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Some Factors which Influence the Catalytic Activity of Pancreatic Cholesterol Esterase. (26091)

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(Introduced by C. W. Pettinga)
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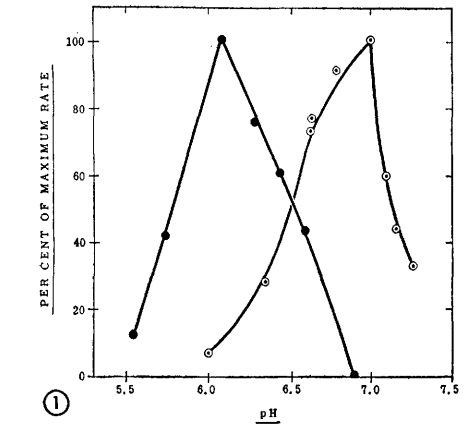
Extracts of beef pancreas catalyze a rapid synthesis and hydrolysis of cholesteryl oleate (1,2,3). Differences in some of the requirements for optimal activity of the two reactions have been noted(3). The influence of pH, electrolyte concentration, and β-sitosterol upon catalytic activity of the enzyme is considered further in the present report.

Methods and materials. Preparation of substrates and enzyme and procedure for manometric estimation of cholesterol esterase activity have been described(1). Briefly, the composition of the standard test substrate for synthesis was 0.04 M cholesterol or other sterol, 0.12 M lithium oleate, and 0.02 M cholic acid; for hydrolysis it was 0.07 M cholesteryl oleate, 0.014 M cholic acid, and 0.035 M lithium oleate. The reaction mixture contained substrate, $NaHCO_3$, and $(NH_4)_2SO_4$ in amounts indicated with the data of each experiment. The enzyme was a 10% aqueous or 0.01 M NaCl extract of acetone-dried beef pancreas clarified by filtration. The protein content ranged from 15 to 20 mg per ml(4). Approximately 0.3 ml was used in each reaction vessel. The reaction was conducted in conventional Warburg flasks at 37°C. After appropriate periods of gassing with CO_2 and temperature equilibration, the reaction was started by pouring the enzyme solution from the side-arm into the main compartment. The first reading was made 3 minutes later, to allow for non-specific gas exchange, and thereafter at desired intervals. An increase in the pressure of CO_2 occurs during ester hydrolysis; this is indicated by a plus sign. The pressure of CO_2 decreases during ester synthesis; this is shown by a minus sign. Results are reported either as relative activities or as μl CO_2 exchanged, *i.e.* released (+) or absorbed (−), per unit time.

Results. The effect of pH on rate of synthesis and hydrolysis of cholesteryl oleate at the concentration of electrolyte which is optimal for each reaction is seen in Fig. 1. It is evident that the optima are distinct and that at the optimal pH for each reaction the opposite reaction would not occur or its rate would be negligible. At intermediate values of pH both reactions take place although at reduced rates. Nearly 50% of the maximal rate at optimal pH occurs in the vicinity of pH 6.5. This pH is taken to be the single optimum for both reactions.

The data of Fig. 2 show the effect of electrolyte concentration at optimal pH for each reaction. It is seen that the concentration of electrolyte which is optimal for synthesis is inadequate for hydrolysis. Addition of electrolyte even in amounts slightly greater than required for optimal synthetic activity causes immediate precipitation of the substrate for synthesis, resulting in diminished reaction rate. Extent of decline in rate depends upon completeness of precipitation,



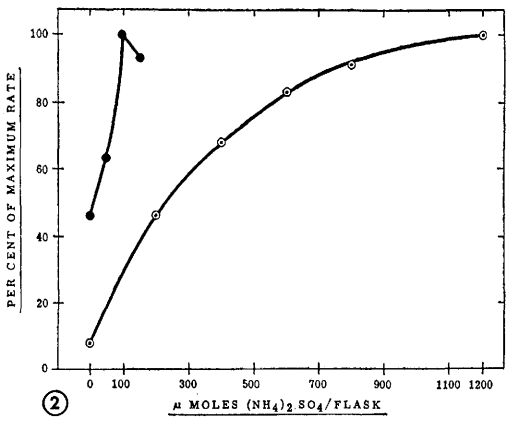


FIG. 1. Activity as a function of pH. The standard test substrates for synthesis (●) and hydrolysis (○) of cholesteryl oleate were prepared at approximately the pH values indicated. Test systems consisted of 0.8 ml of the appropriate standard test substrate with 0.045M-$(NH_4)_2SO_4$ for synthesis and 0.72M-$(NH_4)_2SO_4$ for hydrolysis. $NaHCO_3$ concentration was altered in accordance with the Henderson-Hasselbach equation to obtain the various pH values. Appropriate amounts of NaCl were added to all reaction mixtures containing less than the maximum amount of $NaHCO_3$ used in the system to maintain a constant uni-univalent electrolyte concentration. Total reaction volume 2.2 ml; gas, CO_2. The pH values recorded are those found after the 30-min. experimental period, but do not differ from initial pH values by more than 0.1 pH unit.

FIG. 2. Effect of electrolyte concentration on rate of synthesis (●) and hydrolysis (○) of cholesteryl oleate. Each reaction mixture consisted of 0.8 ml of appropriate standard test substrate. Concentration of $NaHCO_3$ was 0.014M in the system for synthesis and 0.18M in that for hydrolysis; gas, CO_2. Amount of $(NH_4)_2SO_4$ was varied over the range shown on abscissa. Total reaction vol, 2.2 ml.

