Optimization and validation of assays to estimate pancreatic esterase activity using well-characterized micellar solutions of cholesteryl oleate and tocopheryl acetate

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Summary Studies have been carried out to determine the maximal solubilization of cholesteryl oleate and tocopheryl acetate within mixed bile salt-fatty acid micelles and to establish reproducible assays for pancreatic esterase activity using these micellar substrates. At pH 8.5, using 30 mM sodium taurocholate and 10 mM oleic acid, reproducible micellar solutions of the esters could be prepared giving micellar concentrations of 0.4 mM and 0.1 mM for tocopheryl acetate and cholesteryl oleate, respectively. Conditions were then optimized to estimate pancreatic esterase activity using these micellar-solubilized substrates. Maximal activity was obtained at pH 8.5 with 2–4 mM oleic acid and 15–30 mM sodium taurocholate, and gave coefficients of variation for the assays of 7.4% and 20.2% using tocopheryl acetate and cholesteryl oleate, respectively, as substrates. Micellar-solubilized cholesteryl oleate and tocopheryl acetate, together with a non-micellar system using p-nitrophenyl acetate, were used to estimate esterase activity in human duodenal aspirates and rat pancreatic homogenates. Esterase activity in children with cystic fibrosis was greatly reduced and paralleled tryptic and pancreatic lipase activity, which suggested that the esterase activity was pancreatic in origin. The results of this study, therefore, provide a basis for future investigations concerned with the hydrolysis and absorption of dietary esters.—**Mathias, P. M., J. T. Harries, and D. P. R. Muller.** Optimization and validation of assays to estimate pancreatic esterase activity using well-characterized micellar solutions of cholesteryl oleate and tocopheryl acetate. *J. Lipid Res.* 1981. **22:** 177–184.

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Two classes of enzymes (hydrolases) are able to hydrolyze carboxylic esters. These are the lipases which act at the water/lipid interface of emulsions formed by water-insoluble substrates and the esterases that require their substrates to be water-soluble or solubilized in micellar solutions (1). Many important dietary constituents are carboxylic esters such as triglyceride, cholesteryl esters, and esters of the fat soluble vitamins, and these are hydrolyzed in the lumen of the small intestine prior to uptake by the enterocyte. Triglyceride is hydrolyzed to monoglyceride and free fatty acids by pancreatic lipase (2), whereas pancreatic esterase (also known as cholesterol esterase, cholesteryl ester hydrolase, sterol ester hydrolase, or carboxylic ester hydrolase) appears to be the principal hydrolytic enzyme for cholesteryl esters (3) and tocopheryl (vitamin E) esters (4).

In order to be able to detect, estimate, and separate lipase and esterase activities, it is essential to use appropriate substrates and conditions (5). The most commonly used assays for pancreatic lipase activity use either an emulsion of trioleoyl glycerol (5) or tributyryl glycerol (6). The former substrate, being a naturally occurring long chain triglyceride, fulfills all the necessary criteria for a substrate for pancreatic lipase, but the shorter chain length tributyryl glycerol is especially convenient as it can be easily dispersed in water by shaking or stirring to give

Abbreviations: CO, cholesteryl oleate; TA, tocopheryl acetate; pNPA, p-nitrophenyl acetate; TAME, p-tosyl-l-arginine methyl ester; TLC, thin-layer chromatography.

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a maximal surface area for lipase to act on without the addition of emulsifying agents. In addition butyric acid, the product of the reaction, is relatively water-soluble which facilitates its partition into the aqueous phase, thereby preventing inhibition of the enzymatic reaction at the water/lipid interface.

Various methods have been used to measure esterase activity. The simplest and most convenient methods use unnatural substrates such as p-nitrophenyl acetate (pNPA) which can be easily solubilized (7). Natural substrates such as cholesteryl esters have also been used as substrates and have been dispersed in various ways, for example, in ethanolic, albumin-stabilized, and bile salt dispersions (8–10). Other workers have prepared the substrate in a more physiological form by solubilizing it in mixed micellar solutions (3, 11, 12). None of these studies have, however, strictly defined the mixed micellar system used, especially with regard to the exact micellar concentration of the substrate presented to the enzyme, nor have they discussed the optimization, validation, and reproducibility of their assay systems.

