DEMONSTRATION OF CARBONIUM ION INTERMEDIATE DURING LYSOZYME CATALYSIS*

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X-ray analysis studies[1, 2] on the relative modes of association of various inhibitors and substrates with lysozyme have led to a proposal for the catalytic mechanism of the enzyme.[3, 4] This proposal suggests that the enzyme catalyzes the formation of a carbonium ion at C_1 of the glycosidic bond undergoing hydrolysis and, further, provides it with steric and electrostatic stabilization. To date, little evidence of a chemical nature has been available to test the mechanistic proposal.

It has recently been shown[5] that hen egg-white lysozyme-catalyzed hydrolysis of a glycosidic bond proceeds with quantitative retention of configuration (>99.7%). This result eliminates a single displacement mechanism for the enzyme[6] and leaves the following: (1) a carbonium-ion mechanism whose enzyme-bound orientation would allow reaction with solvent from only one side (i.e., frontal attack); (2) any mechanism involving an even number of displacements at C_1 of the glycoside being cleaved, e.g., the double-displacement mechanism[7] which could involve a covalent enzyme-substrate intermediate; and (3) a variation of (2) in which the acetamido carbonyl group of the glycoside would displace the aglycone with formation of an oxazoline intermediate. Evidence for this mechanism has been obtained in model studies of N-acetyl-D-glucosamine derivatives[8] and in aryl-2-acetamido-2-deoxy-β-D-glucopyranoside spontaneous hydrolysis.[9, 10] Such a scheme has been suggested as a mechanism to explain lysozyme catalysis.[9-13]

We have recently shown that lysozyme catalyzes the hydrolysis of aryl-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranosides (I) with release of the aglycone.[14] Furthermore, participation by the C_2—OH as a nucleophilic neighboring group was ruled out since the analogous 2-deoxy-D-glucose
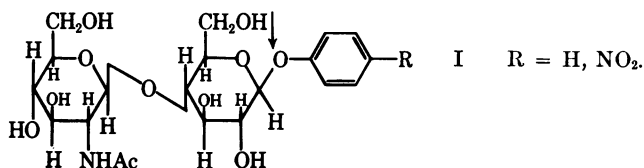
derivatives were also hydrolyzed by the enzyme. Therefore, for type-I substrates, two possible mechanistic pathways for lysozyme-catalyzed hydrolysis exist: (1) a carbonium-ion mechanism, or (2) an even number of displacements involving covalent enzyme-substrate intermediate(s).

This communication describes the use of α-deuterium kinetic isotope effects to distinguish between the mechanistic alternatives for lysozyme-catalyzed hydrol-

ysis of substrates of type I. This represents a unique approach to studies of enzyme mechanisms, since the isotopically substituted substrates employed should have identical *orientations* with respect to the catalytic groups on the enzyme. The method described has distinct advantages over the use of substrate analogues, an approach which can give rise to serious complications due to slight changes in enzyme-bound *orientation* of the various derivatives.

Isotope effects have been used to determine the degree of nucleophilic participation of solvent in the rate-determining steps of many solvolytic reactions.[15] Typical displacement reactions show an effect near unity, while carbonium-ion reactions show an isotope effect with $k_H/k_D \sim 1.14$. As model reactions, the acid- and base-catalyzed hydrolyses of phenyl-β-D-glucoside were studied. The acid-catalyzed reaction is generally considered to involve a carbonium-ion intermediate.[16–18] The base-catalyzed reaction is considered[19, 20] to involve C-2 oxyanion participation, with formation of a 1,2-epoxide which is opened by the C-6 hydroxyl to give as final product 1,6-anhydro-β-D-glucopyranose in good yield, depending on the conditions used.

Experimental.—Phenol-^{3}H, generally labeled, and phenol-^{14}C, uniformly labeled, were purchased from Nuclear Chicago Co. Glucose-1-d was obtained from Merck Co. Phenyl-β-D-glucoside, phenyl (^{14}C)-β-D-glucoside-1-d, and phenyl (^{3}H)-β-D-glucoside were synthesized according to published procedures.[21, 22] Chitobiose was isolated from chitin partial acid hydrolysates.[23] The disaccharide phenyl-4-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside was synthesized enzymatically, using lysozyme, from chitobiose and phenyl-β-D-glucopyranoside and isolated by gel-filtration methods.[14] The same disaccharide containing ^{3}H and ^{14}C radioisotopically labeled aglycone was similarly prepared using a mixture of the labeled phenyl glucosides.

Acid hydrolysis of labeled phenyl glucoside was conducted in 2.0 N HCl at 50°C, with $1 \times 10^{-2} M$ substrate. The reactions were allowed to proceed to 1–3% completion (5–15 min). Gel filtration was used to estimate relative amounts of starting material and liberated phenol. Basic hydrolysis was conducted in 3.0 N sodium methoxide in methanol at 70°C for periods of time (30 min–1 hr) sufficient to produce similarly small fractions of reaction.

Lysozyme-catalyzed hydrolysis of substrate ($1 \times 10^{-2} M$) was carried out at 40°C in 0.1 M citrate buffer, pH 5.5, with $3.5 \times 10^{-3} M$ enzyme. The extent of reaction was determined by chromatography on Bio-gel columns and fractions were monitored by scintillation counting.

Radioisotopically labeled phenol released in all reactions was extracted into ether from mixtures at pH 5.5, the acid- and base-catalyzed reaction mixtures having been adjusted to this pH. Such ethereal solutions were back-extracted and dried before an aliquot (1 ml) was mixed with Bray's solution,[24] and the ^{3}H/^{14}C ratio of the phenol determined in a Packard model 3324 Tricarb scintillation spectrometer.

