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EFFECT OF ALCOHOLS ON THE HYDROLYSIS CATALYZED BY HUMAN PANCREATIC CARBOXYLIC-ESTER HYDROLASE

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Summary

Transfer reactions catalyzed by human pancreatic carboxylic-ester hydrolase (EC 3.1.1.1) were studied in the presence of methanol and butanol as nucleophiles. The addition of alcohols produced an increase in the total rate of 4-nitrophenyl acetate and *n*-propylthiol acetate disappearance and a concomitant slow decrease of the hydrolysis rate. These results indicate a competitive partitioning of an acyl-enzyme intermediate between water and nucleophile. Moreover, a strong inhibition of the rates of hydrolysis of methyl butyrate and triacetin by nucleophiles is in agreement with a rate-limiting acylation step. The kinetic data and a trans-ester characterization argue in favor of the formation of an acyl-enzyme intermediate and a two-step reaction mechanism, acylation and deacylation both being rate-limiting. The experiments performed with 4-nitrophenyl acetate show the existence of a nucleophile binding site.

Introduction

The catalysis mechanism of a large number of esterases is known to function via the formation of an acyl-enzyme intermediate. If the intermediate acyl-enzyme has been isolated with chymotrypsin [1] and lipase [2], in many cases kinetic data have provided evidence for the formation of this intermediate. Esterases have been found to catalyze transfer of a substrate acyl group to certain amine acceptors [3] and alcohols have been shown to be able to serve as

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

acyl acceptors. The effect of alcohols on the hydrolysis reactions can be interpreted in terms of competitive partitioning of the intermediate between water and nucleophile. Such a procedure has been used with chymotrypsin [4—6] and pig and beef liver esterases [7—9]. The mechanisms of many other esterases have been found to involve an acyl-enzyme intermediate, e.g., ribonuclease [10], cholinesterase [11,12], subtilisin [13], trypsin [14] and papaïn [15].

The aim of the present work is to provide some evidence for the formation of an acyl-enzyme intermediate in the reactions catalyzed by pancreatic carboxylic-ester hydrolase. This enzyme isolated from human pancreatic juice is a serine esterase inhibited by diisopropyl phosphorofluoridate. It exhibits a broad substrate specificity and covers a great number of lipolytic activities formerly ascribed to different pancreatic enzymes. It is, in part, responsible for the hydrolysis of cholesterol esters and lipid-soluble vitamin esters [16,17].

Materials and Methods

Materials

Triacetin (glycerol triacetate), methyl butyrate and cholesterol were puris products from Fluka (Switzerland). 4-Nitrophenyl acetate was a Merck product (F.R.G.). Cholesterol oleate came from Sigma (U.S.A.), [4-^{14}C]cholesterol from CEA (France) and sodium taurocholate (A grade) from Calbiochem (U.S.A.). *n*-Propylthiol acetate was synthetized in our laboratory from *n*-propylmercaptan (Fluka) esterified with a 2-fold molar excess of acetic anhydride (Fluka) in the presence of 10% pyridine. After at least 1 day of reaction at 25°C, the mixture was washed with distilled water and the final product was dried on anhydrous Na_2SO_4 or $CaCl_2$. We have checked that the reaction was complete and that no free sulfhydryl group was detectable using the Ellman reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB from Fluka).

All organic compounds used for these studies were the best available commercial products.

Pure human pancreatic carboxylic-ester hydrolase (EC 3.1.1.1) was obtained from pancreatic juice according to the method previously described [18].

Enzyme assays

The reaction rates were measured spectrophotometrically and titrimetrically.

Spectrophotometric experiments were carried out at 25°C using a Gilford 2400 spectrophotometer with thermostatically controlled cell compartments. 0.010—0.025 ml enzyme solution were added to 3 ml substrate. The enzyme concentration was 0.3—1.6 mg/ml. Substrate was in solution in a 33 mM sodium phosphate, pH 7.4, buffer. The total rate of hydrolysis of 4-nitrophenyl acetate was measured at 348 nm by 4-nitrophenol liberation using the molar absorption coefficient of 5150 $M^{-1} \cdot cm^{-1}$ [8]. We have checked that this molar absorption coefficient was independent of pH at 348 nm. This wavelength corresponds to the isosbestic point of 4-nitrophenol and its anion, 4-nitrophenolate. The total rate of hydrolysis of *n*-propylthiol acetate was determined by the liberation of *n*-propylmercaptan at 412 nm in the presence of 0.33 mM Ell-

man's reagent using the molar absorption coefficient of 14 150 $M^{-1} \cdot cm^{-1}$ [19].

