Mechanism for Lysozyme-Catalyzed Hydrolysis

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Abstract: Secondary α-tritium kinetic isotope effects have been utilized to probe the nature of the transition state in the lysozyme-catalyzed hydrolysis of chitotriose. A general synthesis of specifically labeled chitin oligomers (in particular 1,1′,1″-[^{3}H]chitotriose, the substrate used in these studies) is described. Injection of *Drosophilia melanogaster* larvae with labeled *N*-acetyl-D-glucosamine yields chitin, which can be hydrolyzed to give a range of chitin oligomers from chitobiose to chitoheptose. The value of k_H/k_T obtained for the lysozyme-catalyzed hydrolysis of chiotriose was found to be 1.19. This result indicates very considerable carbonium ion character in the transition state, and thus the mechanistic alternatives for lysozyme hydrolysis become distinguishable.

X-Ray analysis studies[2,3] on the association of various inhibitors and substrates with lysozyme have led to a proposed mechanism of catalysis.[3,4] The suggested mechanism involves catalytic production of a carbonium ion which is electrostatically and sterically stabilized by groups on the enzyme. This proposal has been lent credence by the work of Dahlquist, *et al.*,[5,6] which demonstrated that for aryl glucoside substrates the mechanism for hydrolysis involves a carbonium ion intermediate. However, the possibility still remained that a more natural substrate, that is, one with an *N*-acetyl moiety on the 2 position of the sugar ring, might hydrolyze *via* a different mechanism. The alternate mechanistic possibilities for a substrate of the chitin oligomer $(GlcNAc)_n$ type include (1) any mechanism involving an even number of displacements at C_1 of the glycoside being cleaved, *e.g.*, a double displacement mechanism[7] which implies a covalent enzyme–substrate intermediate; (2) a displacement mechanism in which the acetamido carbonyl group of the glycoside displaces the aglycone forming an oxazoline intermediate.[8–11] Evidence for this mechanism has been obtained in model studies[12] of *N*-acetyl-D-glucosamine[13] and in studies of the spontaneous hydrolysis of aryl 12-acetamido-2-deoxy-β-D-glucopyranoside derivatives.[8] Any mechanistic possibilities involving a single displacement have been eliminated by the results obtained by Dahlquist, *et al.*,[14] showing that hen egg white lysozyme catalyzed hydrolysis of a glycosidic bond proceeds with quantitative retention of configuration (>99.7%).

This communication describes (1) a novel synthesis of specifically labeled β 1,4-linked GlcNAc oligomers and (2) α-tritium secondary isotope studies of $(GlcNAc)_3$ (Figure 1) which make distinguishable the mechanistic alternatives for lysozyme-catalyzed hydrolysis of natural substrates.

Experimental Section

Materials. Both chitin (lot 80-C H60) and hen egg white lysozyme (lot L6876) were purchased from Sigma Chemical Co. The Oregon-R-stock of *Drosophila melanogaster* (kindly supplied by H. K. Mitchell) was used in all experiments. The mass culture technique described by Mitchell and Mitchell[15] with 2-hr egg collection was used. About 1000 eggs were collected at a time (per feeding tray) to ensure synchronous animals. Good feeding under noncrowded conditions was necessary for synchrony. The larvae were injected under a microscope 74 hr from egg laying.

All radioactive measurements were done on a Packard TriCarb liquid scintillation spectrometer (Model 3375/3380) using a modified Bray's solution.[16]