Results and Discussion.—For the model studies the following compounds were synthesized: phenyl (^{14}C)-β-D-glucopyranose-1-d (II) and phenyl (^{3}H)-β-D-glucopyranose (III) by the methods used to synthesize the unlabeled compounds.[21, 22]
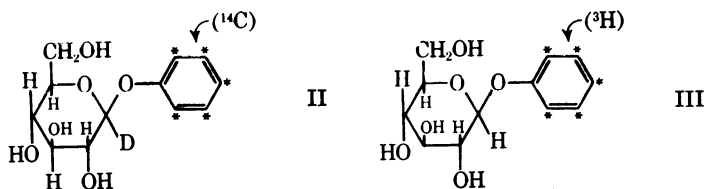
The relative rates of hydrolysis of the two compounds, as a mixture, were easily and accurately determined by measurement of the $^3H/^{14}C$ ratio of the liberated phenol. The observed kinetic isotope effects, k_H/k_D obs., were determined from a comparison between the $^3H/^{14}C$ ratio of the liberated phenol after completion of a small percentage (1–3%) of reaction and the $^3H/^{14}C$ ratio of the initial mixture of ^3H- and ^{14}C-labeled phenyl glucosides. Correction of the observed isotope effect, k_H/k_D obs., for the fraction of compound II which was deuterated (determined from a 220-MHz nuclear magnetic resonance spectrum) gave the secondary α-deuterium kinetic isotope effect, k_H/k_D. The equations used to calculate k_H/k_D obs. and k_H/k_D[25] are similar to those described elsewhere for such calculations.[26]

Table 1 shows the results obtained from the model studies in acid and base. The isotope effect (k_H/k_D = 1.13) determined for the acid-catalyzed reaction agrees well with the accepted value of *ca.* 1.14 for typical S_N1 reactions.[15] Thus, the results presented here further substantiate the accepted mechanism for the acid-catalyzed reaction. The isotope effect (k_H/k_D = 1.03) for the methoxide-catalyzed reaction suggests that the mechanism entails some carbonium-ion character rather than being exclusively one involving nucleophilic attack by the C-2 oxyanion. This is not unexpected, since it has been shown that the yield of the final product, 1,6-anhydro-β-D-glucopyranose, decreased due to competing bimolecular nucleophilic aromatic substitution as the electron-withdrawing character of the leaving group was increased.[27, 28] This is consistent with the notion that the base-catalyzed reaction involves some carbonium-ion character and that the extent of this is a function of the leaving group. However, the model studies described here serve to show that S_N1 and S_N2 mechanisms for the hydrolysis of phenyl-β-D-glucoside can be distinguished by measurement of secondary α-deuterium kinetic isotope effects.

For the application of the kinetic isotope method to lysozyme catalysis the disaccharide I (R = H) was synthesized enzymatically[14] from chitobiose and a mixture of II and III. The radioisotopically doubly labeled disaccharide IV ($^3H/^{14}C$ dpm ratio $\sim$ 7) was purified by gel filtration on Bio-gel P-2 and was shown to have a constant $^3H/^{14}C$ ratio across the chromatographic peak. The saccharide was shown to have equivalent amounts of glucose and N-acetyl-D-glucosamine. Gel filtration of the hydrolysate obtained from treatment with lysozyme showed that only the aryl glycosidic bond was cleaved by the enzyme.

The isotope effect for lysozyme-catalyzed hydrolysis of IV was determined to be k_H/k_D = 1.11. This suggests that the enzyme-catalyzed reaction proceeds through an intermediate with considerable carbonium-ion character. This result clearly distinguishes between the mechanistic alternatives, regarding the nature of the transition state, for lysozyme-catalyzed hydrolysis of IV.

Table 1.   *Hydrolysis of phenyl-β-D-glucoside* (1-H and 1-D) in acid and base.

Hydrolysis conditions	k_H/k_D obs.	k_H/k_D (corr.)
2 N HCl, 50°C	1.080 ± 0.004	1.13
3 N NaOCH₃ in CH₃OH, 70°C	1.021 ± 0.002	1.03

* A mixture of compounds II (^{14}C-labeled) and III (^3H-labeled), with a $^3H/^{14}C$ dpm ratio of approximately 7, was used. Total concentration of the mixture was $1 \times 10^{-2} M$.

It is possible that the carbonium-ion intermediate that we have demonstrated could collapse in a *post*-rate-determining step to form a covalent enzyme-substrate complex which would govern the stereochemistry of the reaction with solvent. Further work is necessary to decide this question. In this regard, it is also possible that the carbonium ion could be stabilized in the transition state as an ion pair by a base on the enzyme. Collapse to a covalent intermediate might be prevented by steric factors. This might account for the slightly smaller value for the isotope effect observed for the lysozyme-catalyzed reaction ($k_H/k_D = 1.11$) when compared to the value obtained for the acid-catalyzed reaction ($k_H/k_D = 1.13$).

It should be noted that substrates having acetamido side chains in the C-2 position (e.g., chitin oligosaccharides or cell wall constituents) could be hydrolyzed by the mechanism we have demonstrated or by a mechanism involving acetamido group participation in the rate-limiting step. It is also possible that a carbonium-ion intermediate *on the enzyme* could react with the acetamido side chain in a *post*-rate-determining step, with consequences for the stereochemistry of reaction with solvent. Further experiments designed to answer these questions are in progress.

In conclusion, it is felt that the work presented in this communication has demonstrated the nature of the transition state in an enzyme-catalyzed reaction.

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