Secondary Valence Force Catalysis. 16. Melittin-Catalyzed Hydrolysis of *p*-Nitrophenyl Dodecanoate

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Melittin, a cationic surface active polypeptide containing 26 amino acid residues isolated from the venom of the common honeybee *Apis mellifera*, is an effective catalyst for the hydrolysis of *p*-nitrophenyl dodecanoate. Catalytic activity reflects the capacity of melittin to (1) disperse aggregates of this ester which form in aqueous solution and (2) facilitate attack of hydroxide ion on the dispersed ester. An unexpected observation is the finding that a complex of dodecanoate and melittin is a more effective catalyst than is melittin itself. This is a unique example of the facilitation of the attack of an anionic nucleophile by an anionic surfactant.

The rates of many organic reactions are influenced by the nature and the concentration of micelle-forming amphipathic, or surfactant, molecules or ions.[1-5] Such molecules generally consist of a straight hydrocarbon chain, usually 8–18 carbon atoms in length, to which a polar head group is attached. In some cases the head group only provides a specific chemical environment for the reaction; in others it participates directly in the reaction as, for example, a nucleophile. The similarities between micelles and globular proteins and the utility of micelles in organic synthesis[6] have stimulated considerable interest in micelle-catalyzed reactions. Previous investigations have provided significant information concerning the principal features of reactions catalyzed by structurally simple surfactants. Consequently, it appears appropriate to deviate from established patterns in order to probe the possible catalytic properties of more complex surfactants, especially those which are polypeptides and, hence, which may shed new light on enzymatic reaction mechanisms.

One example of a complex polypeptide amphipath is the low molecular weight polypeptide, melittin,[7] which is the major component of venom obtained from the common honeybee, *Apis mellifera*. The primary structure is provided below:[8]

Gly-Ile-Gly-Ala-Val$_5$-Leu-Lys-Val-Leu-Thr$_{10}$-Thr-Gly-Leu-

Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln(NH_2)
$_{15}$ $_{20}$ $_{25}$

A single melittin subunit consists of 26 amino acids. Since the two γ-carboxyl groups of residues 25 and 26 and the C terminus occur as the corresponding amides, there are no negatively charged centers. In contrast, there are six free basic groups: the N terminus, the lysine residue at position seven, and the sequence of four residues near the blocked C terminus. The amino acid sequence is worthy of note: predominantly hydrophobic residues occur in positions 21–26, and, as a consequence, melittin has amphipathic character analogous to that of simpler cationic surfactants. In aqueous solution melittin forms micellelike, highly surface-active[9] structures which contain four polypeptide molecules per aggregate.[10-12] Since cationic surfactants catalyze the hydrolysis of certain aliphatic esters,[13] we chose to probe the possible catalytic activity of melittin for the hydrolysis of *p*-nitrophenyl dodecanoate. The results are presented herein.

Experimental Section

Materials. *p*-Nitrophenyl dodecanoate[14] was prepared by a slight modification of the general procedure of Bender and Nakamura:[15] 0.125 mol of *p*-nitrophenol, 0.10 mol of dodecanoyl chloride, and 0.10 mol of pyridine were dissolved in 100 ml of toluene and refluxed for 1 h. The reaction mixture was then neutralized with saturated $NaHCO_3$ and washed consecutively with water, 5% NaOH, 0.1 N HCl, and finally with water. The resulting solution was dried over anhydrous $MgSO_4$ and the solvent was evaporated. The ester, a light yellow, waxy solid, mp 40–41 °C (lit.[14] 46 °C), carbonyl stretching frequency (liquid film) at 1755 cm^{-1}, was not purified further.

Melittin was purified from the venom of the common honeybee (*Apis mellifera*).[12] One gram of the whole lyophilized crude venom, obtained from Sigma Chemical Co., was dissolved in 2 ml of a 0.1 M ammonium formate buffer, pH 4.5, and applied to a Sephadex G-50 column (1.1 × 300 cm) previously equilibrated with the ammonium formate buffer. The melittin-rich fractions were pooled and lyophilized. The brown powder thus obtained was dissolved in 15 ml of distilled water and divided into ten fractions. A saturated picric acid solution (1.5 ml) was added to each of the ten fractions causing the melittin to precipitate as the picrate complex. The precipitate was washed twice with a 70% picric acid solution and collected by centrifugation. The recovered picrate was dissolved in acetone and dissociated by the addition of concentrated HCl. The precipitation step was repeated and the protein was collected and washed twice with a 1% HCl–acetone solution. The melittin was dissolved in water, the pH was adjusted to 3.0 by the addition of HCl, and the solution was passed through a Dowex AG-1-X8 column (Cl^- form) (0.8 × 10 cm) in order to remove trace amounts of picric acid. The purified protein, 350–400 mg/g crude venom, was stored in aqueous solution at 4 °C.[16]

Other reagents were obtained commercially. Inorganic salts were used without further purification. Glass-distilled water was employed throughout.

