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Hydrolysis of tocopheryl and retinyl esters by porcine carboxyl ester hydrolase is affected by their carboxylate moiety and bile acids

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Abstract

The objective of this study was to examine the in vitro hydrolysis of vitamin E esters (α-tocopheryl acetate, α-tocopheryl succinate and α-tocopheryl nicotinate) by pancreatic carboxyl ester hydrolase (CEH) at the concurrent presence of different bile acids at different concentrations. The assay was performed by measuring the amount of α-tocopherol released by porcine pancreatic juice upon addition to different solutions of α-tocopheryl esters, which were dispersed in bile acid mixed micelles at 37°C, pH 7.4. The CEH activity was 10 U in the final assay, and the optimal concentration of cholate in this in vitro-system was determined to 30 mM for the hydrolysis of α-tocopheryl acetate. The hydrolysis of α-tocopheryl esters required presence of pancreatic juice and bile acids, and the results showed furthermore that the ability of pancreatic CEH towards hydrolysis of different α-tocopheryl esters increased with increasing lipophility, irrespective of the type or concentration of bile acid present in the assay. Likewise, retinyl palmitate was hydrolyzed at a faster rate than retinyl acetate. The structure of the bile acid influenced the rate of hydrolysis. Thus, cholate followed by glycodeoxy- and glycochenodeoxycholate were the most effective activators of CEH among the bile acids tested in this assay. The presence of γ-tocopherol or all-*trans*-retinyl acetate in the assay showed a non-competitive inhibition of the hydrolysis rate of α-tocopheryl acetate.

Keywords: α-Tocopheryl acetate; α-Tocopheryl succinate; α-Tocopheryl nicotinate; Pancreatic enzymes; Carboxyl ester hydrolase; Michaelis-Menten kinetic

1. Introduction

Both vitamin E and A are absorbed in the small intestine only as free alcohols [1]. However, supplements of vitamin E are generally given in the form of all-*rac*-α-tocopheryl acetate (Toc-Ac) in which the reactive hydroxyl group of α-tocopherol is esterified, rendering the molecule more stable than the free phenol form. In addition, succinate (Toc-Suc) and nicotinate (Toc-Nic) esters of α-tocopherol are also commercially available sources of vitamin E. Likewise, vitamin A (retinol) is provided primarily as the acetate ester, but the palmitate ester is also commercially available. Recently it was found in an experiment with broilers that the absorption coefficient for Toc-Suc was significantly lower than that of the Toc-Ac [2].

Several reports have shown a higher uptake of α-tocopherol when the free alcohol form was supplemented compared with Toc-Ac [3–7]. In some of these animal experiments, the secretion of pancreatic enzymes or bile acids was considered to be a limiting factor for hydrolysis and subsequent absorption of α-tocopherol. The pancreatic enzyme, responsible for the hydrolysis of α-tocopheryl esters, has commonly been referred to as pancreatic esterase, or carboxyl ester hydrolase, CEH. The ester form of vitamin A is hydrolyzed by retinyl ester hydrolase, which also is secreted by the pancreas [8]. However, evidence revealed that porcine retinyl ester hydrolase is most likely an isoform of CEH [9].

Bile acids are required for activation of the esterases, and for the formation of the lipid micelle, which carries the vitamins from the emulsified dietary lipids to the microvillus. Beside their role as detergents and CEH-activators, bile acids furthermore modulate CEH's chiral selectivity [10]. In addition to the chiral properties of the tocopherol molecule, the rate of hydrolysis of α-tocopheryl esters will depend on the affinity of the CEH toward the ester, and this may depend on the presence of other dietary molecules, e.g. retinol or tocopherol derivatives. Jensen et al. [2] showed

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that the lower absorption of Toc-Suc by broilers compared with Toc-Ac was caused by a limited activity in the gut, and a higher capacity of CEH for hydrolysis of Toc-Ac compared with Toc-Suc, as well as a limited activity of this enzyme in the broilers intestine.

For animals and humans with insufficient secretion of pancreatic juice or bile acid it is of utmost importance to achieve the most optimal physical and chemical conditions in the digestive tract in order to obtain the highest possible absorption rate of nutrients. It is therefore of interest to elucidate the importance of CEH activity and bile acid secretion on the hydrolysis and subsequent absorption capacity of various tocopheryl esters. The objective of the present study was to investigate the in vitro hydrolysis of different ester forms of α-tocopherol and retinol by pancreatic CEH with emphasis on the role of different bile acids.

