Carboxyl Group Participation in Acetal Hydrolysis. Hydrolysis of Disalicyl Acetals[1]

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Abstract: The rates of appearance of salicylic acid from the disalicyl acetals of formaldehyde, benzaldehyde, and *p*-nitrobenzaldehyde have been measured in the solvents H_2O and 50% dioxane–H_2O (v/v). Bell-shaped pH–rate constant profiles are obtained. Hydronium ion catalysis is not observed even at hydronium ion concentrations as high as 1 *M*. The rates of hydrolysis of these compounds are extremely fast in comparison with suitable reference compounds. The maximum difference in k_{obsd} for benzaldehyde disalicyl acetal and that calculated for its corresponding dimethyl ester is 2.7×10^9. Intramolecular general acid catalysis is most likely taking place in the hydrolysis of these compounds. In 50% dioxane–H_2O, the monoanionic species has a rate constant for intramolecular general acid catalysis that is greater than that for the completely un-ionized species by a factor of 65 in the case of the benzaldehyde derivative, 260 with the *p*-nitrobenzaldehyde acetal, and 25 with formaldehyde disalicyl acetal. *p*-Nitrobenzaldehyde *o*-carboxyphenyl *p*-carboxyphenyl acetal has a pH–rate constant profile that is also bell shaped, but the magnitude of the bell is greatly reduced in comparison with the acetal having two ortho carboxyl groups, there being a difference of only 5 between the rate constants for the monoanion and the un-ionized species. While the two un-ionized species hydrolyze at about the same rate, the rate constant for the monoanion is 233 times greater in the case of the di-*o*-carboxyl-substituted compound. Thus, while changing substituent effects due to ionization of one of the carboxyl groups in combination with general acid catalysis by the un-ionized carboxyl group can give rise to a bell-shaped pH–rate constant profile, still the relatively fast rate of the monoanionic species cannot be completely accounted for in this manner. It is possible, therefore, that the carboxylate anion is also participating in the reaction, *i.e.*, bifunctional catalysis is taking place. However, the contribution of this type of catalysis to the overall rate enhancement in comparison with the dimethyl ester is small. Most of this rate enhancement is achieved by intramolecular general acid catalysis by one carboxyl group. The significance of these findings in regard to the mechanism of action of the enzyme, lysozyme, is discussed.

The three-dimensional structure of the glycosidic enzyme lysozyme has been elucidated by X-ray crystallographic analysis,[3] and the complete amino acid sequence of the enzyme has been determined.[4] Carboxyl groups from glutamic acid-35 and aspartic acid-52 presumably form part of the active site. The pH–rate profile for the enzyme is bell shaped, indicating that two groups are possibly involved.[5] Several mechanisms have been proposed,[6,7] which differ in detail, but all involve glutamic acid-35 acting as a general acid. A mechanism receiving recent support involves general acid catalysis by glutamic acid-35 and electrostatic stabilization of a developing carbonium ion by the aspartate anion.[7]

In view of the detailed information concerning the structure of lysozyme, a number of physical organic studies have been carried out to determine the structural features in an acetal that will lead to general acid catalyzed hydrolysis reactions.[8-12] Buffer acid catalysis is observable when the leaving group is good (a phenol) in cases where a moderately stable carbonium ion intermediate is formed.[8] General acid catalysis is also found when the leaving group is poor (an aliphatic alcohol) when an exceedingly stable oxotropylium ion intermediate is produced,[9] or in cases where C–O bond breaking is easy due to relief of ground state steric strain in the transition state.[12]

Intramolecular facilitation of acetal hydrolysis by one carboxyl group has been observed in several cases.[13-15] Intramolecular general acid catalysis was suggested by Capon, *et al.*,[13] to take place in hydrolysis of *o*-carboxyphenyl β-D-glucoside and *o*-methoxymethoxybenzoic acid. Dunn and Bruice,[14] however, presented evidence that with the latter compound the mechanism is A-1. In hydrolysis of *o*-carboxyphenoxytetrahydropyran and benzaldehyde methyl *o*-carboxyphenyl acetal, rate enhancements of 10^5–10^6 are observed in comparison with suitable reference compounds.[15] With these compounds the mechanism most probably involves intramolecular general acid catalysis since mechanistically similar buffer acid catalysis is seen with the unsubstituted compounds.[8,13] Bifunctional catalysis of acetal hydrolysis by two carboxyl groups has not been observed.