The effect of electrolyte over a wide range of concentrations can be evaluated at pH 6.5-6.6. At this pH the dispersed character of the substrate for synthesis is not destroyed even by the high electrolyte concentrations required in the hydrolytic system. The data are given on Table I. At pH 6.5-6.6 the response of the two systems in presence of either substrate alone (Nos. 1 and 3) is qualitatively similar to that seen at the optimal pH for each reaction (Fig. 2). In the presence of both substrates a complete reversal of gas pressure change from negative (synthesis) to positive (hydrolysis) is brought about by increase of electrolyte concentration from 0 or 100 μmoles $(NH_4)_2SO_4$. to 1200 μmoles $(NH_4)_2SO_4$.

TABLE I. Selective Effect of Electrolyte Concentration. Standard test substrate for synthesis of cholesteryl oleate was used in Exp. 1. For Exp. 2 this was modified by addition of cholesteryl oleate equivalent in amount to cholesterol. For Exp. 3 cholesteryl oleate was substituted for cholesterol. Each reaction flask contained 0.8 ml of one of the substrates in a total fluid vol of 2.2 ml. Reaction mixture was maintained at pH 6.5-6.6 by 0.047M $NaHCO_3$ and CO_2. Minus and plus signs indicate ester synthesis and hydrolysis respectively.

		μl CO_2/60′		
No.	μmoles $(NH_4)_2SO_4$:	0	100	1200
1.	Cholesterol	−86	−107	− 22
2.	" + cholesteryl oleate	−30	− 18	+ 38
3.	Cholesteryl oleate	+58	+ 89	+127

Both cholesterol and β-sitosterol are esterified with oleic acid although the optimal conditions are not the same(3). The data of Table II show that the percent of $4C^{14}$-cholesterol which is esterified decreases as the absolute amount of β-sitosterol is increased.

Discussion. Although the pH optima for synthesis and hydrolysis of cholesteryl oleate differ by approximately one pH unit, it has been shown that both reactions can occur at a single intermediate pH. In the presence of both substrates the reaction which predominates at this pH, as determined by the direction of gas exchange, is governed by the electrolyte concentration. At low concentrations of electrolyte the esterification reaction is favored, whereas at high concentrations of electrolyte hydrolysis is dominant. These results are in accord with the effect of electro-

TABLE II. Effect of β-Sitosterol upon Rate of Esterification of Cholesterol. 0.1 ml of the standard substrate for synthesis of $4C^{14}$ cholesteryl oleate was present in all flasks. A similar substrate, but prepared with β-sitosterol, was added in amounts to give the ratios indicated; fluid vol, 2.2 ml. Concentration of $(NH_4)_2SO_4$ was 0.023M and of $NaHCO_3$, 0.014M; gas CO_2. Reaction mixture was extracted with 1:1 acetone:alcohol (v/v) and free sterol was removed as the digitonide. Activity of the supernate, combined with washings of the digitonide, was determined by a Packard Tri-Carb Scintillation Counter.

Ratio, cholesterol/β-sitosterol	% $4C^{14}$-cholesterol in ester
1:0	60.0
1:1	41.9
1:2	37.5
1:4	23.4
1:6	21.9
1:8	15.0
1:10	13.0

lyte concentration on reaction rate with each substrate alone.

The magnitude of gas exchange in the presence of both substrates does not appear to be related in any obvious way to the magnitude of change observed with each substrate alone. At low electrolyte concentrations the rate in the presence of both substrates is approximately equal to the algebraic sum of the individual rates. If it can be assumed that the concentration of each reactant when both are present is sufficient to saturate the enzyme, as is the case when each is used alone, then the data may suggest that a unique catalytic site is required for synthesis and hydrolysis. At high electrolyte concentration summation of activity does not occur; hence it would appear that the hydrolytic reaction is inhibited by cholesterol. It may be that only at high electrolyte concentration cholesterol alters the colloidal properties of the substrate for hydrolysis making it less susceptible to enzymic attack.

The inhibitory effect of β-sitosterol upon intestinal absorption of cholesterol has been amply documented(5). β-Sitosterol is not absorbed in significant amounts(5) although it is readily esterified *in vitro*(3,5) and presumably *in vivo*. Thus it can be anticipated that rate of cholesterol esterification will be influenced by amount of β-sitosterol present, as is verified by the data. The function of pancreatic cholesterol esterase is associated with the process of cholesterol absorption(5). Interference with esterification of cholesterol by an esterifiable but non-absorbable sterol may be one important mode of action of β-sitosterol.

It is suggested that conflicting data on the type of reaction catalyzed by pancreatic cholesterol esterase may be attributed to insufficient regard for the factors which have been shown to influence the catalytic properties of this enzyme.

Summary. These data indicate that pancreatic cholesterol esterase catalyzes both synthesis and hydrolysis of cholesteryl oleate at a single pH, but the reaction which predominates in presence of both substrates is governed by the electrolyte concentration. Rate of synthesis of $4C^{14}$-cholesteryl oleate is decreased in proportion to relative concentration of β-sitosterol.

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