUBSTRATES BY PANCREATIC JUICE MAINTAINED PRIOR TO ASSAY AT THE pH INDICATED FOR 1 HR AT 40°C

pH	Methyl Butyrate	2,3-Dioleoyl Butane	Cholesteryl Linolenate	Triolein
		% of control rate		
3	0	0	0	9
4	96	65	64	16
5	100	74	68	89
6	105	105	100	103
7	109	104	100	101
8	59	66	78	97
9	0	0	0	101
Control rate (μeq/min per mg of enzyme)	1.4	0.55	0.46	29

TABLE 2 RETENTION OF HYDROLYTIC ACTIVITY TOWARD VARIOUS SUBSTRATES BY PANCREATIC JUICE MAINTAINED PRIOR TO ASSAY FOR THE TIMES INDICATED AT pH 4.0 and 40°C

Time	Methyl Butyrate	2,3-Dioleoyl Butane	Cholesteryl Linolenate	Triolein
hr		*% of control rate*		
0	100	100	100	100
1/4	100	72	72	76
1/2	96	72	79	36
3/4	87	70	72	30
1	87	66	72	24
1 1/2	79	65	76	15
2	79	—	80	6
Control rate (μeq/min per mg of enzyme)	1.6	0.63	0.41	33

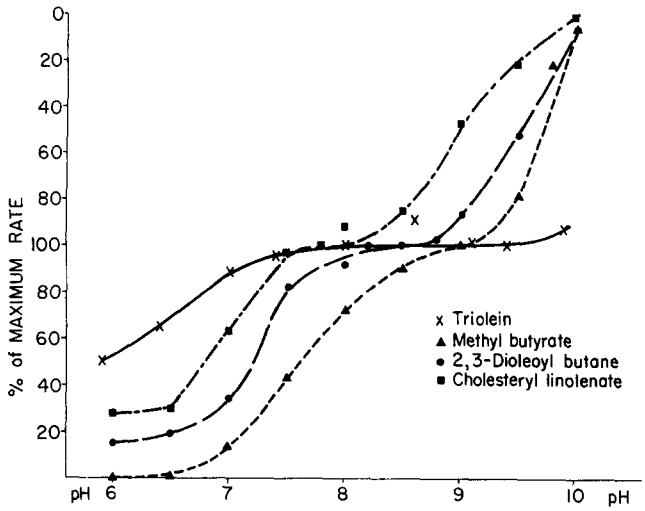

FIG. 3. Influence of the pH of the digest on the hydrolysis of various substrates by pancreatic juice. Digestion conditions were as described under Methods except for the pH, the replacement of Tris with histidine, and the addition of 1 mg of ovomucoid per 20 mg of pancreatic juice powder. Rate, μeq/min per mg of enzyme, equivalent to 100% was: methyl butyrate, 2.4; 2,3-dioleoyl butane, 0.88; cholesteryl linolenate, 0.83; triolein, 40.

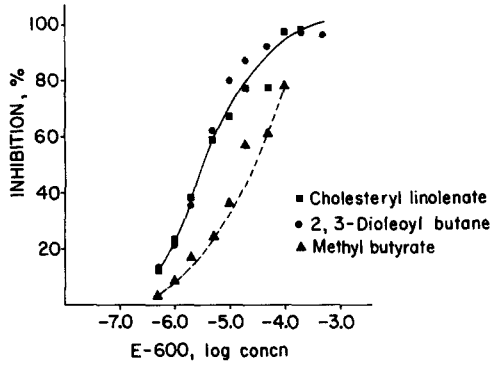

FIG. 4. Hydrolysis of various substrates by pancreatic juice that had been treated with diethyl *p*-nitrophenyl phosphate (E-600). To an aqueous solution, pH 7.0, of pancreatic juice powder, 0.6 mg/ml, the E-600 was added. After 1 hr at 25°C, hydrolytic activity was determined using the digestion conditions described under Methods. Rate, μeq/min per mg of enzyme, equivalent to 100% was: methyl butyrate, 1.7; 2,3-dioleoyl butane, 0.81; cholesteryl linolenate, 0.66; triolein, 40.

substrates was determined. The activity of the enzyme that splits triolein was essentially unaltered by the highest concentration of inhibitor that was tested (5×10^{-4} M). The greater resistance of this enzyme to E-600 has been reported earlier (19). As shown in Fig. 4, the enzymes that hydrolyzed the other substrates were very sensitive to this material, but all were not equally sensitive. A 50% inhibition of the methyl butyrate hydrolysis was given by a solution of 4×10^{-5} M E-600. Approximately one-tenth this concentration, 3×10^{-6} M, was required to cause a comparable inhibition of the hydrolysis of cholesteryl linolenate and of 2,3-dioleoyl butane.