Titrimetric experiments were performed at 25°C using a Radiometer TTT 60 titrator with magnetic stirring and 20 mM NaOH as titrant. A gentle stream of nitrogen was passed over the surface except for reactions with highly volatile esters. Rates of hydrolysis were followed at pH 7.4 in a 10 ml reaction mixture containing 10 mM NaCl and the following substrates: 4-nitrophenyl acetate, *n*-propylthiol acetate, methyl butyrate and triacetin. In all experiments the substrate was equilibrated with alcohol before the addition of 0.02—0.2 ml enzyme solution.

Total rates of reactions (measured spectrophotometrically) and hydrolysis rates (measured titrimetrically) by acid release were generally followed for 2—10 min. The rates were found to be linear with respect to enzyme concentration and corrected for non-enzymatic hydrolysis measured under the same experimental conditions. The titrimetric and spectrophotometric measurements were standardized against each other in experiments carried out in the absence of alcohol. The observed rate of acid liberation was corrected for the proton released from 4-nitrophenol, which was found to be 74% ionized at pH 7.4.

The alcoholysis rate was given by the difference between the total and the hydrolysis rates for a given nucleophile concentration.

Specific activities were expressed as μmol hydrolyzed/min per mg protein (μmol $min^{-1} \cdot mg^{-1}$).

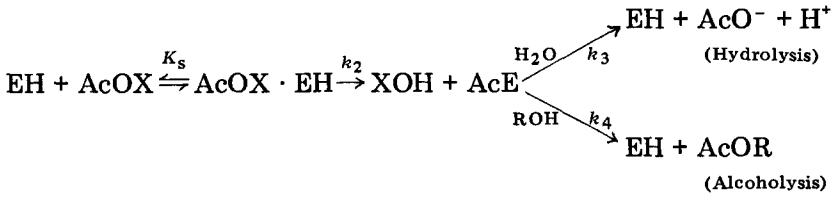
Protein concentration was determined by the absorbance at 280 nm using $E^{1\%}_{1cm} = 14.5$.

Acyl-acceptor characterization

The acyl-acceptor was characterized by trans-esterification using [4-^{14}C]cholesterol as the nucleophile agent and cholesterol oleate as substrate. [4-^{14}C]-Cholesterol oleate (trans-ester) should appear if an oleate-enzyme complex is formed during the hydrolysis of cholesterol oleate. The substrate was prepared as described by Hyun et al. [20] for the measurement of cholesterol ester hydrolase activity but using a 0.1 M sodium phosphate buffer, pH 7.4. Experiments were performed in the presence of 2.35 mM cholesterol oleate and 3 mM [^{14}C]cholesterol (specific radioactivity $60 \cdot 10^6$ dpm/mmol) as nucleophile agent. 450 μl substrate was incubated with the enzyme in a final volume of 600 μl. After 1 h equilibrium at 37°C in a metabolic shaker the medium was extracted by the method of Folch et al [21]. Then the organic phase was evaporated under nitrogen and solubilized by 200 μl chloroform/methanol (2 : 1, v/v). This phase was submitted to TLC in petroleum ether/diethyl ether/acetic acid (90 : 10 : 1, v/v). Spots of cholesterol and cholesterol oleate were scraped quantitatively into separate scintillation vials and were counted in a scintillation counter (Intertechnic).

Kinetic treatment of data

When an enzymatic hydrolysis involves an acyl-enzyme intermediate there are at least two possible modes of action for a nucleophile. First, there is a simple mechanism according to Scheme I as proposed by Bender et al. [4], where



Scheme I

enzyme (EH) and substrate (AcOX) form the enzyme substrate complex AcOX · EH which further reacts to release the alcohol moiety (XOH) and produces the acyl-enzyme (AcE) intermediate. The latter can react either with water (rate constant k_3) to yield the acylate ion (AcO^-) regenerating the enzyme, or with a nucleophile (ROH, rate constant k_4) producing the alcoholysis product (AcOR).

