The procedure we have described makes pure cyclopropenone conveniently available for chemical and physical studies, and many such studies are under way. However, the properties already observed are striking. The remarkably high boiling point of this compound indicates its very polar character. This, and the fact that such a strained molecule can be prepared and handled as a neat liquid or solid, again confirm the idea that cyclopropenone shares some of the aromatic stabilization of the cyclopropenyl cation, to which it is related.

Acknowledgment. Support of this work by the National Institutes of Health is gratefully acknowledged.

Ronald Breslow,* Masaji Oda
Department of Chemistry, Columbia University
New York, New York 10027
Received April 15, 1972

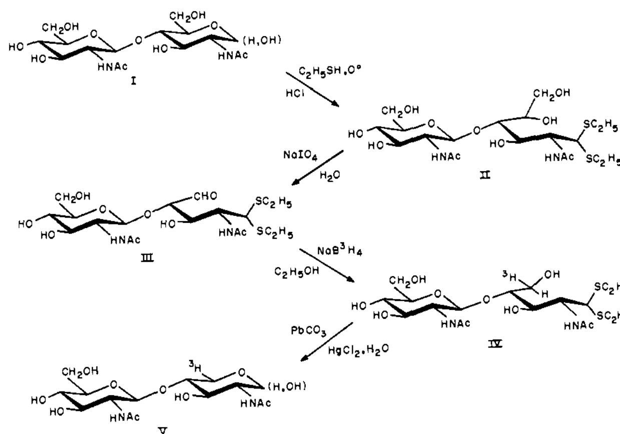
Substrate Distortion in Catalysis by Lysozyme. Interaction of Lysozyme with Oligosaccharides Containing *N*-Acetylxylosamine

Sir:

Studies of hen egg white lysozyme have given the strongest support available to date for the theory that enzymes may catalyze reactions by binding substrates in the geometry of the transition state more strongly than in that of the ground state.[1,2] On the basis of crystallographic studies and model building, Phillips and his coworkers have proposed that, in subsite D of the enzyme, steric hindrance to the C-6 hydroxymethyl group of an *N*-acetylglucosamine (GlcNAc) residue prevents it from being bound in the "chair" conformation, and requires that it adopt the "half-chair" conformation to fit into the active site.[3,4] The expected oxonium ion-like transition state for cleavage of the glycosidic bond at this residue[5] will prefer the half-chair conformation, which allows overlap between oxygen lone-pair electrons and C-1.[6] Several studies have provided indirect evidence for hindrance to binding in subsite D,[4,7] and recently Secemski and Lienhard have demonstrated strongly enhanced binding for an *N*-acetylglucosamine tetramer with its terminal residue oxidized to a δ lactone,[8] which should be stable in the half-chair conformation. We wish to report here studies of oligosaccharides containing *N*-acetylxylosamine (XylNAc) which provide further support for Phillips' theory. The model[3] would predict that a XylNAc residue, in which the C-6 CH_2OH of GlcNAc has been replaced by a proton, may be bound in subsite D without distortion.

The saccharides $(GlcNAc)_n$XylNAc, $n = 1 \ldots 3$, all linkages $\beta(1\rightarrow4)$, were prepared by two different procedures: chemical synthesis from $(GlcNAc)_{n+1}$, and lysozyme-catalyzed transglycosylation.[9] For example, $(GlcNAc)_2$[10] (I, Scheme I) was converted to the

Scheme I



diethyl dithioacetal II by reaction with ethanethiol and concentrated HCl at 0°.[11] II was isolated as a crystalline solid, mp 152–159°, $[\alpha]^{23}D$ −13.4° (*c* 0.82, ethanol). II was oxidized by treatment with a 30% excess of sodium metaperiodate at 0° for 5 min[12] and the reaction quenched with barium hydroxide. III was not isolated, but was directly reduced with ^3H–$NaBH_4$ in ethanolic solution and demercaptalated with mercuric chloride and lead carbonate to yield crude V. A similar procedure yielded $(GlcNAc)_2$XylNAc. Details of the syntheses will be published elsewhere.