Studies by others of the hydrolysis of water-soluble esters, like methyl butyrate, have employed digestion conditions covering a wide range of pH. To be certain that our inability to obtain hydrolysis of methyl butyrate at pH 8.0 in the absence of taurocholate was not attributable to the use of this particular pH, we attempted to hydrolyze methyl butyrate at unit intervals between pH 4.0 and 9.0. In the absence of taurocholate, no hydrolysis occurred. When taurocholate was added (0.44 mg/ml), hydrolysis took place in the pattern shown in Fig. 2.

Certain of the proteolytic enzymes of the pancreas are reported to have esterolytic activity. We were unable to find published reports of studies with proteolytic enzymes that employed substrates of the types investigated here. Therefore we attempted hydrolysis of the four model substrates using our standard digestion conditions, but with the pancreatic juice replaced by 10 mg of trypsin, α-chymotrypsin, or carboxypeptidase. None of the substrates was hydrolyzed by any of these proteolytic enzymes. Nor were these substrates hydrolyzed when 10 mg of *Crotalus adamanteus* venom replaced the pancreatic juice in the incubation mixtures.

The solubility of methyl butyrate in water is about 1.5 g/100 ml. The rate of hydrolysis of this material when it was present at a level of 0.18 g/100 ml and of 3.6 g/100 ml was determined. The results are shown in Table 3. When methyl butyrate was added at the lower concentration and thus was soluble in water, it was hydrolyzed by pancreatic juice only if taurocholate was present. This hydrolytic activity toward a water-soluble substrate in the presence of taurocholate was lost if a solution of the pancreatic juice at pH 9.0 was maintained at 40°C for 1 hr. When the level of methyl butyrate (3.6 g/100 ml) exceeded its solubility in water, the ester was hydrolyzed by pancreatic juice whether taurocholate was present or not. Maintenance of the juice at pH 9.0 before use in the

TABLE 3 HYDROLYSIS OF SOLUBLE AND INSOLUBLE METHYL BUTYRATE BY FRESH AND PARTIALLY INACTIVATED PANCREATIC JUICE

	Pancreatic Juice			
	Untreated		Treated*	
	Taurocholate Added†			
	+	−	+	−
	μeq/min per mg of enzyme			
Methyl butyrate‡				
Soluble	1.82	0	0	0
Insoluble	1.70	1.66	0.66	1.31
Triolein	6	38	2	33

* Reconstituted powder (0.6 mg/ml) maintained at pH 9.0, 40°C for 1 hr before assay.

† Where added, at a concentration of 0.44 mg/ml. Mostly taurocholate, with other bile salts as contaminants (see Materials).

‡ "Soluble," methyl butyrate at a concentration of 0.18 g/100 ml; "insoluble," methyl butyrate at a concentration of 3.6 g/100 ml.

TABLE 4 EFFECT OF TAUROCHOLATE ON THE HYDROLYSIS OF METHYL BUTYRATE BY PANCREATIC TISSUE AND JUICE*

	Tissue	Juice
	μeq butyrate released/min per g wet weight	
No taurocholate	21	0
Plus taurocholate (0.2 mM)	110	1800

* Methyl butyrate present in the digest at a concentration of 0.18 g/100 ml.

assay with the insoluble substrate resulted in only a small diminution in activity (1.31 vs. 1.66) when taurocholate was absent. However, addition of taurocholate to this system resulted in a marked decrease in the activity (0.66 vs. 1.31). The hydrolysis of triolein by both of these pancreatic juice preparations was markedly suppressed by the presence of taurocholate in the digest, but there was little loss in hydrolytic activity as the result of the initial maintenance of the enzyme solution at pH 9. These results are explainable on the basis of two enzymes being present in pancreatic juice. One splits water-soluble methyl butyrate, requires taurocholate, and is inactivated at pH 9. The other enzyme splits triolein and methyl butyrate, but the latter only when its concentration exceeds its solubility, is inhibited by taurocholate, and is stable at pH 9. These properties of this second enzyme correspond to those of glycerol-ester hydrolase (EC 3.1.1.3) (6).

