A Model System for the Study of Equilibrium Hydrophobic Bond Formation[1,2]

Rosalind G. Shorenstein,[3] Carol S. Pratt,[3] Chen-Jung Hsu, and Thomas E. Wagner[4]

Contribution from the Department of Chemistry, Wellesley College, Wellesley, Massachusetts 02181. *Received April 29, 1968*

Abstract: Long-chain N-acylhistidine derivatives cause a marked acceleration in the rate of release of *p*-nitrophenol from the *p*-nitrophenyl N-dodecyl-N,N-dimethylammonioethyl carbonate ion. The formation of a complex between the N-acylhistidine derivative and the *p*-nitrophenyl carbonate ester is due to strong hydrophobic bonding between the interactants' hydrocarbon components. It is assumed that equilibrium complex formation determines the observed initial rate of hydrolysis of the *p*-nitrophenyl carbonate ester according to the Michaelis–Menten rate expression. Dissociation (equilibrium) constants are obtained by kinetic methods for an N-acylhistidine derivative–*p*-nitrophenyl carbonate ester complex, as well as for a complex between the N-acylhistidine derivative and a detergent which functions as a competitive inhibitor. The process of hydrophobic bond formation for both complexes is characterized by a large negative free energy change. The variation of the equilibrium constants with temperature is used to determine enthalpy and entropy changes. For both complexes the entropy change for hydrophobic bond formation is large and positive, while the enthalpy change is close to zero. These results are incorporated in a model for hydrophobic bonding as a partial reversal of the solution process, accompanied by a net increase in the disorder of the aqueous system and by little net change in the strength of hydrogen bonding and dipole interactions between water molecules. The observed decrease in the initial rate of the long-chain N-acylhistidine-catalyzed hydrolysis of the *p*-nitrophenyl carbonate ester when alkyl-substituted urea or ethanol is present is also interpreted by this model for hydrophobic bonding.

The concept of apolar or hydrophobic bonding has been applied to complex biochemical problems such as protein structure, protein-binding reactions, and the denaturation of proteins. Several definitions of the apolar or hydrophobic bond based on the behavior of simple models have been offered.

Frank and Evans[5] studied the vaporization of small nonpolar gases, including hydrocarbons and inert gases, from an aqueous solution. The positive entropy change for this process (ΔS_{vap}) is unusually large, as is the slope of a plot of ΔS_{vap} *vs.* ΔH_{vap}. Since the reverse process is return to solution, it was concluded that the observed decrease in solubility of larger nonpolar molecules in water is due to an unfavorable negative entropy change. In order to account for the increased order in such solutions, the formation of "iceberg" regions in the solvent were postulated.

Frank and Wen[6] suggested a model for the structure of liquid water which could explain the formation of "icebergs." "Flickering clusters" of hydrogen-bonded water molecules are postulated to be in equilibrium with nonhydrogen-bonded water. The half-life of any cluster in pure water is very short since local energy fluctuations are transmitted efficiently by water molecules, but nonpolar solutes are suggested to partially isolate neighboring water clusters from these fluctuations, increasing the local half-life of these clusters and increasing the total "icelikeness" of the solution in a statistical sense.

Klotz[7] was among the first to formulate a general theory of the apolar bond. This theory, however, differed from the work of Frank and Evans about the relative magnitudes of ΔS and ΔH characterizing the interaction of water with nonpolar molecules. Klotz maintains that a negative ΔH counterbalances a negative ΔS and results in a favorable negative free energy change. This position stems from the choice of crystalline hydrates of nonpolar molecules as models. In crystalline hydrates, polyhedra of tetrahydrogen-bonded water molecules form cages around trapped solute; without solute in the cavities, the host lattice would be thermodynamically unstable.[8] A variety of solutes, including chlorine gas, ethane, methyl mercaptan, and even the nonpolar side chains of tetra-*n*-butylammonium salts,[9] form clathrates with water. A large negative ΔH of about -16 kcal/mol accounts for the spontaneous formation of many crystalline hydrates.[10] The uniformity of this value for the enthalpy change for a range of solutes suggests that a change in water structure produces the major energy contribution, rather than van der Waals interactions between water and specific solutes. Although a negative entropy change resulting from increased order in the system is observed, this parameter does not control the process of hydrate formation.

Since amino acid side chains found in proteins include the nonpolar groups which form clathrate compounds, Klotz[10] suggests that water molecules form clathrate-type lattices around proteins in solution. Here the term "apolar bond" refers to an enthalpic stabilization of proteins in water resulting from the cooperative formation of hydrogen-bonded water lattices by neighboring nonpolar side chains. The clathrate cages are suggested to be responsible for the masking of sulfhydryl

(1) This research was supported by grants from the Petroleum Research Fund of the American Chemical Society.

(2) A preliminary report of this research has been published: T. E. Wagner, C-J. Hsu, and C. Pratt, *J. Am. Chem. Soc.*, **89**, 6366 (1967).

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(4) Author to whom inquiries should be sent at the Department of Biochemistry, Sloan-Kettering Division, Cornell University Medical College, New York, N. Y.

(5) H. Frank and M. Evans, *J. Chem. Phys.*, **13**, 507 (1945).

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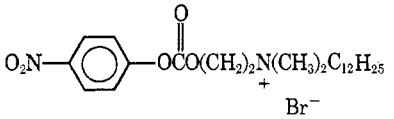
groups in proteins to silver reagents, and the binding of detergents to serum albumin.[11]

The views of Kauzmann[12] on the behavior of nonpolar groups in water are the inverse of the definition of apolar bonding offered by Klotz. Kauzmann defines the term "hydrophobic bond" as the tendency of nonpolar groups on proteins to adhere to one another in aqueous environments, thereby forming intramolecular micelles and avoiding contact with water molecules. This idea is based on an analogy with the transfer to aliphatic hydrocarbons from an aqueous solution to a nonaqueous one. In this system the favorable free energy change for transfer to a nonpolar solvent is due to a large positive entropy change which compensates generously for a small unfavorable enthalpy change. The entropy change is presumably due to breakdown in the organization of water solvent molecules which occurs when the hydrocarbon (or nonpolar side chain) is removed from exposure to water and enters a hydrophobic solvent (or hydrophobic region of the protein). Thus according to this model, the most stable structure a protein can adopt in water solution requires the packing of the largest number of nonpolar residues in the interior of the protein.

A general quantitative theory of hydrophobic bonding has been formulated by Némethy and Scheraga.[13] The foundation of this theory is a statistical mechanical treatment of the properties of liquid water itself,[14] based on the "flickering cluster" model of Frank and Wen.[6] The thermodynamic properties of aqueous hydrocarbon solutions are then analyzed in terms of this theoretical framework.[15] The Némethy–Scheraga theory predicts increased "order" in water induced by the presence of nonpolar species.