For Scheme I, the steady-state total rate is

$$v_T = \frac{d(XOH)}{dt} = \frac{k_2(k_3' + k_4[ROH])[E_0][S]}{K_s(k_3' + k_4[ROH]) + [S](k_2 + k_3' + k_4[ROH])}$$

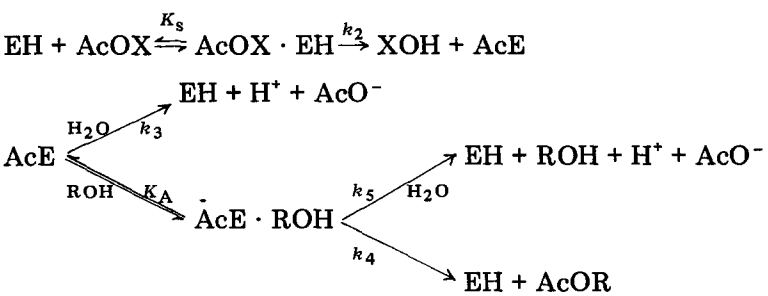
and the steady-state hydrolysis rate is

$$v_W = \frac{d(AcO^-)}{dt} = \frac{k_2 \cdot k_3'[E_0][S]}{K_s(k_3' + k_4[ROH]) + [S](k_2 + k_3' + k_4[ROH])}$$

where $k_3' = k_3[H_2O]$.

It can be seen that as nucleophile concentration increases v_W will decline continously and eventually become zero. A plot of $v_W^0/(v_W^0 - v_W)$ vs. The reciprocal of alcohol concentration should yield a straight line with an intercept of unity on the ordinate (v_W^0 being the hydrolysis rate in the absence of nucleophile).

Another mechanism can be proposed, which postulates the existence of a binding site for the nucleophile (Scheme II).



Scheme II

where $K_A = [AcE \cdot ROH]/[AcE][ROH]$ is the association constant for the acyl-enzyme-nucleophile complex.

Here the hydrolysis rate is $v_W = k_3'[AcE] + k_5'[AcE \cdot ROH]$ with $k_3' = k_3[H_2O]$ and $k_5' = k_5[H_2O]$ and the alcoholysis rate $v_N = k_4[AcE \cdot ROH]$. The ratio

$$\frac{v_W}{v_N} = \left(\frac{k'_3}{k_4 \cdot K_A}\right)\left(\frac{1}{[ROH]}\right) + \frac{k'_5}{k_4}$$

gives a straight line which does not pass through the origin when plotted vs. 1/[ROH], and which has an ordinate intercept equal to k'_5/k_4 [9].

The important additional feature of Scheme II compared to Scheme I is the ability of the acyl-enzyme-nucleophile complex to undergo either alcoholysis in a monomolecular reaction or hydrolysis by reacting with water. Thus, even when saturation of the enzyme by alcohol is complete, hydrolysis still exists. Both formulations assume that the trans-ester (AcOR) does not react further with the enzyme under experimental conditions, because only initial rates are measured.

Results

I. Evidence for the formation of an acyl-enzyme intermediate

(a) Kinetic parameters of the hydrolysis of different acetate esters. The kinetic parameters of the hydrolysis of 4-nitrophenyl acetate, *n*-propylthiol acetate and glycerol triacetate catalyzed by carboxylic-ester hydrolase are given in Table I. The rate constants are not the same for the three acetate esters, which indicates that, if catalysis pass through an acyl-enzyme intermediate, the acylation step is rate-limiting. Consequently the intermediate does not accumulate and the isolation of an acyl-enzyme seems improbable. In the case of 4-nitrophenyl acetate the higher k_{cat} value suggests that with this substrate the deacylation step could become rate-limiting.

(b) Kinetic evidence by nucleophilic competition. We have studied the effects of nucleophiles like *n*-butanol and methanol on the reaction of human carboxylic-ester hydrolase with different esters.

The addition of low amounts of *n*-butanol or methanol affected the rate of cleavage of 4-nitrophenyl acetate (Fig. 1). As *n*-butanol concentration increased, the total rate increased to a maximum of about 2-fold greater than the rate in the absence of nucleophile and above 0.3 M *n*-butanol the total rate decreased slightly. In contrast, in the presence of methanol, the total rate increased progressively at least for the concentrations used. These effects were accompanied by small initial decreases in the corresponding hydrolysis rates

TABLE I

KINETIC CONSTANTS FOR THE HYDROLYSIS OF ACETATE ESTERS BY CARBOXYLIC-ESTER HYDROLASE

The kinetic constants were determined at pH 7.4.

Ester	k_{cat} (s^{-1})	K_m (mM)	Method
4-Nitrophenyl acetate	170	4.5	Spectrophotometry
n-Propylthiol acetate	30	10.5	Spectrophotometry and titrimetry
Glycerol triacetate	30	105.0	Titrimetry

followed by levelling off. The differences between total rates and hydrolysis rates represent the conversion of 4-nitrophenyl acetate into methyl acetate or butyl acetate. In the insert of Fig. 1, the variations of k_{cat1} (catalytic constant for alcohol release) and k_{cat2} (catalytic constant for acid release) are presented. k_{cat1} increased about 30-times with *n*-butanol concentration to reach a plateau value corresponding to k_2. The ratio k_{cat1}/K_m, equal to $2.27 \cdot 10^6\ M^{-1} \cdot min^{-1}$, was constant whatever the *n*-butanol concentration. The k_{cat2} value decreased less than 2-fold and remained constant.