In the attempt to obtain rate enhancements in simple chemical systems of the magnitude seen in enzymatic reactions and to determine whether bifunctional catalysis is indeed a chemically reasonable mechanism for lysozyme, we have been engaged in a study of dicarboxyl substituted acetals of various types. The present paper deals with hydrolysis of disalicyl acetals.

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(2) Postdoctoral Fellow, Department of Biochemistry, University of Southern California.
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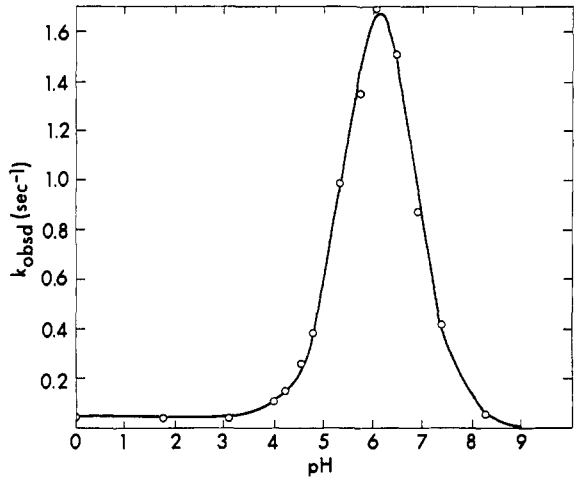
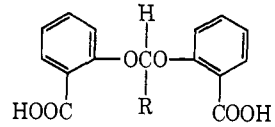


Figure 1. Plot of k_{obsd} *vs.* pH for release of salicylic acid from benzaldehyde disalicyl acetal in 50% dioxane–H_2O (v/v) at 25°, μ = 0.05 (with KCl).



I, R = C_6H_5
II, R = H
III, R = *p*-$NO_2C_6H_4$

Experimental Section

Materials. Benzaldehyde Dimethylsalicyl Acetal. A solution of α,α-dichlorotoluene (0.05 mol) and sodium methyl salicylate (0.1 mol) in DMSO (100 ml) was heated at 135° for 3 hr. The solution, which was by then neutral, was allowed to cool and was then poured into water and extracted with dichloromethane. The dichloromethane solution was washed with 0.05 *N* aqueous sodium hydroxide until the organic layer showed no methyl salicylate on tlc. The solution was concentrated *in vacuo* and distilled (bp 210–220° (0.05 mm)) to yield a viscous oil which would not crystallize (40%). Infrared showed typical ester carbonyl and acetal absorption. *Anal.* Calcd for $C_{23}H_{20}O_6$: C, 70.40; H, 5.14. Found: C, 70.46; H, 5.03.

Because of the high rates of hydrolysis below pH 9, solutions of the sodium salt of the carboxylic acid (I) were obtained by a previously published technique.[15]

Formaldehyde Disalicyl Acetal (II). Diiodomethane (0.05 mol) and sodium methyl salicylate (0.1 mol) were heated in DMSO (100 ml) at 145° for 24 hr. The solution was worked up in a similar manner to the benzaldehyde acetals to yield the acetal (bp 190–195° (0.05 mm), mp 42–43°). *Anal.* Calcd for $C_{17}H_{16}O_6$: C, 64.55; H, 5.10. Found: C, 64.59; H, 5.09.

The free acid was prepared by the hydrolysis of the ester in 75% aqueous ethanolic sodium hydroxide, followed by dilution with water, acidification to pH 3.0, filtration, and recrystallization from acetonitrile, mp 159–162°. *Anal.* Calcd for $C_{15}H_{12}O_6$: C, 62.50; H, 4.20. Found: C, 62.57; H, 4.20.

***p*-Nitrobenzaldehyde Dimethylsalicyl Acetal.** α-Bromo-*p*-nitrobenzyl methyl salicylate ether was prepared by the bromination of *p*-nitrobenzyl methyl salicylate ether in carbon tetrachloride by the procedure of Eliel and Rivard.[16] The product is rather unstable and was characterized in the following manner. The infrared spectrum in CCl_4 showed a typical ester carbonyl peak at 1740 cm^{-1} and no aldehyde peak. Hydrolysis in moist ethanol yielded HBr (95% by titration), methyl salicylate (glc and tlc), and *p*-nitrobenzaldehyde (tlc and characterized as the 2,4-dinitrophenylhydrazone). The α-halo ether was treated with methyl salicylate to yield the desired product by the general procedure of Fife and Anderson,[15] mp 103–105° (from methanol). *Anal.* Calcd for $C_{23}H_{19}NO_8$: C, 63.16; H, 4.38; N, 3.20. Found: C, 63.25; H, 4.41; N, 3.19. The sodium salt of the dicarboxylic acid (III) was prepared as before.[15]