The arbitrariness in selecting a particular model process (clathrate formation, transfer from an aqueous to a nonpolar solvent, or a theoretical statistical mechanical treatment) as an analogy for apolar or hydrophobic bonding of biological significance is reflected in the controversy involving the thermodynamic driving force for the formation of the apolar interaction. The present investigation was undertaken to measure the thermodynamic parameters characterizing hydrophobic bonding in a system which models the function of enzymes. Unusually large rate enhancements are observed in the N-acylhistidine-catalyzed hydrolysis of E_{12} due to hydrophobic bond formation between the acyl portion of the histidine



E_{12}

derivative and the hydrocarbon component of E_{12}. This system is also characterized by several enzyme-like qualities: Michaelis–Menten kinetics, competitive inhibition, and urea denaturation.[2] It is felt that the direct observation of the thermodynamic parameters involved in the formation of the prereaction complex between an N-acylhistidine and E_{12} (a hydrophobic interaction) will provide a sound basis for the development of a less arbitrary theory of hydrophobic bonding.

Experimental Section

Materials. Stearyltrimethylammonium bromide was a commercial sample recrystallized by the procedure of Duynstee and Grunwald.[16] N-Methyl-, N-ethyl-, and N-butylureas were obtained from the K & K Laboratories, Inc., and the N-acetylhistidine used was a product of Calbiochem. Inorganic salts used in making up buffer solutions were Baker Analyzed Reagent Grade.

N-Dodecyl-N,N-dimethylhydroxyethylammonium bromide was prepared by the following procedure. To a solution of 1-bromododecane (5.98 g, 0.02 mol) in 25 ml of anhydrous methanol was added 1.78 g (0.02 mol) of N,N-dimethylaminoethanol (Pennsalt Corp.). After refluxing for 24 hr the reaction solution was allowed to cool to room temperature, and the product appeared as a white crystalline material. Successive crystallizations from acetone–methanol yielded 6.2 g (80% yield) of N-dodecyl-N,N-dimethylhydroxyethylammonium bromide, mp 198–199°. *Anal.* Calcd for $C_{16}H_{36}NOBr$: C, 56.81; H, 10.65; N, 4.14; Br, 23.67. Found: C, 56.63; H, 10.81; N, 4.12; Br, 24.10.

***p*-Nitrophenyl Chloroformate.** To 100 ml of 0°, 12.5% solution of phosgene in benzene (City Chemical) 19.7 g (0.1 mol) of dried, finely ground sodium *p*-nitrophenolate (Eastman) was added with stirring over a period of 1 hr. The resulting mixture was filtered to remove all sodium chloride and after evaporation of the solvent yielded 15 g (75% yield) of *p*-nitrophenyl chloroformate. This product was purified by sublimation, mp 80–81°. *Anal.* Calcd for $C_7H_4NO_4Cl$: C, 41.58; H, 1.98; N, 6.93. Found: C, 41.61; H, 1.92; N, 6.98.

***p*-Nitrophenyl N-Dodecyl-N,N-dimethylammonioethyl Carbonate Bromide (E_{12}).** Equimolar amounts of *p*-nitrophenyl chloroformate (2.02 g) and N-dodecyl-N,N-dimethylhydroxyethylammonium bromide (3.38 g) were stirred in 10 ml of dry amine-free pyridine (distilled sequentially from phthalic anhydride and calcium hydride) for 12 hr. Solvent was removed *in vacuo* and the residue recrystallized from dioxane, 4.1 g (80% yield), mp 168–169°. *Anal.* Calcd for $C_{23}H_{39}O_5N_2Br$: C, 54.86; H, 7.81; N, 5.56. Found: C, 54.72; H, 7.78; N, 5.62.

N-Stearoylhistidine. To a 50-ml dry benzene solution of ethyl chloroformate (5.43 g, 0.05 mol) (Eastman), stearic acid (14.2 g, 0.05 mol) (K & K Laboratories), and triethylamine (9.09 g, 0.09 mol) (Eastman) was added 4.82 g (0.02 mol) of histidine methyl ester dihydrochloride (Calbiochem). After stirring for 24 hr at 0° the reaction mixture was filtered to remove the insoluble methyl ester of N-stearoylhistidine. This product was dried, saponified with 0.05 *M* NaOH, and acidified with HCl to yield the hydrochloride of N-stearoylhistidine, mp 250–252°. *Anal.* Calcd for $C_{24}H_{44}O_3N_3Cl$: C, 56.97; H, 8.76; N, 8.30. Found: C, 57.12; H, 8.68; N, 8.36.

Kinetics of Hydrolysis of *p*-Nitrophenyl N-Dodecyl-N,N-dimethylammonioethyl Carbonate Bromide (E_{12}). The reactions were carried out by following the appearance of *p*-nitrophenol spectrophotometrically (400 mμ) using a Cary Model 14 recording spectrophotometer equipped with a thermostated cell compartment, a thermostated cell holder, and a 0–0.1 absorbance slide wire. The reaction medium, generally 3.00 ml of pH 7.3 0.01 *M* potassium phosphate buffer containing N-stearoylhistidine and in some cases an inhibitor, was placed in a stoppered 1-cm silica cell and thermostated for 15 min. The reaction was initiated by the addition of 0–25 μl of a stock solution of carbonate ester in acetonitrile (Eastman Spectro Grade) using a plastic flat-tipped stirring rod (Calbiochem). The final acetonitrile concentration varied from 0.5 to 1.3%. The time necessary to complete the mixing of the reactants and begin recording the spectral changes did not exceed 5 sec. The hydrolysis of the *p*-nitrophenyl carbonate ester followed initial (pseudo) first-order kinetics. Times were determined from the recorded chart divisions by calibration using a Precision Scientific Time-It electric clock and are accurate to ±1%. The infinite absorbance values were obtained after at least seven half-lives, and the reactions were recorded during the initial (0–20%) changes. The rate constants were calculated by electronic computation of the linear least-squares slope of the line formed by the set of points [time, ln (infinite absorbance − absorbance)]. The reported rate

(11) I. Klotz and S. Luborsky, *J. Am. Chem. Soc.*, **81**, 5119 (1959).
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(16) E. F. J. Duynstee and E. Grunwald, *J. Am. Chem. Soc.*, **81**, 4542 (1959).

constants are averages of at least five determinations which agreed within 5%.

Determination of Dissociation Constants by Kinetic Methods. The observed first-order rate constants for the hydrolysis of the *p*-nitrophenyl carbonate ester (as measured by *p*-nitrophenol release) in the absence (k_{un}) and presence (k_{obsd}) of added N-stearoylhistidine were determined. At least five points were obtained at *p*-nitrophenyl carbonate ester (E_{12}) concentrations spanning (whenever possible) the value of K_{diss} (the dissociation constant of the *p*-nitrophenyl carbonate ester (E_{12})–N-stearoylhistidine complex assuming a 1:1 stoichiometry). The values of K_{diss} and k_2[N-stearoylhistidine]$_0$ (where k_2 is the maximal catalyzed rate due to the decomposition of the fully complexed N-stearoylhistidine molecule) were obtained as the slope and *Y* intercept of the line[17-19] formed by plotting $k_{obsd} - k_{un}$ against $(k_{obsd} - k_{un})$/[*p*-nitrophenyl carbonate ester (E_{12})]. Although Lineweaver–Burk plots are presented, Eadie-type plots are used in all calculations because they are statistically preferable.[20] The slope and intercept were obtained by electronic computation of the least-squares line. The error limits of K_{diss} were calculated from the experimental data by application of Student's t test for 95% probability of fit to the least-squares line. The error in k_2[N-stearoylhistidine] may be expected to parallel the error in K_{diss}.