The effect of *n*-butanol on the total rate and hydrolysis rate of an aliphatic thiol ester was determined using *n*-propylthiol acetate (Fig. 2). The activity of carboxylic-ester hydrolase increased with substrate concentration and reached V near 17 mM. With 4 mM substrate concentration, butanol caused a small increase in the observed total rates, 1.3-fold, and this activation was followed by a severe inhibition. The variation of V values obtained by extrapolation showed a more important increase of total rates (4-fold) with *n*-butanol con-

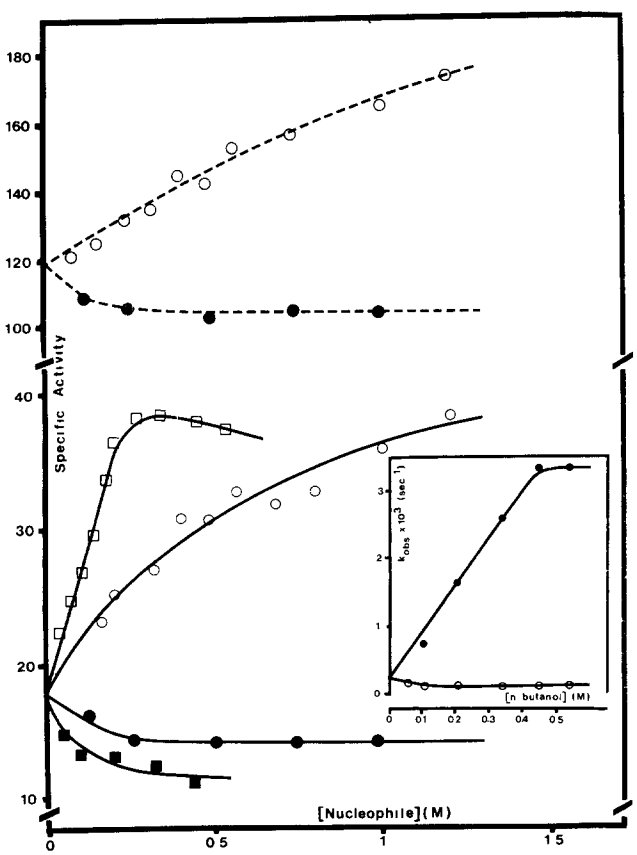


Fig. 1. Effect of nucleophiles on 4-nitrophenyl acetate hydrolysis by carboxylic-ester hydrolase. Lower part: Effect of methanol (○, ●) and *n*-butanol (□, ■) on the total reaction rate (open symbols) and hydrolysis rate (closed symbols). Substrate concentration 2 mM. Upper part: effect of methanol (○, ●) on the total rate (open symbols) and hydrolysis rate (closed symbols) in the presence of 1 mM sodium taurocholate. Substrate concentration 2 mM. Insert: Effect of *n*-butanol concentration on the kinetic constant (k_{obs}); for the total reaction rate ($k_{obs} = k_{cat1}$, ●) and for the hydrolysis rate ($k_{obs} = k_{cat2}$, ○).

