Digestion in vitro of erythritol esters by rat pancreatic juice enzymes

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Abstract The mechanism of the digestion of erythritol esters was determined using rat pancreatic juice and purified pancreatic lipase (EC 3.1.1.3). Conditions of hydrolysis were used that would selectively activate or inactivate nonspecific lipase or lipase. It was shown that erythritol tetraoleate was hydrolyzed by nonspecific lipase but not by lipase. The initial digestion product was a triester, predominantly erythritol-1,2,3-trioleate. Thus, nonspecific lipase preferentially hydrolyzed the ester of a primary alcohol. In contrast to the results obtained with the tetraester, lipase could remove a fatty acid from the triester but the resulting erythritol-2,3-dioleate was not hydrolyzed by lipase. The selectivity of this hydrolysis and the inability to hydrolyze the diester are attributed to the known specificity of this enzyme to act only on esters of primary alcohols. Nonspecific lipase completely hydrolyzed erythritol tetraoleate to free erythritol in a stepwise manner. The relative rates of these reactions were

$$\text{tetraester} \xrightarrow{0.1} \text{triester} \xrightarrow{1.1} \text{diester} \xrightarrow{0.8} \text{monoester} \xrightarrow{0.1} \text{erythritol}$$

Because of the specificity of pancreatic lipase and the lack of specificity of nonspecific lipase it is likely that this latter enzyme is the primary agent for the hydrolysis of erythritol esters in the intact animal.

Supplementary key words erythritol tetraoleate · erythritol partial esters · thin-layer and column chromatography · pancreatic lipase · pancreatic nonspecific lipase · synthesis of erythritol-1,2,3-trioleate

We have reported (1) that the complete esters of erythritol and adonitol are not hydrolyzed by pancreatic lipase (EC 3.1.1.3). However, these compounds are hydrolyzed by another enzyme which is present in pancreatic juice. We have suggested the trivial name nonspecific lipase for this enzyme (2).

Abbreviations: TLC, thin-layer chromatography.

Although pancreatic lipase has been isolated, nonspecific lipase is not available in pure form. It is possible to distinguish between the activities of these two enzymes because nonspecific lipase has an absolute requirement for bile salts and is rapidly inactivated by proteolytic enzymes, whereas lipase is inhibited by bile salts under the conditions of digestion employed here and is relatively more resistant to inactivation by proteolysis (1, 3). This report is a further elucidation of the mechanism by which erythritol esters are hydrolyzed by pancreatic juice. For this, we used purified lipase and the selective inactivation and activation of the two enzymes in pancreatic juice to determine their ability to hydrolyze various esters of erythritol.

MATERIALS AND METHODS

[U-^{14}C]Erythritol was purchased from Amersham/Searle, Arlington Heights, Ill. The radiochemical purity was determined by TLC, using $CHCl_3$–MeOH–H_2O 70:30:4 to develop the chromatogram. 96% of the radioactivity was associated with a single spot that had an R_F of 0.5. The remaining activity was uniformly dispersed behind this spot.

Oleic acid was isolated from olive oil by low temperature solvent crystallization and urea adduction; it was 98% pure as judged by gas–liquid chromatography. A portion of the oleic acid was converted to the methyl ester with H_2SO_4 as the catalyst. Oleoyl chloride was obtained by reacting oleic acid with oxalyl chloride (4).

[U-^{14}C]Erythritol tetraoleate was prepared by base-catalyzed interesterification of 0.01 mole of the labeled erythritol and 0.06 mole of methyl oleate (4). The tetraester was obtained by crystallization from 20 vol of acetone at −35°C with further purification by chromatography on a Florisil column; the complete ester eluted with ethyl ether–hexane 15:85. TLC showed the sample

to be homogeneous. The final product had a specific activity of 4200 cpm/μmole.

