Table II. Sample Run: Base-Catalyzed Hydrolysis of I at 25.0°[a]

Time, sec	Absorbance at time t, A_t	Concn of I remaining at time t, $A = A_\infty - A_t$	Log A + 1.00
0	0.277	0.433	0.637
30	0.330	0.380	0.580
60	0.373	0.337	0.528
70	0.413	0.297	0.473
120	0.447	0.263	0.420
150	0.477	0.233	0.367
180	0.501	0.209	0.320
∞	0.710	...	...

[a] 0.01 N NaOH, initial concentration of phosphate *ca.* 3×10^{-4} M, reaction run under nitrogen.

Table III. Pseudo-First-Order Rate Constants for the Base-Catalyzed Hydrolysis of *o*-Phenylene Phosphate at 25.0°[a]

Hydroxide ion concn, M	$10^3 k_{obsd}$, sec^{-1}
0.0050	3.49
0.0075	5.53
0.0100	6.72
0.0125	8.92

[a] Ionic strength 0.40. When the initial concentrations of the phosphate ester were varied over the range 3.7×10^{-4} to 7.64×10^{-4} M, the agreement between the observed first-order rate constants was generally better than 5%.

k_{OH} for the alkaline hydrolysis of diphenyl phosphate at an ionic strength of 1.0. The second-order rate constant measured at 25.0° and at an ionic strength of 1.0 for the hydroxide ion catalyzed hydrolysis of *o*-phenylene phosphate was 9.21×10^{-1} M^{-1} sec^{-1}. Therefore, it can be concluded that the five-membered cyclic aromatic ester, *o*-phenylene phosphate, undergoes alkaline hydrolysis about 6×10^6 times as fast as does the acyclic compound, diphenyl phosphate. This rate acceleration is similar in magnitude to that

Table IV. Effect of Ionic Strength on the Base-Catalyzed Hydrolysis of *o*-Phenylene Phosphate at 25.0°

Ionic strength[a]	$10k_{OH}$
0.01	4.05
0.20	5.30
0.40	6.72
0.70	8.12
1.00	9.21

[a] The ionic strength was adjusted with sodium perchlorate. The initial concentrations of the phosphate esters were varied over the range 3.7×10^{-4} to 7.64×10^{-4} M. The hydroxide ion concentration was maintained at 0.01 M in these runs.

Table V. Base-Catalyzed Hydrolysis of Diphenyl Phosphate. Pseudo-First-Order Rate Constants[a]

Hydroxide ion concn, M	Temp, °C	$10^5 k_{obsd}$, sec^{-1}
1.00	100	3.42
0.75	100	2.68
0.50	100	1.36
0.25	100	0.61
1.00	75	0.70

[a] Ionic strength 1.0. The concentration of diphenyl phosphate was varied over the range 2×10^{-4} to 5×10^{-4} M. At a given hydroxide ion concentration the agreement between observed first-order rate constants was better than 10%.

reported by Westheimer[4] for the hydroxide ion catalyzed hydrolysis of the five-membered cyclic aliphatic ester, ethylene phosphate, relative to its acyclic analog, dimethyl phosphate.

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Intramolecular Catalysis in a Nonhydroxylic Solvent and Its Relationship to the Enzymatic Active Site

R. L. Snell, Wo-Kong Kwok,[1] and Young Kim[1]

Contribution from the Department of Chemistry, East Tennessee State University, Johnson City, Tennessee 37601. *Received May 24, 1967*

Abstract: Evidence is presented to show that intramolecular catalysis of ester aminolysis is possible in a nonhydroxylic solvent and that the required acid–base catalytic species can be derived from the reactants themselves. This evidence is used in support of a new concept of the structure and function of the active sites of certain classes of enzymes.

There are apparently no reported studies of intramolecular catalytic processes in nonhydroxylic media. We wish to report here the results of one such study, that of the amidation of methyl salicylate by *n*-butylamine in dry dioxane, and to show how these results support a new concept of the structure and function of the active sites of certain types of enzymes.

