Kinetic Characterization of Bile Salt Micelles

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Abstract: Bile salts were found to perturb the hydrolysis rates of fatty acid esters. This observation was utilized to investigate the structure and binding properties of bile salt micelles. Hydrolysis rates were determined as a function of ester chain length, number and position of hydroxy substituents on the bile salt, ionic strength, presence of urea, etc. Both binding constants and hydrolysis rates of adsorbed ester were evaluated from the observed data. For example, the association constant for *p*-nitrophenyl octanoate and deoxycholate micelles is $5.3 \times 10^4\ M^{-1}$, and the reaction rate of bound ester is within experimental error of zero. Dihydroxy bile salts bind an order of magnitude more strongly than trihydroxy analogs, whereas conjugation of the steroids to glycine or taurine has only a small affect on binding. An attempt was also made to synthesize an enzyme "mimic" by attaching a good nucleophile, an oxime group, to the 7 position of deoxycholate. Micelles of this compound have both binding and catalytic sites. It was found that the bile salt derivative is a considerably better catalyst than acetone oxime in the hydrolysis of *p*-nitrophenyl dodecanoate. A mechanism is proposed in which dodecanoate uncoils when adsorbed onto a steroid surface, thus exposing its ester functionality to a catalyzed hydrolysis.

Bile salts are steroidal surfactants which are emptied from the gall bladder into the upper intestines for the purpose of solubilizing dietary lipids and aiding their absorption. We present below a study of bile salt/lipid complexation using kinetic methods (methods which have been applied with great success to synthetic surfactants[3,4] but never to bile salts). Although the main reason for this work was our interest in elaborating the nature of micellar reactions, the practical importance of understanding bile salt interactions cannot be ignored. For example, atherosclerosis has been treated with cholestyramine,[5] a drug which binds to bile salts and thus reduces cholesterol absorption. Dissolution of gallstones is assisted by oral administration of a bile salt, chenodeoxycholic acid.[6]

The literature on bile salt chemistry is so vast that we can only summarize the essentials and suggest that other articles[7-9] be consulted for details. Bile acid is a name given to several cholanic acid derivatives found in bile which differ in the number or position of α-hydroxy substituents (Figure 1). Human bile contains principally cholic acid (3α, 7α, and 12α), deoxycholic acid (3α and 12α), and chenodeoxycholic acid (3α and 7α). In the native state, bile acids are "conjugated," *i.e.*, linked *via* an amide bond to either glycine ($H_2NCH_2COO^-$) or taurine ($H_2NCH_2CH_2SO_3^-$), thereby imparting greater water solubility to the steroids. The stereochemistry of bile salts is particularly noteworthy. Since the hydroxy groups are all α (Figure 1), they are directed toward the concave side of the carbon framework (which is crescent-shaped owing to the cis A/B ring juncture). Hence, two regions of different polarity can be identified: a hydrophobic side containing the angular methyl groups, and a more polar face bearing the hydroxy substituents.

When bile salts are dissolved in water, they form aggregates (micelles) above a critical concentration (the CMC) as do linear ionic surfactants. However, bile salt micelles have large hydroxylated surfaces (not merely ionic functionalities at the ends of chains) exposed to the aqueous phase. The micelles can be crudely depicted by broad cylinders: the hydrophobic sides of the steroids comprise the interiors whereas the hydroxy and carboxylate groups project from the outside walls. Although the properties of the micelles vary considerably with conditions, the following values are useful guideposts.[10] At pH 9.0 and 0.10 *M* NaCl, the CMC for cholic acid and deoxycholic acid are 3.3 and 1.0 m*M*, respectively. The number of bile salt units per micelle are 5 and 15, respectively. Lowering the pH decreases the CMC and increases the aggregation number especially for deoxycholic acid. The conjugated bile salt, sodium taurocholate, has a pH-independent CMC of 3.2 m*M* (0.15 *M* NaCl, 20°) and an aggregation number of 5. Increasing the ionic strength decreases the CMC and increases the aggregation number.

We present below rate constants for the basic hydrolysis of *p*-nitrophenyl esters of fatty acids (ranging in chain length from 2 to 18) in the presence of a variety of bile salts (Figure 1). The observed rate perturbations produced by the steroidal surfactants were measured as a function of ester size, bile salt structure, presence of urea, etc. Analysis of the rate data showed that the micelles bind the long-chain esters tightly (with association constants equaling or exceeding those between many enzymes and their specific substrates). Reactivities of ester molecules within the micelles could also be determined from the observed rate constants.

(1) Recipient of a Camille and Henry Dreyfus Foundation Teacher–Scholar Grant and a National Institutes of Health Career Development Award.

(2) Participant in the Emory University Medicinal Chemistry Program supported by the National Institutes of Health (Grant GM01970).