2. Materials and methods

Pancreatic juice was obtained from pigs surgically fitted with a catheter in the pancreatic duct for continuous collection of pure pancreatic juice [11]. The activity of CEH was determined in the pancreatic juice using p-nitrophenylacetate (Sigma, St. Louis, MO 61378) as substrate according to the procedure described previously [11]. One unit of enzyme activity was defined as the hydrolysis of 1 μmol of substrate in 1 min. Prior to use, the pancreatic juice was diluted to an activity of 100 U/L with respect to CEH-activity with an isotonic saline solution (0.9% NaCl).

The in vitro hydrolysis of α-tocopheryl esters was performed in a solution of 5 mM sodium acetate, 6 mM sodium cholate and 0.3 M Tris/HCl buffer, pH 7.4. The amounts of α-tocopheryl esters ranged from 0 up to 260 μM. The final volume of the solution was adjusted to 2.0 ml with Tris/HCl buffer. The hydrolysis was initiated by addition of 0.200 ml pancreatic juice with an activity of 100 U/L and took place for 20 min at 37°C, while the samples were frequently shaken. The reaction was stopped by adding 1.5 ml ethanol to the mixture, where after α-tocopherol was extracted using heptane prior to quantification on HPLC as described by Jensen et al. [2]. The in vitro hydrolysis of the retinyl esters was performed under the same conditions as described above and was also quantified on HPLC [12].

In the experiment with different bile acids and concentrations thereof, the conditions described above for the hydrolysis of α-tocopheryl esters were used. However, in addition to sodium cholate, other deconjugated (deoxycholate, chenodeoxycholate), tauroconjugated (taurocholate, taurodeoxycholate, and taurochenodeoxycholate), and glycoconjugated (glycocholate, glycodeoxycholate, and glycochenodeoxycholate) as well as hyodeoxycholate and lithodeoxycholate were tested (all obtained from Sigma). The concentration of sodium taurodeoxycholate, cholate and chenodeoxycholate was furthermore varied within the range from 0 and up to 60 mM bile acid. All bile acids were dissolved in the Tris/HCl buffer (pH 7.4), except hyodeoxycholate and lithocholate, which failed to give clear solutions. These bile acids were therefore dissolved in water with 300 μl (hyodeoxycholate) and 500 μl (lithocholate) 0.5 M NaOH, after which pH was adjusted with HCl to 7.4. In the final assay, Tris (pH 7.4) was added to the sodium acetate.

Table 1
Hydrolysis constants (K_M and V_{max}) according to Michaelis-Menten equation for the hydrolysis of tocopheryl ester, retinyl palmitate and retinyl acetate by pancreatic carboxylic ester hydrolase (CEH) at 37°C, pH 7.4, in the presence of sodiumcholate (6 mM) and Tris-buffer, means (SD).

Substrate	Number of assays	K_M (μM)	V_{max} (nmol/h)	R^2
Toc-Ac	3	9.0 (1.1)	44.1 (4.0)	0.794–0.807
Toc-Nic	2	3.7 (2.7)	22.7 (8.4)	0.832–0.976
Toc-Suc	1	1.0	1.7	0.988
Retinyl palmitate	1	33.0	203.6	0.900
Retinyl acetate	1	4.1	32.5	0.989

The effect of increasing activity of CEH in the assay was investigated by adding 0.200 ml of pancreatic juice with an activity ranging from 0 and up to 200 U/L CEH using three different concentrations of cholate (6, 30, and 60 mM) and 30.9 mM Toc-Ac.

The results determined in the in vitro hydrolysis experiments with pancreatic CEH were treated according to Michaelis-Menten kinetic. Plots of the substrate concentration ([*s*], μmol/L α-tocopheryl or retinol ester) against the substrate concentration divided with the amount of α-tocopherol or retinol liberated (nmol/h) (Hanes plot) were analyzed by linear regression. From these plots the Michaelis-Menten constant, K_M (μmol/L substrate), was calculated as the intercept divided with the slope, and V_{max} (nmol/h of reaction product liberated) was calculated as the reciprocal slope.