The *o*-hydroxyl group in salicylic acid derivatives has been shown by Bender[2] and by Bruice[3] to function in its phenoxide form as an intramolecular catalytic

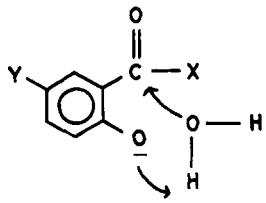
(1) Taken in part from the M.S. Thesis of W.-K. Kwok, East Tennessee State University, 1963, and from work to be submitted by Y. Kim in his M.S. Thesis, East Tennessee State University.

(2) M. L. Bender, F. J. Kèzdy, and B. Zerner, *J. Am. Chem. Soc.*, **85**, 3017 (1963).

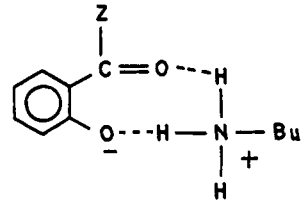
(3) T. C. Bruice and D. W. Tanner, *J. Org. Chem.*, **30**, 1668 (1965).

group for the aqueous basic hydrolysis of these derivatives. Mechanism **1a** was selected by these investigators as best representing the process and was designated as an example of intramolecular general base catalysis.

Fuller[4] studied the aqueous base hydrolysis of phenyl



1a, X = NH_2; $OC_6H_4NO_2$
Y = H; NO_2



1b, Z = OMe

chloroacetate and found it to be subject to nucleophilic catalysis by the monoanion of catechol in the pH range 6.0–7.5. Evidence was presented to support the hypothesis that the first step in the process was transfer of the acyl group to the catalytic body, catechol monoanion, followed by hydrolysis (probably catalyzed intramolecularly) of the acylated catalytic body. This is one of the few reported examples of an over-all hydrolytic process catalyzed by a nonenzymatic molecule to give an acylated intermediate which is then hydrolyzed by an intramolecularly catalyzed process.

There is apparently only one pertinent study in a nonhydroxylic solvent. Vartak, *et al.*,[5] studied the amidation of variously substituted methyl benzoates by aniline in nitrobenzene and found the reaction to be first order in ester and first order in aniline. In this case the *o*-hydroxy group failed to catalyze the reaction, for the second-order rate constant was essentially the same as that found with the *o*-methoxy group. It is obvious that there was no acidic catalysis by the *o*-hydroxy group or an anilinium ion nor base catalysis by the *o*-phenoxide ion (presumably because aniline is a weak base).

Experimental Section

Materials. Methyl salicylate was washed with aqueous sodium carbonate solution, then with water, dried over anhydrous magnesium sulfate, and distilled, collecting from 122 to 123° (30 mm). The purification of methyl *p*-hydroxybenzoate was achieved by recrystallization. Pulverized potassium hydroxide pellets were used to dry *n*-butylamine, which was then distilled in an apparatus protected from water and carbon dioxide by a soda lime tower. The reagent grade dioxane used in obtaining the data for Table II was dried by passage through chromatographic alumina and found to be water free by infrared examination and shown to be peroxide free with aqueous acidic potassium iodide. The reagent-grade dioxane used in obtaining data for Table I was purified and dried by the method described by Wiberg.[6] Perchloric acid in glacial acetic acid was treated with enough acetic anhydride to remove most of the water (but not all since an excess of acetic anhydride would acetylate an amine under these conditions) and standardized against potassium acid phthalate.

Authentic N-*n*-butylsalicylamide was best made by amidation of the ester, followed by a water wash, drying, and distillation.

Kinetics. The data in Table II were obtained by withdrawal of aliquots from the reaction mixture, diluting these with acetic acid, and titrating to the potentiometric end point of 670 mv using glass *vs.* calomel electrodes.