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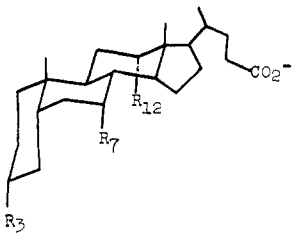

Figure 1. Bile salts used in this work: cholic acid (CA), R_3 = R_7 = R_{12} = OH; deoxycholic acid (DCA), R_3 = R_{12} = OH, R_7 = H; chenodeoxycholic acid (CDA), R_3 = R_7 = OH, R_{12} = H. Glycine and taurine conjugates of cholic acid (GCA, TCA) and deoxycholic acid (GDCA, TDCA) were also investigated.

The idea occurred to us that it might be possible to synthesize an enzyme mimic by attaching a good nucleophile to a bile salt. The resulting compound would have, as do all enzymes, both a catalytic site and a binding site. We investigated, therefore, the "esterase activity" of a deoxycholic acid derivative bearing a nucleophilic oxime group.

Experimental Section

Materials. *p*-Nitrophenyl acetate (p-NPA) was prepared by the method of Chattaway[11] and recrystallized repeatedly until it was nearly colorless, mp 77–78° (lit.[11] mp 77.5–78°). (All our melting points are corrected.) *p*-Nitrophenyl hexanoate (p-NPH) was prepared in the same way and distilled two times through a 4-cm vacuum-jacketed Vigreux column, bp 106–111° (0.01 mm) (lit.[12] bp 174.6° (6 mm)). *p*-Nitrophenyl octanoate (p-NPO) and *p*-nitrophenyl dodecanoate (p-NPD) were secured from Pierce Chemical Co. and used without further purification. Sigma Chemical Co. supplied the *p*-nitrophenyl esters of myristic acid (p-NPM), palmitic acid (p-NPP), and stearic acid (p-NPS). Mono-*p*-nitrophenyl dodecanedioate (p-NPDD) had been prepared previously.[13]

Urea (Fisher) was crystallized from water, dried *in vacuo* at 80°, and stored in a desiccator. Acetone oxime (Eastman) melted at 59.5–60.5° (lit.[14] mp 59°) after recrystallization from petroleum ether. Acetonitrile was distilled twice over phosphorus pentoxide and once over anhydrous potassium carbonate through a 30-cm Vigreux column. Alternatively, we used Eastman spectroanalyzed material stored over type 4A molecular sieve with no difference in results.

Deoxycholic acid (DCA) from Mann Research Labs or Sigma Chemical Co. was extracted with refluxing carbon tetrachloride;[15] the residue was then crystallized twice from acetone followed by drying under reduced pressure at 151° to constant weight, mp 175.4–176.2° (lit.[16] mp 176–178°). Cholic acid (CA) from Pierce Chemical Co. was crystallized two times from absolute ethanol and dried *in vacuo* at 151° to constant weight, mp 197.7–198.5° (lit.[16] mp 198°). Chenodeoxycholic acid (CDA) and all conjugated bile salts were obtained from Calbiochem and used as received.

The synthesis of 3α,12α-dihydroxy-7-keto-5β-cholanic acid oxime involved initial conversion of cholic acid into the ethyl ester of 7-keto-DCA according to the method of Fieser and Rajagopalan.[17] Subsequent hydrolysis of the ester by the method of Hoehn and Linsk[18] gave the corresponding acid. The final step of the sequence, based on the work of Redel, *et al.*,[19] will be described in detail.

3α,12α-Dihydroxy-7-keto-5β-cholanic Acid Oxime. Hydroxylamine hydrochloride (5 g, 0.072 mol) was dissolved in 6 ml of water and mixed with 12 ml of 5 *N* NaOH and then 50 ml of ethanol. The mixture was cooled in an ice bath, filtered to remove NaCl, and added to a round-bottomed flask containing 10.5 g (0.026 mol) of 7-keto-DCA dissolved in 50 ml of absolute ethanol. After boiling under reflux for 6 hr, the solution volume was reduced to 30 ml with the aid of a rotary evaporator. Precipitation of the oxime occurred upon dilution to 200 ml with water. The product was collected by filtration; concentrating the filtrate to 75 ml and cooling yielded more material. Since the oxime could not be purified by crystallization, it was precipitated as the sodium salt from 100 ml of 1 *N* NaOH–ethanol. The material was filtered, dissolved in 400 ml of water, and acidified with HCl to produce 6.3 g (58%) of solid 7-keto-DCA oxime. Purification (including removal of ketone) was effected by two crystallizations from 20% water in methanol followed by drying *in vacuo* at 100° for 12 hr, mp 158–160° (lit.[19] mp 159–160°).

Anal. Calcd for $C_{24}H_{39}O_5N$: C, 68.37; H, 9.33; N, 3.32. Found: C, 68.16; H, 9.34; N, 3.26.