Determination of Inhibition Constants. The dissociation constant for the N-stearoylhistidine–inhibitor complex (K_i) was determined by measuring the rate of *p*-nitrophenyl carbonate ester (E_{12}) hydrolysis in the presence of a fixed amount of N-stearoylhistidine and varying concentrations of inhibitor. The concentration of the carbonate ester was 1.33×10^{-5} *M* and the N-stearoylhistidine was 1.85×10^{-5} *M*. Added inhibitor at the highest concentrations used did not have any significant effect on the rate of the uncatalyzed reaction. The method of Dixon[21] was used in graphically determining the values of K_i. The inhibitor concentration [In] was plotted as a function of $1/(k_{obsd} - k_{un})$. The intersection of the line $1/(k_{obsd} - k_{un}) = 1/k_2$[N-stearoylhistidine]$_0$ with the line formed by the set of points [In], $1/(k_{obsd} - k_{un})$ occurs at a value of [In] $= -K_i$. The values of k_2 were obtained previously from Eadie-type plots.

Determination of Rate Constants in the Presence of Urea Derivatives and Alcohols. Buffer solutions 0.01 *M* in potassium phosphate and containing varying concentrations of either absolute ethanol (Eastman Spectro Grade) or a urea derivative were made up and the pH was adjusted to 7.3. The initial (pseudo) first-order rate constants for reactions carried out in these solutions were determined as previously described.

Results

The Hydrolysis of *p*-Nitrophenyl N-Dodecyl-N,N-dimethylammonioethyl Carbonate Bromide (E_{12}) in the Presence of N-Acetyl- and N-Stearoylhistidines. The pseudo-first-order rate constants for the solvent- (k_{un}), N-acetylhistidine-, and N-steroylhistidine-catalyzed hydrolysis of E_{12} are shown in Table I. N-Stearoylhistidine is observed to catalyze this reaction *ca.* 2000 times faster than an equivalent amount of N-acetylhistidine at 25°. This rate enhancement is apparently due to the formation of a prereaction, hydrophobically bonded, 1:1 complex between N-stearoylhistidine and the *p*-nitrophenyl carbonate ester (E_{12}).[2] Within this complex the ensuing reaction may take place with a much smaller loss of translational entropy than the bimolecular reaction between E_{12} and N-acetylhistidine.[22] The complex is assumed to be 1:1 since surface tension measurements do not indicate mixed micelle formation at the concentrations and conditions utilized in these reactions (the concentrations of detergent-like reactants used in these experiments are well below the CMC for compounds similar in structure[23]). Pair formation between detergent-like molecules has previously been demonstrated below the CMC by conductometric means.[24]

Table I. Reaction of N-Acylhistidine Derivatives with the *p*-Nitrophenyl Carbonate Ester (E_{12}) at 25.0°, pH 7.3

N-Acylhistidine	Concn × 10^5 *M*	$10^5 k_{obsd}$, sec^{-1}	$(k_{obsd} - k_{un})$/[N-acylhistidine derivative], l. mol^{-1} sec^{-1}
None	0	5.5	
N-Acetylhistidine	100.0	34.0	0.285
N-Stearoylhistidine	3.3	2120.0	641.0

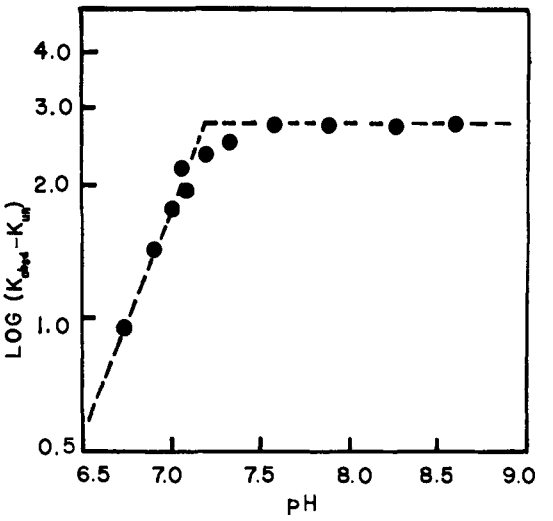

Figure 1. The log $(k_{obsd} - k_{un})$ *vs.* pH profile for the N-stearoylhistidine-catalyzed hydrolysis of the ester E_{12}; [N-stearoylhistidine] = [E_{12}] = 3.3×10^{-5} *M*, μ = 0.01, 25°.

The determined value of $(k_{obsd}^{H_2O} - k_{un}^{H_2O})/(k_{obsd}^{D_2O} - k_{un}^{D_2O})$ for the N-stearolyhistidine-catalyzed reaction (0.85 ± 0.10 at 25°) is as anticipated for a nucleophilic reaction by an imidazole function unassisted by general acid or general base catalysis.[25] Since attempts to observe an intermediate were not carried out we rely only on the isotope effect to distinguish between the imidazole function in N-stearoylhistidine acting as a general base catalyst for ester hydrolysis and as a nucleophile.

The effect of pH on the rate of reaction between N-stearoylhistidine and E_{12} was investigated over the range pH 6.5–8.5. A plot of pH *vs.* log $(k_{obsd} - k_{un})$ is shown in Figure 1. The pK_{app} for the N-stearoylhistidine-catalyzed reaction (pK_{app} = 7.2) was obtained from the intersection of the two straight lines drawn through the rising and stationary portions of the curve. This observed pK_{app} is in agreement with the pK_a of N-stearoylhistidine determined by half-neutralization.

In order to study the formation of a prereaction complex between N-stearoylhistidine and E_{12} within which the reaction has been proposed to take place, the concentration dependence of the catalytic action

(17) A. K. Colter, S. S. Wang, G. H. Megerle, and P. S. Ossip, *J. Am. Chem. Soc.*, **86**, 3106 (1964).
(18) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).
(19) R. L. VanEtten, J. F. Sebastian, G. A. Clowes, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 3242 (1967).
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(25) Reference 22, p 56.

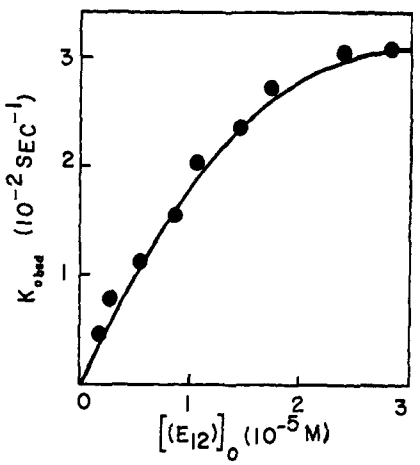


Figure 2. The initial pseudo-first-order rate constant for the N-stearoylhistidine-catalyzed release of *p*-nitrophenol from E_{12} at pH 7.30 plotted as a function of added E_{12}. The concentration of the catalyst, N-stearoylhistidine, remained constant at 3.3×10^{-5} *M*; 0.5% (v/v) acetonitrile–water, 25°, $\mu = 0.01$.