Kinetic measurements were performed spectrophotometrically with the aid of a Zeiss PMQII spectrophotometer equipped with a thermostated cell holder. All measurements were made at 25 °C. Reactions were monitored at 400 nm, near the absorption maximum of the *p*-nitrophenolate anion. Each reaction mixture initially contained 3×10^{-2} M triethylamine–ammonium ion buffer. Measured quantities of solid *p*-nitrophenyl dodecanoate were dissolved in acetonitrile in such proportions that the addition of 20 μl of this solution to 3.0 ml of reaction mixture gave the desired ester concentration. First-order rate constants were calculated using the initial straight-line portions of plots of log ($OD_\infty - OD_t$) vs. time in the usual manner. Values of pH were measured with the aid of a Radiometer PHM4c pH meter equipped with a glass electrode.

Results

Hydrolysis of *p*-nitrophenyl dodecanoate at 25 °C and pH 10.0 in the presence of melittin was monitored by observing the appearance of the *p*-nitrophenolate anion. No reaction occurs in the absence of added polypeptide. In Figure 1, the data for this reaction in the presence of melittin are plotted in the usual manner for a pseudo-first-order reaction. It is obvious that the reaction does not obey simple first-order kinetics and that the rate of ester hydrolysis increases with increasing time. Using data from the initial straight-line portion of this curve, an approximate first-order rate constant of 0.056 min^{-1} can be calculated. If, on the other hand, one employs data taken near the terminus of the reaction, the corresponding rate constant is 0.21 min^{-1}.

Following completion of the hydrolysis of 3×10^{-5} M *p*-nitrophenyl dodecanoate in the presence of 1×10^{-4} M melittin, addition of a second aliquot of ester to give again an ester

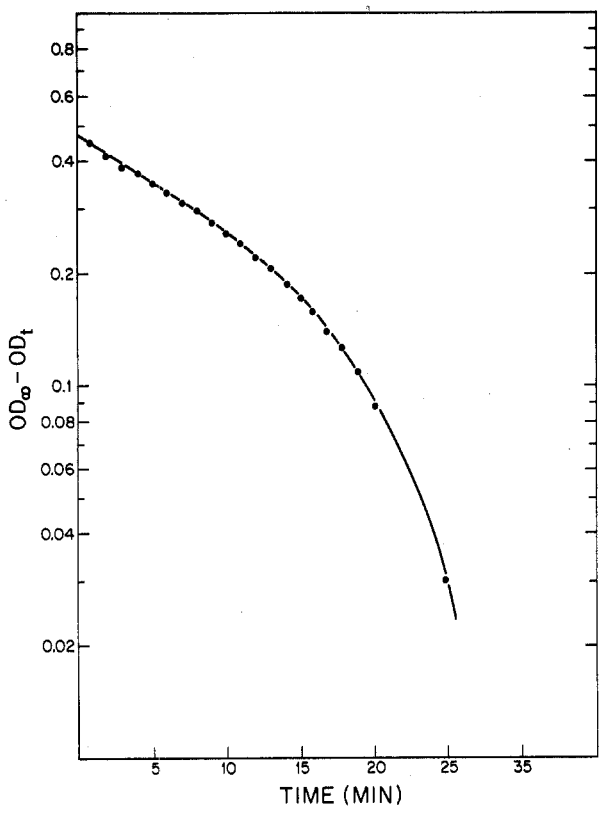

Figure 1. Data for the hydrolysis of 3×10^{-5} M p-nitrophenyl dodecanoate at pH 10.0 and 25 °C in the presence of 1×10^{-4} M melittin plotted according to the usual method for first-order reactions. Employing the data points near the beginning of the reaction yields a first-order rate constant of 0.056 min^{-1}.