***N,N′,N″*-Triacetyl[^{14}C]chitotriose [(^{14}C-GlcNAc)$_3$].** The synthesis of the unlabeled compound has been described.[17] A modification of this procedure was employed for the labeled material. Chitosan was prepared from chitin by de-N-acetylation and was then subjected to acid hydrolysis as described. The acid hydrolysate (500 mg) in water (12.5 ml) was treated at 0° with methanol (1.25 ml), Amberlite IRA-400 (CO_3^{2-} form) (15 ml), and acetic [2-^{14}C]anhydride (0.33 ml, 0.5 mCi) purchased from New England Nuclear, lot No. 555-241 (specific activity 10 Ci/mol). The stirred mixture was stored at 0–5° for 1.5 hr. Thereafter the resin was removed and replaced by Amberlite IR-120 (H^- form) (1 ml) to remove any free amino sugars. The deionized solution was concentrated to 10 ml, insoluble material removed, and the supernatant concentrated by lyophilization. The material was then applied to a Bio-Gel P-4 (200–400 mesh) column (5 cm × 150 cm) in water with 10-ml fractions collected. The separated oligosaccharides (GlcNAc to $(GlcNAc)_7$) (Figure 2) were then rechromatographed on Bio-Gel P-4 in water.

***N*-Acetyl-[D-glucosamine-*1*-^{3}*H*].** Tritiated GlcNAc (*N*-acetyl-[D-glucosamine-*1*-^{3}*H*]), specific activity 4.4 Ci/mmol, was obtained from Amersham/Searle (code TRK·376 batch 1) (1 Ci/l.). The ^{3}H-GlcNAc was injected into 74 hr (from egg laying) *Drosophila* larvae at a concentration of 1.0 Ci/l. using 0.1 μl per animal. A total of 20,000 animals were injected. For injection, larvae were

(1) (a) IBM Predoctoral Fellow 1971–1972; (b) National Institutes of Health Predoctoral Trainee; (c) National Institutes of Health Career Development Awardee.

(2) C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature (London)*, **206**, 757 (1965).

(3) (a) C. C. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc., Ser. B*, **167**, 378 (1967); (b) *ibid.*, **167**, 365 (1967).

(4) D. C. Phillips, *Proc. Nat. Acad. Sci. U. S.*, **57**, 484 (1967).

(5) F. W. Dahlquist, T. Rand-Meir, and M. A. Raftery, *Proc. Nat. Acad. Sci. U. S.*, **61**, 1195 (1968).

(6) F. W. Dahlquist, T. Rand-Meier, and M. A. Raftery, *Biochemistry*, **8**, 4214 (1969).

(7) D. E. Koshland, Jr., *Biol. Rev. Cambridge Phil. Soc.*, **28**, 416 (1953).

(8) (a) D. Piskiewicz and T. C. Bruice, *J. Amer. Chem. Soc.*, **89**, 6237 (1967); (b) *ibid.*, **90**, 2156 (1968).

(9) G. Lowe, *Proc. Roy. Soc., Ser. B*, **167**, 431 (1967).

(10) G. Lowe, G. Sheppard, L. M. Sinnott, and A. Williams, *Biochem. J.*, **104**, 893 (1967).

(11) G. Lowe and G. Sheppard, *Chem. Commun.*, 529 (1968).

(12) T. Osawa, *Chem. Pharm. Bull.*, **8**, 597 (1960).

(13) Abbreviations used are: *N*-acetyl-D-glucosamine, GlcNAc; chitin, $(GlcNAc)_n$; *N,N′,N″*-triacetyl[^{14}C]chitotriose, (^{14}C-GlcNAc)$_3$; β 1,4-linked polymer of *N*-acetyl-[D-glucosamine-*1*-^{3}*H*], [^{3}H]chitin; 1,1′,1″-[^{3}H]chitotriose, ([^{3}H]-GlcNAc)$_3$.

(14) F. W. Dahlquist, C. L. Borders, G. Jacobson, and M. A. Raftery, *Biochemistry*, **8**, 694 (1969).

(15) H. K. Mitchell and A. Mitchell, *Drosophila Inform. Serv.*, **39**, 135 (1964).

(16) L. H. Mohr, L. E. H. Smith, and M. A. Raftery, *Arch. Biochem. Biophys.*, in press.