Bile Salt Purity. All the bile salts and bile salt derivatives used for the kinetics were found to be homogeneous by tlc using Eastman silica gel chromatogram sheets and ether–acetone (9:1 v/v), chloroform–ethanol (1:9 v/v), or ethanol.

Buffers and Bile Salt Solutions. Buffers of pH 10.53, 11.50, and 12.04 were prepared as directed by Bates and Bower[20] from 0.012 *M* $Na_2B_4O_7$, 0.025 *M* Na_2HPO_4, and 0.025 *M* Na_2HPO_4, respectively. Sodium chloride was used to maintain ionic strength. Buffers were stored in a refrigerator in polyethylene bottles. Stock solutions of bile salts were prepared by (1) mixing a bile acid with an equimolar amount of sodium hydroxide in a beaker, (2) adding 10–15 ml of one of the buffers, (3) adjusting the pH with small quantities of aqueous NaOH or HCl, and (4) transferring the solution quantitatively to a volumetric flask and diluting to the mark with more buffer. Lower concentrations of bile salt were secured by serial dilution of the stock solutions. Final pH adjustments were made immediately prior to the kinetic runs. Periodically, a solution was tested for pH constancy by comparing its pH before and after a kinetic run; the largest deviation was 0.03 pH unit. A Corning Model 12 pH meter with an expanded scale and a low sodium error glass electrode was used in this work.

Kinetics. The following procedure, given for one particular substrate and bile salt, is typical of that used throughout. A 3.00-ml solution of 0.1 *M* sodium deoxycholic acid (pH 12.04, I = 0.23) was pipetted into a 1-cm cuvette. The cuvette was stoppered and placed within the thermostated chamber (25.0 ± 0.1°) of a Cary 14 or Beckman Acta II spectrophotometer. After about 20 min, 25 μl of a solution of *p*-nitrophenyl octanoate in acetonitrile was added to the cuvette (with the aid of a stirring rod flattened at one end) such that the initial ester concentration was 4.4×10^{-6} *M*. The increase in absorbance at 400 nm (0.1 slidewire) was then traced as a function of time until no absorbance change could be detected for at least 1 half-life. Pseudo-first-order rate constants were calculated from the absorbance–time data by means of a least-squares program using either a PDP-9 or an RCA Spectra 70/55 digital computer. Duplicate runs, including those involving different spectrophotometers, agreed to better than 3%. Repeat experiments performed 3 years apart agreed to within 10%.

Binding Constants. Binding constants of esters to bile salt micelles were determined by the method of Menger and Portnoy.[13] The assumptions in this treatment and the need to use small rate differences caused considerable uncertainty in the K values (see Discussion).

Results

We determined the hydrolysis rates of ten fatty acid esters in the presence of varying concentrations of eight natural and three modified bile salts. Since it is obviously difficult to cite hundreds of rate constants,[21] we shall only summarize the salient observations.

(1) The rate constants for basic hydrolysis of *p*-nitrophenyl esters of chain length greater than six decrease in the *absence* of bile salts with increasing ester concentration. This is consistent with previous ob-

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Table I. Observed Rate Constants (in sec^{-1}) for the Basic Hydrolysis of *p*-Nitrophenyl Esters in Aqueous Solutions of Sodium Deoxycholate at 25.0°

[DCA] × 10^2, *M*	p-NPA[a]	p-NPH[b]	p-NPO[c]	p-NPD[d]
0.00	4.29×10^{-3}	1.86×10^{-2}	7.95×10^{-2}	9.78×10^{-4}
0.10	4.24×10^{-3}	1.51×10^{-2}	7.62×10^{-2}	9.46×10^{-4}
0.30	4.21×10^{-3}	1.43×10^{-2}	5.18×10^{-2}	1.73×10^{-3}
0.60	4.05×10^{-3}	8.09×10^{-3}	9.25×10^{-3}	1.22×10^{-3}
1.0	3.93×10^{-3}	5.08×10^{-3}	3.98×10^{-3}	9.01×10^{-4}
2.0	3.71×10^{-3}	2.62×10^{-3}	1.70×10^{-3}	
3.0	3.46×10^{-3}	1.73×10^{-3}	1.20×10^{-3}	6.02×10^{-4}
6.0	3.39×10^{-3}	9.37×10^{-4}	7.53×10^{-4}	6.12×10^{-4}
10.0	3.06×10^{-3}	6.78×10^{-4}	6.74×10^{-4}	5.46×10^{-4}