The data in Table I were obtained differently. Solutions were made up by weight and aliquots pipetted into necked-down test tubes (flushed with dry nitrogen) which were corked temporarily until they could be sealed off with a torch and placed in the water bath. One tube was taken at this time for determination of *n*-butylamine concentration and a corrected value of reactant concentrations was thus obtained. When the samples in the bath had come to temperature equilibrium, a tube was withdrawn for the time-zero sample. Each tube contained enough solution to be divided into two aliquots. An aliquot was taken from the opened tube, diluted with 50 ml of glacial acetic acid, and titrated potentiometrically.

Second-order rate constants were calculated from

$$k_2t = \frac{2.303}{(b - a)} \log \frac{a(b - x)}{b(a - x)}$$

The reaction has the A + B → C + D stoichiometry so that the corresponding third-order kinetic equation becomes

$$k_3t = \frac{1}{(b - a)}\left[\frac{x}{a(a - x)}\frac{2.303}{(b - a)} \log \frac{b(a - x)}{a(b - x)}\right]$$

where a = initial concentration of amine and b = initial concentration of ester.

Salts of Methyl Salicylate and of N-*n*-Butylsalicylamide. Purified methyl salicylate and purified *n*-butylamine were mixed with immediate shaking in anhydrous ether at −7° in such a way as to obtain white needles, mp 26–29°. This salt was not particularly stable, apparently liberating *n*-butylamine and being converted to the amide, but by titrating samples (recrystallized from anhydrous ether) in glacial acetic acid at various time intervals it was possible to extrapolate back to the time of isolation of the recrystallized material and obtain a molecular weight of 227.6, whereas the calculated molecular weight is 225.28.

In a similar manner, N-*n*-butylsalicylamide was treated with *n*-butylamine to give another salt, white crystals, mp 63–68°. This salt was quite stable, remaining essentially unchanged (as indicated by infrared) in a screw-cap vial for 3 months. Titration of this salt gave a molecular weight of 279.6 whereas the calculated molecular weight is 266.39.

These salts were examined for conductivity in purified dioxane, using an Industrial Instruments conductivity bridge, Model RC 1B, and a specially made conductivity cell of large surface area and 1/8-in. electrode spacing.

Infrared Studies. All infrared studies were made on a Perkin-Elmer 521 infrared spectrophotometer. Using a pair of variable space cells for solvent cancelation and either dioxane or cyclohexane as solvent, an attempt was made to observe interactions of *n*-butylamine with methyl salicylate in concentration ranges up to 2 *M* in methyl salicylate and 4 *M* in *n*-butylamine. Infrared curves were obtained for the *n*-butylamine salt of methyl salicylate (Nujol mull), the *n*-butylamine salt of N-*n*-butylsalicylamide (solid), and *N*-*n*-butylsalicylamide (liquid).

Results

In the study reported here the methyl esters of salicylic acid and of *p*-hydroxybenzoic acid were allowed to react with *n*-butylamine in dry dioxane. The reaction with methyl salicylate was second order in *n*-butylamine and first order in ester as determined by substitution into the appropriate forms of the integrated second- and third-order rate equations. These results are shown in Tables I and II. The data in Table II were obtained with higher concentrations of reactants but are of the same kinetic order (with straight line kinetic plots up to 70% reaction) but there is, from run to run, a linear increase in the k_3 value with respect to the amine/ester ratio. The values of ΔH^* and ΔS^* for aminolysis of methyl salicylate by *n*-butylamine are 8.2 kcal/mole and −48.5 eu, respectively.

It was shown that, under the conditions used for the Table II data, methanolysis of N-*n*-butylsalicylamide was not detectable after 115 hr at 50° nor ethanolysis after 96 hr at 70°, and that methyl *p*-hydroxybenzoate

(4) E. J. Fuller, *J. Am. Chem. Soc.*, **85**, 1777 (1963).
(5) N. T. Vartak, N. L. Phalnikar, and B. V. Bhide, *J. Indian Chem. Soc.*, **24**, 131A, (1947).
(6) K. B. Wiberg, "Laboratory Technique In Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p 245.