In a typical transglycosylation experiment, 17 mg of $(GlcNAc)_4$[10] (20 μmol) and 17 mg of XylNAc-5-^3H[12] (85 μmol, 3.1 × 10^6 dpm/mg) were incubated with 2 mg of lysozyme in 2 ml of pH 5.2 acetate buffer at 39.5° for 25 hr. The mixture was chromatographed on a 1 × 30 cm charcoal–Celite column,[10,13] with a 2-l. 0–40% linear water–ethanol gradient, and the effluent monitored for ^3H and uv absorption (227 nm, amide end absorption). GlcNAc–XylNAc and $(GlcNAc)_2$ were readily resolved, but the pairs $(GlcNAc)_2$XylNAc–$(GlcNAc)_3$ and $(GlcNAc)_3$XylNAc–$(GlcNAc)_4$ could not be completely resolved.

Oligosaccharides produced either synthetically or enzymically were purified by rechromatography one or more times on longer charcoal–Celite columns with more gradual gradients. Crude synthetic GlcNAc–XylNAc was found to be contaminated with a saccharide containing *N*-acetylarabinosamine,[14] pre-

(1) J. H. Quastel, *Biochem. J.*, 20, 166 (1926); H. von Euler and K. Josephson, *Z. Physiol. Chem.*, **157**, 122 (1926); L. Pauling, *Amer. Sci.*, **36**, 58 (1948).

(2) For penetrating recent discussions of this notion see: W. P. Jencks in "Current Aspects of Biochemical Energetics," N. O. Kaplan and E. P. Kennedy, Ed., Academic Press, New York, N. Y., 1966, p 273; W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p 282ff; D. M. Blow and T. A. Steitz, *Annu. Rev. Biochem.*, **39**, 63 (1970).

(3) C. C. F. 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).

(4) L. N. Johnson, D. C. Phillips, and J. A. Rupley, *Brookhaven Symp. Biol.*, **21**, 120 (1968).

(5) J. A. Rupley, V. Gates, and R. Bilbrey, *J. Amer. Chem. Soc.*, 90, 5633 (1968); F. W. Dahlquist, T. Rand-Meir, and M. A. Raftery, *Biochemistry*, **8**, 4214 (1969).

(6) R. U. Lemieux and G. Huber, *Can. J. Chem.*, **33**, 128 (1955); J. T. Edward, *Chem. Ind. (London)*, 1102 (1955); B. Capon, *Chem. Rev.*, **69**, 407 (1969).

(7) D. M. Chipman, V. Grisaro, and N. Sharon, *J. Biol. Chem.*, **242**, 438 (1967).

(8) I. I. Secemski and G. E. Lienhard, *J. Amer. Chem. Soc.*, **93**, 3550 (1971).

(9) J. J. Pollock, D. M. Chipman, and N. Sharon, *Biochem. Biophys. Res. Commun.*, **28**, 779 (1967).

(10) J. A. Rupley, *Biochim. Biophys. Acta*, **83**, 245 (1964).

(11) M. L. Wolfrom and K. Anno, *J. Amer. Chem. Soc.*, **74**, 6150 (1952).

(12) M. L. Wolfrom and M. W. Winkley, *J. Org. Chem.*, **31**, 1169 (1966).

(13) S. A. Barker, A. B. Foster, M. Stacey, and J. M. Webber, *J. Chem. Soc.*, 2218 (1958).

(14) R. Kuhn and G. Baschang, *Justus Leibigs Ann. Chem.*, **628**, 193 (1959).

Table I. Association Constants of Saccharides with Lysozyme, pH 5.2, 0.1 *M* Sodium Acetate–Acetic Acid Buffer, 25°, M^{-1}

Saccharide[a]	This study: Equilibrium dialysis	This study: Fluorescence	Others[b]
XylNAc	$(1.03 \pm 0.23) \times 10^2$		
GlcNac–XylNAc	$(2.43 \pm 0.11) \times 10^4$	$(2.4 \pm 1.6) \times 10^4$	
$(GlcNAc)_2XylNAc$	$\geq 1.5 \times 10^5$		
$(GlcNAc)_3XylNAc$		$(5.5 \pm 1.3) \times 10^6$	
GlcNAc–*N*-acetylarabinosamine	<15		
GlcNAc	~20		20–50
$(GlcNAc)_2$		$(5.5 \pm 2.1) \times 10^3$	5.0×10^3
$(GlcNAc)_3$		$(1.4 \pm 0.4) \times 10^5$	1.0×10^5
$(GlcNAc)_4$			1.0×10^5
GlcNAc–MurNAc–GlcNAc			2.8×10^5
GlcNac–MurNAc–GlcNAc–MurNAc			2.1×10^3

