HYDROLYTIC ENZYME MODELS

I. EFFECT OF NEIGHBORING CARBOXYL ON THE REACTIVITY OF ESTER AND ANILIDE GROUPS*

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INTRODUCTION

The understanding of the mode of action of a number of hydrolytic enzymes has advanced greatly in recent years. Tracer techniques and studies of the stereochemistry of enzymic reactions have been used to differentiate between some glucosidases in which an intermediate compound is formed by group transfer from substrate to enzyme and others, whereby the reactants are merely adsorbed onto the molecular surface of the enzyme[1,2]. The action of acetylcholine esterase on a number of substrates and inhibitors over a wide pH range has led to the concept of two essential sites on the enzyme molecule, an "anionic site" with a negative charge attracting the quaternary ammonium group of the substrate and an "esteratic site", containing an acid and a basic group, responsible for the attack on the ester[3]. A particularly detailed picture has been obtained for the chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate. In a fast initial reaction, the hydroxyl of a serine residue of chymotrypsin is acetylated[4]. The enzyme is then reactivated by splitting off the acetate in a step catalyzed by the imidazole group of a histidine residue in its basic form[5].

A search for model systems exhibiting specific catalytic activity analogous to that of enzymes has been carried out by a number of investigators in the hope that it may provide information suggestive of possible pathways of enzymic reactions[6,7,8]. Since the mode of enzyme action may be represented in simplified form by the Michaelis-Menten mechanism[9]

$$E + S \rightleftharpoons ES \qquad (1)$$

$$ES \longrightarrow E + P \qquad (2)$$

where E, S, ES and P stand for enzyme, substrate, enzyme-substrate complex and the product of the enzymic reaction, respectively, the enzyme models aimed at in previous studies would have had to combine several enzyme characteristics, *i.e.* (1)

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a high affinity for the substrate, (2) the ability to activate the sensitive substrate bond and (3) a low affinity for the reaction product, so as to regenerate the catalyst.

It has been frequently demonstrated that there is no general correlation between the affinity of an enzyme for various substrates and the rates with which the corresponding enzyme–substrate complexes react[10]. An extreme example is the fact that the affinity of chymotrypsin for its specific substrates and their *d*-eniantomorphs is of comparable magnitude, although the enzyme can decompose only the *l*-compounds[11]. It would, therefore, seem reasonable, in considering enzyme model studies, to investigate first possible mechanisms of substrate activation before any attempt is made to meet the exacting requirements for specific complex formation. Molecules containing both the sensitive bonds characteristic of a given class of substrates and functional groups, such as occur in proteins, may be thought of as models of the enzyme–substrate complex. A study of the reactivity of such compounds should show which neighboring functional groups are able to accelerate reactions of the sensitive bond and which relative steric configuration is most effective in producing such activation.

EXPERIMENTAL

Materials

The preparation of copolymer A/Es containing 9 mole % of *p*-nitrophenyl methacrylate and 91 mole % acrylic acid has been described previously[12]. The preparation of two acid copolymers containing *p*-nitroanilide groups will be reported elsewhere[13]. The first one, designated A/An-1, contained 97 mole % acrylic acid and 3 mole % *p*-nitroacrylylanilide. Copolymer A/An-2 was derived from a methacrylic acid copolymer containing one third maleic anhydride residues which were partially converted to the corresponding *p*-nitroanilide. Mono-*p*-nitrophenyl glutarate and the mono-*p*-nitroanilides of succinic and glutaric acid were prepared from the corresponding anhydride. To synthesize N-*p*-nitrophenyl succinimide, equimolar proportions of succinic acid, *p*-nitroaniline and potassium hydrogen sulphate were ground together and fused. The thickened melt was cooled, extracted with water, dried and recrystallized from glacial acetic acid (m.p. 211-212° C, literature value[14] 210° C).