Table I. Rate Constants[a] for the Reaction of Methyl Salicylate and *n*-Butylamine in Specially Purified Dioxane

Amine concn, *M*	Ester concn, *M*	$k_2 \times 10^4$, l. mole^{-1} min^{-1}	$k_3 \times 10^4$, l.^{2} mole^{-2} min^{-1}
	At 70°		
0.1786	1.4258	0.558	3.12
0.2272	1.4212	0.625	3.01
0.8823	1.3273	3.12	4.13
0.9538	1.2251	3.05	3.46
0.3309	0.4069	1.06	3.28
1.7760	0.5419	6.32	3.94
1.2182	0.4517	4.01	3.57
0.4814	0.1116	1.74	3.68
0.7072	0.1092	2.22	3.18
1.1814	0.1795	3.39	2.98
			Av 3.42
	At 50°		
0.3889	1.1211		1.42
0.4982	1.3100		1.61
1.0053	0.5763		1.80
0.9735	0.3658		1.63
			Av 1.62

[a] This table contains half the runs made and the constants have been selected so as to contain the maximum and minimum values of k_3. The average values given are for all the runs.

Table II. Rate Constants for the Reaction of Methyl Salicylate and *n*-Butylamine in Dioxane Passed through Alumina

Amine concn, *M*	Ester concn, *M*	$k_3 \times 10^4$, l.^{2} mole^{-2} min^{-1}
	At 70°	
1.3070	2.0268	4.40
1.3127	2.0411	4.25
2.2216	1.4246	5.66
2.3988	1.4486	6.15
2.7889	1.4381	6.50
2.7324	1.4007	6.10
4.0382	1.4538	7.47
4.0350	1.4520	8.0
	At 50°	
0.5859	1.5250	1.85
0.6010	1.5310	1.83
1.1599	1.6135	1.80
1.1599	1.6007	1.82
1.1138	0.9860	3.25
1.2754	0.9870	2.80
2.0487	1.4990	2.10
2.1360	1.4956	2.32
1.2664	0.5538	2.74

had not reacted with *n*-butylamine to a detectable amount after 30 hr at 50° or 190 hr at 70°.

Infrared studies were made to determine what ester–amine interactions could be detected. In dioxane solution, at concentrations up to 2 *M* in ester and 4 *M* in amine, no significant interactions between methyl salicylate and *n*-butylamine were detectable. The *n*-butylammonium ion was clearly present in equimolar mixtures of phenol and *n*-butylamine. When methyl salicylate and *n*-butylamine were combined in approximately equimolar amounts between NaCl plates a solid resulted which also showed the ammonium ion peak. This solid slowly liquefied in the sample beam over a period of an hour and showed drastic changes in its spectrum during this process.

It was possible to prepare a solid salt, mp 63–68°, in cold, anhydrous ether which titrated as the *n*-butylamine salt of N-*n*-butylsalicylamide. The infrared spectrum of this compound showed the broad butylammonium ion peak in the 2000–3600-cm^{-1} region and had the distinctive feature of a strong pip at 3350 cm^{-1} superimposed on the broad peak. A solid salt, mp 26–29°, also prepared in cold, anhydrous ether, was identified by titration with acid as the *n*-butylamine salt of methyl salicylate. This salt does not show any great change in the carbonyl region as compared to that of the ester itself but it is quite unstable and may have decomposed in the sample beam of the infrared machine. Neither of these salts showed a hydroxyl stretch. The other infrared data are summarized below.

Methyl salicylate and its *n*-butylamine salt have a peak at 1300 cm^{-1} which we attribute to the C–O–CH_3 grouping. N-*n*-Butylsalicylamide also exhibits a peak at 1300 cm^{-1} which we have assigned to the amide III band, for this peak is no longer present in the *n*-butylamine salt of the amide.

N-*n*-Butylsalicylamide shows a peak at 1360 cm^{-1} which appears to be due to the O–H deformation for it is not present in the *n*-butylamine salt of either methyl salicylate or N-*n*-butylsalicylamide. The relatively broad 1327-cm^{-1} peak in methyl salicylate appears to contain the O–H deformation.