This communication presents studies on the solubilization of cholesteryl oleate (CO) and tocopheryl acetate (TA) within mixed bile salt-fatty acid micelles. Assays using these micellar substrates were then optimized and validated and used together with pNPA to estimate esterase activity in human duodenal juice and rat pancreatic homogenate.

MATERIALS

Oleic acid, DL-alpha-tocopherol, DL-alpha-tocopheryl acetate, cholesterol, cholesteryl oleate, and pNPA were obtained from the Sigma Chemical Company Limited and were all 99% pure. Sodium taurocholate was prepared by the method of Norman (13) as modified by Hofmann (14). Its purity was determined enzymatically (15) after TLC (amyl acetate–n-propanol–propionic acid–water 4:3:2:1 (v/v)) (16), and found to be 94% pure with sodium taurodeoxycholate as the major impurity. D-alpha-[5-methyl-^{3}H]tocopherol, [4-^{14}C]cholesterol, and cholesterol [1-^{14}C]oleate were obtained from the Radiochemical Centre, Amersham. Tritiated tocopheryl acetate was prepared from the labeled free tocopherol (17). The free and esterified tocopherols and cholesterols were all more than 95% pure after TLC.

Scintillation counting was performed on an LKB Wallac 1210 beta-counter with external standardization using a dioxane-based scintillation mixture.

Human duodenal juice was collected following stimulation of the pancreatico-biliary system by a standardized test meal (18). The meal was administered via a nasogastric tube following an overnight fast, and a 2-hr postprandial collection was obtained by siphonage on ice and stored at −20°C prior to enzymatic analysis.

Pancreata from male Sprague Dawley rats weighing 250–300 g were homogenized in 10 ml of 0.15 M KCl. The homogenates were stored as 1-ml aliquots at −20°C prior to assay of enzymatic activity.

METHODS

Preparation of micellar solubilized substrates

A dispersion of cholesteryl oleate (0.5 mM), [^{14}C]-cholesteryl oleate (0.05 μCi/ml), oleic acid (4 mM) and sodium taurocholate (15 mM) in phosphate saline buffer (pH 8.5, 37.5 mM, and 75 mM in respect of phosphate and sodium ions, respectively) was prepared, by mixing on a vortex mixer and sonication (Ultrasonics Limited) for a minute at maximum power. This dispersion was then filtered to completion through a 220 nm Millipore filter under nitrogen pressure (75 psi) using a stirred ultrafiltration cell (19).

A dispersion of TA was prepared exactly as that for CO except that 30 mM sodium taurocholate was used.

The filtrates were judged to be isotropic using the criteria of Carey and Small (20). They were clear to the eye in reflected light, had an absorbance of <0.025 at 750 nm with water as a reference, and were stable for at least 48 hr. After 3 weeks storage at 4°C, the filtrates containing radiolabeled substrates were centrifuged at 40,000 rpm for 18 hr at 20°C. The contents of the tube were then divided into five equal aliquots and the radioactivity was measured. A concentration gradient was formed down the tube without any increase in radioactivity in the uppermost fraction.

The presence of micelles within the isotropic phase was confirmed by a shift in the absorption maximum of rhodamine 6G (21).

The mean concentration (n = 10) of TA in the 220 nm filtrate was 0.26 mM ± 0.02 (1 SD) and for CO (n = 10) was 0.08 mM ± 0.01, which corresponded to the solubilization of approximately 52 and 16%, respectively, of that in the dispersion. The recovery of radiolabeled taurocholate and oleate and sodium ions in the filtrate was always greater than 90%, and on ultracentrifugation a concentration gradient down the tube was produced. Filtration of the 220 nm filtrate through a 50 nm filter did not significantly alter the composition of the isotropic phase.

Assay of esterase activity

Esterase activity was determined using the isotropic phases containing CO and TA and also with pNPA as substrate. Results are expressed as nmol product liberated $\cdot min^{-1} \cdot ml^{-1}$ or $\cdot mg\ protein^{-1}$, depending on whether duodenal juice or pancreatic homogenate was used as enzyme source. Blanks (water substituted for enzyme) and quality control specimens were included with each batch of estimations.