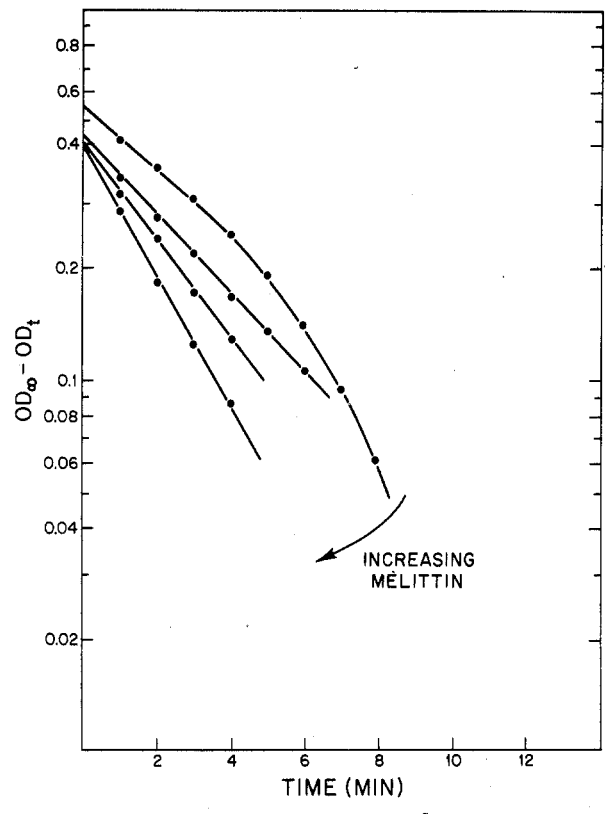

Figure 3. Data for the hydrolysis of 3×10^{-5} M p-nitrophenyl dodecanoate at pH 10.0 and 25 °C in the presence of various concentrations of melittin plotted in the usual manner for first-order reactions. The concentrations of melittin, in increasing amounts as indicated, are 2.5×10^{-4}, 5.0×10^{-4}, 7.5×10^{-4}, and 1.0×10^{-3} M.

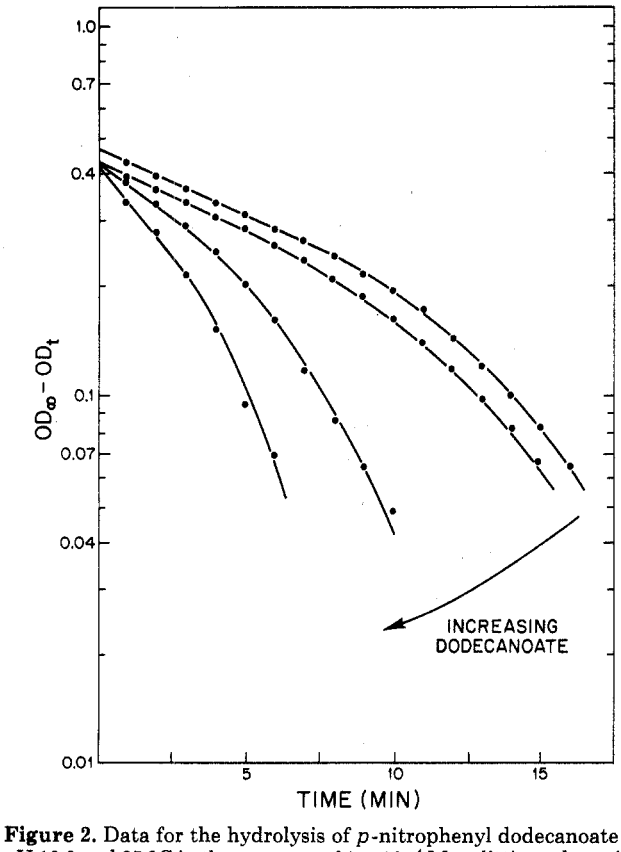

Figure 2. Data for the hydrolysis of p-nitrophenyl dodecanoate at pH 10.0 and 25 °C in the presence of 1×10^{-4} M melittin and varying concentrations of dodecanoate plotted according to the usual method for first-order reactions. The concentrations of dodecanoate, in increasing amounts as indicated, are 7.5×10^{-6}, 1.5×10^{-5}, 3.0×10^{-5}, and 4.5×10^{-5} M.

concentration of 3×10^{-5} M results in an initial rate of ester hydrolysis equal to the terminal rate observed for the first reaction. As before, the rate of ester hydrolysis increases with increasing time. These results establish that the melittin-dependent hydrolysis of p-nitrophenyl dodecanoate is autocatalytic.