The strong amide I and II bands in N-*n*-butylsalicylamide at 1630 and 1540 cm^{-1}, respectively, are decreased greatly in intensity in the *n*-butylamine salt of the amide and the amide III band at 1300 cm^{-1} has very nearly disappeared or else shifted so as to become a shoulder on the low side of the 1320-cm^{-1} peak.

The *n*-butylamine salts of either methyl salicylate or N-*n*-butylsalicylamide were nonconducting in anhydrous dioxane solution. In commercial absolute ethanol either salt showed more conductivity than *n*-butylamine alone or methyl salicylate alone.

Discussion

The fact that the reaction of methyl salicylate with *n*-butylamine in dry dioxane is first order in ester and second order in amine is somewhat unusual.[7] It is clear that *n*-butylamine in dry dioxane reacts differently from aniline in nitrobenzene in the aminolysis of methyl salicylate,[5] for here we find not only intramolecular catalysis but also third-order kinetics. At present we attribute this difference to the low basicity of aniline.

A steady-state treatment of this system, assuming formation of an undissociated ion pair (or some other kinetically equivalent aggregate), gives a third-order kinetic expression. This does not define the structure of the interaction product of methyl salicylate and the first *n*-butylamine molecule, nor does it define the type of catalysis as acidic, basic, electrostatic, or a combination of these. The fact that dioxane solutions of the reactants or of the *n*-butylamine salt of methyl salicylate are not conductive allows no choice to be made between the possibilities of no salt formation, formation of undissociated ion pairs, or formation of hydrogen-bonded species. Infrared studies of dioxane solutions of the reactants showed only slight effects

(7) Other kinetic behavior is possible under these conditions for catechol monoacetate shows second-order kinetics, first order in amine: unpublished work done by Carl Chandler in this laboratory.

on the spectra of the reactants and the presence of a salt could not be detected. Infrared studies were then made in the absence of solvent where the increased concentration of the interaction product would allow its detection. A mixture of methyl salicylate and *n*-butylamine without solvent showed the presence of the *n*-butylammonium ion in the resulting solid. This solid slowly liquefied in the sample beam, accompanied by a considerable change in the spectrum. The *n*-butylammonium salt of methyl salicylate also showed the ammonium ion peak. There is no doubt that proton transfer is possible for these reactants though we have not yet succeeded in detecting a hydrogen bond between the *n*-butylammonium ion and the phenoxide ion. Hydrogen bonding (or something with an equivalent effect on the spectrum) between the *n*-butylammonium ion and the ester carbonyl oxygen may be inferred since the carbonyl absorption in the salt is at the same position as in methyl salicylate itself. The isolation of the ionic *n*-butylamine salt of methyl salicylate shows the possibility that it is this species, present in low concentration, that is subject to nucleophilic attack by a second *n*-butylamine molecule. If so, this accounts for the third-order kinetics, defines the structure of the adduct, and defines the type of catalysis as acid catalysis by the *n*-butylammonium ion. This interpretation has not yet been proven to be the correct interpretation.

The amide I band is greatly decreased in intensity in the *n*-butylamine salt of N-*n*-butylsalicylamide and the amide II band has virtually disappeared while the amide III band has either disappeared completely or shifted so as to be obscured by some other absorption. Such drastic changes in going from the amide to its salt deserve consideration. It is tempting to assume an equilibrium system between two different interaction products in the salt because there are residual, unshifted peaks corresponding to the amide I and II bands, but the data do not support a definite conclusion. Hydrogen bonding of the *n*-butylammonium ion to the amide carbonyl oxygen hardly seems an adequate explanation of these effects, especially since there was no other effect on the ester group frequencies in the *n*-butylamine salt of methyl salicylate. There is no way for the 1:1 salt to consist of the *n*-butylammonium ion and the phenoxide ion and to also have the carbonyl oxygen protonated. The amide hydrogen may hydrogen bond to the phenoxide ion which, without considering any other factors, forces the amide into the *trans* configuration. It is supposedly this configuration[8] which is responsible for the amide II and III bands. It is not clear that this type of hydrogen bonding alone should provide so much constraint to the system as to so drastically alter the spectrum. It is conceivable, however, that the positive charge on the *n*-butylammonium ion could polarize the carbonyl group, decreasing the double-bond character and increasing the basicity of the carbonyl oxygen. If this effect is present in the spectrum, the lowered carbonyl absorption is hidden by the ammonium ion absorption. We had hoped to detect this electrostatic polarization in the *n*-butylamine salt of either the amide or the ester. Bender[9] has proposed an amine polarization of the ester carbonyl bond in the 2-aminopyridine-catalyzed aqueous hydrolysis of the mono-*p*-nitrophenyl ester of oxalic acid.