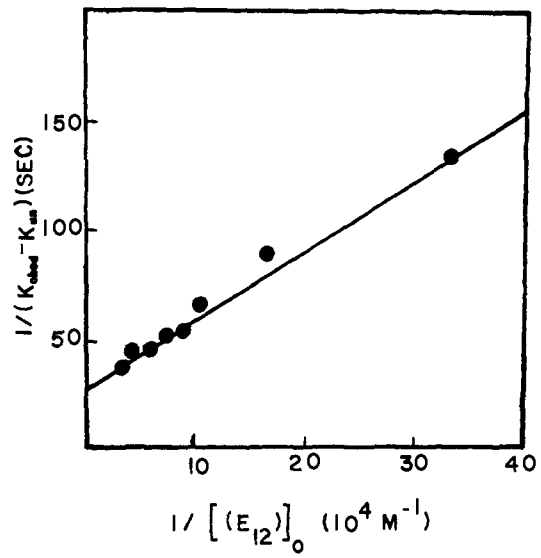


Figure 3. $1/(k_{obsd} - k_{un})$ for the N-stearoylhistidine-catalyzed decomposition of E_{12} plotted *vs.* the reciprocal of the E_{12} concentration (data from Figure 2).

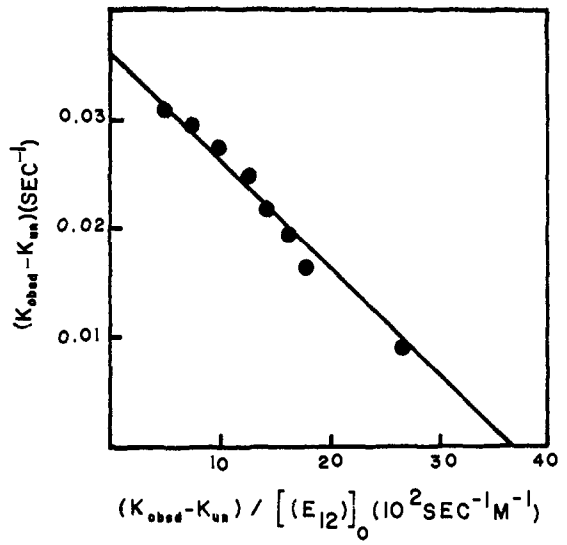


Figure 4. $k_{obsd} - k_{un}$ for the N-stearoylhistidine-catalyzed decomposition of E_{12} plotted as a function of $(k_{obsd} - k_{un})/[E_{12}]$ (data from Figure 2).

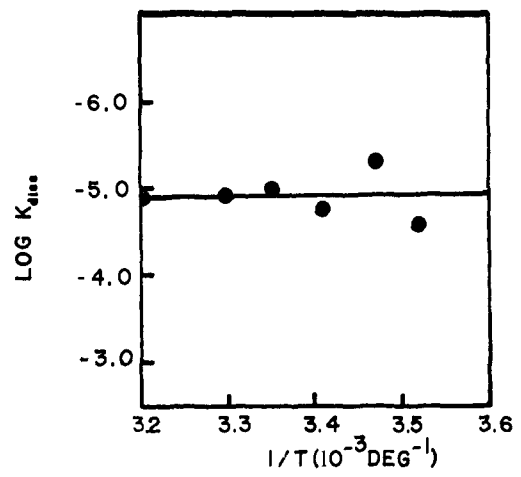


Figure 5. Plot of log K_{diss} *vs.* the reciprocal of the absolute temperature. The data are that of Table II for the dissociation of the N-stearoylhistidine–E_{12} complex.

of N-stearoylhistidine was examined. The effect of varying *p*-nitrophenyl carbonate ester (E_{12}) concentration on the pseudo-first-order rate constant for the hydrolysis of this ester in the presence of a constant amount of N-stearoylhistidine is shown in Figure 2. Because the system under investigation shows marked product inhibition by the N-dodecyl-N,N-dimethylhydroxyethylammonium ion,[2] only initial (through 10–15% completion) pseudo-first-order rate constants are shown in Figure 2. As is observed in enzyme kinetics, the rate accelerations approach a maximum saturation value. The data were treated by using a reciprocal form of Michaelis–Menten kinetics which has been previously shown to be useful for investigating reactions involving complex formation. By plotting the data obtained from the N-stearoylhistidine-catalyzed hydrolysis of the *p*-nitrophenyl carbonate ester (E_{12}) in the form of $1/(k_{obsd} - k_{un})$ *vs.* the reciprocal of the concentration of *p*-nitrophenyl carbonate ester (E_{12}) a straight line is obtained (Figure 3) having a slope of K_{diss}/k_2 and a Y intercept equal to $1/k_2$. The term K_{diss} refers to the dissociation constant for the N-stearoylhistidine–*p*-nitrophenyl carbonate ester (E_{12}) complex and k_2 to the rate constant for the reaction of the entirely complexed N-stearoylhistidine ($k_{obsd} - k_{un}$ at infinite *p*-nitrophenyl carbonate ester concentration).[26]

Data treated by a variant of Michaelis–Menten kinetics due to Eadie[18] (a statistically preferable form[21]) was used to evaluate more accurately the values of k_{diss} and k_2. By plotting $k_{obsd} - k_{un}$ against $(k_{obsd} - k_{un})/[p\text{-nitrophenyl carbonate ester } (E_{12})]$ a straight line is obtained (Figure 4) with slope $-K_{diss}$ and a Y intercept of k_2.

Temperature Dependence of the N-Stearoylhistidine–*p*-Nitrophenyl Carbonate Ester (E_{12}) Dissociation Constant. The kinetic dissociation constant of the N-stearoylhistidine–*p*-nitrophenyl carbonate ester (E_{12}) was determined at temperatures between 10 and 45°. An average value of at least five determinations at each temperature is presented in Table II. The

(26) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

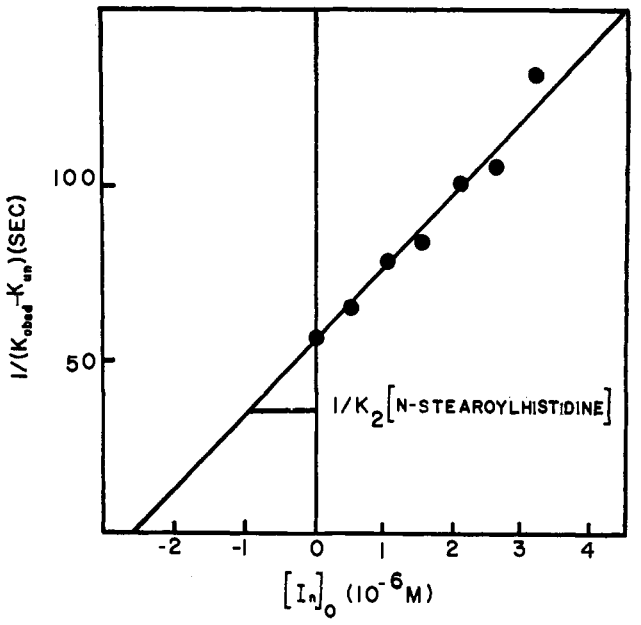


Figure 6. Added inhibitor concentration [In] graphed as a function of $1/(k_{obsd} - k_{un})$ for inhibition by stearyltrimethylammonium ion of the acceleration of E_{12} decomposition by N-stearoylhistidine; [N-stearoylhistidine] = 1.85×10^{-5} M, [E_{12}] = 1.33×10^{-5} M, pH 7.30, 25°, μ = 0.01, 0.85% (v/v) acetonitrile–water.