***p*-Nitrobenzaldehyde *o*-carbomethoxyphenyl *p*-carbomethoxyphenyl acetal** was prepared as the di-ortho isomer, substituting sodium methyl *p*-hydroxybenzoate for sodium methyl salicylate, mp 143–144° (from methanol). *Anal.* Calcd for $C_{23}H_{19}NO_8$: C, 63.16; H, 4.38; N, 3.20. Found: C, 63.08; H, 4.42; N, 3.14. The sodium salt of the dicarboxylic acid (IV) was prepared as before.[15]

Formaldehyde bis(2-carboxy-4-methoxyphenyl) acetal (V) was prepared by an analogous method (mp 173–174°). *Anal.* Calcd for $C_{17}H_{16}O_8$: C, 58.62; H, 4.63. Found: C, 58.85; H, 5.09.

Since all disalicyl acetals have bell-shaped pH–rate constant profiles, it may be safely presumed that both ester groups were cleaved during hydrolysis to the final product. In no case could any evidence be found for methyl salicylate as a product.

Kinetic Measurements. The rates of hydrolysis were determined spectrophotometrically, employing either a Durram-Gibson Model D110 stopped-flow spectrophotometer or a Gilford Model 2000 recording spectrophotometer. Reactions were followed by monitoring appearance of either salicylic acid or the appropriate aldehyde. The solvents employed were 50% dioxane–H_2O (v/v) and H_2O. Constant temperature was maintained in the stopped-flow measurements by suspension of the drive syringes, mixing chamber, and cuvette in a water trough whose temperature was maintained constant by circulating the water through a heat exchanger immersed in a thermostat. Optical density changes after mixing were recorded on a Hewlett-Packard storage oscilloscope (Model 1207B). Relatively stable solutions of the disalicyl acetals were prepared at high pH and placed in one of the drive syringes. In the other was placed an acidic solution so that the appropriate pH would be obtained upon rapid mixing of the solutions. In the vast majority of cases the buffering capacity of the acidic solutions was sufficient that the mixing resulted in a negligible difference between its pH and the resultant pH. pH measurements were made with a Radiometer pHM 22. The glass electrode gives the correct pH reading in 50% dioxane–H_2O.[17]

All data were analyzed on an IBM 360/50 computer by rigorous nonlinear least-squares analysis using programs developed by Dr. Edwin Anderson. Copies of the source listings can be made available on request.

Product Characterization. The formaldehyde acetals released 2 mol of salicylic acid (spectrophotometric determination). The possible intermediate from the hydrolysis of formaldehyde disalicyl acetal, 4,5-benzo-1,3-dioxan-6-one, is known to be stable under the hydrolytic conditions,[13] and cannot be an intermediate. We assume the same to be true for the bis(4-methoxysalicyl) acetal where 2 mol of 4-methoxysalicylic acid was released.

In the case of benzaldehyde bis(methylsalicyl) acetal, the appearance of either benzaldehyde or methyl salicylate may be used for kinetic measurements yielding identical experimental rate constants; hence hydrolysis is simple acetal hydrolysis.

No increase in absorbance is observed at 246 mμ during hydrolysis of benzaldehyde disalicyl acetal, and hence benzaldehyde, which has a high absorbance at 246 mμ, is not being formed. One equivalent of salicylic acid is produced (by uv estimation). A large-scale hydrolysis in 50% dioxane–H_2O was run at pH 6.00. The solution was extracted with ether. Salicylic acid is fully ionized at this pH in 50% dioxane and is not extracted. Evaporation of the ether left a waxy solid with a carbonyl peak at 1755 cm^{-1} and mp 60° (yield 65%) as the sole product. 4,5-Benzo-2-phenyl-1,3-dioxan-6-one has reported mp 58° and infrared carbonyl peak at 1750 cm^{-1}.[18] This compound was prepared by reacting salicylic acid with benzaldehyde diacetate[18] and had mp 59–61°. A mixture melting point revealed no depression. The infrared spectra of the synthetically prepared material and the isolated intermediate were identical. The spectrophotometrically measured yield of salicylic acid was independent of the pH of hydrolysis.

p-Nitrobenzaldehyde is not formed from *p*-nitrobenzaldehyde disalicyl acetal, and only 1 mol of salicylic acid is produced. It must therefore react in the same manner as the unsubstituted acetal.