(17) S. A. Barker, A. B. Foster, M. Stacey, and J. M. Weber, *J. Amer. Chem. Soc.*, **80**, 2218 (1958).

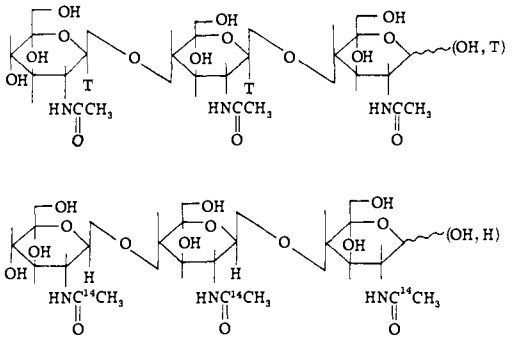

Figure 1. The substrate used in the lysozyme hydrolysis studies: 1,1′,1″-[^{3}H]chitotriose and *N*,*N*′,*N*″-triacetyl[^{14}C]chitotriose.

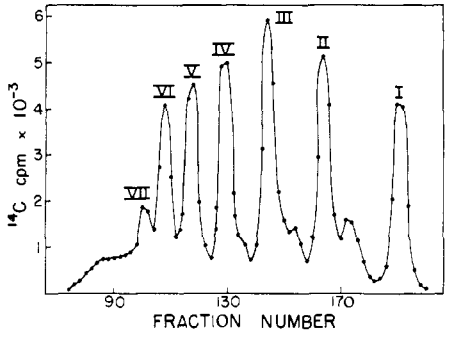

Figure 2. Chromatography on Bio-Gel P-4 in water of the reaction mixture for the synthesis of ^{14}C labeled chitin oligomers. The various peaks were identified as ^{14}C-GlcNAc (I), (^{14}C-GlcNAc)$_2$ (II), and so on up to (^{14}C-GlcNAc)$_7$ (VII).

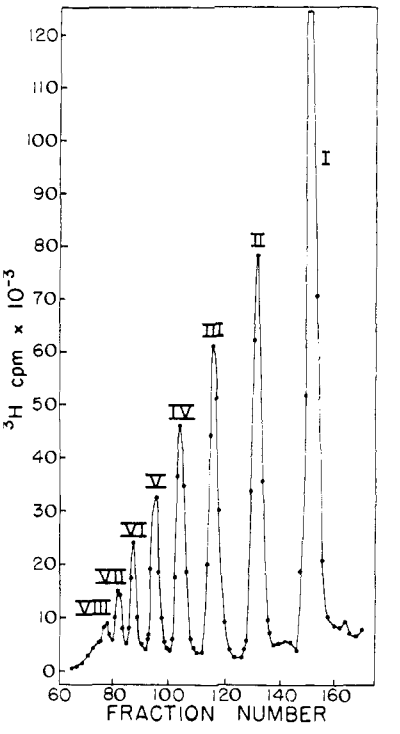

Figure 3. Chromatography on Bio-Gel P-4 of the acid hydrolysis reaction mixture of [^{3}H]chitin. The peaks (I–VIII) were identified as [^{3}H]chitin oligomers ^{3}H-GlcNAc (I) to (^{3}H-GlcNAc)$_8$ (VIII).

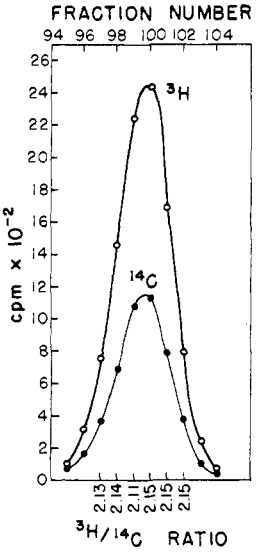

Figure 4. Chromatography on Bio-Gel P-4 of the mixture of [^{3}H]- and [^{14}C]chitotriose to check for purity. The ratio observed was constant within the counting error.

washed from feeding trays with water; the animals were washed again in water to remove yeast and food particles, then in 1% trichloroacetic acid for 2–3 min followed by a thorough washing in a dilute streptomycin and penicillin solution. The animals were then etherized and mounted on double sticky Scotch Taped microscope slides for injection. Injections under a microscope were made into the hemolymph, three segments from the end of the animal. The larvae were allowed to remain on the tape at least 10 min after injection and were then washed off the slide with a wet sterile brush and transferred to a thick yeast–cornmeal suspension for subsequent growth.