3. Results

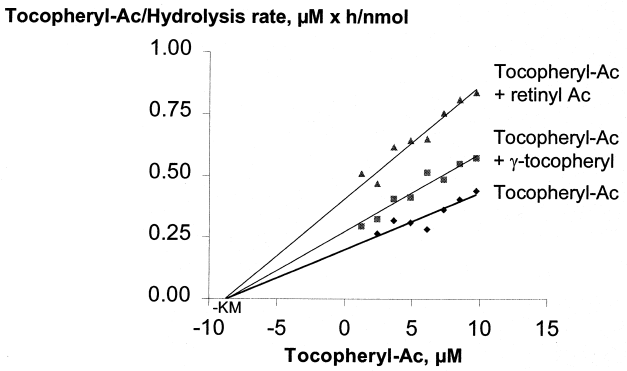
3.1. Hydrolysis of esters of vitamin E and vitamin A

Table 1 shows the kinetic values of the hydrolysis of tocopheryl and retinyl esters. It is clear that the capacity of pancreatic CEH to hydrolyze Toc-Nic and Toc-Suc is much lower than the capacity to hydrolyze Toc-Ac. In the same manner, the hydrolysis rate of retinyl acetate was only 16% of the corresponding hydrolysis rate of retinyl palmitate.

3.2. Inhibition of the hydrolysis of α-tocopheryl acetate

Addition of γ-tocopherol or retinyl acetate to a reaction mixture containing Toc-Ac decreased the hydrolysis rate of Toc-Ac significantly. From Figure 1 it is seen that the effect

a



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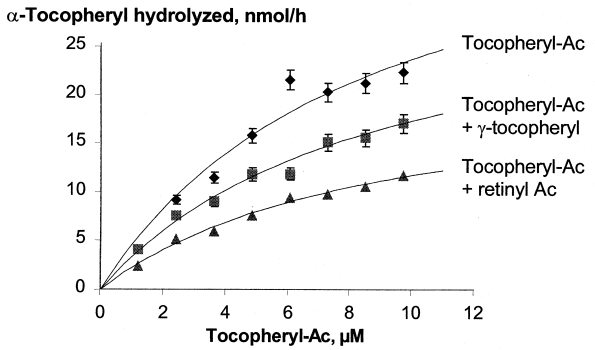

Fig. 1. Non-competitive inhibition of the hydrolysis of Toc-Ac by CEH at the concurrent presence of γ-tocopherol (4.83 μM), or retinyl acetate (4.65 μM). Each assay was performed twice with eight Toc-Ac concentrations each time. a) Hanes plots of the observed data with K_M = 8.77 μM. b) Observed and predicted hydrolysis rate according to the Michaelis-Menten equation. Toc-Ac: V = 43.8 nmol/h × X μM/(8.77 + X) μM; R^2 = 0.805; Toc-Ac + γ-tocopherol (4.83 μM): V = 32.0 nmol/h × X μM/(8.77 + X) μM; R^2 = 0.940; Toc-Ac + retinyl acetate (4.65 μM): V = 21.7 nmol/h × X μM/(8.77 + X) μM; R^2 = 0.931.

of γ-tocopherol and retinyl acetate on the hydrolysis rate of Toc-Ac can be described as a non-competitive inhibition effect according to the Michaelis-Menten equation.

3.3. Activation of CEH by bile acids

The activating effect of different bile acids on the activity of CEH was assessed by testing the deconjugated, glycoconjugated and tauroconjugated bile acids of cholate, deoxycholate and chenodeoxycholate. The obtained kinetic parameters are summarized in Table 2. Deconjugated cholate showed the highest $V_{max,}$ but also a high K_M value. The bottom line was achieved with taurodeoxycholate, as this bile acid was not able to facilitate any hydrolysis of Toc-Ac. A common feature for the dihydroxy bile acids was

Table 2
Influence of type of bile acid (6 mM) on hydrolysis constants (K_M and V_{max}) according to Michaelis-Menten equation for the CEH-catalyzed hydrolysis of Toc-Ac (37°C, pH 7.4)