The simplest explanation for this autocatalytic behavior is that one or both of the reaction products combine with melittin to form a more effective catalyst than melittin alone. In an effort to probe this possibility, measured quantities of dodecanoic acid were added to the reaction mixture prior to its initiation. In Figure 2 the time course plots for ester hydrolysis in the presence of four concentrations of added dodecanoate are shown. In each case, significant deviations from first-order kinetics are observed. Both initial and final rate constants increase regularly with increasing dodecanoate concentration. An approximately linear relationship is obtained between the initial first-order rate constants and dodecanoate concentration; the addition of 4.5×10^{-4} M dodecanoic acid increases the rate of reaction approximately threefold.

In the presence of higher melittin concentrations the reaction kinetics for the basic hydrolysis of p-nitrophenyl dodecanoate are simpler. In Figure 3, first-order plots for this reaction at melittin concentrations ranging from 2.5×10^{-4} to 1.0×10^{-3} M are shown. There is significant deviation from simple first-order kinetics only at the lowest melittin concentration employed. The respective rate constants for these reactions, evaluated from data near the beginning of the reaction for the lowest concentration of melittin for which deviations from first-order kinetics were observed, are plotted as a function of melittin concentration in Figure 4. Although some scatter in the data is evident, the rate constants increase regularly with increasing concentration of melittin. The

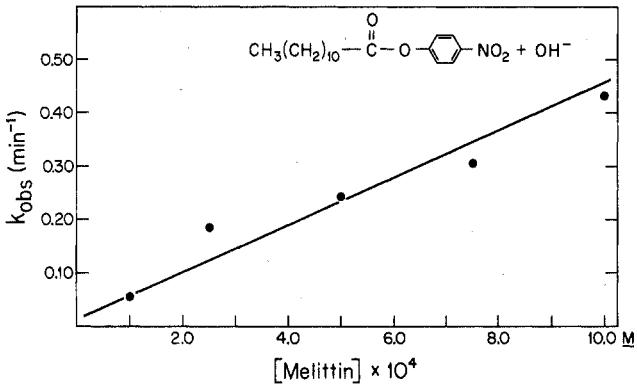

Figure 4. First-order rate constants for melittin-dependent hydrolysis of *p*-nitrophenyl dodecanoate at pH 10.0 and 25 °C plotted as a function of melittin concentration. For the lower concentration of melittin, the first-order rate constants were evaluated from data taken in the first half-life of the reaction only.

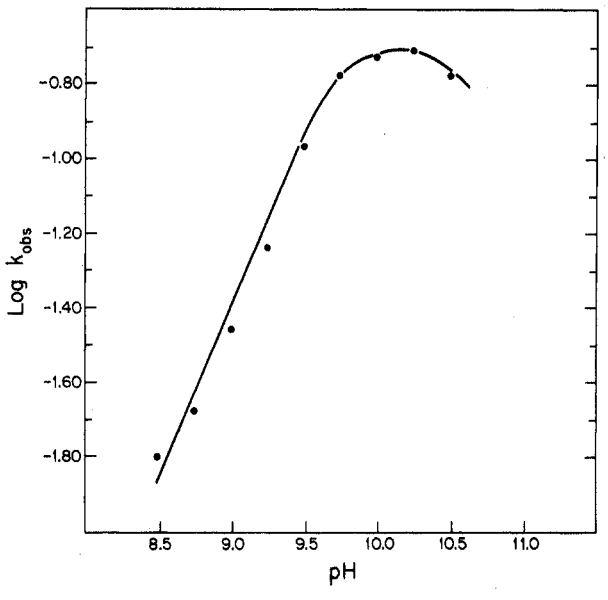

Figure 5. Logarithms of first-order rate constants for hydrolysis of 3×10^{-5} M *p*-nitrophenyl dodecanoate at pH 10.0 and 25 °C and in the presence of 2.5×10^{-4} M melittin plotted against pH.

maximal rate constant obtained, 0.434 min^{-1}, is approximately eightfold greater than that observed at the lowest melittin concentration employed.