The reaction mixture after hydrolysis of *p*-nitrobenzaldehyde *o*-carboxyphenyl *p*-carboxyphenyl acetal is identical with a synthetic equimolar mixture of *p*-nitrobenzaldehyde, salicylic acid, and *p*-hydroxybenzoic acid.

Results

In Figure 1 is shown a plot of k_{obsd} for release of

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Table I. Rate Constants for Hydrolysis of Disalicyl Acetals

Compd	Solvent	$k_1 \times 10^{-4}$, M^{-1} sec^{-1}	$k_2 \times 10^{-4}$, M^{-1} sec^{-1}	k_{H_2A}, sec^{-1}	k_{H_1A}, sec^{-1}	pK_a	pK_a'
Benzaldehyde disalicyl acetal[a]	50% Dioxane–H_2O	1.83	1170	0.042	2.74	5.64	6.63
Benzaldehyde disalicyl acetal[b]	H_2O	0.0219	9.75	0.105	3.23	3.32	4.48
p-Nitrobenzaldehyde disalicyl acetal[c]	50% Dioxane–H_2O	0.0033	6.17	0.0000904	0.024	5.56	6.41
p-Nitrobenzaldehyde salicyl *p*-carboxyphenyl acetal[c]	50% Dioxane–H_2O	0.0023	0.0167	0.0000214	0.000103	6.04	6.21
Formaldehyde disalicyl acetal[d]	H_2O	0.00000126	0.000879	0.0000118	0.000305	3.03	4.46
Formaldehyde bis(2-carboxy-4-methoxy)phenyl acetal[a]	50% Dioxane–H_2O	0.000807	0.383	0.0000244	0.0042	5.52	5.96

[a] At 25°; μ = 0.05 (with KCl). [b] At 25°; μ = 0.1 (with KCl). [c] At 45°; μ = 0.05 (with KCl). [d] At 65°; μ = 0.1 (with KCl).

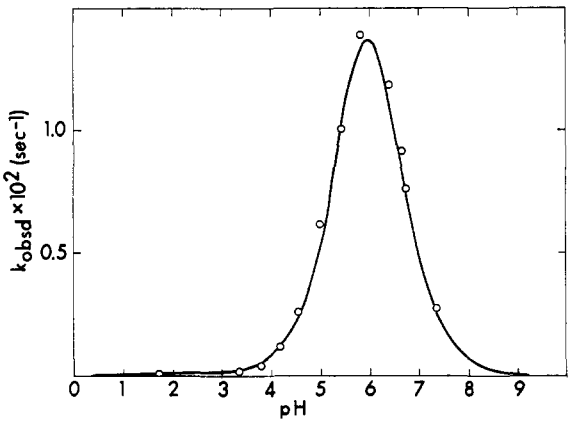


Figure 2. Plot of k_{obsd} *vs.* pH for release of salicylic acid from *p*-nitrobenzaldehyde disalicyl acetal in 50% dioxane–H_2O (v/v) at 45°, μ = 0.05 (with KCl).

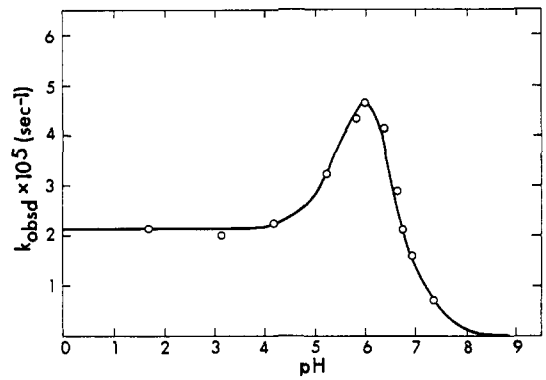


Figure 3. Plot of k_{obsd} *vs.* pH for hydrolysis of *p*-nitrobenzaldehyde *o*-carboxyphenyl *p*-carboxyphenyl acetal in 50% dioxane–H_2O (v/v) at 45°, μ = 0.05 (with KCl).