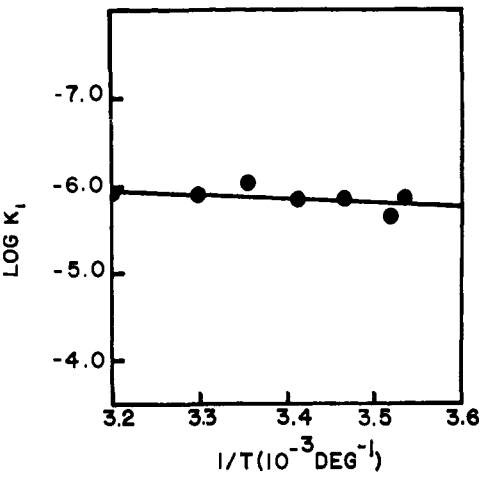
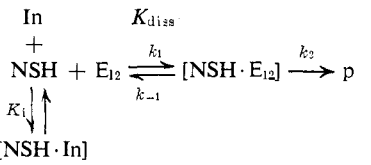


Figure 7. Plot of log K_i *vs.* the reciprocal of the absolute temperature. The data are that of Table III for the dissociation of the N-stearoylhistidine–stearyltrimethylammonium ion complex.



thermodynamic parameters for the dissociation process were calculated from the least-squares slope of the line formed by plotting log K_{diss} against the reciprocal of the absolute temperature (Figure 5). Assuming that the heat capacities of reactants and products are not significantly different (entirely justified here since the average error in K_{diss} is ~15%) one may obtain $\Delta H°$ and $\Delta S°$ from the relationship $d[\ln K_{eq}]/d(1/T) = -\Delta H°/R$. The enthalpy change, $\Delta H°$, is $-R$(slope) and the entropy change, $\Delta S°$, is given by R (intercept). The electronically computed line was also used to calculate the average value of $\Delta G°_{298}$.

Table II. Temperature Dependence of the N-Stearoylhistidine–*p*-Nitrophenyl Carbonate Ester (E_{12}) Dissociation Constant[a]

Temp, °K	K_{diss}, 10^{-5} M
284	2.3 ± 0.26
288	0.8 ± 0.10
293	1.45 ± 0.15
298	1.1 ± 0.12
303	1.25 ± 0.14
312	1.23 ± 0.16

[a] At pH 7.30, I = 0.01 potassium phosphate buffer with 0.5% acetonitrile–water. [b] Error limits computed from the experimental data by application of Student's t test at 95% probability level.

Competitive Inhibition of N-Stearoylhistidine Catalysis by Stearyltrimethylammonium Bromide. The system under study allows for the investigation of hydrophobic bonding between N-stearoylhistidine and molecules other than the substrate *p*-nitrophenyl carbonate ester (E_{12}) due to competitive inhibition of the reaction by molecules similar in structure to E_{12}. This possibility arises from the formation of an N-stearoylhistidine (NSH)–inhibitor (In) complex in the presence of an inhibitor.

The dissociation constant (K_i) for the formation of this complex may be determined by the method of Dixon[21] (see Experimental Section). The Dixon plot for determination of K_i for the N-stearoylhistidine–stearyltrimethyl ammonium ion complex at 25° is shown in Figure 6. From the value of K_i ($9.5 \pm 0.4 \times 10^{-7}$ M) determined it is apparent that the 18-carbon–18-carbon hydrophobic association is significantly stronger than the 18-carbon–12-carbon association observed in the formation of the N-stearoylhistidine–*p*-nitrophenyl carbonate ester (E_{12}) complex. In order to evaluate the thermodynamic parameters for this 18-carbon–18-carbon hydrophobic interaction the temperature dependence of K_i in the stearyltrimethylammonium ion inhibited reaction between N-stearoylhistidine and E_{12} was investigated between 10 and 45°. An average value of at least five determinations of K_i at each temperature studied are presented in Table III. Figure 7 shows a plot of ln K_i *vs.* the reciprocal of the absolute temperature for the stearyltrimethylammonium ion inhibited reaction. From the slope and intercept of this plot the values of $\Delta H°$ and $\Delta S°$ for the 18-carbon–18-carbon hydrophobic interaction have been calculated (see Discussion section).

The Effects of Some Protein Denaturing Reagents on the Rate of Reaction between N-Stearoylhistidine and E_{12}. In order to further investigate the nature of the forces existing between apolar groups in water an investigation of the effects of two types of reagents thought to interfere with hydrophobic bond formation in protein systems was undertaken. The effect of increasing concentrations of urea and mono-N-alkylurea derivatives on the rate of the N-stearoylhistidine-catalyzed hydrolysis of E_{12} is shown in Figure 8. A similar plot showing the effect of increasing ethanol concentration on the rate of the reaction is presented in Figure 9. Both urea derivatives (especially those containing apolar N-alkyl groups) and ethanol appear

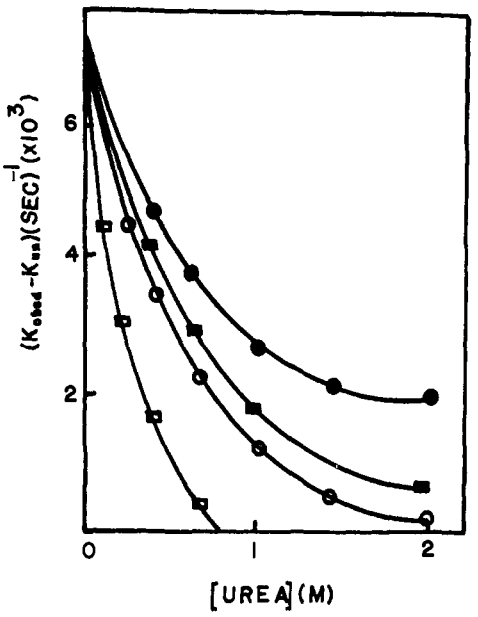


Figure 8. $k_{obsd} - k_{un}$ for the N-stearoylhistidine-catalyzed hydrolysis of E_{12} plotted as a function of increasing concentration of N-alkylurea derivatives: ●, urea; ■, N-methylurea; ○, N-ethylurea; □, N-butylurea. [N-stearoylhistidine] = [E_{12}] = 3.3×10^{-5} *M*, pH 7.30, 25°, μ = 0.01.

to have a large negative effect on the formation of hydrophobically bonded complexes in this system.