The function of the *n*-butylammonium ion would seem to be that the ion (as an ion pair) effectively serves to transfer a phenolic hydrogen to the ester carbonyl oxygen, providing acid catalysis for nucleophilic attack by the second amine molecule. Whatever the precise reaction path, it is clear from the kinetics that *n*-butylammonium ion does not substitute for water in structure **1a** and that its function is probably represented in simplified form in structure **1b**.

There are few ΔE^* and ΔS^* values reported for the amminolysis of esters. Vartak, *et al.*,[5] give ΔE^* values ranging from 4.2 to 6.2 kcal/mole where aniline is the amine. For the specific reaction of methyl salicylate with aniline their value of ΔE^* is 5.4 kcal/mole with a ΔS^* of -55.0 eu. Where the amine is ammonia and the esters simple aliphatic esters, Miller and Day[10] give ΔE^* values from 5.2 to 8.2 kcal/mole and ΔS^* values from negative 46 to 65 eu. These values were obtained in dioxane solutions containing high concentrations of ethylene glycol or amides to catalyze the otherwise exceedingly slow reaction. Their ΔE^* values are probably about those expected for these aminolysis reactions since it was shown that hydrolysis of these esters under these conditions gave fairly normal ΔE^* values of 13–14 kcal/mole. Entropy of activation for these ester hydrolyses was shown to vary from negative 46 to 51 eu. Thus the values of ΔH^* (8.2 kcal/mole) and of ΔS^* (-48.5 eu) found here for the amminolysis of methyl salicylate by *n*-butylamine are normal when compared with the limited data available.

While we cannot yet specify a detailed mechanism for the third-order reaction (second order in amine) of methyl salicylate with *n*-butylamine in dry dioxane, we have shown that acid–base species not in equilibrium with water can intramolecularly catalyze a nucleophilic displacement, that the catalytic function can be generated by the reactants themselves and that (in view of the findings of Vartak[5]) the relative acid–base strengths of functional groups in the reactants is significant. We believe that these findings support our views, presented below, of the nature of the "active site" in certain types of enzymes.

A New Concept of the Enzymatic Active Site

Most studies of intramolecularly catalyzed solvolyses, or of nucleophilic displacement reactions, of esters and of amides have been carried out in aqueous solution with the expectation that the results could be applied to an understanding of enzyme-catalyzed reactions of these types, for such processes are known to occur in an aqueous macroenvironment. On this basis, the goal has been to study the various mechanistic aspects of acid and base catalysis by groups in Lowry–Brønsted equilibrium with water or aqueous acid or base. However, these need not be the only types of acid–base equilibria that are involved in catalysis nor is a gross aqueous environment the only one that needs be considered, as the following discussion shows.

(8) L. J. Bellamy, "The Infra-Red Spectra of Complex Molecules," 2nd ed, Methuen and Co., Ltd., London, 1958, pp 214, 217.

(9) M. L. Bender and Y.-L. Chow, *J. Am. Chem. Soc.*, 81, 3929 (1959).

(10) G. R. Wolf, J. G. Miller, and A. R. Day, *ibid.*, 78, 4372 (1956).