	Cholate (3α,7α,12α)	Deoxycholate (3α,12α)	Chenodeoxycholate (3α,7α)
Deconjugated	K_M = 7.3 V_{max} = 13.4 R^2 = 0.96	K_M = 2.5 V_{max} = 0.31 R^2 = 0.91	K_M = 13.5 V_{max} = 3.8 R^2 = 0.58
Tauroconjugated	K_M = 0.88 V_{max} = 2.4 R^2 = 0.97	No reaction	K_M = 6.7 V_{max} = 1.3 R^2 = 0.84
Glycoconjugated	K_M = 0.01 V_{max} = 1.8 R^2 = 0.97	K_M = 2.2 V_{max} = 6.7 R^2 = 0.96	K_M = 13.7 V_{max} = 5.9 R^2 = 0.92

that the glycoconjugated bile acids showed the highest V_{max} values followed by deconjugated and tauroconjugated bile acids. The K_M value were in all cases highest for the chenodeoxycholates. As mentioned above the highest V_{max} value for cholate was achieved with deconjugated cholate followed by taurocholate and glycocholate. However, glycocholate showed a very low K_M value. Lithocholate (3α) gave some activation of the CEH-induced hydrolysis of Toc-Ac (Lithocholate: K_M = 7.4 μM, V_{max} = 0.7 nmol/h, R^2 = 0.816), whereas hydroxycholate (3α,6α) failed to give a proper hydrolysis reaction.

The activating effect of different bile acids on the activity of CEH was also assessed for Toc-Suc. In all cases, the kinetic parameters demonstrated that the lower capacity of pancreatic CEH to hydrolyze Toc-Suc compared with Toc-Ac was independent of the bile acid used (taurocholate: K_M = 0.8 μM, V_{max} = 0.99 nmol/h, R^2 = 0.95; taurochenodeoxycholate: K_M = 0.27 μM, V_{max} = 0.41 nmol/h, R^2 = 0.92; chenodeoxycholate: K_M = 3.2 μM, V_{max} = 0.9 nmol/h, R^2 = 0.96; cholate: K_M = 1.1 μM, V_{max} = 2.2 nmol/h, R^2 = 0.995; deoxycholate: No proper reaction).

3.4. Effect of bile acid concentration

Based on the previous results the importance of varying concentrations (0–60 mM) of cholate, taurodeoxycholate and chenodeoxycholate were studied in assays containing 30.9 μM Toc-Ac (Figure 2). There was no detectable reaction in the complete absence of bile acid, but the reaction could be readily detected upon the addition of as little as 3 mM of these bile acids. The amount of α-tocopherol liberated was much higher in the presence of cholate than in the presence of taurodeoxycholate or chenodeoxycholate at any concentration. Furthermore, the hydrolysis of Toc-Ac increased with increasing concentration of cholate up to 30 mM, whereas tauro- and chenodeoxycholate peaked below a concentration of 10 mM. Varying amounts of the three bile acids were also tested with 14.3 μM Toc-Suc as substrate. The amount of α-tocopherol liberated from Toc-Suc

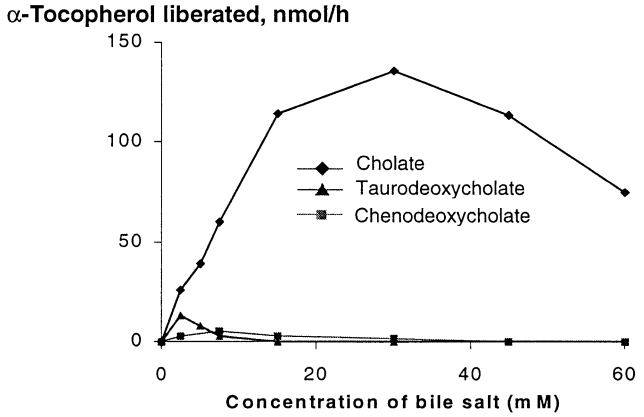

Fig. 2. Hydrolysis of α-tocopheryl acetate (30.9 μM) with increasing concentration of cholate (N = 8), taurodeoxycholate (N = 8), or chenodeoxycholate (N = 7). The reaction was initiated with 200 μl pancreatic juice (CEH activity: 100 U/L).

increased with cholate concentrations up to 8 mM, but there was no proper activation by the increasing amount of taurodeoxycholate and chenodeoxycholate (results not shown).