As shown in Table 4, the hydrolysis by pancreatic juice of methyl butyrate at a concentration less than its solubility limit occurs only if taurocholate is present. On the other hand, this water-soluble substrate is hydrolyzed by homogenized whole pancreatic tissue in the absence of taurocholate. The addition of taurocholate to the digest containing pancreatic tissue results in an increase in the rate at which methyl butyrate is hydrolyzed. These results point to the presence in pancreatic tissue of two water-soluble ester hydrolases, one requiring taurocholate and the other being a taurocholate-independent enzyme.

DISCUSSION

There are numerous reports on the hydrolysis of a wide variety of esters by pancreatic tissue. In the studies reported here, we selected substrates that are model compounds resembling the important types of esters that might be found in the diet. The three classes of esters investigated were: the water-soluble esters of primary alcohols, e.g., methyl butyrate; the water-insoluble esters of primary alcohols, e.g., those involving the 1- and 3 positions of triolein; and the water-insoluble esters of secondary alcohols, e.g., 2,3-dioleoyl butane. The replacement of triolein by methyl oleate or of 2,3-dioleoyl butane by isopropyl oleate gave the same patterns of enzymatic activity toward esters of primary and secondary alcohols, respectively, that are reported here. However, the rate of hydrolysis of these monoesters is so much slower than the rate of hydrolysis of the di- and triesters that it was more convenient to use triolein and dioleoyl butane as the model substrates. Cholesteryl linolenate, although it is a member of the secondary alcohol group, was included because of the extensive studies by others. Moreover, the presence of the cyclopentanophenanthrene ring could modify the substrate specificity.

A number of differentiating properties reported here suggest the presence in pancreatic juice of three enzymes that bring about the hydrolysis of the classes of esters that were investigated. One of the distinguishing characteristics among these enzymes is the form in which the substrate must be present. Water-insoluble esters of secondary alcohols such as cholesterol and butane-2,3-diol must be converted by sodium taurocholate to a form that is dispersed in water, possibly as micelles. On the other hand, the hydrolysis of water-insoluble esters of primary alcohols, e.g. triolein, is inhibited if it is dispersed by means of this surface-active agent. For this substrate, under these conditions, a simple oil/water interface is required. The hydrolysis of water-soluble esters, although dependent on the presence of taurocholate, does not require either of these types of surfaces. Thus the critical micelle concentration of the taurocholate used in this system was 0.5 mg/ml (0.93 mM) (see Materials) and hence the appearance of activity at 0.2 mM taurocholate cannot be due to micellization of the methyl butyrate. Moreover, the rate of hydrolysis decreases when the level of taurocholate exceeds the critical micelle concentration. Other evidence that will be presented in a subsequent publication suggests an interaction between the taurocholate and the enzyme that splits methyl butyrate.

Sterol ester hydrolase is active in vitro only if bile salts are present (20, 21). More recently this requirement has been shown to be filled solely by the trihydroxy bile acids (22). The hydrolysis of dioleoyl butane shows a similar dependence on the presence of taurocholate. However, the quantity of taurocholate required for the maximum rate of hydrolysis of the cholesterol and butanediol esters is in excess of the critical micelle concentration. Thus, it seems possible that the taurocholate has two functions when the substrate is a water-insoluble ester of a secondary alcohol: dispersion of the substrate, and interaction with the enzyme. Vahouny, Weersing, and Treadwell (22) have suggested the formation of a specific bile acid–sterol ester hydrolase complex.