[U-^{14}C]Erythritol-1,2,3-trioleate was prepared by acylation of erythritol in which one primary hydroxyl group had been blocked with trityl. The procedures used, except for the removal of the trityl group, have been reported by Barker (5). Chlorotriphenylmethane, trityl (Matheson Coleman & Bell, Norwood, Ohio), was purified by crystallization from petroleum ether containing acetyl chloride (6). 0.010 mole of [U-^{14}C]erythritol in 50 ml of dry pyridine was allowed to react with 0.011 mole of chlorotriphenylmethane for 24 hr at 27°C. 0.04 mole of oleoyl chloride was then added and the sample was maintained for an additional 24 hr at 27°C. The reaction mixture was diluted with 100 ml of ethyl ether and washed with water three times. The bulk of the ether was removed under a stream of N_2 and the residue was steam-deodorized for 30 min at 100°C and 1 mm Hg pressure. The sample was charged to a 70-g Florisil column, and the tritylated erythritol triester was eluted with 250 ml of ethyl ether–petroleum ether 10:90. The trityl group was removed with a minimum of isomerization by passing this compound through a column of 70 g of acid-washed Florisil (Supelco, Bellefonte, Pa.). Before use, the column was washed with 50 ml of petroleum ether that had been sparged for 1 min with anhydrous HCl. The sample was added to the column in 50 ml of petroleum ether. The first elution was with 300 ml of ethyl ether–petroleum ether 10:90 to remove trityl chloride and any erythritol tetraoleate that might have been formed. This was followed by 200 ml of ethyl ether–petroleum ether 20:80. The final wash with 200 ml of ethyl ether eluted the erythritol-1,2,3-trioleate. The separation was monitored by TLC. The yield was 2.4 g, 26%. The predominance of one compound can be seen on the photographs of the TLC plates (Fig. 1, lane *5*, and Fig. 2, lane *4*). Identification of the structure of this compound was on the basis of the positions of the free and hydrogen-bonded OH stretching frequencies in the 3600 to 3400 cm^{-1} region of their infrared spectra (7).

Since the 1- and 4-positions of erythritol are identical, as are the 2- and the 3-positions, there are two possible positional isomers of the triester, four of the diester, and two of the monoester. Esterification of erythritol with an amount of a single fatty acid that is insufficient to esterify all of the hydroxyl groups will result in the formation of some tetraester as well as all of these possible isomers in a random fashion. Such a random mixture was prepared by reacting 0.01 mole of erythritol and 0.04 mole of methyl oleate with sodium methoxide as the catalyst. Thin-layer chromatograms of the resulting mixture of esters after removal of the residual methyl oleate by crystallization from 10 vol of acetone at −35°C are shown in Fig. 1, lane *3*, and Fig. 2, lane *2*. Erythritol tetraoleate is the most mobile compound (Fig. 1, lane *1* vs. *3*). Identification of several of the remaining components in the random mixture was based on primary alcohols being more reactive than secondary alcohols, the R_F of the compound, and infrared spectroscopy of the isolated compound. Thus, the 1,2,3-triester was identifiable by its mobility relative to that of the synthetic reference material (Fig. 1, lane *5* vs. *3*, and Fig. 2, lane *4* vs. *2*). The spot preceding this was assigned the 1,2,4-triester structure on the basis of its similar, although slightly greater, mobility and its presence in a relatively larger quantity, which would be expected because of the greater reactivity of primary hydroxyl groups. Separation of the various ester classes of the randomized mixture was accomplished on a silica gel column with successive small fractions of the effluent being collected and monitored by TLC (petroleum ether–ethyl ether–acetic acid 60:40:0.05). The column was washed first with ethyl ether–benzene 15:85, which eluted the tetra- and triesters. The mixture of diesters then eluted in two fractions with ethyl ether–benzene 50:50 as the solvent. One of the diesters was ob-