In Figure 5, logarithms of first-order rate constants for hydrolysis of *p*-nitrophenyl dodecanoate catalyzed by 2.5×10^{-4} M melittin are plotted as a function of pH. At this concentration of melittin, ester hydrolysis yields a satisfactory approximation to first-order kinetics through at least 1 half-life; rate constants were calculated from data taken during the first half-life of the reactions. In the pH range in which rate constants increase with increasing hydroxide ion concentration, the slope of the line in this plot is somewhat less than unity. At higher values of pH, rate constants become relatively independent of this variable.

In Figure 6, first-order plots for the melittin-catalyzed hydrolysis of *p*-nitrophenyl dodecanoate at pH 10.0 in the presence of 1×10^{-4} M are shown for three concentrations of added sodium bromide. With increasing salt concentration, deviations from first-order kinetics become less noticeable and, moreover, the rate of ester hydrolysis decreases modestly. Similar behavior was observed for addition of sodium chloride and sodium nitrate, the former being less effective and the latter more effective than sodium bromide in eliciting the indicated changes.

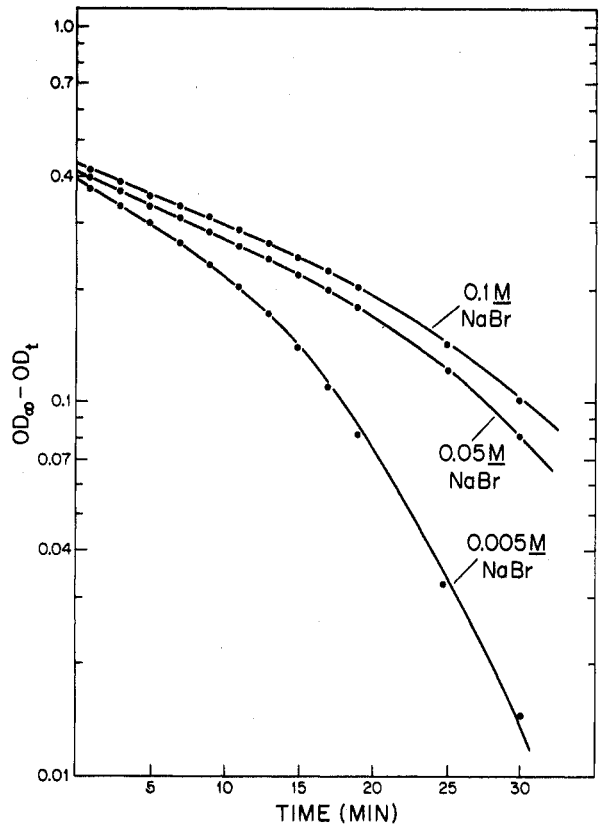

Figure 6. Data for hydrolysis of *p*-nitrophenyl dodecanoate at pH 10.0 and 25 °C and in the presence of 1×10^{-4} M melittin plotted according to the usual method for a first-order reaction at three concentrations of added sodium bromide.

Discussion

Several features of the hydrolysis of *p*-nitrophenyl dodecanoate in aqueous solution have been probed in detail by Menger and Portnoy.[17] These workers have established that this ester forms aggregates in water; the individual molecules in such aggregates are extremely unreactive toward hydroxide ion. Reactivity is so low that no reliable second-order rate constants for attack of hydroxide ion on this substrate in water are available. Agents which disperse the *p*-nitrophenyl dodecanoate aggregates lead to greatly increased rates of base-catalyzed hydrolysis.[13,17]

Previous reports from this laboratory have established that simple *n*-alkyltrimethylammonium bromides are effective catalysts for the basic hydrolysis of *p*-nitrophenyl dodecanoate and structurally related esters.[13] Since rate constants for the hydrolysis of this ester in the presence of such surfactants are greater than those for hydrolysis of water-soluble esters of inherently equal reactivity, such as *p*-nitrophenyl hexanoate and acetate, in the absence of surfactants, it follows that factors other than dispersal of ester aggregates must intervene. The electrostatic field at the cationic micellar surface, which will have the effects of stabilizing the negatively charged transition state and of concentrating hydroxide ion at the micellar surface, must be responsible for a portion of the total catalytic effect.