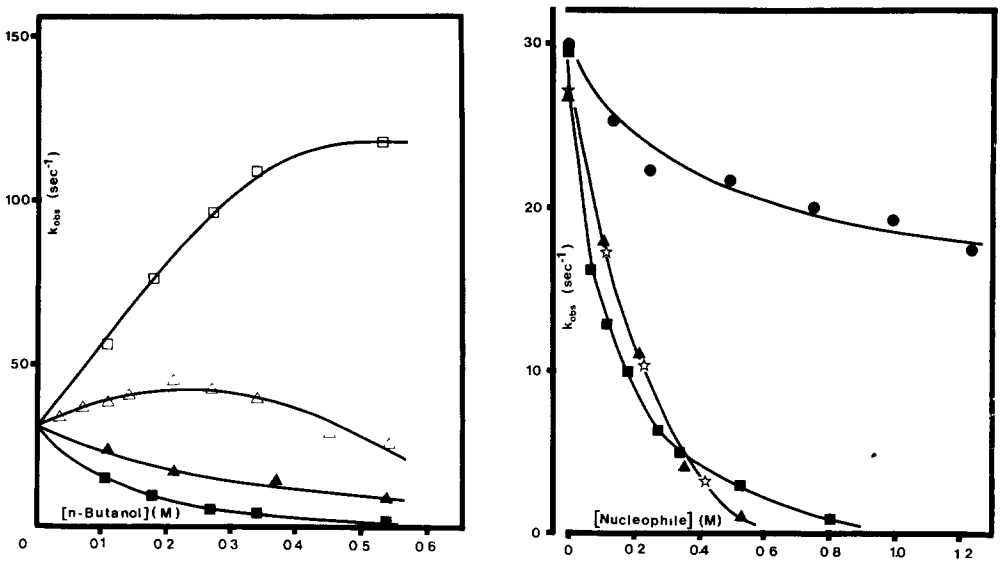


Fig. 2. Effect of *n*-butanol on *n*-propylthiol acetate hydrolysis by carboxylic-ester hydrolase. Effect on the total reaction rate (□, △) and on the hydrolysis rate (■, ▲). Substrate concentrations: 4 mM (▲, △), 17 mM (■). Open squares indicate the *V* extrapolated values.

Fig. 3. Effect of nucleophiles on triacetin and methyl butyrate hydrolysis by carboxylic-ester hydrolase. Effect of methanol (●) and *n*-butanol (■) on triacetin hydrolysis. Substrate concentration 210 mM. Effect of *n*-butanol on methyl butyrate hydrolysis (▲). Substrate concentrations 153 mM. Stars indicate the *V* extrapolated values.

centration and no inhibition at high concentrations. The inhibition observed with 4 mM *n*-propylthiol acetate was probably due to competition between substrate and nucleophile. The effect of *n*-butanol on the hydrolysis rate consists of a progressive inhibition which leads to completion. This phenomenon is more pronounced with 17 mM than with 4 mM substrate concentration.

The effects of nucleophiles on the hydrolysis rates of esters such as triacetin and methyl butyrate are presented in Fig. 3. *n*-Butanol produced a very strong inhibition of the hydrolysis rate of triacetin. A 210 mM triacetin concentration was used for the determination of the maximal rates [17]. In contrast the inhibitory effect of methanol which is a better nucleophile was weaker. At 0.4 M alcohol concentration, triacetin hydrolysis was 90% inhibited by butanol and only 30% by methanol.

Methyl butyrate hydrolysis rates were measured at 153 mM substrate concentration, a value close to the substrate saturation which permits one to measure *V* [17]. As shown in Fig. 3, *n*-butanol produced a very important decrease of the hydrolysis rate. But it is known that at substrate saturation the added nucleophile acts not only on the enzyme mechanism but also on the substrate which can form aggregates surrounded by the nucleophile, whose concentration in the aqueous phase becomes uncertain [22]. Therefore, the values of *V* have been determined by extrapolation and as shown on the diagram these values are located on the same curve as that determined at 153 mM methyl-butyrate concentration.

(c) Trans-ester characterization. Since human pancreatic carboxylic-ester hydrolase is a cholesterol esterase [16] an attempt to characterize the formation of a trans-ester has been made in the presence of [^{14}C]cholesterol using cholesterol oleate as substrate. The results show that 0.12—0.4% of [^{14}C]cholesterol oleate was present in the mixture after incubation with the enzyme and less than 0.03% in the mixture without enzyme. This low incorporation of [^{14}C]cholesterol was not negligible considering the high recovery of radioactivity (greater than 95%) after all operations including incubation, Folch extraction, chromatography and plate extraction. Consequently the presence of radioactive cholesterol oleate demonstrates the formation of the acyl-enzyme intermediate and shows that [^{14}C]cholesterol plays the nucleophile role in the deacylation step of the acyl-enzyme. However, the physical state of this substrate did not permit us to perform kinetic studies in the presence of alcohol.

II. Evidence for a nucleophile binding site

The effect of *n*-butanol on the rates of hydrolysis of the different substrates is shown in Fig. 4 by the plotting of $v_W^0/(v_W^0 - v_W)$ vs. the reciprocal of *n*-butanol concentration. A straight line is obtained with all substrates, which is in agreement with the acyl-enzyme mechanism. However, three out of four lines give an intercept of unity on the ordinate. The values obtained with 4-nitrophenyl acetate give a line with an intercept close to 2.5, which may be explained by the existence of a nucleophile binding site, apparent only in the experiments performed with 4-nitrophenyl acetate. The existence of such a site is confirmed by the effect of acetone on 4-nitrophenyl acetate alcoholysis. As shown in Fig. 5, during the reaction of carboxylic-ester hydrolase with 4-nitrophenyl acetate in the presence of *n*-butanol, the alcoholysis rate is inhibited by acetone. The same concentrations of acetone have no effect on the hydrolysis rate either in the presence or in the absence of *n*-butanol, so that this inhibition