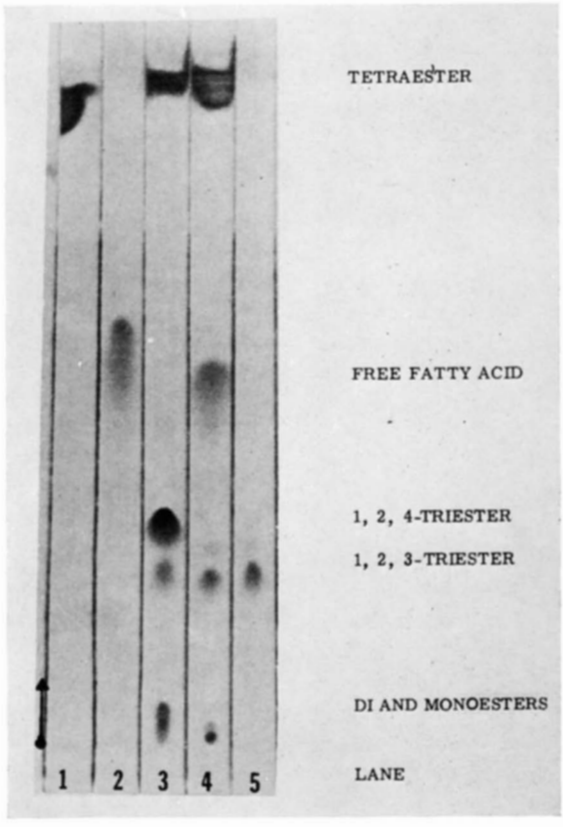

FIG. 1. Thin-layer chromatogram (petroleum ether–ethyl ether–acetic acid 75:25:0.5). Lane *1*, erythritol tetraoleate; lane *2*, oleic acid; lane *3*, products of base-catalyzed rearrangement of methyl oleate and erythritol; lane *4*, products of erythritol tetraoleate digestion by pancreatic juice in the presence of sodium taurocholate; lane *5*, synthetic erythritol-1,2,3-trioleate.

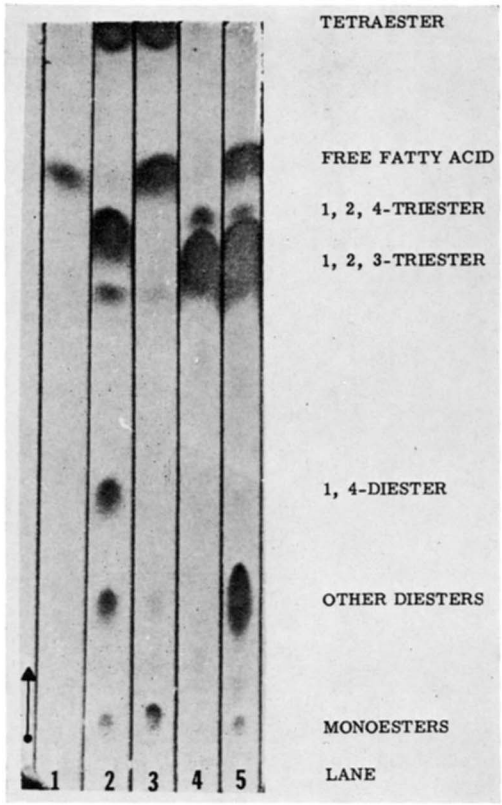

FIG. 2. Thin-layer chromatogram (petroleum ether–ethyl ether–acetic acid 60:40:0.5). Lane *1*, oleic acid; lane *2*, products of base-catalyzed rearrangement of methyl oleate and erythritol; lane *3*, products of erythritol tetraoleate digestion by pancreatic juice in the presence of sodium taurocholate; lane *4*, synthetic erythritol-1,2,3-trioleate; lane *5*, products of erythritol-1,2,3-trioleate digestion by pancreatic juice in the absence of sodium taurocholate.

tained from the first fraction by crystallization from hexane at 0°C. It was identified by infrared spectroscopy as the 1,4-diester. The spot on the chromatogram (Fig. 2, lane *2*) labeled "Other Diesters" was not resolved into its individual components. Based on R_F and the relative reactivity of the hydroxyl groups of erythritol, it probably consisted of the 1,2- and 1,3-diesters. This spot would contain also the 2,3-diester. However, in this random mixture of esters, smaller amounts of it would be present because of the lesser reactivity of secondary hydroxyl groups. The monoesters were not resolved or identified.

Sodium taurocholate that was purchased from Maybridge Chemical Co., Launceston, Cornwall, U.K., was shown to be at least 98% pure by TLC (8).

Rat pancreatic juice free of bile was collected at 4°C. This material was freeze-dried and reconstituted to 6 mg/ml in 0.01 M histidine, pH 7.0, on the day it was used (2). This preparation contains both lipase and nonspecific lipase. In some instances the nonspecific lipase was inactivated by chymotrypsin (1). Lipase (EC 3.1.1.3) was isolated from rat pancreatic juice using the method of Vandermeers and Christophe (9). The purified enzyme had an activity of 2900 units/mg of protein as measured by the method of Marchis-Mouren, Sarda, and Desnuelle (10).