Table III. Temperature Dependence of the N-Stearoylhistidine–Stearyltrimethylammonium Ion Dissociation Constant[a]

Temp, °K	K_i,[b] 10^{-6} *M*
282	1.4 ± 0.20
284	1.16 ± 0.13
288	1.3 ± 0.18
293	1.5 ± 0.20
298	0.95 ± 0.17
303	1.45 ± 0.20
312	1.48 ± 0.18

[a] At pH 7.30, I = 0.01 potassium phosphate buffer with 0.65% acetonitrile–water. [b] Error limits computed from the experimental data by application of Student's t test at 95% probability level.

Discussion

N-Stearoylhistidine causes a marked acceleration in the rate of *p*-nitrophenol release from the *p*-nitrophenyl carbonate ester (E_{12}). That this acceleration is due to the formation of a prereaction complex between N-stearoylhistidine and E_{12} is strongly indicated by the saturation kinetics observed. It is highly unlikely that the complementary electrostatic interaction possible between N-stearoylhistidine and E_{12} contributes significantly to the stability of the complex since N-acetylhistidine has little or no effect on the rate of hydrolysis of E_{12}. The presence of the complementary electrostatic interaction may nevertheless be a useful element in the observed rate enhancement. The electrostatic interaction may aid the orientation of the two reactants so that the imidazole nucleophile has a favorable geometry to attack the ester carbonyl. By favoring productive binding, the electrostatic interaction may lower the ΔG^* and thus increase K_2, the hydrolysis rate within the complex. The formation of

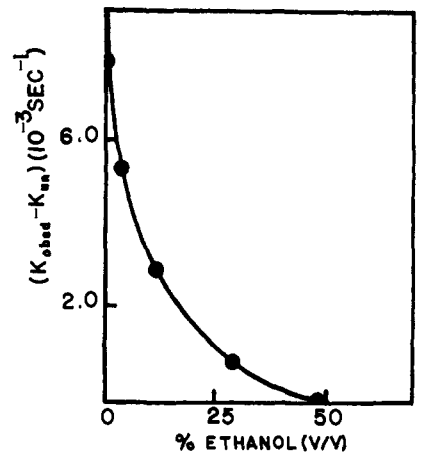


Figure 9. $k_{obsd} - k_{un}$ for the N-stearoylhistidine-catalyzed hydrolysis of E_{12} plotted as a function of increasing ethanol concentration; [N-stearoylhistidine] = [E_{12}] = 3.3×10^{-5} *M*, pH 7.30, 25°, μ = 0.01.

the prereaction complex between N-stearoylhistidine and E_{12} appears therefore to be solely due to a hydrophobic interaction between the long-chain hydrocarbon components of the reactants. The 12-carbon–18-carbon interaction between N-stearoylhistidine and E_{12} as well as the 18-carbon–18-carbon interaction between N-stearoylhistidine and the inhibitor, stearyltrimethylammonium ion, have been studied. The thermodynamic parameters for these interactions are presented in Table IV.

Table IV. Thermodynamic Changes for the Association of N-Stearoylhistidine Complexes[a]

Complexing molecule	$\Delta G°_{298}$, kcal/mol	$\Delta H°$, kcal/mol	$\Delta S°$, gibbs/mol
p-Nitrophenyl carbonate ester (E_{12})	−6.7 ± 0.5	0.0 ± 1.7	+23 ± 8
Stearyltrimethyl-ammonium ion	−8.2 ± 0.7	+0.2 ± 2	+28 ± 6

[a] The error limits given above were obtained by applying Student's t test to the experimental data in Tables II and III.

Hydrophobic bonding is defined by Kauzmann, Némethy, Scheraga, and others as the spontaneous tendency for nonpolar groups to adhere to one another in aqueous solution, thereby decreasing contact with water molecules. In the two examples of hydrophobic bonding studied the equilibrium greatly favors the hydrophobic bonding in the complex over the dispersed reactants exposed to water. The negative free energy change for association could be the result of two factors. The first involves attractive forces between solute molecules considered as transient dipoles. These "London dispersion forces" depend on the inverse sixth power of distance (r), provided that the distance between molecules is much larger than the size of the transient dipole. However, for 12- and 18-carbon atom groups, the assumption that the size of the dipole is negligible is not valid. Recent theoretical work indicates that the interaction energy may vary inversely as a power of r smaller than 6 as the size of the dipole increases and distance between molecules decreases.[27]

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Thus, since the nonpolar group of a solute is larger than the water molecule, London dispersion forces should strengthen the intracomplex solute–solute interaction more than the dispersed solute–water interaction because the former forces would be effective over a greater dipole area. Although at the present time no quantitative theory is available for predicting the magnitude of this factor in hydrophobic bonding, it is probably small. The energy of solute–water van der Waals attraction was treated as an empirically adjustable parameter in the Némethy–Scheraga theory. The second factor which contributes to the negative free energy change for hydrophobic complex formation is an *unfavorable* interaction of water molecules with dispersed N-stearoylhistidine, E_{12}, or inhibitor. There are unusually strong mutual interactions between water molecules, as indicated by the large surface tension of water. During the solution process, work is required to provide a cavity for accommodation of the solute; the amount of work depends on the surface area of the cavity and the internal pressure of water.[28] A mechanism for hydrophobic bonding would logically arise with the tendency of water to "squeeze out" solute from the small dispersed cavities into fewer large cavities. One large cavity has a smaller surface area than two small cavities. Thus when hydrophobic bonding brings together the catalyst and substrate in one cavity, the free energy of the system is lowered by a part of the energy originally used to dissolve the separate species. Thus, some water molecules which initially bordered on a hydrocarbon component are now released to the bulk water phase and can interact more strongly with other water molecules.

Since this model claims that hydrophobic bond formation is a partial reversal of the solution process, it is interesting to compare the free energy changes for the N-stearoylhistidine–E_{12} and N-stearoylhistidine–inhibitor complexes with the free energy changes for complete reversal of the solution process (*i.e.*, the transfer of a nonpolar solute from aqueous solution to a nonpolar liquid). At 298°K, the average $\Delta G°_{tr}$ is -0.8 kcal/mol for each methylene group in a linear nonpolar chain transferred from water to a nonpolar solvent.[29] Since the strength of hydrophobic bonding between N-stearoylhistidine and E_{12} is limited by the length of the shorter (12 carbon) hydrocarbon component, an average ΔG_{tr} for the transfer of separated N-stearoylhistidine and E_{12} molecules from water to a nonpolar liquid would be about -9.6 kcal/mol for 12 methylene units. The observed average $\Delta G°_{assoc}$ for complex formation is -6.7 kcal/mol. As expected, the free energy change for the pairwise hydrophobic bond is smaller than the hypothetical estimate of $\Delta G°_{tr}$ for the complete removal of E_{12} and N-stearoylhistidine from water. This result is understandable since the hydrophobic complex still shares surface area with water molecules, a situation which is unfavorable for water dipole–dipole interactions and less favorable for solute molecules than solute–nonpolar liquid interactions would be.