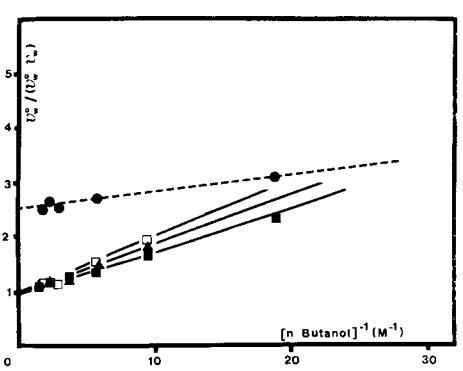


Fig. 4. Effect of *n*-butanol on the rates of hydrolysis of various substrates. Plotting of $v_W^0/(v_W^0 - v_W)$ vs. the reciprocal of *n*-butanol concentration. v_W and v_W^0 are hydrolysis rates in the presence and in the absence of nucleophile. Substrate concentrations: 17 mM *n*-propylthiol acetate (▲), 153 mM methyl butyrate (□) and 210 mM triacetin (■). For 4-nitrophenyl acetate (●, broken line) extrapolated values to V are used.

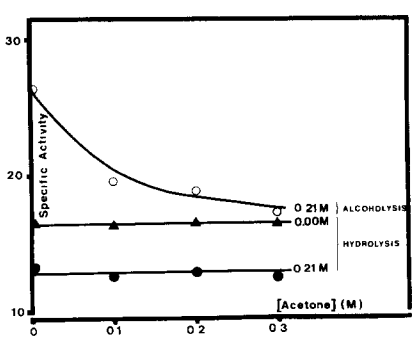


Fig. 5. Effect of acetone on the reaction of carboxylic-ester hydrolase with 4-nitrophenyl acetate. Effect of acetone on the alcoholysis (○) and the hydrolysis (●) rates in the presence of 0.21 M *n*-butanol. Effect of acetone on the hydrolysis rate in the absence of *n*-butanol (▲). Substrate concentration 2 mM.

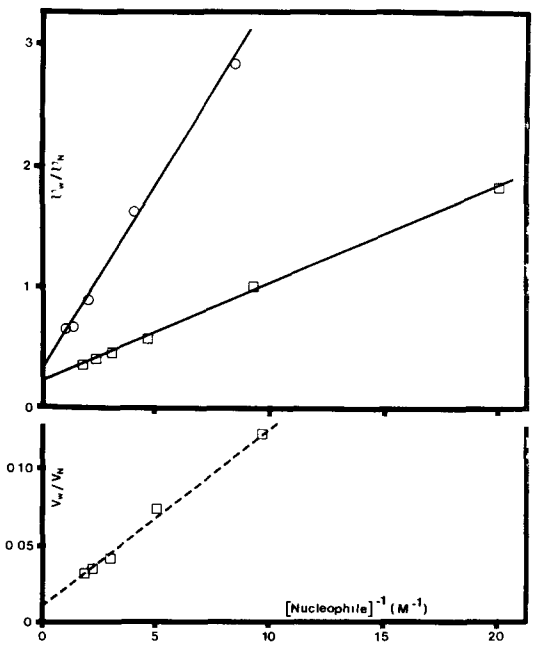


Fig. 6. Effect of methanol and *n*-butanol on 4-nitrophenyl acetate alcoholysis. v_W/v_N ratio vs. the reciprocal of methanol (○) and *n*-butanol (□) concentration. v_W and v_N are the rates of hydrolysis and alcoholysis, respectively. Substrate concentration 2 mM. The broken line has been obtained from V extrapolated values.

specific of alcoholysis may be explained by a competition between alcohol and acetone for the nucleophile binding site. This alcohol binding site is not required for water attack.

As explained in Materials and Methods, if the reaction with 4-nitrophenyl acetate involves the existence of a binding site, the plotting of v_W/v_N vs. 1/[ROH] should give a straight line which does not pass through the origin. Fig. 6 which shows the effect of methanol and *n*-butanol on the v_W/v_N ratio is in good agreement with this proposition. The plotting obtained with extrapolated values of V gives a similar result.