It is presently considered[11] that those enzymes dealing with ester or peptide bonds have an "active site" (usually only one) composed of two substituted amino acids adjacent to each other along the chain and a third substituted amino acid remote along the enzyme chain but near in space to the first two groups by virtue of the gross configuration assumed by the enzyme. Presumably other polar substituents on the surface of the enzyme may participate in the process by bonding the substrate to the enzyme through hydrogen bonding and dipole–dipole interactions in such a way as to place the appropriate substrate bond at the active site, with the correct orientation for reaction. The formation of this enzyme–substrate complex may be accompanied by some distortion of the enzyme surface and perhaps a distortion of substrate bonds in the region of the active site. Within the active site one group is a reactant and at least one group is an acidic or basic catalytic group. The function of the third group is less obvious but it may stabilize the transition state (by solvation or by electrostatic interaction), or it may be the second catalytic component in difunctional catalysis, or it may function in another step of the over-all process.

Consider a hypothetical enzyme, "specific" for transamidation, having in its "active site" a carboxyl group and an amine group of inherently weaker base strength than a second amine group, also present. Assume also that the relative acid and base strengths of these groups (and the environment as a whole) is such that the carboxyl group is a carboxylate ion, the stronger base is an ammonium ion, and the weaker base is a neutral amine. There are present then, a nucleophile, a proton acceptor, and a proton donor, the usual groups required for hydrolysis or nucleophilic displacement of carboxylic acid derivatives. Now assume that a substrate peptide becomes attached to the surface of the enzyme and in the process all water is excluded, at least in the region of the active site. During this process there may or may not be some distortion of the enzyme surface and of the substrate bonds but the net result is a substrate bond, correctly spaced and oriented with respect to the groups in the active site so that catalysis is performed along the optimum reaction coordinates through proton transfer with minimum atom rearrangement, through electrostatic effects, or a combination of these in conjunction with hydrogen bonding. The displacement also takes place along the optimum reaction coordinate and there is again no great relocation of atoms with respect to one another. Under these conditions the active site itself functions in terms of a "microstereochemically oriented system." It is conceivable that the active site could retain as much as one molecule of water per functional group, so that an oriented water molecule could serve as an acid–base transfer agent. However, the presence of such water would decrease the stereochemical specificity of the active site and replace groups with inherent differences in acid–base strengths with hydrogen ion or hydroxyl ion. This should decrease "specificity" by providing a leveling effect on the relative rates of the different reactions catalyzed by a given enzyme.

This argument presumes that only a part of the so-called enzyme "specificity" is derived from the fit of substrate to the enzyme surface, the remainder being derived from the properties of the "active site." Such a presumption is valid since most enzymes acting on carboxylic acid derivatives, *in vitro* at least, are not really specific in their choice of a particular substrate nor of a particular bond within the substrate, nor even specific for only a single bond type. These enzymes may hydrolyze a "specific" bond in a given substrate most efficiently and still carry out transamidations, ester hydrolyses, or plastein syntheses in other substrates. It is for these reasons that acid–base species in equilibrium with water cannot be responsible for catalyzing any step in the enzymatic process, unless it is the step regenerating the functional groups of the active site. The less efficiently catalyzed processes must be less efficient because of one or more of the following factors: (a) poor fit of substrate to enzyme, (b) nonoptimum relations of acid–base strengths, or (c) nonoptimum "microstereochemical" requirements.

Many intramolecularly catalyzed hydrolytic reactions involve a nucleophilic attack on the substrate bond as the first step (other than acid–base reactions) in the over-all hydrolysis.[12] These reactions are acylation or transacylation processes. One recent example of such a reaction, due to Fuller,[4] has already been cited. More recently, Bender[13] has shown that transacylation is the first step in the cycloamylose-catalyzed aqueous base hydrolysis of various esters.