For nonpolar chains *longer* than 12-carbon atoms, the free energy change for the water to nonpolar liquid transfer process becomes more negative, but the increment to the free energy loss per *methylene group* in the chain *decreases*. In solubility studies it is also found that very long chain hydrocarbons are more soluble (have a less positive $\Delta G°_{solvation}$) than would be expected from an extrapolation of the $\Delta G°_{solvation}$ values of smaller hydrocarbons.[30] This trend is also observed in the present investigation of hydrophobic bond formation between N-stearoylhistidine and the stearyl inhibitor, each of which contains a hydrocarbon component with 18-carbon atoms. The $\Delta G°_{assoc}$ is only about 1 kcal/mol more negative than $\Delta G°_{assoc}$ for the N-stearoylhistidine–E_{12} complex although E_{12} contains six fewer carbon atoms in its nonpolar component. The increment to $\Delta G°_{assoc}$ for the first 12 carbons is roughly estimated as $-6.7/12 = -0.55$ kcal/mol per methylene group, while the increment to $\Delta G°_{assoc}$ for the 12th through 18th methylene groups is roughly about $(-8.2 + 6.7)/6 = -0.25$ kcal/mol per methylene group. The geometry of very long chain nonpolar groups in water may account for this behavior. A single long chain could curl up in a "globule" and thus be partly removed from contact with water.[15] As a result, the observed driving force for further elimination of mutual surface area with water molecules would be a nonadditive function of the number of constituent methylene groups.

The prediction of enthalpy and entropy changes for the interaction between two nonpolar groups in an aqueous environment has played a central role in several theories which attempt to interpret hydrophobic or apolar bonding on the molecular level. Klotz considers apolar bonding between two groups to be an extension of the events which occur when a single apolar group is dissolved in water. The formation of a strongly hydrogen-bonded water lattice with a specific clathrate-like structure is postulated. Such a model required that apolar bond formation be characterized by a large negative enthalpy change, comparable in size to the enthalpy changes observed for the formation of clathrate compounds ($\Delta H° = -16$ kcal/mol), and by a negative entropy change. However, the results of the present investigation of hydrophobic complex formation suggest that Klotz's model process is not valid. The average enthalpy change for hydrophobic bonding between N-stearoylhistidine and E_{12} is about 0. This parameter is $+0.2$ kcal/mol for N-stearoylhistidine and the stearyl inhibitor. These small enthalpy changes reflect little net change in the extent of hydrogen bonding by water molecules; even the negative extreme of the statistical error in $\Delta H°_{assoc}$ does not allow for an enthalpy change of clathrate-like proportions. Furthermore, the average entropy changes are positive ($\Delta S°_{assoc}$ for the N-stearoylhistidine–E_{12} complex $= +23$ eu; $\Delta S°_{assoc}$ for the N-stearoylhistidine–stearyl inhibitor complex $= +28$ eu) and indicate a decrease in the order of the system. These average enthalpy and entropy changes indicate a rejection of Klotz's concept of apolar bonding for this system.

The observed average $\Delta S°_{assoc}$ for both N-stearoylhistidine–inhibitor and N-stearoylhistidine–E_{12} complexes is compatible with data for hydrophobic interactions in other model systems.[31–34] Since the average

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$-T\Delta S^{\circ}_{assoc}$ term provides a major contribution to the favorable negative free energy change for hydrophobic bonding, the predictions of the statistical mechanical theory proposed by Némethy and Scheraga are fulfilled. The positive entropy change would result from an increase in the number of energetically equivalent orientations available to each water molecule which is released into the bulk solvent when the hydrophobic bond forms. Two types of evidence suggest that there is also some validity in interpreting this positive entropy change as a net decrease in the "structure" of the system in the sense that the freedom of movement of water molecules is restricted. It is known that the viscosity of aqueous solutions of long-chain alcohols is greater than the viscosity of pure water or pure alcohol.[35] Furthermore, the average time necessary for water dipoles to orient themselves in an applied alternating electric field is increased when substances containing nonpolar groups are added to water.[36] These facts indicate that the presence of dispersed nonpolar groups (the *initial* state for the hydrophobic bonding process) restricts the free movement of water molecules in comparison with the behavior of pure water. The increase in entropy when the system goes from the initial state to the final state in the hydrophobic bonding process could thus reflect a partial removal of this restriction to the rotations and vibrations of water molecules.

The very small or zero values of the average enthalpy change in hydrophobic bond formation are again compatible with results for other model systems involving nonpolar groups containing four or more carbon atoms.[32-34] This enthalpy change indicates that very little *net* change in the strength or extent of hydrogen bonding among water molecules occurs when the hydrophobic complex forms. The thermodynamic change alone, however, tells us nothing about the detailed mechanism involved. Values of ΔH^{*} for the dissociation and for the association of the complex would be of much interest in this regard. Nevertheless, on the level of hypothesis, one model appears especially helpful in explaining both the small average enthalpy change and the positive average entropy change observed in the present study. The evidence for the restriction of motion of water molecules in contact with nonpolar groups has already been discussed, and this restriction of motion ("structure") can be attributed to strong hydrogen bonds between water molecules. In this initial state, effective hydrogen bonding will be achieved only if water molecules assume a small number of specific geometrical orientations with respect to the nonpolar solute. When two nonpolar groups form a hydrophobic bond and cohabit one cavity in the solvent, some water molecules will be released from the hydrogen-bonded structure surrounding the nonpolar groups. The breaking or weakening of hydrogen bonds in this step will contribute a positive enthalpy change. As these released water molecules join the bulk solvent phase, hydrogen bonds and dipole interactions can be re-formed with other water molecules, which produces a negative enthalpy contribution. The net observed enthalpy change would turn out to be close to zero. Furthermore, in the bulk water phase hydrogen bonds and dipole interactions of maximum strength could be achieved with water molecules in a large number of possible orientations, and a net positive entropy change would be predicted.

Before any model for the molecular process of hydrophobic bonding can be evaluated critically, however, the nature of hydrogen bonds in water must be understood more fully. Since a hydrogen bond can be defined as a largely electrostatic interaction between two dipoles which includes a hydrogen atom as part of one dipole, it seems difficult to distinguish sharply between dipole–dipole interactions and hydrogen bonding in water as Némethy and Scheraga have done.[12]

An explanation of the effectiveness of urea molecules, especially large nonpolar ones, in depressing the rate of the N-stearoylhistidine-catalyzed hydrolysis of E_{12} may involve several factors. The urea molecules would be expected to provide a more favorable environment for the dispersed nonpolar groups in water, thereby decreasing the magnitude of the free energy change for complex formation. The large, relatively hydrophobic, urea molecule in an aqueous solution should interact more effectively than a water molecule with nonpolar groups since London dispersion forces should be greater for the larger urea molecule. The restriction of motion of a urea molecule next to a hydrophobic group would involve a smaller loss of entropy than would the restriction of motion of several molecules which, in pure water solution, would occupy the same volume.