Assays using solubilized CO were performed in triplicate by incubating 10–50 μl of enzyme source with 1 ml of the isotropic phase for 10 min at 37°C. The reaction was stopped by the addition of 200 μl of concentrated HCl and the lipids were extracted by the procedure of Galanos et al. (22, 23) which gave greater than 99% recovery of labeled CO and oleic acid. The lipids were separated by TLC (cyclohexane–ethyl acetate 60:40 (v/v)) and the fatty acid and CO zones were scraped directly into 10 ml of scintillant and counted. The substrate concentration which gave half maximal velocity was found to be 0.49 mM. The reaction was linear with time and enzyme concentration up to the hydrolysis of 30% of the substrate, which was equivalent to the release of 2.3 nmol of oleic acid per incubation.

The assay using TA was performed as above for CO except that the liberated free tocopherol was routinely estimated colorimetrically by a modification of the Emmerie-Engel reaction (24) as described previously (25). The substrate concentration at half maximal velocity was 0.16 mM and the reaction was linear with respect to time and enzyme concentration up to an absorbance of 0.7 at 534 nm; this was equivalent to the release of 5.8 nmol of tocopherol per incubation.

The method using pNPA as substrate was performed as described by Erlanson (7). The molar extinction coefficient of p-nitrophenol at pH 7.4 was found to be 1.35×10^4 and, using normal human duodenal juice, the Km of the reaction was 0.36 mM. The reaction was linear with time and enzyme concentration at least up to the liberation of 100 μmol of p-nitrophenol (equivalent to an absorbance of 1.35).

The reproducibility of the three assay procedures was assessed by assaying a control human duodenal juice on ten occasions over an 8-week period. The mean activities against CO, TA, and pNPA were 89.0, 76.9, and 3200 $nmol \cdot min^{-1} \cdot ml^{-1}$, respectively, with coefficients of variation of 20.2, 7.4, and 9.7%.

Miscellaneous estimations

Pancreatic lipase and trypsin were both estimated titrimetrically at pH 8.0 using glycerol tributyrate (6) and p-tosyl-l-arginine methyl ester (26), respectively, as substrates. Protein was estimated by the method of Lowry et al. (27) using bovine serum albumin as a standard (grade V—Sigma Chemical Company Limited).

SUBJECTS

Enzyme activities were estimated in the duodenal juice of six patients (one male and five female) with cystic fibrosis. Their ages at the time of study were 1 month to 15 years. The diagnosis of cystic fibrosis was established by the finding of sweat sodium and chloride concentrations of >70 mM on at least two occasions on volumes of sweat >100 mg. All the patients had the typical clinical features of the condition. Pancreatic extracts and antibiotics were discontinued 48 hr before administering the test meal. The enzyme activities of a child aged 4 years with biliary obstruction were also estimated. She was found to have extrahepatic biliary atresia on the basis of an operative cholangiogram and liver biopsy. The major fraction of her raised serum bilirubin was conjugated; bile pigments were undetectable in the stools but excess bile pigment was present in the urine. The control group (six male and three female) consisted of children whose ages ranged from 1 to 16 years who were being investigated for suspected malabsorption but who were subsequently proved to be normal.

RESULTS

Micellar solubilization of CO and TA

*Concentration of oleic acid (**Fig. 1**).* The effectiveness of oleic acid to form mixed micelles and in turn solubilize highly non-polar lipids such as CO and TA was investigated. The micellar concentration of both esters increased with increasing oleic acid concentrations (1–10 mM) and at a concentration of 10 mM oleic acid approximately 80% of TA and 20% of the CO was solubilized giving micellar concentrations of approximately 0.4 mM and 0.1 mM, respectively.

Variation of pH. The effect of varying the pH of the buffer on micellar solubilization was studied at bile salt concentrations of 5 and 30 mM. Varying the pH and concentration of sodium taurocholate had no effect on the solubilization of CO, the maximum micellar concentration obtained being approximately 0.1 mM. The micellar concentration of TA, however, increased with increasing pH at both bile salt concentrations. Using 5 mM sodium taurocholate, it increased from