III. Effect of sodium taurocholate on nucleophilic competition

We have shown that the addition of bile salts increased the activity of carboxylic-ester hydrolase on 4-nitrophenyl acetate [18]. In the presence of 1 mM sodium taurocholate or taurodeoxycholate, k_{cat} was unaffected but the K_m value decreased 10-times. Therefore, it was interesting to investigate the effects of methanol on the rates of reaction with 4-nitrophenyl acetate in the presence of 1 mM sodium taurocholate, conditions which permitted us to work at saturating substrate concentration ([S] > K_m). As shown in the upper part of Fig. 1, the effect of methanol parallels the results obtained in the absence of bile salts. The decrease in hydrolysis rate was not modified significantly. The only difference was a smaller increase of the total rate of hydrolysis, 1.4 instead of 2.1 at 1 M methanol concentration.

Discussion

All these results provide some evidence in favor of the formation of an acyl-enzyme and a two-step reaction mechanism, as shown in Scheme I and II. The first step is the acylation of the enzyme (constant rate k_2) and the second step is the hydrolysis of the acyl-enzyme in the absence of nucleophile (constant rate k_3). The trans-ester characterized with cholesterol oleate as substrate and radioactive cholesterol as nucleophile argues in favor of the formation of an acyl-enzyme intermediate. The small effect of cholesterol on acyl-acceptor may be explained by the fact that the formed trans-ester is accessible to enzyme at pH 7.4 [23]. This small effect is not comparable in magnitude to cholesterol esterification by human pancreatic carboxylic-ester hydrolase which has been shown to appear at a much lower pH (5.2) and lead to 30% of total cholesterol esterification during the same time of incubation [23]. At pH 7.4 no ester synthesis occurs with human enzyme in contrast to results obtained using rat enzyme [20,24].

The kinetics of hydrolysis catalyzed by carboxylic-ester hydrolase in the presence of nucleophiles, can be interpreted in terms of a competitive partitioning of the acyl-enzyme intermediate between water and the added nucleophile. The strong inhibition of the rates of hydrolysis of methyl butyrate and triacetin by nucleophiles, indicates that the acylation step is rate determining as shown for pig liver esterase catalyzing the hydrolysis of aliphatic esters [8]. The decrease of the hydrolysis of 4-nitrophenyl acetate and *n*-propylthiol acetate, related to an increase in total rates in the presence of butanol suggests that k_2 and k_3 are comparable in magnitude. Both k_2 and k_3 are partly rate determining for the substrate hydrolysis in the absence of nucleophile, as described by Greenzaid and Jencks [8]. In addition, in the case of *n*-propylthiol acetate hydrolysis, k_2 becomes rate-limiting in the presence of *n*-butanol.

To demonstrate a nucleophilic competition, it is important to know the variations of K_m, k_{cat1} (catalytic constant for alcohol release) and k_{cat2} (catalytic constant for acid release) with nucleophile concentration. As discussed by Seydoux and Yon [25], these variations can indicate which step is rate limiting. When k_2 and k_3 are both partly rate limiting, k_{cat1} and K_m values increase with nucleophile concentration and reach plateau values corresponding to k_2 and K_s, respectively, (when $k_4[ROH] >> k_2$), while k_{cat2} decreases rapidly. For dilute solution the ratio k_{cat1}/K_m is constant. Our results are in agreement with partly rate-determining acylation and deacylation steps. The results obtained with low concentration of 4-nitrophenyl acetate (2 mM) and *n*-propylthiol acetate (4 mM) show that the total rate of hydrolysis is dependent upon nucleophile concentration even if $[S] \simeq K_m$, while when $[S] << K_m$ this rate must become independent [25]. By contrast, with all substrates, the hydrolysis rate is inhibited by the addition of nucleophile.

Following the principle of microscopic reversibility [26] the reaction of an alkyl ester with an enzyme to form an acyl-enzyme and the reaction of the corresponding alcohol with the acyl-enzyme to form the ester must pass through the same transition state according to the equilibrium:

$$AcOX + EH \rightleftharpoons AcE + HOX$$

with the equilibrium constant K_1

$$K_1 = \frac{[AcE][HOX]}{[AcOX][EH]}$$

which is equal to $(k_2/K_s)/k_{ROH}$ [8].