[a] [p-NPA] = 4.13×10^{-5} *M*, pH 10.53, $I = 0.18$. [b] [p-NPH] = 4.40×10^{-6} *M*, pH 11.50, $I = 0.23$. [c] [p-NPO] = 4.40×10^{-6} *M*, pH 12.04, $I = 0.23$. Identical rate constants were obtained with 4.44×10^{-5} *M* p-NPO at 0.006 *M* DCA or higher; below 0.006 *M* DCA the latter rate constants were 3–4-fold smaller. [d] [p-NPD] = 4.37×10^{-6} *M*, pH 12.04, $I = 0.23$.

servations[22,23] and can be ascribed to an aggregation or coiling of the substrate molecules which impairs hydrolysis. In the presence of a bile salt *above* its CMC, where the bulk of our data was gathered, the rates varied only slightly with *p*-nitrophenyl octanoate or dodecanoate concentration (3×10^{-6} to 3×10^{-5} *M*). The bile salt solubilizes the esters so that substrate aggregation is no longer an important factor.

(2) In the presence of conjugated or free bile salt, the *p*-nitrophenyl esters of acetic acid (p-NPA), hexanoic acid (p-NPH), octanoic acid (p-NPO), and dodecanedioic acid (p-NPDD) invariably display diminished reactivity toward hydroxide ion (Figure 2 and Table I). On the other hand, *p*-nitrophenyl dodecanoate (p-NPD) hydrolysis is accelerated by cholic acid (CA), chenodeoxycholic acid (CDCA), and 7-keto-DCA (Figure 1).[21] Both the inhibitions and accelerations become appreciable only above the CMC of the bile salts (with the breaks in the curves corresponding to known CMC values[7]). Therefore, the rate perturbations are micellar in origin. We see from Table I that 0.1 *M* DCA inhibits hydrolysis of p-NPA by only 29%. In contrast, the longer chained esters, p-NPH and p-NPO, are inhibited 27- and 118-fold, respectively, by 0.1 *M* DCA.

(3) Rate changes depend on the structure of the bile salt as well as the chain length of the substrate. Both the number and the position of the hydroxy group substituents on the steroid nucleus are important. Without exception, dihydroxy bile salts show a greater inhibitory effect (or smaller catalysis in the case of p-NPD) than their trihydroxy analogs. Moreover, DCA, a dihydroxy bile salt substituted in the 3α and the 12α positions, inhibits ester hydrolysis to a greater extent than CDCA with its 3α- and 7α-hydroxy groups. By way of comparison, the hydrolysis of p-NPO at pH 12.04 is inhibited by 0.1 *M* bile salt to the following extents: DCA, 118×; CDCA, 58×; CA, 22×. Selective oxidation of CA at the 7 position (to give 7-keto-DCA) reduces the value to 15×. Conjugation of the bile salts with either taurine or glycine lessens the inhibitory effect,[21] but does not change the order of the sequence: DCA > CDCA > CA.

(4) Urea accelerates the hydrolysis of p-NPO in solutions of DCA at concentrations near the CMC. For example, k_{obsd} for hydrolysis of 4.40×10^{-6} *M* p-NPO ([DCA] = 0.01 *M*, pH 11.50, $I = 0.23$, $T = 25.0°$) increases from 8.86×10^{-4} to 8.68×10^{-3} sec^{-1} upon addition of 6.0 *M* urea.[21] Urea (6 *M*) has only a minor effect at high levels of DCA (0.1 *M*).

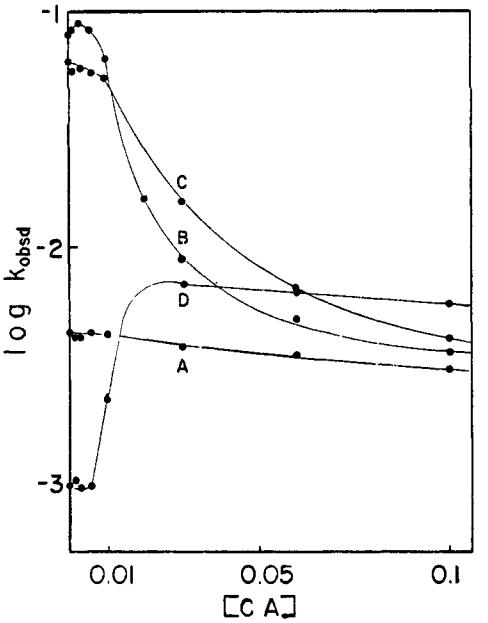

Figure 2. Plots of log k_{obsd} in sec^{-1} *vs.* concentration of cholic acid for the basic hydrolysis of (A) p-NPA, (B) p-NPO, (C) p-NPDD, and (D) p-NPD at 25.0°. Reaction conditions: (A) pH 10.53, $I = 0.18$; (B, C, and D) pH 12.04, $I = 0.23$.

(5) The rate of micellar hydrolysis of p-NPO in DCA solutions is insensitive to ionic strength, increasing only 19% when the ionic strength was elevated from 0.23 to 0.90. As will be shown, this observation bears on the validity of a currently advanced structural theory of bile salt micelles.