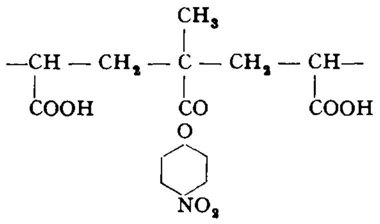
Kinetic runs

One volume of solutions of 0.01 N copolymer A/Es in anhydrous dioxane was added to eleven volumes of aqueous buffers containing 0.10 N Na^+. The ester hydrolysis was studied at 0° C and the reaction was stopped by mixing aliquot samples with an equal volume of 0.1 N HCl. The extent of the hydrolysis was determined by measuring the optical density at 320 mμ (ε_{max} = 9800 for *p*-nitrophenol). The hydrolysis of copolymer A/An-1 was studied in dilute HCl solutions and in acetate and phosphate buffers at 135.4° C. The pH of the buffer solutions was estimated from measurements at 25° C and the relation given by HARNED AND OWEN[15] for the temperature dependence of the pK of these buffers. Another series of kinetic runs was carried out in solutions containing 0.0015 N carboxyl belonging to copolymer A/An-1 and 0.01 N partially neutralized poly(acrylic acid). The solution samples were placed in sealed tubes which were thoroughly degassed before sealing to eliminate oxidation. One tube was used for each kinetic point. The reaction was quenched by immersing the tube in cold water, the solution was made alkaline by mixing with an equal volume of 0.4 N NaOH and the optical density was read at 380 mμ (ε_{max} = 13,300 for *p*-nitroaniline).

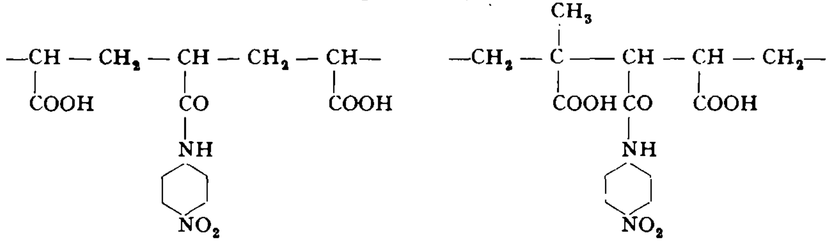
The imidization reaction of copolymer A/An-2 at 84.4° C in the pH range 1.95-7.6 was followed by the decrease of the optical density at 315 mμ (ε_{max} = 11,700 for *p*-nitroanilides) and the increase in the optical density at 270 mμ (ε_{max} = 9500 for N-*p*-nitrophenylimides).

RESULTS AND DISCUSSION

The sections of the chain molecule adjoining the ester and anilide groups, respectively, in the three copolymers investigated in this study are represented on Fig. 1.



Copolymer A/Es



Copolymer A/An-1 Copolymer A/An-2

Fig. 1. Structure of copolymers investigated.

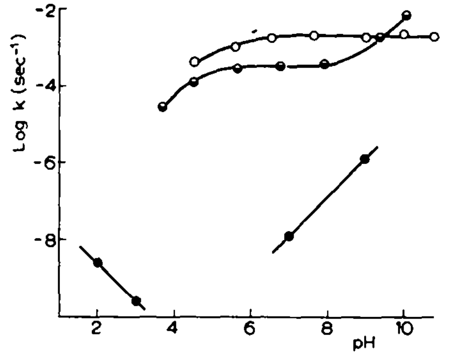


Fig. 2. Hydrolysis of *p*-nitrophenyl esters at 0° C. ● *p*-Nitrophenyl pivalate; ◒ Mono-*p*-nitrophenyl glutarate; ○ Copolymer A/Es.

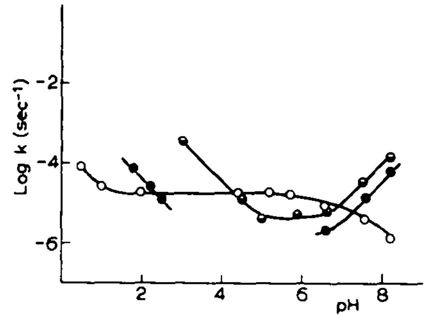
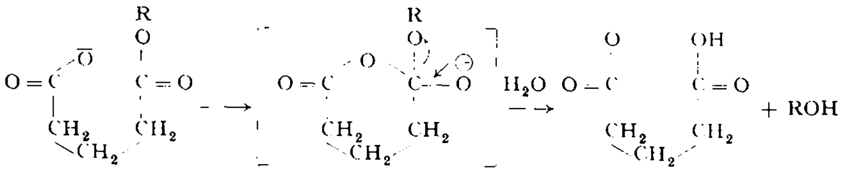


Fig. 3. Hydrolysis of *p*-nitroanilides at 135.4° C. ● *p*-Nitroanilide of pivalic acid; ◒ Mono-*p*-nitroanilide of glutaric acid; ○ Copolymer A/An-1.