The second-order rate constant k_2/K_s for the formation of the acetyl-enzyme from 4-nitrophenyl acetate is equal to k_{cat1}/K_m according to Bender and Kézdy [27]. This constant was calculated and equals $2.27 \cdot 10^6$ $M^{-1} \cdot min^{-1}$. The second-order rate constant, k_{ROH}, for the reaction of *n*-butanol with the acyl-enzyme was estimated from the initial slope of the plotting of k_{cat1} vs. *n*-butanol concentration under conditions in which the nucleophile concentration was not saturating for the enzyme (the deacylation step in the reaction with 4-nitrophenyl acetate is almost completely rate determining). The second-order rate constant, k_{ROH}, was found to be equal to $0.46 \cdot 10^6$ $M^{-1} \cdot min^{-1}$. From these calculated values K_1 is equal to 5 which indicates that the acyl-enzyme formation from 4-nitrophenyl acetate is favored only by a factor 5. This value of the equilibrium constant K_1 is small compared to that obtained with pig liver esterase whose acetyl-enzyme formation from ethyl acetate is favored by a factor 30 [8].

The results obtained with 4-nitrophenyl acetate as substrate are not consistent with a single acyl-enzyme intermediate according to Scheme I. First, the rate of hydrolysis does not approach zero when nucleophile concentration increases as required by the mechanism of Scheme I if k_2 becomes rate-limiting. Second, the variation of the alcoholysis to hydrolysis rates ratios with *n*-butanol and methanol concentrations increase and lead to a constant value. Third, the plotting of $v_W^0/(v_W^0 - v_W)$ vs. the reciprocal of *n*-butanol concentration gives a line with an intercept different from unity. Fourth, acetone inhibits alcoholysis rate without modifying hydrolysis rate. All these results are not compatible with a simple acyl-enzyme mechanism according to Scheme I but imply the existence of a binding site for nucleophile, according to Scheme II, as shown for beef and pig liver esterase [8,9]. Nevertheless, this nucleophile binding site is apparent only with 4-nitrophenyl acetate, a nonspecific substrate for human pancreatic carboxylic-ester hydrolase [18]. The presence or the absence of this binding site cannot be due to the existence of more than one catalytic site on the enzyme since the pseudo-first-order constants of inhibition of carboxylic-ester hydrolase by diisopropyl phosphorofluoridate have been found identical, whatever the substrate used, 4-nitrophenyl acetate or *n*-propylthiol acetate (unpublished data). This binding site may always be present on the enzyme but may be ineffective in the experimental conditions used with other substrates. The fact that *n*-butanol which is a reagent less nucleophilic than methanol [28] is a better inhibitor of triacetin hydrolysis supports this latter hypothesis. Similar results have been obtained with chymotrypsin which showed no evidence of alcohol binding when a specific substrate was used [4] and evidence of alcohol binding site with a nonspecific substrate [9].

While the effect of solvent on the dielectric constant of the reaction medium cannot be ignored, our results tend to show that such effects are, in general, small. Indeed, in experiments with triacetin as substrate we have not found any correlation between the dielectric constant of the solvent (such as hexane,

dioxane and so on . . .) and the percentage of inhibition.

A formal reactivity ratio between alcohol and water, r, can be estimated from the V_W/V_N ratio under non-saturating conditions, at low alcohol concentration, where the rate of alcoholysis is roughly proportional to nucleophile concentration. According to Wynne and Shalitin [9]

$$r \simeq \frac{V_N[H_2O]}{V_W[ROH]}$$

The value obtained with carboxylic-ester hydrolase and *n*-butanol is 4000. This result shows that *n*-butanol is 4000-fold superior to water in the solvolysis of the acetyl-enzyme. This value is higher than that obtained for chymotrypsin catalyzed solvolysis by methanol [29,30] and lower than that obtained by Wynne and Shalitin who have shown very high reactivity ratios for solvolysis of beef liver esterase with methanol and *n*-butanol [9].

It is worthwhile noting that bile salts such as sodium taurocholate do not modify the mechanism of action of human pancreatic carboxylic-ester hydrolase. In the presence of bile salts, the plotting of $v_W^0/(v_W^0 - v_W)$ vs. the reciprocal of methanol concentration yields a straight line with an intercept on the ordinate different from unity, which shows that the nucleophile is still recognized by carboxylic-ester hydrolase. However, the extension in total rate in the presence of bile salts is small compared to the extension in total rate in the absence of bile salts, while the hydrolysis rate is not affected. This result shows that bile salts probably modify the alcoholysis rate. This effect may be due to a competition between the nucleophile and the detergent which is also recognized by carboxylic-ester hydrolase [16,17], or to any steric hindrance.

In conclusion, the effects of alcohols on the hydrolysis reactions catalyzed by human pancreatic carboxylic-ester hydrolase indicate a competition between water and nucleophile at the level of the deacylation step of the acyl-enzyme. Acylation and deacylation steps are both partially rate-limiting even in the presence of bile salts. The existence of a nucleophile binding site can be postulated.

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