After the larvae had all pupated (>140 hr from egg laying) they were removed from the feeding box, washed thoroughly to remove food particles, and placed in a ventilated box without food until emergence (>220 hr). The empty pupal cases were collected, washed several times in water, then ground in a mortar and pestle with liquid nitrogen to a fine powder.

Purification of [^{3}H]Chitin (*N*-**Acetyl-[D-glucosamine-1-^{3}H]$_n$**). The purification of [^{3}H]chitin was achieved by KOH (5%) extraction at 100° for 2 days[18] of the ground pupal cases. After extraction the chitin was centrifuged and washed eight times with water, twice with ethanol, and twice with ether to yield an off-white powder (484 mg of pupal cases yielded 255 mg of purified chitin).

Acid Hydrolysis of [^{3}H]Chitin. [^{3}H]Chitin (255 mg) was dissolved in 3.5 ml of concentrated HCl at 0° for 2 hr, then hydrolyzed at 40° for 1 hr. The mixture was neutralized with a slight excess of lead carbonate and filtered over Celite. The salt ($PbCl_2$) was washed several times with water. The combined filtrate was reduced in volume by lyophilization and run on a column of Bio-Gel P-4 (as before) in water. Two hundred 10-ml fractions were collected over a period of 36 hr (Figure 3). A series of oligomers of ^{3}H-GlcNAc were isolated. (^{3}H-GlcNAc)$_3$ centered at fraction 103. The fractions for each oligomer were pooled, lyophilized, and rerun on Bio-Gel P-4.

Purification of a Mixture of (^{3}H-GlcNAc)$_3$ and (^{14}C-GlcNAc)$_3$. The ^{3}H and ^{14}C trisaccharides were pooled and spotted on Whatman No. 3 mm paper in an 8-in. strip for descending chromatography.[19] The paper was developed with pyridine. 2-pentanol, and water (1:1:1 by volume) and run against the machine direction for 12 hr.

The product was visualized on the paper by a Varian Aerograph thin-layer scanner (Model 6000). The paper was cut perpendicular to the direction of development in 0.5–1-cm wide strips; each strip was eluted with water. An aliquot was counted and the ^{3}H/^{14}C ratio recorded. The eluents from strips with a constant ratio were pooled, lyophilized, and rerun on a Bio-Gel P-4 column in H_2O. Again the fractions were analyzed for ^{3}H/^{14}C ratio and fractions with a constant ratio were pooled and lyophylized (see Figure 4).

(18) G. Fraenkel and K. M. Rudall, *Proc. Roy. Soc., Ser. B*, **129**, 1 (1940).

(19) A. Jeanes, C. S. Wise, and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).

Lysozyme Hydrolysis of Chitotriose [(^{3}H-GlcNAc)$_3$ and (^{14}C-GlcNAc)$_3$]. The enzyme hydrolysis of labeled chitotriose was conducted at 40° at pH 5.5 in 0.1 *M* citrate buffer. The enzyme concentration was 2.5×10^{-3} *M*; that of the substrate was 5.1×10^{-3} *M*. The hydrolysis was allowed to proceed for 5 min at 40° (<5% of completion), then quenched with 100 μl of 10% ammonia. The reaction mixture (total volume of 0.5 ml) was run on a P-4 column (0.9 cm × 90 cm) in 0.05 *M* ammonium formate buffer (pH 9.5). The fraction size was 0.3 ml. The runs were first analyzed by checking the ^{3}H/^{14}C ratio for an aliquot from each fraction. When it had been determined that the ratio was again constant across each peak, aliquots were taken from fractions in each peak (I, II, III of Figure 4) to make up 1 ml of solution each which was counted in 15 ml of modified Bray's.[16] An aliquot of starting material was also made up to 1 ml with 0.05 *M* ammonium formate buffer and counted as described.