salicylic acid from benzaldehyde disalicyl acetal (I) *vs.* pH in 50% dioxane–H_2O (v/v) at 25°. A similar profile resulted when H_2O was the solvent. It can be seen that a bell-shaped plot is obtained. The product of the reaction of I is the stable acylal which was isolated and characterized. Salicylic acid (1 equiv) is released. In comparison with the corresponding dimethyl ester (k_H = 4.37 × 10^{-3} M^{-1} sec^{-1} in 50% dioxane–H_2O), the maximum difference in k_{obsd} at any pH is 2.7 × 10^9, thereby clearly showing that intramolecular carboxyl group participation is occurring. Unfortunately, the dimethyl ester is not sufficiently soluble in H_2O to measure its rate of hydrolysis in that solvent. Bell-shaped pH–rate constant profiles were also observed for the acetals II–V. Figure 2 shows the profile obtained for *p*-nitrobenzaldehyde disalicyl acetal (III), and in Figure 3 is shown the profile for the mixed acetal *p*-nitrobenzaldehyde *o*-carboxyphenyl *p*-carboxyphenyl acetal (IV). Hydronium ion catalysis is not observed with any of these acetals, even at a hydronium ion concentration of 1 *M*. Rate constants for hydrolysis of the acetals I–V are given in Table I.

The curves in Figures 1–3 were calculated from eq 1

$$k_{obsd} = \frac{k_{H_2A}a_H^2 + k_{H_1A}a_H K_a}{a_H^2 + K_a a_H + K_a K_a'} \quad (1)$$

and the rate constants reported in Table I. The constants k_{H_2A} and k_{H_1A} are rate constants for intramolecular general acid catalysis of the hydrolysis of the unionized and monoanionic species, and K_a and K_a' are the first and second acid dissociation constants. The values of the rate constants and K_a and K_a' are computer calculated values, from a rigorous nonlinear least-squares procedure, which gives the best fit to the experimental data. Note that the following kinetic equivalencies hold

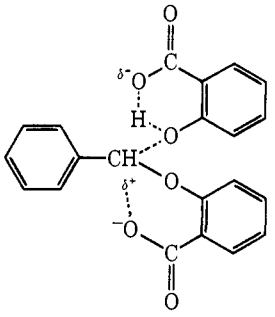
$$k_{H_2A} = k_1 K_a$$
$$k_{H_1A} = k_2 K_a' \quad (2)$$

with k_1 and k_2 rate constants for hydronium ion catalyzed hydrolysis of the monoanion and dianion species, respectively.

Buffer acid catalysis by dichloroacetic acid is observed with the dimethyl ester of I at 45°, k_{HA} being 1.5 × 10^{-2} M^{-1} sec^{-1}. Therefore, it is likely that mechanistically similar intramolecular general acid catalysis is occurring with I rather than a kinetic equivalent possibility.

Discussion

The bell-shaped pH–rate constant profiles obtained for hydrolysis of the disalicyl acetals and the extremely large rate enhancements in comparison with the dimethyl ester (3 × 10^9 in the case of I) might be taken to indicate that the mechanism for hydrolysis of the monoanion involves bifunctional catalysis as in VI. In this mechanism the un-ionized carboxyl group acts as an intramolecular general acid while the carboxylate anion provides electrostatic stabilization of the incipient carbonium ion. This mechanism is exactly analogous to one of the mechanisms suggested for lysozyme.[6,7] Intramolecular general acid catalysis is most likely occurring rather than a kinetically equivalent possibility since buffer acid catalysis is observed in the hydrolysis of the dimethyl ester of I. It is a reasonable assumption that