(6) 3α,12α-Dihydroxy-7-keto-5β-cholanic acid oxime (7-keto-DCA oxime) catalyzes the release of *p*-nitrophenol from several esters (Figure 3). However, the catalysis differs dramatically from that of acetone oxime. Acetone oxime gives linear k_{obsd} *vs.* [oxime] plots at constant pH with *p*-nitrophenyl esters of eight carbons or less.[21] This is a manifestation of simple nucleophilic catalysis by oxime anion.[24] On the other hand, none of the esters, even p-NPA, has a rate linearly related to the concentration of 7-keto-DCA oxime at constant pH. We also see from Figure 3 that the log k_{obsd} *vs.* [7-keto-DCA oxime] plots for p-NPH, p-NPO, and p-NPDD (but not p-NPA) possess

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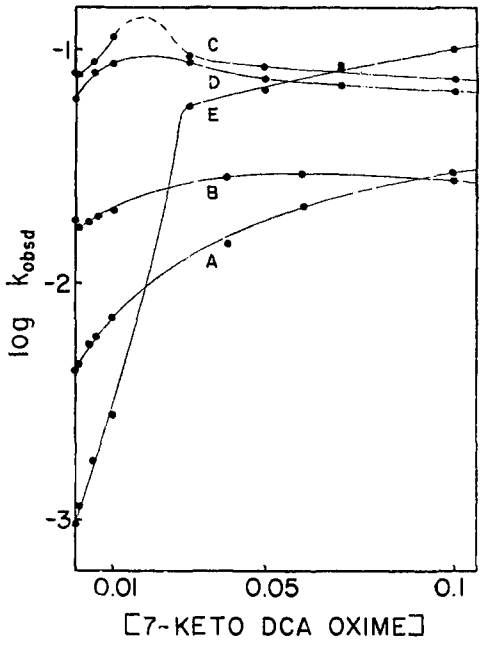

Figure 3. Plots of log k_{obsd} in sec^{-1} *vs.* concentration of 7-keto-DCA oxime for the reaction with (A) p-NPA, (B) p-NPH, (C) p-NPO, (D) p-NPDD, and (E) p-NPD at 25.0°. Reaction conditions: (A) pH 10.53, I = 0.18; (B) pH 11.30, I = 0.23; (C, D, and E) pH 12.04, I = 0.23–0.26.

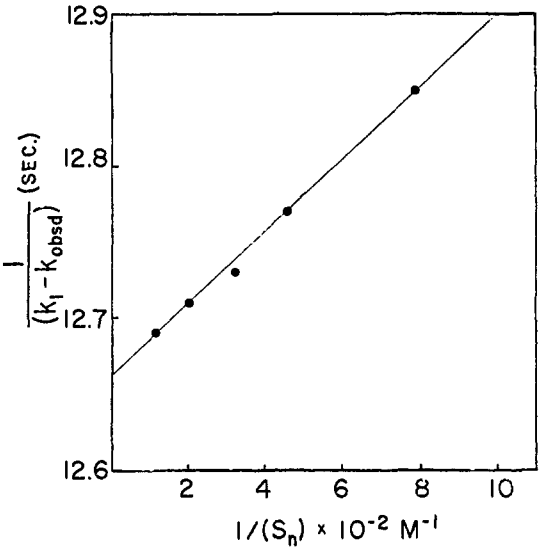

Figure 4. Determination of the association constant for p-NPO and DCA micelles in aqueous solutions of pH 12.04, I = 0.23, 25.0°. See eq 2.

maxima. Although these four esters react with steroidal oxime more slowly than with acetone oxime at the same pH (the pK_a values for the two nucleophiles being 12.5[25] and 12.4,[24] respectively), the difference is not substantial. For example, k_{obsd} for the hydrolysis of p-NPH (pH 11.30, I = 0.23, 25.0°) in 0.006 M 7-keto-DCA oxime is 1.93×10^{-2} sec^{-1}; the corresponding value in 0.006 M acetone oxime is 3.26×10^{-2} sec^{-1}. Importantly *p*-nitrophenyl dodecanoate (p-NPD) reacts much *faster* with 7-keto-DCA oxime than with the nonmicellar oxime of acetone: 0.1 M steroid increases the rate 102-fold above background hydrolysis (pH 12.04, I = 0.26), whereas the increase under equivalent conditions for acetone oxime is only 7. The bulk of the 102-fold increase arises from catalysis *below* the CMC (Figure 3).