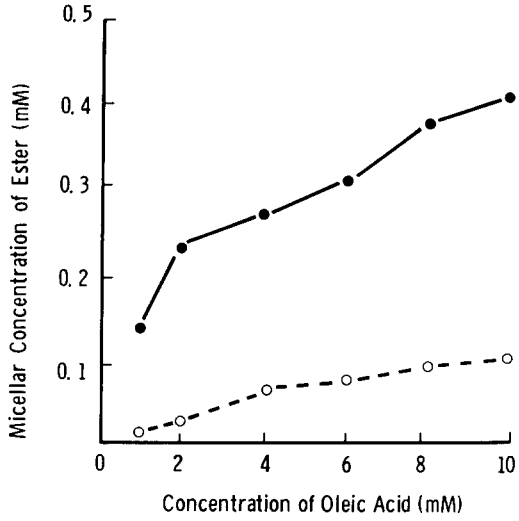

Fig. 1. Effect of increasing concentrations of oleic acid on the micellar solubilization of tocopheryl acetate (● —— ●) and cholesteryl oleate (○ – – – ○). The initial dispersions consisted of 10 mM sodium taurocholate, 0.5 mM carboxylic ester plus radiolabeled ester (0.05 μCi/ml), from 1 to 10 mM oleic acid made up in 5 ml phosphate saline buffer (pH 8.5), 37.5 mM and 75 mM in respect of phosphate and sodium ions, respectively. The micellar solution was prepared from this dispersion as described under Methods. Each point represents the mean of at least three estimations.

approximately 0.1 mM at pH 6.0 to approximately 0.25 mM at pH 7.5–8.5 and at the higher bile salt concentration it increased from approximately 0.2 to 0.25 mM.

Concentration of carboxylic ester. Increasing the concentration of the carboxylic ester in the initial dispersion from 0.1 to 2.0 mM resulted in an increasing micellar concentration, the maximum being 0.3 and 0.1 mM for TA and CO, respectively, with a 1.0 mM concentration of carboxylic ester.

Optimization of esterase activity

Having studied the solubilization of the substrate, the conditions for optimal enzyme activity were investigated.

Concentration of oleic acid (***Fig. 2***). Using human duodenal juice, it was found that for both substrates there was an optimal oleic acid concentration after which activity rapidly decreased. For TA the optimal oleic acid concentration was 4 mM and for CO, 2 mM. Use of 2 mM oleic acid in the dispersion, however, resulted in a micellar concentration of CO of only 0.025 mM (Fig. 1) which would be severely limiting. An oleic acid concentration of 4 mM was therefore chosen for both assays as this increased the micellar concentration of CO to around 0.08 mM with only a 15–20% decrease in enzymatic activity.

Variation of pH. Increasing the pH resulted in increasing enzymatic activity with both enzyme sources and substrates. With human duodenal juice as enzyme source, a plateau of activity was reached at pH 8.0. With CO as substrate, there was only trace activity at pH 6.0 which rose to 40 nmol·min^{-1}·ml^{-1} juice at pH 8.0. The activity against TA increased from 12 to 60 nmol·min^{-1}·ml^{-1} over the same pH range. With rat pancreatic homogenate there was increasing activity over the whole pH range studied (6.0 to 8.5); the activity with CO as substrate increased from 5.4 to 8.5 nmol·min^{-1}·mg protein^{-1}, and with TA from 2.0 to 6.5 nmol·min^{-1}·mg^{-1}. There was no spontaneous hydrolysis at alkaline pH, blanks (water substituted for enzyme) being used throughout. A pH of 8.5 was therefore routinely used.

Concentration of sodium taurocholate (***Fig. 3***). Increasing the concentration of the bile salt from 5 to 30 mM resulted in increasing enzymatic activity against both CO and TA with both human duodenal juice and rat pancreatic homogenate. A bile salt concentration of 30 mM was routinely used for the TA assay. However, at this concentration, 90% of the CO was hydrolyzed and thus 15 mM sodium taurocholate was used in the CO assay, giving approximately 30% hydrolysis with a normal human duodenal juice, although 90% of the CO substrate was still hydrolyzed when rat pancreatic homogenate was used as enzyme source. All the reaction rates were linear under the stated conditions except for CO and rat pancreatic homogenate where the rate was curvilinear.