The pH dependence of the hydrolysis rates of *p*-nitrophenyl pivalate, mono-*p*-nitrophenyl glutarate and copolymer A/Es at 0° C are shown in Fig. 2, while Fig. 3 gives a similar comparison for the hydrolysis of *p*-nitroanilide groups in copolymer A/An-1 and its low molecular weight analogs at 135.4° C. The pseudo-first order rate constants of the pivalic acid derivatives are, as would be expected, given by expressions of the form $k_1 = k_H(H^+) + k_{OH}(OH^-)$ but the groups attached to the polycarboxylic acids behave in a strikingly different fashion. With the ester groups of copolymer A/Es the hydrolysis rate is constant between pH 8 and 11, but falls off at lower pH. The reaction was shown to be proportional to the degree of ionization of the carboxyl groups attached to the polymeric chain[12], and the hydrolysis rate of the glutaric acid monoester depends, in a similar fashion, on the degree of ionization of the free carboxyl. This effect, which results in an acceleration of ester hydrolysis around pH 5 by a factor of about 10^6, may be accounted for by the following reaction mechanism:



The pH dependence of the p-nitroanilide hydrolysis of copolymer A/An-1 is entirely different. Here the rate constant falls with increasing pH to level off to a constant value over the pH range 2 to 5.5, to decrease again with a further pH rise. Very significantly, the mononitroanilide of glutaric acid does not behave like the polymer, but follows the simple pattern to be expected from hydrogen and hydroxyl catalysis. Since the pH estimate cannot be considered precise at the reaction temperature of 135.4° C and the degree of ionization of the polymeric acid in any given buffer is highly uncertain, a series of runs was also carried out in partially neutralized polyacrylic acid solution where the fraction of ionized carboxyl may be assumed to be the same in the polyacrylic acid and the copolymer molecules. The results given in Table I show that the anilide hydrolysis rate of copolymer A/An-1 remains about constant up to half-neutralization of the carboxyl groups, but decreases rapidly on further carboxyl ionization. The evidence seems to suggest that the copolymer hydrolysis at pH 2-8 is due to direct water attack which is not affected appreciably by the ionization of one neighboring carboxyl, but that the attack by both water and hydroxyl are effectively inhibited by the ionization of both carboxyl neighbors. This inhibition is believed to be due to hydrogen bonding which has been reported previously to interfere with hydrolyses[16]. Such an interpretation is supported particularly by analogy with PINNER's observation[17] that the amide group in methacrylic acid-methacrylamide copolymers is not hydrolyzed even on refluxing with 4 N sodium hydroxide, provided the amide group is flanked by two carboxyl neighbors. The exact analysis of the experimental data obtained with copolymer A/An-1 is rendered difficult by the fact that the probabilities of the ionization of the two carboxyl groups adjoining the anilide are not independent of each other, so that a knowledge of the overall degree of ionization is insufficient for the calculation of the fraction of anilide groups placed between two ionized carboxyls*.

TABLE I

HYDROLYSIS RATES OF COPOLYMER A/An-1 IN PARTIALLY NEUTRALIZED POLYACRYLIC ACID SOLUTIONS AT 135.4° C

Degree of carboxyl neutralization α	*pH (measured at 24° C)*	*$10^6\ k$ (sec^{-1})*
0.045	3.55	29.7
0.120	3.91	27
0.308	4.71	29.7
0.510	5.22	27
0.780	6.16	14.5
0.90	6.86	4.9

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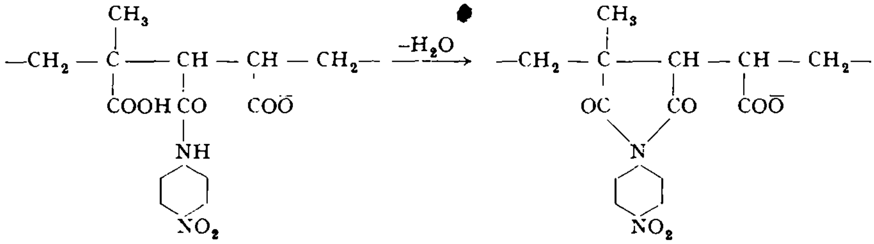
* An alternative interpretation of the experimental data previously suggested by one of us (E. W. WESTHEAD, Jr. *Ph.D. Thesis*, Polytechnic Institute of Brooklyn, 1955) assumes that the rate of nitroanilide hydrolysis in the investigated pH range is governed by the attack of an *unionized* neighboring carboxyl group. This interpretation has been recently strengthened by the observation (M. L. BENDER, *J. Am. Chem. Soc.*, 79 (1957) 1258) that the hydrolysis rate of phthalamic acid at pH 2–5 is proportional to the fraction of unionized carboxyl.