The above considerations identify two catalytic mechanisms by which melittin may increase the rate of *p*-nitrophenyl dodecanoate hydrolysis: promotion of ester dispersal, with formation of ester–polypeptide aggregates, in the aqueous environment and electrostatic facilitation of the attack of hydroxide ion. In addition, it is possible that one or more of

the nucleophilic groups present in the melittin molecule may attack the ester substrate directly, increasing the rate of p-nitrophenolate liberation. That the rate of p-nitrophenyl dodecanoate hydrolysis is readily measureable at pH 10.0 in the presence of dilute solutions of melittin is proof that the first mechanism at least is operative. For example, the rate constant for ester hydrolysis at pH 10.0 in the presence of 1×10^{-4} M melittin, based on the data points early in the reaction, is 0.05 min^{-1}. This value is, coincidentally, almost the same as that measured for the hydrolysis of p-nitrophenyl hexanoate, an ester which does not aggregate, in the absence of melittin or surfactants at the same pH and temperature.[13] The observation that the first-order rate constants for ester hydrolysis increase about eightfold at higher concentrations of melittin (Figure 4) establishes that melittin facilitates ester hydrolysis by some mechanism other than ester dispersal. The magnitude of the catalytic effect is similar to that elicited by simple cationic surfactants and suggests that the electrostatic field present at the surface of the melittin micelle may underlie the catalytic effect. Were nucleophilic participation by basic groups on the melittin molecule involved, greater rate increases might have been expected.

One of the intriguing aspects of melittin-dependent p-nitrophenyl dodecanoate hydrolysis is the autocatalytic behavior observed at low melittin concentrations and the rate enhancement elicited by the addition of the anionic product of the reaction, dodecanoate. Previous work has shown that anionic surfactants alone inhibit base-catalyzed ester hydrolysis.[13] Consequently, the discovery that the addition of dodecanoate to melittin solutions, either through product accumulation or exogenous addition, produces increased rates for ester hydrolysis is unexpected and surprising. The results strongly suggest that a complex between dodecanoate and melittin is formed which is a better catalyst than melittin alone. In addition, the observation that higher melittin concentrations produce reactions which are kinetically first order requires that autocatalysis depends critically upon the ratio of the number of moles of melittin to the number of moles of dodecanoate. To the best of our knowledge, this is the only case in which an anionic reagent increases the capacity of a cationic reagent to catalyze an organic reaction.

Aside from ester dispersion, melittin catalysis for p-nitrophenyl dodecanoate decomposition may be viewed as either (1) electrostatic facilitation by cationic melittin of the attack of hydroxide ion on the ester substrate or (2) direct nucleophilic attack of melittin on the ester with formation of acylated melittin (at serine, threonine, or lysine). The fact that added dodecanoate elicits the same catalytic effect as is observed in the course of the reaction at low melittin concentrations is consistent with the former observation, since dodecanoate is a reaction product. In the latter case, however, dodecanoyl–melittin would be expected to be stable under the reaction conditions and the autocatalytic reaction would have to be ascribed to acylated melittin. The fact that added dodecanoate elicits the same rate effects would, then, have to be coincidental.

The complex pH–rate profile for melittin-catalyzed hydrolysis of p-nitrophenyl dodecanoate (Figure 5) is almost certainly the consequence of a combination of effects. The effect on reaction rate of increasing hydroxide ion concentration may be partially or completely offset by a decreasing positive charge on the melittin molecule reflecting neutralization of the cationic lysine residues. To the extent that the catalytic activity of melittin depends on its cationic character, catalysis will diminish with increasing pH.

Finally, added inorganic anions have two effects on the kinetics of the melittin-catalyzed hydrolysis of p-nitrophenyl dodecanoate. The first is to cause the reactions to become somewhat more nearly first order at low melittin concentrations, and the second is to cause a slight reduction in rate. The fact that the reactions approach first-order kinetics may reflect the decreased ability of melittin to bind dodecanoate in the presence of inorganic ions. Sites on the protein molecule which might have been previously available to carboxylate ions are instead occupied by added inorganic anions. The observation that rates of ester hydrolysis are somewhat slower may also be a result of binding inorganic anions to the melittin molecules. To the extent that the positive charges on the protein aggregates are neutralized via association with the inorganic anions, that component of the melittin catalysis which depends upon electrostatic stabilization will be diminished. Inorganic anions are known to be potent inhibitors of the cationic surfactant catalysis for ester hydrolysis.[13]

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