In Figure 2 it was shown that the optimal concentration of cholate for the hydrolysis of α-tocopheryl acetate was 30 mM. Therefore a new assay with the tocopheryl esters (Toc-Ac, Toc-Nic and Toc-Suc) was performed at 30 mM cholate instead of the 6 mM cholate used in the first experiment. Figure 3 shows the order of the three esters with respect to hydrolysis rate was the same as observed in the experiment with 6 mM cholate. The hydrolysis rate increased for Toc-Ac, but decreased for Toc-Suc and Toc-Nic. Thus, this was an optimal assay for Toc-Ac (K_M = 59 μM and V_{max} = 190 nmol/h), while the corresponding kinetic parameters for Toc-Nic and Toc-Suc were much lower (K_M = 19.5 μM and V_{max} = 0.5 nmol/h; K_M = 2.3 μM and V_{max} = 0.5 nmol/h, respectively).

3.5. Effect of CEH-activity

The effect of CEH-activity was studied at 6, 30 and 60 mM cholate and 30.9 μM Toc-Ac. The hydrolysis rate of Toc-Ac increased from zero without any activity of CEH in the assay and reached the highest rate at 100 U/L for all three concentrations of cholate (Figure 4). The highest hydrolysis rate was obtained in the assay using 30 mM cholate.

4. Discussion

The biological activity of the acetate and the succinate form of vitamin E is considered to be equal on molar basis [13]**.** However, reports demonstrated lower plasma concentrations of α-tocopherol after similar oral doses of Toc-Suc compared with Toc-Ac, apparently due to lower absorption of Toc-Suc [2,14–15]. The objective of this study was to investigate the relative role of porcine CEH and bile acids on the in vitro hydrolysis of various tocopheryl and retinyl esters, since the hydrolysis reaction is a prerequisite prior for in vivo absorption.

The present experiments showed that pancreatic CEH had a decreasing affinity towards hydrolysis of tocopheryl esters with increasing polarity (Toc-Ac > Toc-Nic > Toc-Suc) irrespective of the type or concentration of bile acid present in the assay. In addition, retinyl palmitate hydrolyzed 6.3 times more rapidly than retinyl acetate. However, it cannot be excluded that other enzymes than CEH are of importance with respect to the hydrolysis of retinyl esters. CEH is known to be rather nonspecific with respect to substrate specificity [16]. The difference between the vitamin esters in the susceptibility to CEH-activated hydrolysis can therefore most likely be ascribed to their different physical properties. CEH requires its lipophilic substrates to be solubilized in the aqueous phase in the lumen. This can only be performed in mixed micelles, and the difference in sol-

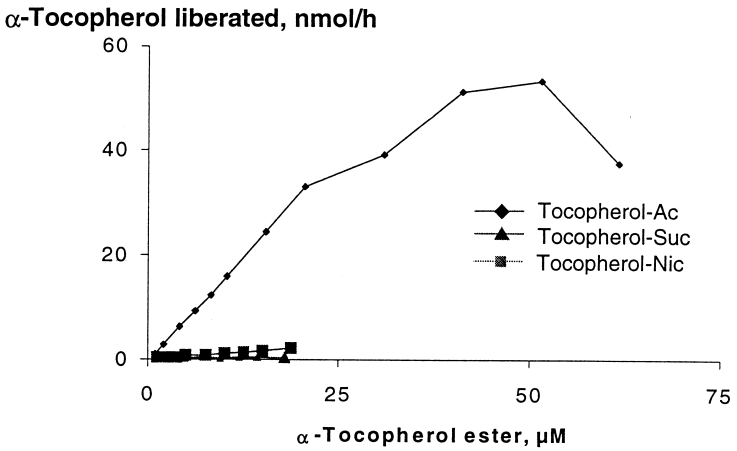

Fig. 3. Formation of α-tocopherol from Toc-Ac, Toc-Suc, and Toc-Nic in the presence of 30 mM cholate and Tris-buffer (pH 7.4). The reaction was initiated with 200 μl pancreatic juice (CEH activity: 100 U/L).