Discussion

We begin this discussion with a summary of current thought on the structure of bile salt micelles. Micelles of trihydroxy bile salts (free or conjugated) and of dihydroxy bile salts at low ionic strength contain less than 20 units.[7,8] The steroid molecules aggregate with their hydrophobic sides (*i.e.*, the β sides in Figure 1) back-to-back in the micelle interior. This arrangement exposes the relatively hydrophilic α faces to the water. The degree of water penetration into the interior is unknown. Addition of sodium chloride to a dihydroxy bile salt in alkaline water can cause the aggregation number to increase manyfold,[26] although cholic acid does not seem to be similarly affected. Small[8] visualizes these large "secondary" micelles to be conglomerates of "primary" micelles which are held together by intermicellar hydrogen bonding between surface hydroxy groups. With either type of micelle there are three possible adsorption sites: the hydrophobic interior, the micelle exterior studied with hydroxy groups, and a polar region in proximity to anionic side chains and their counterions.

A comparison was made of the effect of the more common conjugated and unconjugated bile salts on the rates of hydrolysis of several fatty acid esters, and sample data are presented in Figure 2 and Table I. These show that the hydrolyses of p-NPA, p-NPH, and p-NPO are inhibited above the CMC of the surfactants with an increasing order of effectiveness. (p-NPD shows unusual behavior and will be considered later.) Since the water solubilities of the esters decrease in the same order, at least part of their differences might be ascribed to variations in the amount of nonmicellar material. We therefore analyzed the kinetics in terms of the scheme shown in eq 1 in which

$$\begin{array}{ccc} S_n + E & \overset{K}{\rightleftharpoons} & S_nE \\ \downarrow k_1 & & \downarrow k_2 \\ P & & P \end{array} \qquad (1)$$

S_n, E, and S_nE denote micellar steroid, free ester, and bound ester, respectively; K is the ester–micelle association constant, and P is hydrolysis product. The scheme leads to eq 2 (a rate law which was first applied to

$$\frac{1}{(k_1 - k_{obsd})} = \frac{1}{(k_1 - k_2)} + \frac{1}{(k_1 - k_2)K[S_n]} \qquad (2)$$

micellar systems by Menger and Portnoy[13] and which has been used successfully in subsequent years[3,4]). Several assumptions underlie eq 2.[13] In particular, eq 2 is valid only if the presence of ester does not modify micellar properties such as the CMC or aggregation number. This was one reason why the initial substrate concentration was kept very low (less than 5×10^{-6} M with most runs). Evaluation of the association constant (K) and the reactivity of adsorbed material (k_2) was accomplished by plotting $1/(k_1 - k_{obsd})$ *vs.* $1/[S_n]$ as shown in Figure 4. The concentration of micelles,

(25) This pK_a was determined by the "half-neutralization" method using 0.07 M 7-keto-DCA oxime, a concentration well above the CMC.

(26) Even larger micelles (aggregation number greater than 1000) can be produced with unconjugated DCA by lowering the pH to 7. Below neutrality the bile salt precipitates.

$[S_n]$, was secured from the concentration of bile salt in excess of the CMC divided by the aggregation number (eq 3).[13] Equation 3 is a satisfactory approximation

$$[S_n] = ([\text{bile salt}] - \text{CMC})/n \quad (3)$$

for simple surfactants, and we assume that the same is true for bile salts although we have no evidence on this point. The observed rate constants in the total absence of bile salt gave the k_1 values for Figure 4 and similar plots.

Equilibrium constants are tabulated in Table II

Table II. Binding Constants of Several Esters to Bile Salt Micelles in Aqueous Solutions at 25.0° [a]

Bile salt	Ester	CMC	Ag. no.[c]	K, M^{-1}
DCA[f]	p-NPA	0.006[b]	15	3.6×10^2
CA[f]	p-NPA	0.012[b]	5	1.1×10^2
DCA	p-NPH	0.006	15	5.4×10^3
DCA	p-NPO	0.006	15	5.3×10^4
CDCA	p-NPO	0.006[b]	11	2.9×10^4
CA	p-NPO	0.012	5	2.2×10^3
TDCA	p-NPO	0.0018[d]	22	3.5×10^4
GDCA	p-NPO	0.0012[d]	19	3.3×10^4
TCA	p-NPO	0.009[e]	5	6.5×10^3
GCA	p-NPO	0.007[e]	6	2.8×10^3

[a] See Table I for reaction conditions. [b] A. F. Hofmann and D. M. Small, *Annu. Rev. Med.*, **18**, 333 (1967). [c] Reference 8 (20°, 0.15 *M* NaCl). [d] Reference 9. [e] A. F. Hofmann, *Biochem. J.*, **89**, 57 (1963). [f] Kinetically determined CMC values were used to calculate *K* for these bile salts.

along with CMC and aggregation numbers from the literature. Owing to the many assumptions, the equilibrium constants are probably accurate only to within a factor of 2. To our knowledge, however, Table II provides to date the only quantitative data on the binding of fatty acid derivatives to bile salt micelles.