The studies reported in Table 2 offer further information as to the number of enzymes involved in the hydrolysis of these various substrates. The stability in slightly alkaline solution and instability in acidic solution of the enzyme that hydrolyzes water-insoluble esters of primary

alcohols clearly distinguish it from the enzymes that act on the other substrates. The relative stabilities at pH 4 differentiate the remaining enzymes. Thus there was an initial rapid decrease in the ability to hydrolyze dioleoyl butane and cholesteryl linolenate when the pancreatic juice was maintained at pH 4.0 for a few minutes. An extension of the time of exposure did not result in any further loss in activity. This pattern of inactivation suggests the presence of two species; one that accounts for 30–50% of the total activity is rapidly destroyed, perhaps by a rapid and irreversible dissociation at this pH; the remaining portion of the enzyme does not undergo this dissociation. The inactivation pattern of the water-soluble ester hydrolase at pH 4.0 did not show this initial drop in activity, or subsequent stability, but followed a more typical pattern of progressive inactivation as the time of exposure was increased.

The results reported here demonstrate that pancreatic juice contains at least two enzymes capable of splitting water-insoluble esters. One of these hydrolyzes esters of primary alcohols, while the other can hydrolyze esters of secondary alcohols. The presence of these two enzymes could explain Borgström's (9) observation of an increase in the hydrolysis of 2-monoglyceride to free glycerol when increasing amounts of human pancreatic juice protein are added to digests in vitro. This he interpreted to mean that the splitting of 2-monoglyceride by lipase is direct, and does not go by way of an intermediate step of isomerization to 1-monoglyceride. Since his digests contained taurodeoxycholate, this hydrolysis could be due to the same enzyme that has been shown in our experiments to split esters of secondary alcohols. This is not the enzyme commonly referred to as lipase.

It has long been recognized that essentially all tissues contain an enzyme capable of hydrolyzing water-soluble esters. For example, Sarda and Desnuelle (6) have detected such an enzyme in pancreatic tissue and demonstrated that its activity does not demand the presence of bile salts. This ubiquitously distributed enzyme is now designated as carboxylic-ester hydrolase (EC 3.1.1.1). The presence of this enzyme in pancreatic tissue is confirmed by the results presented in Table 4. However, pancreatic tissue contains a second enzyme that splits water-soluble esters only if taurocholate is present. The results presented in Table 3 demonstrate that only this second enzyme, the one dependent on taurocholate, is present in pancreatic juice; carboxylic-ester hydrolase (EC 3.1.1.1) activity could not be found. This explains the apparent conflict in the reports by Borgström (12) and Sarda and Desnuelle (6). Whereas the former could find no esterase activity in pancreatic juice, the latter demonstrated its presence in pancreatic tissue.

In many of our experiments we used techniques similar to those reported by Fodor (23). However, pancreatic tissue was the source of the enzymes in his work. Since at least one of these enzymes, carboxylic ester hydrolase, does not appear to be secreted in the pancreatic juice, it is difficult to compare his results with ours. An additional problem in comparing enzymes in pancreatic tissue with those secreted in the juice is indicated by the observation that the enzyme in the tissue that splits water-insoluble esters of primary alcohols has a molecular weight four times greater than that found in the juice (24).

The results presented here demonstrate that pancreatic juice, besides its well-known ability to split water-insoluble esters of primary alcohols and cholesterol esters, can also split esters of secondary alcohols and water-soluble esters. How many enzymes are responsible for these reactions and which, if any, are identical with known enzymes? We had hoped that the experiments described here would answer this question. Unfortunately they do not. It is probable that the answer will be known only when the enzymes have been obtained from pancreatic juice, not tissue, in pure form and tested on the appropriate substrates under proper conditions. Although an unequivocal answer is not possible at this time, we believe it likely that three enzymes of pancreatic juice are responsible for the hydrolysis of nitrogen- and phosphorus-free esters of fatty acids. The first of these is the enzyme presently known as glycerol-ester hydrolase (EC 3.1.1.3) and has the trivial name lipase. A more accurate description is that it splits water-insoluble esters of primary alcohols. The second enzyme splits esters of secondary alcohols, including cholesterol esters. This enzyme, presently known as sterol-ester hydrolase (EC 3.1.1.13), has the trivial name cholesterol esterase. Here too this enzyme does not have the specificity its name would indicate; rather it appears to have little substrate specificity. Evidence that will be reported subsequently shows this enzyme to be capable also of splitting esters of primary alcohols. The third enzyme hydrolyzes water-soluble esters only in the presence of taurocholate. This enzyme is not identical with carboxylic-ester hydrolase (EC 3.1.1.1). The specificity of these enzymes is related not only to the substrate itself but also to the physical form in which the substrate is presented to the enzyme.

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