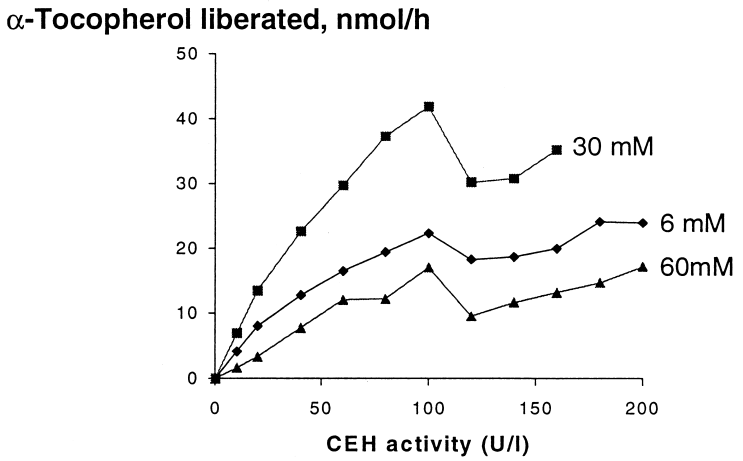

Fig. 4. Effect of adding pancreatic juice (200 μl) with different activity of CEH (0 and up to 200 U/L) on the amount of α-tocopherol liberated (nmol/h) from α-tocopheryl acetate (30.9 μM) incubated with 6, 30 or 60 mM cholate.

ubility of the esters in the core of the mixed micelles or at the surface of the mixed micelles reflects their hydrophobicity. For the esters investigated here, the hydrophobicity increases in the order Toc-Suc < Toc-Nic < Toc-Ac, and likewise, retinyl palmitate is more lipophilic than retinyl acetate. Thus, it seems like the more lipophilic esters are more readily available for the CEH-catalyzed reaction, i.e. give a better physico-chemical status of the substrate for CEH activity which allows a proper binding of CEH to the interface of the micellar suspension regardless of the bile acids involved.

Under most dietary circumstances several vitamin compounds are present in the intestine, and both high levels of vitamin A and γ-tocopherol in the diet are known to impact the α-tocopherol status of the body [7,17]. Our data (Figure 1) regarding the non-competitive inhibition effect on the hydrolysis of Toc-Ac by γ-tocopherol and retinyl acetate are therefore important for the conditions in vivo. It could clarify why competitive antagonisms of vitamin E absorption have been indicated in cases of high dietary levels of vitamin A [7,17]. Though under most practical feeding conditions, the molar concentration of retinyl acetate is normally lower in feed compared to the concentrations of tocopherols.

In absence of bile acids, pancreatic CEH was unable to hydrolyze Toc-Ac, as also previously shown by Lombardo et al. [18]. In vivo, lack of adequate biliary secretion would result in a failure to solubilize and absorb tocopherols. In agreement with Muller et al. [1], the addition of increasing concentrations of bile resulted in increased hydrolysis of Toc-Ac. The optimal concentration of cholate in the present study was 30 mM. It is indistinct how bile acid concentration would affect the hydrolysis of Toc-Ac in vivo. Greaves and Schmidt [19] showed that oral administration of deoxycholate increased the absorption of α-tocopherol in bile-fistulated rats, whereas feeding of sodium taurocholate had no effect on the absorption of α-tocopherol in turkey poults [20]. It is well known that both the production of bile acid and the absorption of α-tocopherol in vivo are influenced by the presence of dietary fat, apparently because the micellar solubilization of the tocopheryl esters is influenced by the concentration of free fatty acids [21].

Chenodeoxycholate (as well as hyocholate) is the primary bile acid in the pig [22]. However, as can be seen from the present data, cholate was the most efficient activator of porcine CEH in the hydrolysis of α-tocopheryl acetate among the bile acids tested in this assay, and in general the trihydroxylated bile acids (3α,7α,12α), and the 3α,7α-bile salt seemed to be more efficient activators than the others. This is in agreement with other studies [23,24], but it is unclear which part of the bile salt is of most importance in the hydrolysis reaction. In conclusion, the presence of pancreatic juice and the concurrent presence of bile acids are necessary for the hydrolysis of tocopheryl esters in the small intestine. As the difference in CEH's hydrolysis capacity against the tocopheryl esters could not be modulated by the different bile acids used (which differed in specific characteristics in terms of hydroxylation- and conjugation-pattern) to activate the enzyme, it is most likely to suggest that the discrimination between the esters could be caused by the enzyme's specific substrate recognition.

We conclude furthermore that our present porcine model system hydrolyses the acetate more rapidly than either of the more water-dispersible succinate or nicotinate forms, and this appears to be consistent with in vivo results obtained for the succinate versus the acetate form in broilers.

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