The reaction rates for adsorbed ester (k_2) were found to be small compared with that for bulk phase ester (k_1). In fact, k_2 for p-NPH and p-NPO is less than the experimental uncertainty in k_1 ($\pm 3\%$), and therefore micellar hydrolysis is experimentally undetectable for these substrates. This observation, along with the prodigious binding constants discussed below, strongly suggests that p-NPH and p-NPO reside within the hydrocarbon interior of the micelle where they would be shielded from hydroxide ion. Since the k_2 for p-NPA in DCA solutions was calculated to be greater than 60% of k_1, this small ester probably adsorbs near the micelle surface.

With regard to binding, the following generalizations can be deduced from Table II. (1) The longer the ester chain, the larger the binding constant. Binding constants approach five orders of magnitude—equalling or exceeding those for complexation between enzymes and many of their specific substrates. (2) Dihydroxy bile salts bind an order of magnitude more effectively than trihydroxy analogs. (3) Conjugation lowers the CMC but has little effect on binding of fatty acid esters.

The behavior of *p*-nitrophenyl dodecanoate (Figure 2D, Table II) is not adequately described by eq 1 and 2. DCA (0.10 *M*) causes only a minor twofold inhibition, and CA actually catalyzes the hydrolysis. The difference between p-NPD and the other shorter esters resides in the necessity of using initial ester concentrations no less than 4×10^{-6} *M* in order to be able to follow the reactions spectrophotometrically. But this concentration, low as it is, nevertheless exceeds the aggregation level for p-NPD.[23] Clearly, the observed rate perturbations for p-NPD reflect the *difference* in environments between coiled or aggregated ester *outside* the bile salt micelles and ester adsorbed *within* the micelles. Ester aggregates must exclude water more effectively than do CA micelles since the latter accelerate hydrolysis. Owing to the complexity of the situation, we made no attempt to analyze the kinetics of p-NPD hydrolysis rigorously.

Urea (6 *M*) removes the inhibition of p-NPO hydrolysis by 0.01 *M* DCA, presumably by elevating the CMC above 0.01 *M*.[27] In solutions containing DCA concentrations greatly exceeding the CMC (0.1 *M*), 6 *M* urea displays only a twofold enhancement. Since urea decreases aggregation numbers,[8] the observed kinetics are not crucially affected by small decreases in the size of the primary micelles.

The above discussion deals mainly with rate *inhibitions* induced by bile salts. Rate inhibitions are, of course, as valuable in characterizing bile salt micelles as are rate accelerations. Any parameter is useful as long as it responds (negatively or positively) to structural changes in the steroids or steroid micelles, and rate constants obviously meet this criterion. Nevertheless, our interest in enzyme action prompted us to search for bile salt derivatives which would manifest substantial catalytic activity. These systems would then possess two properties of enzymes: binding and catalysis. The possibility of regiospecificity and stereospecificity with the chiral steroids also presented itself. This line of thinking led to the synthesis of 3α,12α-dihydroxy-7-keto-5β-cholanic acid oxime (7-keto-DCA oxime). The kinetic behavior of this compound toward several esters is described in part 6 of the Results and in Figure 3. Three substrates, p-NPH, p-NPO, and p-NPDD, display maxima in Figure 3, suggesting the involvement of both catalytic and inhibitory factors. Equation 4 accommodates these two effects. *p*-Nitro-

$$\begin{aligned} E + {}^-OH &\xrightarrow{k_1} P \\ E + S_t &\xrightarrow{k_2} P \\ E + S_n &\underset{}{\overset{K}{\rightleftharpoons}} S_nE \end{aligned} \quad (4)$$

phenyl ester (E) reacts bimolecularly in alkaline solutions with hydroxide ion and oximate. The nucleophile in the latter reaction is *total* steroid (S_t) because micellar and nonmicellar oximate are assumed to possess identical reactivities. Ester can also bind to the micelles where the rate of *p*-nitrophenolate release is assumed to be within experimental error of zero. This simple three-equation scheme is sufficient to describe fully the 7-keto-DCA oxime system. There is no need to invoke a Michaelis–Menten-type mechanism (*i.e.*, an oxime-catalyzed reaction of *bound* ester). Parameters k_2 and K can be evaluated from eq 5 and the

$$k_{obsd} = \frac{k_1[OH^-] + k_2[S_t]}{1 + K[S_n]} \quad (5)$$

following additional assumptions. (1) Equation 3 is

(27) M. F. Emerson and A. Holtzer, *J. Phys. Chem.*, **71**, 3320 (1967).