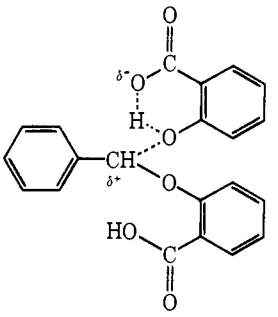
VI

the mechanism of the intramolecular reaction is the same as that of the bimolecular reactions of the compounds not having carboxyl group substitution, *i.e.*, general acid catalysis. Any mechanism involving a completely transferred proton in the transition state (A-1) can be ruled out if basicity of the acetal I is normal. A reasonable dissociation constant for the conjugate acid would be 10^9 since the measured dissociation constant of benzaldehyde diethyl acetal[19] is $10^{5.7}$ and aryl ethers are generally less basic than aliphatic ethers[20] by 10^3. From the magnitude of k_2 in H_2O (9.75×10^4 M^{-1} sec^{-1}), it can then be calculated that if the mechanism were A-1, the rate constant for transfer of a proton from the conjugate acid to H_2O would necessarily be much greater than that for a diffusion controlled reaction. The value of k_r, the rate constant for decomposition of the conjugate acid to salicylic acid and an oxocarbonium ion, would have to be $\sim 10^{14}$ sec^{-1}, if the mechanism were A-1. The rate enhancement in 50% dioxane–H_2O of 2.7×10^9 is considerably greater than found previously with monocarboxyl substituted acetals where rate enhancements compared to the corresponding methyl esters or para carboxyl-substituted isomers are 600 for *o*-methoxymethoxybenzoic acid,[13] 10^4 for *o*-carboxyphenyl β-D-glucoside,[13] and 10^5–10^6 with 2-(*o*-carboxyphenoxy)tetrahydropyran and benzaldehyde methyl *o*-carboxyphenyl acetal.[15]

While it seems reasonably certain that the hydrolysis of the benzaldehyde derivatives proceeds with intramolecular general acid catalysis, it is less conclusive that this is the case with the formaldehyde acetals. In the case of those compounds the leaving group is quite good, but the intermediate oxocarbonium ion is highly unstable. Although the pH–rate constant profiles are bell shaped and exhibit large plateau regions at low pH, still the reactions are slow and the type of reasoning that was employed with the benzaldehyde acetals cannot be utilized. Capon, *et al.*,[13] have preferred intramolecular general acid catalysis as the mechanism of hydrolysis of methoxymethoxybenzoic acids, but Dunn and Bruice[14] have concluded that the mechanism is actually A-1. If the formaldehyde disalicyl acetals hydrolyze by an A-1 mechanism, then the pH-independent reaction at low pH must be hydronium ion catalyzed hydrolysis of the monoionized compound.

The product of the reaction of I is the cyclic acylal (1 equiv of salicylic acid is released), but this does not prove direct involvement of the carboxylate anion in the critical transition state of the monoanion reaction, since the acylal could result from carbonium ion capture by the carboxyl group. With formaldehyde disalicyl acetal, 2 equiv of salicylic acid are released, so if bifunctional catalysis is involved it must be of the type in VI, since an acylal intermediate which would be formed by nucleophilic attack would be relatively stable as is the case with the corresponding acylal derived from I.

It will be noted in Figures 1–3 that hydronium ion catalysis is not seen in the hydrolysis of disalicyl acetals. At low pH, the rate is invariant with increasing hydronium ion concentration to a concentration of 1 *M*. Thus, hydronium ion catalysis cannot compete with facile intramolecular general acid catalysis in the un-ionized species. While the monoanion is the most



VII

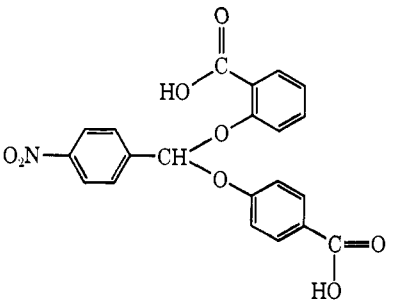
reactive species, still its associated rate constant in the case of I is only 65 times greater than that for the un-ionized compound[21] where bifunctional catalysis of the type shown in VI cannot take place. Part of this difference of 65 times must be due to the changing substituent effect produced by ionization of the carboxyl group. Therefore, if the carboxylate anion in the monoanionic species of I is directly participating in the reaction, its contribution to the rate of the reaction must be small. Most of the rate enhancement of 3×10^9 in comparison with the dimethyl ester is due to intramolecular general acid catalysis by one carboxyl group, as must be the case with the completely un-ionized compound. It is not possible to measure rate enhancements relative to the dimethyl esters of the other acetals since acid-catalyzed hydrolysis of the ester grouping would most certainly be faster than acetal hydrolysis.

With *p*-nitrobenzaldehyde disalicyl acetal (III), the pH–rate constant profile for release of salicylic acid is bell shaped (Figure 2). However, the difference in the magnitude of the rate constants for the monoanion and the un-ionized compound is greater than with the unsubstituted compound I, being a factor of 260. The effect of electrostatic carbonium ion stabilization should increase as carbonium ion stability is decreased by the presence of the para nitro group substituent in the benzaldehyde portion of the molecule.