The conditions for enzymatic hydrolysis were those employed previously (1). The quantities of substrate and of enzyme used are given in the figures. Also, as indicated, 200 mg of sodium taurocholate, 37 mM, was added to certain of the digests. This concentration completely inhibits lipase (1), and as a consequence the hydrolysis observed in the presence of this bile salt is due to nonspecific lipase. The digest was maintained at pH 9 with the aid of a pH stat by the addition of 0.02 N KOH. Thus, the quantity of alkali required for this purpose measured the amount of free fatty acid produced. At the end of the selected period of digestion, hydrolysis was terminated by adjusting the digestion mixture to approximately pH 2 by adding 1 ml of 25% (v/v) aqueous HCl. The acidified digestion mixture was transferred to a separatory funnel with the aid of 20 ml of ethyl ether–ethanol 10:1 and 10 ml of water was added. The aqueous phase was extracted three times with 30-ml portions of ethyl ether. These extracts were combined, washed with water three times, and evaporated to about 2 ml. The original aqueous phase and the water washes were combined and the volume was measured. Preliminary studies established that there was complete recovery of added free erythritol in the aqueous phase with no contamination by erythritol esters or free fatty acid; these were quantitatively recovered in the ether phase.

The relative amounts of complete and partial erythritol esters in the ether phase were determined by radioassay of fractions separated by TLC. 20 μl of the concentrated ether phase, containing free fatty acid and erythritol esters with an activity of about 2000 cpm, was spotted on a TLC plate of silica gel G. The plate was developed with petroleum ether–ethyl ether–acetic acid 60:40:0.5; an example of such a separation is shown in Fig. 2, lane *3*. The compounds were located by brief exposure to iodine, and the areas were outlined. After the iodine had sublimed, the areas corresponding to the free fatty acid and the mono-, di-, tri-, and tetraesters were transferred to vials for radioassay.

The amount of free erythritol was determined by radioassay of 0.5-ml portions of the aqueous phase. Counting of this phase and of the lipid fractions was done on a Packard Tri-Carb liquid scintillation counter (1).

To determine the amount of erythritol esters present after digestion, we subtracted the μmoles of free erythritol found in the aqueous phase from the μmoles of erythritol substrate initially present. The μmoles of the individual esters then was calculated from the percentage distribution of these isomers as described above. Reaction rates are reported as μmoles/min/mg of enzyme preparation.

RESULTS AND DISCUSSION

The chromatogram of the products resulting from the hydrolysis for 30 min of 85 μmoles of erythritol tetraoleate by 12 mg of pancreatic juice solids in the presence of sodium taurocholate is shown in Fig. 2, lane *3*. The values obtained after various periods of digestion are shown in Fig. 3. There was a linear decrease in the amount of tetraester over the course of the digestion. Similarly, there was a constant increase of free fatty acid. At all times, only small amounts of di- and triesters were present. The amount of monoester increased and then became constant at approximately 17 μmoles. Complete hydrolysis to free erythritol occurred at a constant rate (0.12 μmole/min/mg) after 20 min of digestion. Since the free erythritol resulted from the splitting of monoester, the rate of hydrolysis of monoester was the same as the rate of appearance of free erythritol. Moreover, because the amount of monoester was constant after 20 min, the reaction which was the source of monoester, namely the hydrolysis of diester, at that time must have been at the same rate as the hydrolysis of the monoester, 0.12 μmole/min/mg. Since all of these rates of hydrolysis are the same, approximately 0.12, the first of these reactions, tetraester to triester, could be rate limiting.

That the hydrolysis of the tetraester is the rate-limiting step is supported by the evidence presented in Fig. 4. The conditions used in this experiment were the same as those in the preceding one except that the initial substrate was erythritol-1,2,3-trioleate. The quantity of pancreatic juice solids was reduced to one-sixth of that used in the first experiment so as to have measurable quantities of the various esters present at the end of 30 min. The pattern seen is that of a stepwise degradation of triester to diester to monoester to erythritol with free fatty acid released at each step. The rates at which the triester and monoester were split can be obtained from this figure, and the rate of hydrolysis of the diester, once its level became constant, would be equal to the sum of the rates of accumulation of monoester and of free erythritol. These rates are triester $\xrightarrow{1.1}$ diester $\xrightarrow{0.85}$ monoester $\xrightarrow{0.15}$ erythritol. The rapid hydrolysis of the tri- and diesters (Fig. 4) relative to that of the tetraester (Fig. 3) accounts for the failure of these two partial esters to accumulate in the digest where the initial substrate was erythritol tetraoleate. The rate of hydrolysis of the monoester, as measured by the appearance of free erythritol, was approximately the same regardless of whether the initial substrate was the tetraester or the triester.