Calculation of Isotope Effects. In order to account for any possible nonuniformity of label in the reducing end of the substrate ([^{3}H]chitotriose) caused by an isotope effect in the acid hydrolysis of the [^{3}H]chitin, the following scheme was used for calculation of isotope effects: X = ^{3}H/^{14}C ratio of each monosaccharide subunit of chitin before acid hydrolysys; Y = ^{3}H/^{14}C ratio of the monosaccharide product affected by the isotope effect of enzyme hydrolysis; Z = ^{3}H/^{14}C ratio of the reducing end monosaccharide subunit of the substrate trisaccharide affected by acid hydrolysis of chitin. Therefore, assuming cleavage III → I + II

$$\text{starting material (GlcNAc)}_3 \text{ ratio} = (2X + Z)/3 = \text{III}$$

$$\text{product (GlcNAc)}_3 \text{ ratio} = (X + Z)/2 = \text{II}$$

$$\text{product GlcNAc ratio} = Y = \text{I}$$

$$k_H/k_T = X/Y = [3(\text{III}) - 2(\text{II})]/(\text{I})$$

Although Dahlquist, *et al.*,[20] have shown this to be the major mode of hydrolysis, it is not necessary to assume this specific cleavage since calculations based on fission of the bond closest to the reducing end yields the same isotope effect as do calculations based on the weighted averages of the ^{3}H/^{14}C ratios of starting trimer and the sum of the products, *i.e.*

$$k_H/k_T = 3(\text{III})/[2(\text{II}) + \text{I}]$$

Results

Detailed work by Mitchell[21] on the biochemistry of cuticle formation in *Drosophila melanogaster* enabled us to utilize the insect larvae for chitin synthesis from a specifically labeled *N*-acetylglucosamine monomer. The injection method, worked out by Mitchell, proved very efficient, yielding >50% incorporation of label in the final GlcNAc oligomers.

The purity of the substrates was judged primarily by the constancy of the ^{3}H/^{14}C ratio on paper and on gel filtration columns. This method is *very* sensitive to radioactive impurities. Also the monosaccharide GlcNAc isolated from the hydrolysis of [^{3}H]chitin (Figure 5) was oxidized to the *N*-acetylglucosaminic acid with mercuric oxide with release of the ^{3}H label at the C-1 position to show any scrambling of label during the biological synthesis. No scrambling was observed. The purity of the sample was also checked by isolating the starting material from one enzymatic hydrolysis for use as substrate in a subsequent run. Using trisaccharide obtained in this way an isotope effect within the error of previous determinations was obtained.

The pattern of hydrolysis of the substrate used in these studies, (GlcNAc)$_3$, has been examined by Dahlquist, *et al.*,[20] and by Rupley.[22] The cleavage pattern we observed is clean, yielding (GlcNAc)$_2$ and GlcNAc

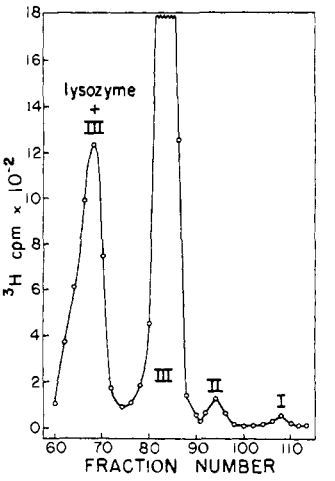

Figure 5. Chromatography on Bio-Gel P-4 in 0.05 *M* ammonium formate of the lysozyme hydrolysis of chitotriose. The peaks identified as GlcNAc (I), (GlcNAc)$_2$, (GlcNAc)$_3$ (III), and a mixture of GlyNAc$_3$ (III) and lysozyme.