[a] GlcNAc, *N*-acetylglucosamine; XylNAc, *N*-acetylxylosamine; MurNAc, *N*-acetylmuramic acid. All oligosaccharides listed are β(1→4) linked. [b] References 7, 17, and J. A. Rupley, L. Butler, M. Gerring, F. J. Hartdegen, and R. Pecoraro, *Proc. Nat. Acad. Sci. U. S.*, **57**, 1088 (1967).

sumably formed by epimerization at the aldehyde (III) stage. Both synthetically and enzymically formed GlcNAc–XylNAc could be completely purified, however, to yield saccharides identical by several chromatographic criteria, and identical in interaction with the enzyme, $[\alpha]^{27}D$ −24.0° (*c* 2, H_2O, final). Elemental analysis was satisfactory, and acid hydrolysis and chromatographic separation[15] showed equimolar amounts[16] of glucosamine and xylosamine, with all radioactivity in the xylosamine. This identity established the structure as GlcNAc-β(1→4)-XylNAc, and by extension established the β(1→4) structure in the other transglycosylation products.

Equilibrium dialysis experiments over a range of concentrations, using absorption at 280 nm to determine enzyme concentrations and radiochemical analysis (liquid scintillation) for saccharide, led to consistent results for the binding of GlcNAc–XylNAc to lysozyme, further demonstrating the purity of the saccharide. Synthetic $(GlcNAc)_2XylNAc$ could not be freed of radiochemical impurities, but equilibrium dialysis experiments could be run on enzymically produced material, under excess enzyme conditions where contaminating nonlabeled $(GlcNAc)_3$ did not interfere.

The change in enzyme fluorescence (excitation 280 nm, emission 320) was also used to determine association constants.[7,17] For an enzymically produced mixture of $(GlcNAc)_3XylNAc$ and $(GlcNAc)_4$, the effect of the impurity must be taken into consideration in an iterative computer fit (Figure 1), but since the $(GlcNAc)_4$ binds much more weakly its effect is actually quite small. The mixture was analyzed for the XylNAc-containing saccharide radiochemically, and for total sugar by weight. The association constants determined in this study are given in Table I, together with relevant data from earlier work.

Oligosaccharides of *N*-acetylglucosamine are apparently bound with their reducing termini in subsite C, avoiding the unfavorable conformational change associated with subsite D.[4,7,18] Thus, the tetramer (and longer saccharides) bind no more strongly than

(15) S. Gardell, *Acta Chem. Scand.*, 7, 207 (1953).
(16) M. J. Crumpton, *Biochem. J.*, 72, 479 (1959).
(17) S. S. Lehrer and G. D. Fasman, *Biochem. Biophys. Res. Commun.*, 23, 133 (1966).
(18) D. M. Chipman and N. Sharon, *Science*, 165, 454 (1969).