The studies that we reported earlier demonstrated that lipase, glycerol ester hydrolase, cannot hydrolyze erythritol tetraoleate (1). Thus, three different preparations of this enzyme, namely, lipase, pancreatic juice that had been treated with chymotrypsin in order to inactivate nonspecific lipase and then used in the presence or absence of taurocholate, and untreated pancreatic juice

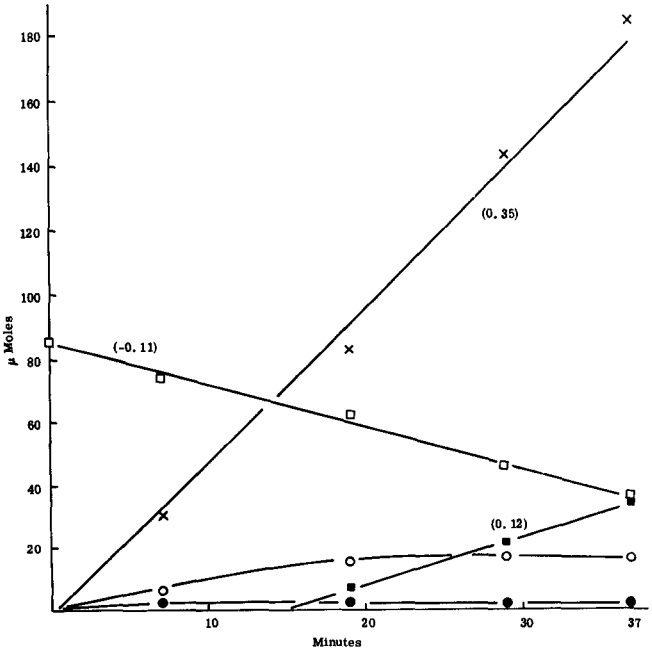

FIG. 3. Hydrolysis products of erythritol tetraoleate formed by the action of 12 mg of rat pancreatic juice solids in the presence of sodium taurocholate. Values in parentheses are μmoles/min/mg of solids. Tetraester, □; di- and triesters, ●; monoester, ○; free erythritol, ■; free fatty acid, ×.

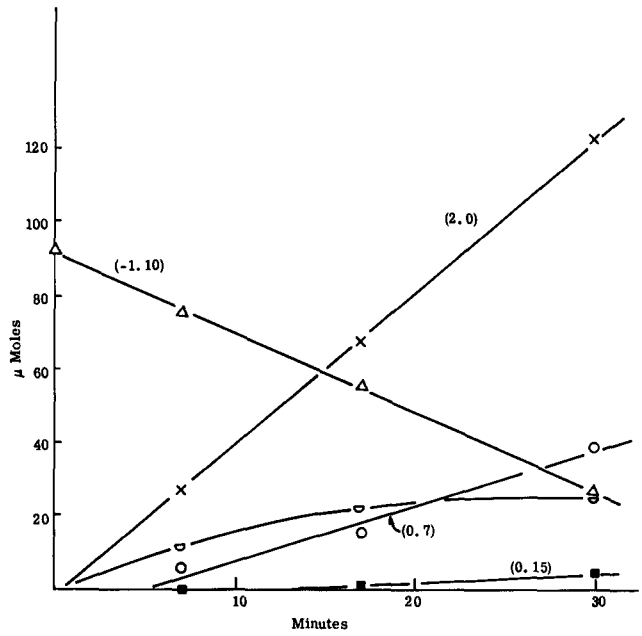

FIG. 4. Hydrolysis products of erythritol-1,2,3-trioleate by the action of 2 mg of rat pancreatic juice solids in the presence of sodium taurocholate. Values in parentheses are μmoles/min/mg of solids. Triester, Δ; diester, ⌓; monoester, ○; free erythritol, ■; free fatty acid, ×.

used in the absence of taurocholate, could not hydrolyze the tetraester. A different situation obtains if the substrate is the triester of erythritol. Fig. 2, lane *5*, shows the hydrolysis products after 30-min digestion of erythritol-1,2,3-trioleate with untreated pancreatic juice in the absence of taurocholate. Under these conditions lipase, but not nonspecific lipase, would be active. The occurrence of hydrolysis is shown by the presence of free fatty acid. However, this resulted in the formation of neither 1,4-diester nor monoester. Since lipase is specific for esters of primary alcohols, the diester that formed and that accumulated was erythritol-2,3-dioleate.