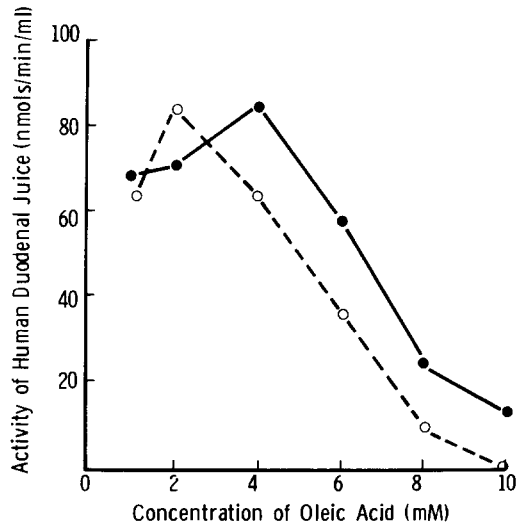

Fig. 2. Effect of increasing oleic acid concentrations on activity of human duodenal juice against micellar-solubilized tocopheryl acetate (● —— ●) and cholesteryl oleate (○ – – – ○). The micellar-solubilized substrates were prepared and the assays run as described under Methods using 20 μl of control human duodenal juice.

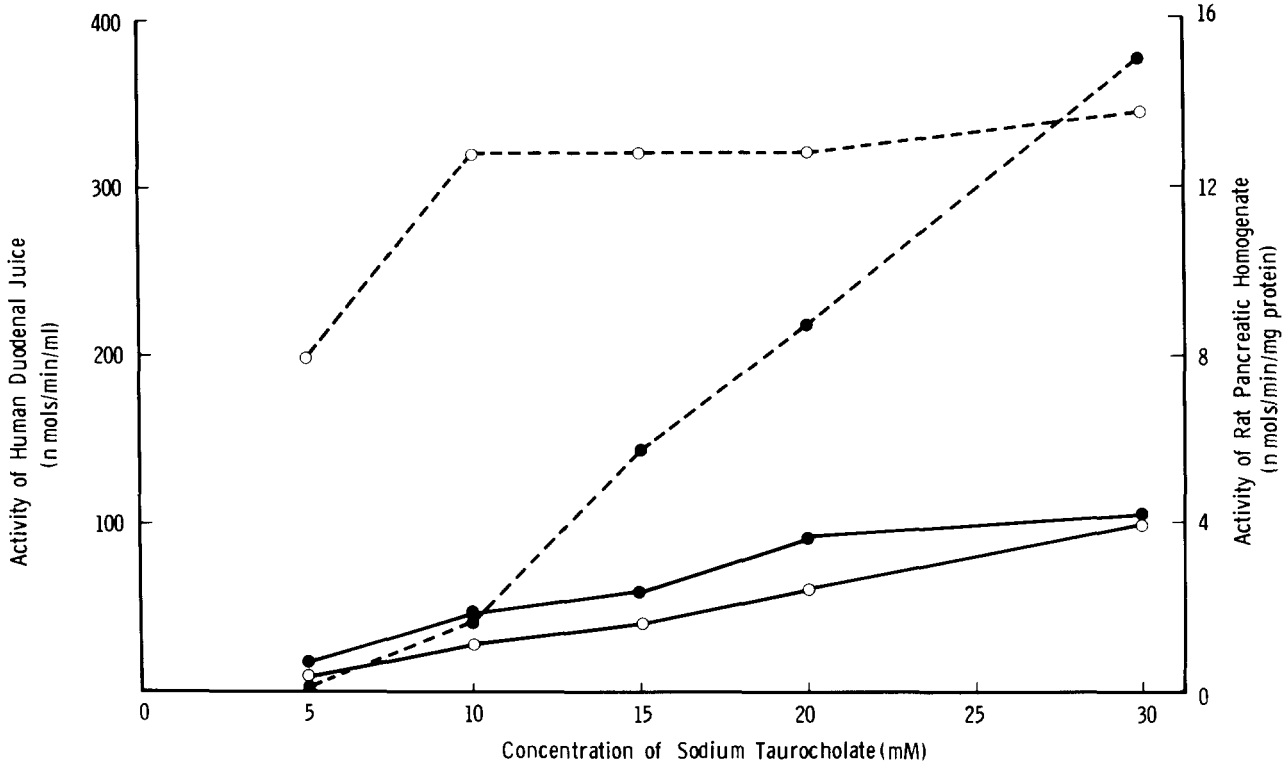

Fig. 3. Effect of increasing concentrations of sodium taurocholate on activity against micellar-solubilized tocopheryl acetate (——) and cholesteryl oleate (– – –) using either 20 μl control human duodenal juice (●) or 50 μl rat pancreatic homogenate (○). The micellar-solubilized substrates were prepared and the assays run as described under Methods.