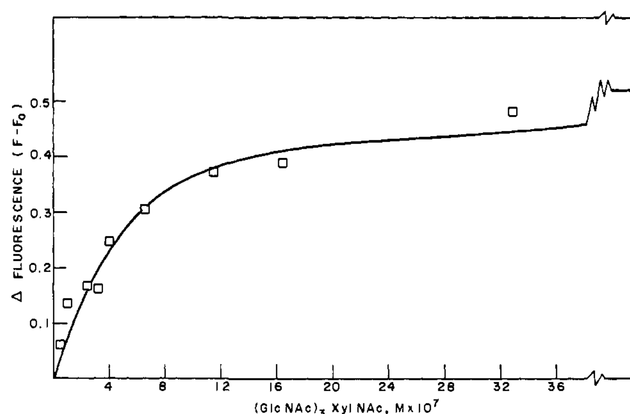


Figure 1. Change in the fluorescence emission of lysozyme at 320 nm (excitation at 280 nm) in the presence of $(GlcNAc)_3$–XylNAc and $(GlcNAc)_4$ at pH 5.2, in 0.1 *M* sodium acetate–acetic acid buffer, 25°. The ratio $[(GlcNAc)_4]:[(GlcNAc)_3XylNAc]^3$ is constant, equal to 5.14. See the text for an explanation of saccharide analysis. The total enzyme concentration is 6.3×10^{-7} *M*. Open squares are experimental points. The curve is that calculated by computer for K_a of $(GlcNAc)_4$ = 1×10^5 M^{-1}, for $(GlcNAc)_3$-XylNAc = 5.5×10^6 M^{-1}; fluorescence of the lysozyme–$(GlcNAc)_4$ complex = 1.40, of the lysozyme–$(GlcNAc)_3XylNAc$ complex = 1.52, when pure enzyme fluorescence (F_0) is taken as 1.034.

the trimer. The *N*-acetylmuramic acid (MurNAc)[19] containing cell wall oligosaccharide GlcNAc–MurNAc–GlcNAc–MurNAc is bound *less* firmly (by a factor of 130) than GlcNac–MurNAc–GlcNAc because a restriction against the binding of MurNAc in subsite C forces the reducing terminus of the tetrasaccharide into subsite D.[4,7,18] On the other hand, $(GlcNAc)_3XylNAc$ is bound 40–50 times *more* firmly than $(GlcNAc)_3$, indicating that a XylNAc residue can be bound in subsite D in a manner leading to a net favorable interaction of some 2.2 kcal/mol. The difference in interactions in subsite D between a XylNAc residue and a MurNAc or GlcNAc residue[20] is thus a matter of some 5 kcal/mol.

We hesitate to suggest what "binding modes" are responsible for the observed association constants for the smaller XylNAc-containing saccharides. Crystallographic studies of the association of various sac-

(19) *N*-Acetylmuramic acid (MurNAc) is the 3-*O*-D-lactic acid ether of *N*-acetylglucosamine.
(20) The 3-*O*-lactyl group of MurNAc probably makes no contact with the enzyme.[3,4,7]

charide derivatives with lysozyme have shown that complexes of short oligosaccharides need not be completely analogous to that of, say, $(GlcNAc)_3$.[21] GlcNAc-β(1→4)-xylose, for instance,[21] is bound in the region C–D, but makes somewhat different contacts than predicted[8] for a productive complex of a GlcNAc polymer.

There can now be no doubt as to the correctness of Phillips' hypothesis that there is steric hindrance to a hydroxymethyl group at C-5 in a pyranose unit bound in subsite D of lysozyme.[3,4] Estimates of the "strain" (unfavorable interaction) range from 4 to 8 kcal/mol.[4,7,18,22,23] The important question which remains is how much such strain contributes to the lowering of the activation energy for cleavage of the glycosidic bond; we hope to approach this problem by studying the enzymic cleavage of glycosides of the XylNAc derivatives reported above.

Acknowledgment. The authors are grateful for a gift of a reference sample of *N*-acetylxylosamine from the late Professor M. L. Wolfrom. Research support from the National Institutes of Health (Public Health Service Grant No. AM-13590) is gratefully acknowledged. P. v. E. also thanks the A. D. Little Co. for a Graduate Fellowship (1970–1971).

(21) C. R. Beddell, J. Moult, and D. C. Phillips in "Ciba Foundation Symposium on Molecular Properties of Drug Receptors," R. Porter and M. O'Connor, Ed., J. and A. Churchill, London, 1970, p 85.

(22) J. A. Rupley and V. Gates, *Proc. Nat. Acad. Sci. U. S.*, **57**, 496 (1967).

(23) D. M. Chipman, *Biochemistry*, **10**, 1714 (1971).

* Address correspondence to this author at the Department of Biology, University of the Negev, Beersheva, Israel.