products in the ratio of ~2:1 (Figure 5). There was little or no transfer observed [(GlcNAc)$_4$ would be the main transfer product] and none expected since the reaction proceeded for less than one turnover. Furthermore, the ratio of (GlcNAc)$_3$ to lysozyme was 2:1, allowing binding of 1 mol of trisaccharide to the nonproductive sites (A,B,C) on the enzyme and thus leaving an enzyme–substrate ratio of 1:1 for catalysis.

The isotope effect for (GlcNAc)$_3$ was 1.19 ± 0.01 calculated from three measurements. The observed counts of ^{3}H and ^{14}C were converted to disintegrations and to isotopic ratios by the methods described by Dahlquist, *et al.*[6]

Discussion

Isotope effects reflect the difference in zero point energy between the gound state and the transition state of a reaction. In particular, α-secondary isotope effects reflect any change in the vibrational energy contribution to the zero point energy of a carbon–tritium (hydrogen) bond α to the reacting center. For a carbonium ion mechanism the reacting carbon changes hybridization from sp^3 to sp^2. The out-of-plane bending vibration of a carbon–hydrogen bond to an sp^2 carbon should be of lower frequency than the corresponding bending vibration of a similar bond to an sp^3 carbon.[23] The difference in frequency more than accounts for the α-deuterium isotope effect typically 10–15% for SN1 reactions. On the other hand, SN2 reactions typically show little or no effect because the proximity of the entering and leaving groups prevents a change in the bending mode that determines the isotope effect for SN1 reactions.

Thus the geometry of the transition state of a reaction at the reacting carbon will govern the α-secondary isotope effect. Any weakening in the transition state of the carbon–hydrogen bond of the reacting carbon will tend to cause an increase in k_H/k_T.

The tritium isotope effect of 19% for lysozyme-catalyzed hydrolysis of GlcNAc trimer suggests a transition state with considerable carbonium ion character.

(20) F. W. Dahlquist and M. A. Raftery, *Nature (London)*, **213**, 65 (1967).

(21) H. K. Mitchell, U. M. Weber-Tracy, and G. Schaar, *J. Exp. Zool.*, **176**, 429 (1971).

(22) J. A. Rupley, *Proc. Roy. Soc., London*, **167**, 416 (1967).

(23) A. Streitwieser, Jr., R. H. Jagow, R. C. Fahey, and S. Suzuki, *J. Amer. Chem. Soc.*, **80**, 2326 (1958).

An isotope effect of this size would appear to rule out both single and double displacement mechanisms involving attack by solvent or by the enzyme itself, at least in any rate-determining or prerate-determining step.

Dahlquist, *et al.*,[5,6] have previously shown that the lysozyme-catalyzed hydrolyses of aryl glucoside substrates exhibit secondary α-deuterium isotope effects of 11% (equivalent to ~15% tritium effect). From this evidence it was concluded that for these substrates the enzyme proceeded *via* a carbonium ion mechanism. However, the possibility still remained that for cell wall substrates or for GlcNAc oligomer substrates the *N*-acetyl group (missing in the aryl glucosides) could provide anchimeric assistance. Also, the aromatic leaving group could have an influence on the isotope effect.

Although it is possible to conclude from our result that the enzyme hydrolysis does *not* proceed *via* anchimeric assistance, it is difficult to judge to a fine degree just how much the transition state resembles a carbonium ion since the isotope effect for the enzyme catalysis is greater than any effect seen in the related model carbonium ion reactions.[19] However, our result (a 19% tritium isotope effect is equivalent to ~14% deuterium effect) is typical of most reactions classified as SN1. We can conclude only that the carbonium ion character of the transition state of the enzymatic reaction is greater than that of the model systems. It may be that the production of the carbonium ion is coincident with the energy maximum on the reaction coordinate for the enzyme reaction and is noncoincident for the model systems. This may be attributable to the large differences in solvation between the two systems.