There is, however, already evidence showing that the active site in aqueous chymotrypsin is a nonaqueous (the authors used the term "nonpolar") region as determined by the effect of solvent on the ultraviolet spectrum of a group (analogous to an acyl group) irreversibly bonded to the active site. It was also shown that when this substituted chymotrypsin molecule (in aqueous solution) was thermally and reversibly denatured, in the denatured state the ultraviolet spectrum of the substituent was that expected in water.[14] In this case, at least, the active site of the undenatured enzyme presents a nonaqueous environment to the irreversibly bonded substrate, and may be considered an analog of an active site ready to carry out a transfer process or else ready to be hydrolyzed back to the original active site.

Phillips, *et al.*[15,16] have completed a three-dimensional structural analysis of the enzyme, lysozyme, and have made some preliminary analyses of lysozyme inhibited at the active site and also of lysozyme inhibited elsewhere. Their interpretation is that a "cleft" exists along the lysozyme surface into which a part of the substrate fits and that the active site is located within the cleft. This picture neither requires the presence of water within the active site nor that the acid–base species within the active site be equilibrated with water once the coniplex has formed. Water, or a species in equilibrium with water, would then enter into the final step of the over-all reaction. The implica-

(11) D. E. Koshland, Jr., *Science*, **142**, 1533 (1963).

(12) For key references see: M. L. Bender, *Chem. Rev.*, **60**, 53 (1960); E. M. Kosower, "Molecular Biochemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962.

(13) M. L. Bender, *et al.*, *J. Am. Chem. Soc.*, 88, 2318, 2319 (1966), and references cited therein.

(14) J. Kallos and K. Avatis, *Biochemistry*, **5**, 1979 (1966), and references cited therein.

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tion is that the same interpretation applies to the action of lysozyme on its normal polysaccharide substrate and only in the final step is a water molecule, or a water-equilibrated, acid–base species, required from bulk solution.

If the preceding speculations are valid the catalytic groups must be preformed zwitterions, possibly hydrogen bonded back to each other prior to complex formation and possibly hydrogen bonded to substrate after complex formation; or else they are free acids and bases with these same hydrogen-bonding capabilities. In the latter case one or both catalytic groups may become ionic upon interaction with the substrate (assuming a water-free active site) and then become hydrogen bonded to each other and/or to the substrate. One group may stabilize, through solvation or hydrogen bonding, charge generated by interaction at the other group, and a charged group may polarize a substrate bond. Some of these possible interactions, those involving hydrogen-bonded structures, have been demonstrated to be realities by infrared studies of acid–base interactions. Tamres,[17] Gordy,[18] and Barrow[19] observed hydrogen bonding between carboxylic acids and amino nitrogen and also hydrogen bonding between protonated amino nitrogen and carboxylate ion. Frankel and Kim[20] have studied the latter interactions in the form of anilinium ion hydrogen bonded back to the gegenion, using nmr techniques.

Considering the preceding speculations and the experimental data cited, it is probable that most intramolecularly catalyzed hydrolytic reactions in the literature are related to the step in the enzymatic process which regenerates the three groups at the active site, and the mechanistic conclusions which are drawn from them are related in only a general way to the other steps. Our own experiments seem to support this conclusion.

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Conclusions

We have demonstrated that intramolecular catalysis in a nonhydroxylic solvent does occur. We have shown that the reactants, by interaction with each other, can generate the acid–base species required for catalysis.

We have postulated that the "active sites" in certain types of enzymes function primarily without the intervention of acid–base species derived from water and in equilibrium with water. We have discussed the reasons for this postulate in terms of a "microstereochemical" requirement for the reacting system and in terms of a method for obtaining acidic and basic catalytic groups of varying catalytic activity from one enzyme to another and of the varying catalytic activity of a single enzyme with respect to various substrates and various processes. These requirements are judged necessary to account for the multiplicity of processes catalyzed by a single enzyme.

We are continuing our studies of intramolecular catalytic effects in nonhydroxylic solvents with both di- and trifunctional molecules and extending the work to include polyfunctional intermolecular catalysis. We expect to determine the steric factors and the relative acid–base strengths required in these systems with the expectation of further application to enzyme theory.

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