Enzyme activity in human duodenal juice and rat pancreatic homogenate

Duodenal juice from nine control children, six children with cystic fibrosis, and one child with intrahepatic biliary atresia was assayed for esterase activity against pNPA and TA, pancreatic lipase, and tryptic activity (**Table 1**). The patients with cystic fibrosis had greatly reduced activities of all the enzymatic activities studied, whereas the activities of the child with biliary atresia fell within the control range except for esterase activity against TA, where the activity of 25.9 nmol·min^{-1}·ml^{-1} was just below the normal range of 33.4–166.3 nmol·min^{-1}·ml^{-1}.

The rat pancreatic homogenate showed appreciable esterase activity against all three substrates, the mean (±1 SD) of the activities for six rats being 140 ± 30, 3.15 ± 0.89, and 15.5 ± 5.4 nmol·min^{-1}·mg protein against pNPA, TA, and CO, respectively.

DISCUSSION

In these studies, substrates for the estimation of esterase activity were prepared by solubilizing CO and TA within mixed bile salt – fatty acid micelles. A pure solution of sodium oleate has a pKa of 8.0 but, under

TABLE 1. The ranges and means (±1 SD) of enzyme activities in a group of nine control subjects, six children with cystic fibrosis, and a child with biliary atresia

	Esterase				Lipase		Trypsin	
	pNPA Mean ± 1 SD	Range	TA Mean ± 1 SD	Range	Mean ± 1 SD	Range	Mean ± 1 SD	Range
	nmol·min^{-1}·ml^{-1}(×10^3)		*nmol·min^{-1}·ml^{-1}*		*μeq·min^{-1}·ml^{-1}*		*μeq·min^{-1}·ml^{-1}*	
Controls (9)	2.9 ± 1.8	1.0–6.3	75.2 ± 44.4	33.4–166.3	690 ± 399.6	280–1320	42.1 ± 20.4	19.4–83.3
Cystic fibrosis (6)	0.2 ± 0.3	0.04–0.5	1.1 ± 1.2	0.4–3.2	7.2 ± 2.7	4–10	1.7 ± 1.0	1.0–3.8
Biliary atresia (1)	1.8		25.9		360		28.7	

the conditions of this study (sodium taurocholate 15 or 30 mM; NaCl 75 mM; pH 8.5), a lower pKa of about 6.5 would be expected (28–30) which should result in the oleate being totally ionized and solubilized within the bile salt micelle. This was confirmed first by recovery and ultracentrifugal studies using labeled sodium oleate where greater than 90% was recovered in the isotropic phase and a concentration gradient was produced down the tube as described by others (31); and second by the fact that increasing concentrations of oleate in the dispersions resulted in increasing concentrations of both TA and CO in the isotropic phase. The difference in solubility of TA and CO reflected their different polarities; CO being a non-polar lipid and TA, with only a two-carbon fatty acid, a class I polar lipid (30). This is in agreement with our previous findings where, under virtually identical conditions, free tocopherol at a concentration of 0.5 mM could be fully incorporated into mixed micelles (32). Similarly Borgström (19), using a bile salt-glyceride dispersion, found the partition ratio between the micellar and emulsion phases for CO to be only 13% of that of free cholesterol.

The effect of varying the pH and bile salt concentration on the solubilization of TA can be explained by changes in the ionization of sodium oleate (29). Acid conditions would be expected to result in a reduced percentage ionization and solubilization of oleic acid and, in turn, reduced solubilization of TA. At alkaline pH values however (i.e., well above the pKa for oleic acid), the pH should not affect micellar solubilization of the ester. With 5 mM taurocholate the pKa of the system would be higher than with 30 mM and thus, using the lower concentration of bile salt at acid pH values, less ionization and solubilization of oleic acid and reduced solubilization of the substrate would be expected. The micellar concentrations of TA responded as predicted, but altering the pH and bile salt concentrations had little effect on the solubilization of CO. These differences between TA and CO can most likely be explained by their different polarities, as non-polar lipids such as CO display different characteristics in mixed micellar solutions compared to more polar lipids (33).