The pH–rate constant profile is also bell shaped with the derivative IV, where one of the carboxyl groups is in the para position (Figure 3). Thus, general acid catalysis by one group in combination with a changing substituent effect due to ionization of a second carboxyl group can give a bell-shaped profile. Bruice and Maugh[22] previously found in ester hydrolysis reactions

(19) T. Pletcher and E. H. Cordes, *J. Org. Chem.*, 32, 2294 (1967).
(20) E. M. Arnett and C. Y. Wu, *J. Amer. Chem. Soc.*, 82, 4999, 5660 (1960).
(21) A statistical correction for the fact that two un-ionized carboxyl groups are present in the un-ionized species, which can act as general acid catalysts, while only one un-ionized carboxyl group is present in the monoanion increases the ratio of the rate constants to 130.
(22) T. Maugh and T. C. Bruice, *J. Amer. Chem. Soc.*, 93, 3237 (1971).

in which bell-shaped pH–rate constant profiles had been observed that these profiles were due to participation by one group and a substituent effect by a second group. With IV, however, the bell is much less pronounced than in the case of the di-ortho-substituted compound, there being a difference of only 5 in the rate constants for general acid catalysis with the monoanion and un-ionized species. This then is the substituent effect for ionization of a carboxyl group. Consequently, a substituent effect is not the only effect being exerted by the carboxylate anion since a factor of 52 in the rate constant is left unaccounted for in this manner. Therefore, the kinetic effects of placing one of the carboxyl groups in the para position and of destabilizing the carbonium ion intermediate with a nitro group in the benzaldehyde portion of the molecule are in accord with monoanion hydrolysis involving bifunctional catalysis. However, a rate constant difference of 52 is also in accord with a steric effect.[23] Ortho-substituted phenyl glycosides[24] and methyl phenyl acetals of formaldehyde[14] hydrolyze faster than corresponding para-substituted compounds. Therefore, it is not conclusive that bifunctional catalysis is occurring. If the mechanism does involve bifunctional catalysis, then it is clear that participation by the carboxylate anion in the monoanion reaction gives only small advantage in comparison to one carboxyl group acting as a general acid in the case of the un-ionized compound.



IV

Bell-shaped pH–rate constant profiles are obtained with the disalicyl acetals, as with lysozyme,[5] and rate enhancements of the magnitude of those in enzymatic reactions take place in comparison with the dimethyl ester of I. However, it is also clear that bell-shaped profiles and extremely large rate enhancements can be observed in cases involving intramolecular general acid catalysis by one carboxyl group, along with a substituent effect exerted by the second carboxyl group, as in IV. Therefore, the role of aspartic acid-52 in lysozyme-catalyzed reactions must be considered an open question. There is no evidence that it is directly involved in the critical transition state. It is possible that it could exert an effect on the reaction by stabilizing a particular conformation of the enzyme. It is clear from the present data that there is no compelling need to propose a direct role for aspartic acid-52, since the kinetics of lysozyme catalyzed reactions can be accounted for in a chemically simpler mechanism involving only intramolecular general acid catalysis.

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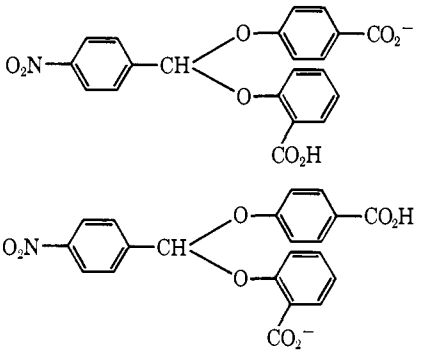
(23) Note that the rate enhancement may be less than 52. Since the pK_a for an ortho carboxyl is always less than that of a para carboxyl, the para carboxyl will be the least acidic group. There are two possible monoionized species of which only one will be catalytically active.



Its concentration will depend on the relative pK_a values of the separate groups, which are, of course, not measurable, but it must be less than 50% of the total because of the above-mentioned relationship between ortho and para carboxyl group pK_a's. This would therefore outweigh the statistical factor of 2 that should be applied to the hydrolysis of the symmetrical un-ionized acetals.

(24) R. L. Nath and H. N. Rydon, *Biochem. J.*, **57**, 1 (1954).