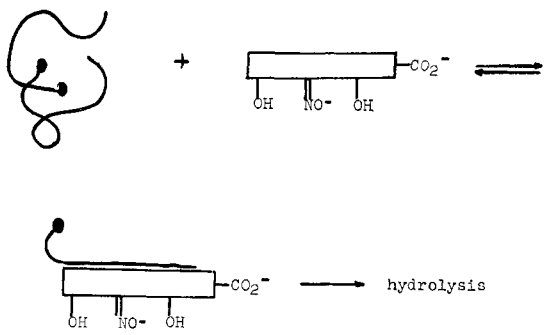

Figure 5. Schematic representation of the catalyzed hydrolysis of *p*-nitrophenyl dodecanoate by monomeric 3α,12α-dihydroxy-7-keto-5β-cholanic acid oxime.

valid. This permits calculation of the micelle concentrations, $[S_n]$, using a CMC = 0.015 *M* (near the maxima in Figure 3) and an aggregation number = 5. (2) The structure of the 7-keto-DCA oxime micelle remains constant within the pH range used in this investigation (10.53–12.04). (3) Micellation occurs precisely at the CMC rather than over a concentration range. (4) Substrate and micelle associate with a 1:1 stoichiometry only. (5) Substrate does not perturb the structure of the micelles. (6) Only anionic steroidal oxime (pK_a = 12.5) reacts. Despite these assumptions and approximations, the curves in Figure 3 can be closely duplicated above the CMC for all esters except p-NPD.[21] The k_2 values for 7-keto-DCA oxime are 2–3 times less than the corresponding bimolecular rate constants for acetone oxime. Association constants *K*, varying from 5.9 M^{-1} for p-NPA to 270 M^{-1} for p-NPO, are considerably smaller than the binding constants to CA and DCA (see Table II). No doubt, this reflects a "looseness" of the steroidal oxime micelle caused by the polar substituent in the 7 position.

The steroidal oxime catalyzed hydrolysis of p-NPD (Figure 3E) is not explained by the preceding model. In contrast to the other esters, p-NPD reacts faster with 7-keto-DCA oxime than with acetone oxime. Thus, the rate in 0.1 *M* 7-keto-DCA oxime is more than two orders of magnitude greater than the rate in buffer alone. This constitutes a 14-fold increase over the catalysis produced by acetone oxime at the same concentration and pH. Owing to coiling or aggregation, p-NPD reacts relatively slowly with acetone oxime anion. When ester complexes with a steroid monomer or dimer (below the CMC) or with a bile salt (above the CMC), the long-chain ester unravels and exposes its carbonyl group to attack. Since acetone oxime *plus* CA was found to give similar rates to steroidal oxime alone under comparable conditions, ester bound to 7-keto-DCA oxime seems to react with external nucleophile. In any event, it is clear from the abrupt leveling of the rate in Figure 3E *above* the CMC that the micelle interior is less conducive to reaction than the surface of a steroid monomer or dimer. Figure 5 depicts monomer-catalyzed hydrolysis of p-NPD. Despite the steroid catalysis, the reactivity of bound p-NPD is still exceeded by that of p-NPA in the bulk solution. Complexation of p-NPD does not completely free the ester functionality from hydrophobic inhibition.

The observed rate constants for hydrolysis of *p*-nitrophenyl octanoate, dodecanoate, myristate, palmitate, and stearate in 0.1 *M* cholic acid (pH 12.04, *I* = 0.23, 25.0°) are all similar: 3.86×10^{-3}, 5.22×10^{-3}, 6.28×10^{-3}, 5.04×10^{-3}, and 1.52×10^{-3} sec^{-1}, respectively. Evidently, the environment of the ester functionality within the micelles is not greatly dependent upon chain length. Since fully extended chains of myristate, palmitate, and stearate are longer than steroid molecules, the chains probably exist in a folded conformation inside the micelles.

The aggregation number of DCA micelles increases from about 5 to 160 upon addition of 0.8 *M* NaCl.[8] According to Small,[8] high salt concentrations cause small *primary* micelles to hydrogen bond into large aggregates called *secondary* micelles. If this "aggregates of aggregates" model is correct, then the rate constants for hydrolysis of p-NPO should be insensitive to ionic strength; agglutination of primary micelles should have no fundamental affect on the nature of the micelle interiors. On the other hand, if the model is incorrect and the structure of the primary micelles is substantially enlarged or otherwise perturbed by changes in ionic strength, then addition of inert salts could modify the rate constants. We found that elevating the ionic strength from 0.23 to 0.90 with sodium chloride increases the rate of p-NPO hydrolysis at pH 12.04 by only 19%. Such a small rate change accompanying a huge increase in aggregation number supports the validity of the secondary micelle concept.[28]

Acknowledgment. This work was supported in part by the National Science Foundation.

(28) An article entitled "Binding Site of Naphthalene to Bile Salt Micelles as Determined by ^1H Nuclear Magnetic Resonance" (F. M. Menger, J. U. Rhee, and L. Mandell) is being published in *Chem. Commun.*