Initial rate constants for the N-stearoylhistidine-catalyzed hydrolysis of E_{12} were also depressed by the presence of ethanol in the reaction medium, although the smaller dielectric constant of an alcohol–water mixture should tend to destabilize the separate ions. Thus ethanol is probably affecting hydrophobic bonding between the hydrocarbon components of N-stearoylhistidine and E_{12}. Since measurements of the viscosity[36] and the dielectric relaxation time[37] indicate that alcohols themselves produce an increase in the average structure of an aqueous solution, the increase in water structure around additional nonpolar solutes is relatively less important. By stabilizing the initially dispersed N-stearoylhistidine and E_{12} in solution, ethanol would dimish the driving force for hydrophobic bonding in the N-stearoylhistidine–E_{12} complex. A decrease in the strength of this bonding would result in a smaller observed rate constant.

Conclusions

The marked acceleration in the rate of hydrolysis of the ester E_{12} caused by N-stearoylhistidine is apparently due to the formation of a prereaction complex between the two reactants. Within this complex the reaction proceeds at a rate more than 2000 times faster than the bimolecular reaction between E_{12} and N-acetylhistidine. The formation of this complex as well as the E_{12}–inhibitor complex studied is due to the formation of strong hydrophobic bonds between the hydrocarbon

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components of the interactants. This hydrophobic bonding is characterized by an enthalpy change of approximately 0 and a large positive entropy change. A model for hydrophobic bonding as the partial reversal of the solution process for hydrophobic molecules is presented.

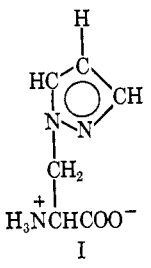
Studies on Polypeptides. XL. Synthetic Routes to Peptides Containing β-(Pyrazolyl-1)- and β-(Pyrazolyl-3)-alanine

Klaus Hofmann, Rudolf Andreatta, and Hans Bohn[1-3]

Contribution from the Protein Research Laboratory, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213. *Received June 5, 1968*

Abstract: Certain derivatives of β-(pyrazolyl-1)- and of β-(pyrazolyl-3)-alanine which are of interest for peptide synthesis are described. These include N^{α}-benzyloxycarbonyl-β-(pyrazolyl-1)-alanine and its *p*-nitrophenyl ester, N^{α}-*t*-butoxycarbonyl-β-(pyrazolyl-1)-alanine, N^{α}-benzyloxycarbonyl-β-(pyrazolyl-3)-alanine and its hydrazide, N^{α}-*t*-butoxycarbonyl-β-(pyrazolyl-3)-alanine and its hydrazide, and N^{α}-N^{pyr}-dibenzyloxycarbonyl-β-(pyrazolyl-3)-alanine and its *p*-nitrophenyl and N-hydroxysuccinimide esters. N^{α}-Benzyloxycarbonyl-β-(pyrazolyl)-3)- and N^{α}-*t*-butoxycarbonyl-β-(pyrazolyl-3)-alanine are shown to form optically active anhydro compounds when treated with N,N′-dicyclohexylcarbodiimide in anhydrous solvents. These anhydro compounds react with nucleophiles to form the respective carboxylic acid derivatives. Anhydro compound formation and ring opening proceed without racemization. The preparation of β-(pyrazolyl-3)-alanylaspartic acid, β-(pyrazolyl-3)-alanylmethionylaspartic acid *d*-sulfoxide, β-(pyrazolyl-3)-alanylphenylalanylarginyltryptophylglycine, and β-(pyrazolyl-1)-alanylphenylalanylarginyltryptophylglycine is described. The similarity between histidine and β-(pyrazolyl-3)-alanine as concerns reactions which are of interest from the point of view of peptide synthesis is discussed.

The replacement, in physiologically active peptides, of histidine by the isosteric pyrazolylalanines provides a useful tool for assessing the importance for biological activity of the characteristic acid–base properties of the imidazole portion of histidine. The rationale for this approach has been presented.[4] To date we have replaced histidine by β-(pyrazolyl-3)-alanine (II) in S-peptide$_{1-12}$ amide,[4] S-peptide$_{1-14}$[5] of ribonuclease A, [5-glutamine]-β-corticotropin$_{1-20}$ amide,[6] and [5-valine]-angiotensin II[7] and have evaluated the effects of this substitution on biological activity.



I

This article describes the preparation of a number of derivatives of β-(pyrazolyl-1)- (I) and particularly of β-(pyrazoly-3)-alanine (II) which are useful for introducing these amino acids into peptides. Also, applications of some of these derivatives to the synthesis of selected peptides are presented. The methyl ester dihydrochlorides and amides of I and II have been described previously.[4]

Preparative Aspects

Exposure of β-(pyrazolyl-1)-alanine (I) to benzyl chloroformate in aqueous sodium hydroxide gives the crystalline N^{α}-benzyloxycarbonyl derivative. This compound forms a crystalline *p*-nitrophenyl ester on treatment with *p*-nitrophenol and N,N′-dicyclohexylcarbodiimide.[8] β-(Pyrazolyl-1)-alanine (I) affords a crystalline N^{α}-*t*-butoxycarbonyl derivative with *t*-butyl azidoformate[9] and magnesium oxide in dioxane–water.

The chemistry of β-(pyrazolyl-3)-alanine (II) (Scheme I) is more complex than that of β-(pyrazolyl-1)-alanine since this unnatural amino acid contains a readily substitutable hydrogen on the pyrazole ring. When β-(pyrazolyl-3)-alanine is treated with 1 equiv of benzyl chloroformate in aqueous sodium hydroxide, the crystalline N^{α}-benzyloxycarbonyl derivative III is formed.

Methyl N^{α}-benzyloxycarbonyl-β-(pyrazolyl-3)-alaninate (V) is obtained as an oil from methyl β-(pyrazolyl-3)-alaninate (IV) and benzyl chloroformate in chloroform containing triethylamine.

(1) The authors wish to express their appreciation to the U. S. Public Health Service for generous support of this investigation.

(2) Except for glycine the amino acid residues in the various peptides and peptide derivatives are of the L variety.

(3) The following abbreviations are used: BOC = *t*-butoxycarbonyl; Z = benzyloxycarbonyl; ONP = *p*-nitrophenylate; ONHS = N-hydroxysuccinimido; Met→O = methionine *d*-sulfoxide; Pyr(3)Ala = β-(pyrazolyl-3)-alanine; Pyr(1)Ala = β-(pyrazolyl-1)-alanine; DCC = N,N′-dicyclohexylcarbodiimide; DMF = dimethylformamide; CMC = carboxymethylcellulose; TEA = triethylamine; AP-M = aminopeptidase M [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)].

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