A comparison of copolymer A/An-1 and A/An-2 was expected to indicate the effect of a variation in the spacing of neighboring carboxyls on the reactivity of *p*-nitroanilide groups. It was found that under conditions which led to the hydrolysis of copolymer A/An-1, water was rapidly split off from copolymer A/An-2, leading to the formation of a cyclic imide:



The nature of the reaction was proved by comparison of the final spectrum with that of N-nitrophenyl succinimide.

The pH dependence of the imidization rate at 84.4° C is shown in Fig. 4, and it can be seen that the reaction is fastest around pH 5, being inhibited both by acid and by base. This behavior, reminiscent of the pH dependence of enzymic reactions, clearly indicates that the reaction requires one neighboring carboxyl, participating in the imidization, to be unionized, while a second carboxyl, which has presumably some catalytic function, has to be ionized. This interpretation is supported by the failure of the mono *p*-nitroanilide of succinic acid to imidize in aqueous solution under comparable conditions.

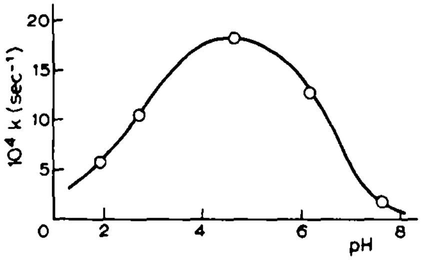


Fig. 4. Imidization of copolymer A/An-2 at 84.4° C.

In considering the various results obtained in this study, no claim is made that they parallel the action of any given enzyme. For instance, although the present study shows that properly spaced carboxylate may greatly accelerate ester hydrolysis, results published since this work was started seem to indicate that imidazole groups are commonly involved in the enzymic attack on ester groups[3–5,18]. However, this need not be true in all cases. In view of the small number of functional groups which can conceivably participate in the activation of enzyme–substrate complexes it appears worth while to determine their effect on the stability of various sensitive bonds, since such information may, in the future, be useful as a guide in enzyme investigations. For instance, the present model studies show that carboxyl groups cannot be involved in the decomposition of the ES complex, if the pH dependence of the enzymic reaction at high substrate concentration is the same for esters and anilides. The results also point to one way in which enzymes could discriminate between two types of hydrolyzable substrates whose response to acid–base catalysis may be quite similar. Thus, if the ES complex forms only at pH values at which an activating carboxyl group is fully ionized, the enzyme would attack esters, but would be without effect on anilides.

The behavior of copolymer A/An-2 may have particularly interesting implications for the mechanism of enzyme action. The imidization reaction of the copolymer is rather similar to condensation and group transfer reactions by which various enzyme-substrate complexes are formed, and the observation that the copolymer reaction is catalyzed by a suitably spaced carboxylate may be suggestive of the manner in which similar results are attained in enzymic reactions. The fact that the interposition of a single methylene group in the spacing of the anilide and its neighboring carboxyl profoundly alters the course of the reaction from the hydrolysis of copolymer A/An-1 to the imidization of copolymer A/An-2, illustrates in a particularly striking manner the influence which the relative spacing of functional groups on the molecular surface of an enzyme may have on the type of reactions in which they participate.

SUMMARY

Polycarboxylic acids containing a small number of *p*-nitrophenyl ester or *p*-nitroanilide groups were prepared and the pH dependence of their hydrolysis rates was investigated. The ester hydrolysis was found to be strongly catalyzed by an ionized carboxyl attached to the γ-carbon, resulting in an acceleration of the hydrolysis rate by a factor of about 10^6 at pH 5–6. The hydrolysis of *p*-nitroanilide groups was apparently affected little by one carboxylate in the γ position, but it was effectively inhibited when the carboxyls attached to both γ-carbons were ionized.

When a *p*-nitroanilide group was placed between two carboxyls attached to the β-carbons, no hydrolysis was observed in the pH range 1.95–7.6, but instead a cyclic imide was formed. The pH dependence of this reaction had a bell-shaped form similar to that characteristic of enzyme reactions, indicating that one neighboring carboxyl had to be ionized and the other unionized for imidization to occur.

It is believed that studies on model systems such as those investigated may indicate the possible role of the functional groups of proteins in the activation of enzyme–substrate complexes.

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