The observation that the composition of the isotropic phase was not significantly altered when passed through a 220 or 50 nm filter suggested that the particle sizes present in the original dispersion were limited to species less than 50 nm and greater than 220 nm. The filtrate from both filters was judged to be isotropic by the criteria of Carey and Small (20) and the presence of micelles was confirmed by a shift in the absorption maximum of rhodamine 6G (21). There was, however, the possibility that this isotropic phase contained a microemulsion (particles of 10–200 nm). Microemulsions behave like emulsions in that they require energy for formation, are unstable, and tend to separate into two phases (34). However, when the isotropic phase was allowed to stand for 3 weeks and then spun at 40,000 rpm for 18 hr, no increase in radiolabeled substrate was found in the top fraction suggesting that an oily phase or microemulsion was not present. Using similar artificial mixtures of lipids and methods of preparation, other workers have also come to the conclusion that their solutions were micellar (19, 20, 31).

Having standardized and characterized the micellar solubilization of the ester, optimal conditions for enzymatic activity were investigated. The optimal pH using either human duodenal juice or rat pancreatic homogenate against both TA and CO was 8.0 to 8.5. Reports for the pH optimum of rat pancreatic esterase have varied, probably as a result of the different methods used to disperse the substrates. Hyun et al. (35), using cholesteryl ester dispersed in bile salt-phospholipid micelles, found the pH optimum to be between 6.6–7.0; whereas pH optima of between 8.0 and 9.0 have been demonstrated by a number of workers using non-micellar dispersions of cholesteryl ester (10, 36–38).

The observation of an optimal oleic acid concentration for activity against both TA and CO suggests that oleic acid inhibits enzyme activity at concentrations greater than 2–4 mM despite increasing the micellar concentration of substrate.

In both assay systems and with either duodenal juice or rat pancreatic homogenate as enzyme source, esterase activity increased with increasing bile salt concentrations. This was not solely the result of increased micellar concentrations, as increasing the bile salt concentration resulted in increasing enzyme activity against CO without any increase in micellar solubilization of the substrate. Similarly, we and others have consistently shown that bile salts, but not other detergents which are able to solubilize lipid esters, are necessary for optimal activity (4, 9, 39). There is, however, conflicting information on the specificity of this stimulation. Hyun et al. (35) and Vahouny, Weersing, and Treadwell (40) found that only cholic acid and its conjugates were effective in activating the enzyme, whereas we and others (4, 12, 38) have found that the dihydroxy bile salts had stimulatory effects similar to the trihydroxy bile salts.

Using the optimized assays for activity against TA and CO, the concentration giving half maximal velocity were approximately one-half and six times the substrate concentrations, respectively. From theoretical

considerations this was not ideal, as substrate concentrations would quickly become limiting especially with CO as substrate. This is presumably reflected in the reproducibility of this assay which has a coefficient of variation almost three times that of the TA assay. This problem can also be seen by comparing the activities presented in Figs. 2 and 3 where, using the same human duodenal juice under the same conditions, activities against CO were 66 and 142 $nmol \cdot min^{-1} \cdot ml^{-1}$ whereas against TA they were 85 and 100 $nmol \cdot min^{-1} \cdot ml^{-1}$. The difference in activity against CO reflected differences in both the percentage solubilization and hydrolysis of the substrate in the two studies and demonstrated the problems working with micellar solutions of highly non-polar substrates and the need for defining the composition and physical state of the substrates used.

Esterase activities in the children with cystic fibrosis paralleled their grossly reduced tryptic and pancreatic lipase activities, which strongly suggests that the activity assayed against the three substrates was pancreatic in origin. The in vitro enzymatic activities of the child with biliary atresia were normal with the exception of the esterase activity against TA which was slightly reduced. However, in vivo, the absence of bile salts would result in a failure to solubilize and thus absorb highly non-polar lipids such as cholesterol and tocopherol, and their esters (41, 42); essential cofactors for optimal esterase activity would also be lacking. This would explain the observation of extremely low serum concentrations of vitamin E in such patients even after the administration of massive doses of the vitamin in a water-miscible form (43).

In conclusion therefore, this study has validated and optimized the necessary conditions for the assay of pancreatic esterase(s) active against TA and CO, and provided a methodological basis for future investigations concerned with the hydrolysis and absorption of dietary esters.

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