The carbonium ion like transition state is almost certainly stabilized electrostatically[2] (probably by the β-carboxylate ion of Asp 52)[24] and also stabilized by binding in subsite D which favors a half-chair conformation over a normal chair.[4] The negative charge of Asp 52 is held at a distance of ~3 Å from both the C_1 carbon of residue D and from the ring oxygen[25] which will share the charge. There would be some covalent character associated with this ion pair[26] which would tend to decrease the observed isotope effect from a maximum possible value.

Protonation of the leaving group *via* general acid catalysis by Glu-35 or by solvent with stabilization of the protonated glycoside by ionized Glu-35 before bond cleavage would tend to decrease the isotope effect since the leaving group would then be stabilized making the transiton state more reactant-like (and less carbonium-like) according to Hammond's (1955) postulate. A concerted protonation would stabilize the leaving group less and the tendency would be toward a larger isotope effect.

The isotope effect observed for the $(GlcNAc)_3$ substrate is slightly larger than that for the aryl glucoside strate.[6] The difference could be attributed to the change in leaving group from aromatic (phenyl and slightly electron withdrawing) to a sugar (electron donating) or possibly to the presence of the *N*-acetyl moiety. From studies of hydrolysis of GlcNAc glycosides in solution[19] an increase in isotope effect would be predicted in going from an electron-withdrawing to an electron-donating group. However, this may or may not be applicable to enzyme hydrolysis. It is impossible to say whether or not the *N*-acetyl group influences the isotope effect for the enzyme reaction one way or another.

This isotope method has inherent limitations common to all measurements of reaction rates. We can know for certain only about the energy of the transition state corresponding to the rate-determining step. It is inherently impossible to draw any conclusions about the mechanism of its formation or its breakdown. And, in fact, it is not always possible to find out what is happening in the whole of the rate-determining step itself. The weakening of the carbon–hydrogen (tritium) bond may not be significantly advanced in the transition state but may still be completed further along the same reaction step. It is well to keep these limitations in mind.

However, the isotope method has the very great advantage of utilizing substrates which have identical orientations with respect to the catalytic groups on the enzyme. This method has large advantages over the use of substrate analogs. Analogs may bind to the enzyme in slightly different orientation or may in fact react with different mechanisms, making interpretation of results very difficult. Isotopic substitution is a much subtler change than the introduction of a substituent. The important feature of isotope effects is that there is no difference in the potential energy surfaces for reactions involving isotopic substitution, in this case tritium and hydrogen. The rate differences observed arise from changes only in the minimum energy levels which are affected by the different vibrational frequencies of hydrogen and tritium containing molecules.

In conclusion, the α-secondary isotope effect observed strongly suggests a carbonium ion mechanism for lysozyme. There still exists the possibility of post-rate-determining collapse of the carbonium ion to a covalent intermediate, either *via* attack by the anion Asp 52 or by the carbonyl oxygen of the *N*-acetyl group to give an oxazoline intermediate.[27] In fact, the observed half-life of the intermediate, which must be long enough to allow for the observed transfer reaction to another saccharide, would suggest some such stabilization.

Acknowledgments. The authors are very grateful to H. K. Mitchell for help and advice on the care, feeding, and injection of *Drosophila* larvae.

(24) S. Parsons and M. A. Raftery, *Biochemistry*, **8**, 4199 (1969).
(25) C. A. Vernon, *Proc. Roy Soc., London*, **167**, 389 (1967).
(26) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, pp 226–229.
(27) W. Beranek, Jr., and M. A. Raftery, unpublished data.