The course of the hydrolysis of erythritol-1,2,3-trioleate by pure pancreatic lipase is shown in Fig. 5. Essentially identical values were obtained with pancreatic juice under conditions that would render nonspecific lipase inactive. As described in the preceding paragraph, the products were free fatty acid and diesters; and for the same reasons the diester is believed to have been erythritol-2,3-dioleate. That the hydrolysis of the triester became progressively slower is probably due to accumulation of the diester at the oil–water interface. If taurocholate was added to the digest in which purified lipase was used, no hydrolysis of the triester occurred.

The erythritol triester formed as the result of the hydrolysis of erythritol tetraoleate (see Fig. 3) could be the 1,2,3 or the 1,2,4 isomer. To determine which isomer was formed, 100 μmoles of erythritol tetraoleate was hydrolyzed by 12 mg of rat pancreatic juice solids in the presence of sodium taurocholate for 30 min. The digestion products were recovered by ether extraction and separated by TLC. These are shown in Fig. 1, lane *4*, and in Fig. 2, lane *3*. Both triester isomers were present, but it is apparent that the 1,2,3 isomer was the predominant species. Nonspecific lipase has been shown to cleave esters of primary alcohols when these are the sole type present (1) and esters of secondary alcohols (2, 3). The results reported here demonstrate that the primary ester is preferred over the secondary, at least in the case of erythritol tetraester. The 1,2,4 isomer probably results from the cleavage of one of the secondary esters, although it could arise by isomerization of the 1,2,3-triester. At present, it is impossible to determine which mechanism is operative.

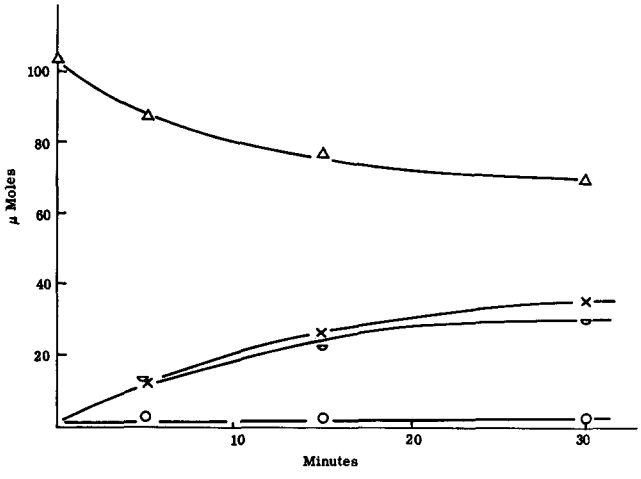

FIG. 5. Hydrolysis products of erythritol-1,2,3-trioleate formed by the action of 100 units of purified lipase in the absence of sodium taurocholate. Triester, Δ; diester, ⊍; monoester, ○; free fatty acid, ×.

The predominant triester formed as the result of the enzymatic hydrolysis of the tetraester is erythritol-1,2,3-trioleate. As shown in Fig. 5, this triester can be hydrolyzed by lipase to erythritol-2,3-dioleate. Since this diester contains only esters of secondary alcohols, the further hydrolysis of it must be by nonspecific lipase. However, if erythritol-1,2,3-trioleate is hydrolyzed by nonspecific lipase, the diesters formed could be the 1,2, 1,3, or 2,3 isomers. We were unable to separate these diesters and hence could not establish the structure of the predominant isomer. That nonspecific lipase preferentially hydrolyzed one of the primary ester groups of erythritol tetraoleate suggests that the predominant diester would be erythritol-2,3-dioleate. If this is the case, further hydrolysis could be only by nonspecific lipase. The primary ester groups of the other possible diesters, 1,2 and 1,3, probably are hydrolyzable by lipase. Consideration of these various routes of hydrolysis and the resulting products points to nonspecific lipase as the enzyme chiefly responsible for the hydrolysis of erythritol esters in the intact animal. The effect that this route of hydrolysis has on the absorbability of erythritol tetraoleate (11) and the appearance of its fatty acids in the lymph (12) have been described.

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