Paul van Eikeren, David M. Chipman*

Department of Chemistry, Massachusetts Institute of Technology Cambridge, Massachusetts 02139

Received February 19, 1972

Near Unit Efficiency of Triplet Production in an Electron-Transfer Reaction

Sir:

Recent reports from this laboratory have indicated the required intermediacy of fluoranthene (FA), and possibly 10-methylphenothiazine (10-MP), triplet states in the chemiluminescent oxidation of the fluoranthene anion radical by the 10-MP cation radical in *N*,*N*-dimethylformamide (DMF)[1–3]

$$FA\cdot^- + 10\text{-}MP\cdot^+ \longrightarrow {}^3FA^* + 10\text{-}MP \quad (1)$$

and possibly

$$FA\cdot^- + 10\text{-}MP\cdot^+ \longrightarrow FA + {}^310\text{-}MP^* \quad (2)$$

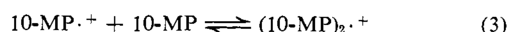
It has also been shown that these triplet states can be intercepted by suitable electrochemically inert triplet energy acceptors, such as anthracene, pyrene, and *trans*-stilbene. In fact, we have utilized this property in a determination of the overall triplet yield of (1) and (2), wherein *trans*-stilbene served as an interceptor and the yield could be calculated from the amount of *cis*-stilbene produced by a known number of charge-transfer events.[2]

(1) D. J. Freed and L. R. Faulkner, *J. Amer. Chem. Soc.*, **93**, 2097 (1971).

(2) D. J. Freed and L. R. Faulkner, *ibid.*, **93**, 3565 (1971).

(3) R. Bezman and L. R. Faulkner, *ibid.*, in press.

Our first such measurements indicated a triplet yield of 0.7% for electron transfer in the fluoranthene–10-MP system.[2] Though we initially accepted this value as characteristic of (1) and (2), recent absolute luminescence measurements carried out in this laboratory by Bezman[3] have led us to reexamine the meaning of this early result. In particular, the absolute measurements suggest a strong inverse dependence of the triplet yield on the concentration of 10-MP used as a substrate. Such behavior is apparently indicative of redox pathways other than (1) and (2) which compete for fluoranthene anion, and it has been ascribed tentatively to the presence of the dimer cation equilibrium[4–6]



$$10\text{-}MP\cdot^+ + 10\text{-}MP \rightleftharpoons (10\text{-}MP)_2\cdot^+ \quad (3)$$

Since several thermodynamic and structural arguments support the idea that the redox reaction involving the dimer cation is incapable of efficient product excitation,[3] one expects the measured triplet yield to decline with concentration of 10-MP, as the dimer cation participates in a progressively larger share of redox events. Bezman's data corroborate the 0.7% triplet yield at 20 m*M* 10-MP (at which the interception measurements were executed), but they indicate quite a high yield for the primary excitation process, (1) and (2). A rough estimate is about 30%.

In a continuing examination of these hypotheses, we have carried out several experiments in which the 10-phenylphenothiazine (10-PP) cation has been substituted for 10-MP+. Because the dimer cations apparently exist in sandwich configurations with a 3–4-Å spacing between moieties,[6] it was felt that the phenyl substitution might sterically hinder the aggregation of $10\text{-}PP\cdot^+$ and that higher triplet yields might be observed at the 20 m*M* concentration levels at which interception measurements can be made most conveniently.[7]

Preliminary investigations showed 10-PP to have all the requisite electrochemical and spectroscopic properties; indeed, its behavior is essentially identical with that of 10-MP (Table I).[1] Figure 1 displays the chemiluminescence spectrum obtained from the 10-PP–fluoranthene system. At short wavelengths, the emission distribution is very similar to the fluorescence of

Table I. Electrochemical and Spectroscopic Data

Compound	$E_p(R/R\cdot^+)^a$	$E_p(R/R\cdot^-)^a$	Lowest triplet, eV	First excited singlet, eV
Fluoranthene	NO[b]	−1.70	2.3	3.0
10-MP	0.90	NR[c]	(2.4)[d]	3.0
10-PP	0.88	NR[c]	(2.4)[d]	3.0

[a] Cyclic voltammetric peak potentials, in volts *vs.* Ag\|AgCl, KCl (saturated), for reversible waves at a Pt disk in DMF with 0.1 *M* tetra-*n*-butylammonium perchlorate. Scan rate = 0.2 V/sec. [b] NO = not oxidized before anodic background limit (+1.3 V). [c] NR = not reduced before cathodic background limit (−2.6 V). [d] Value for phenothiazine from J. M. Lhoste and J. B. Merceille, *J. Chim. Phys. Physicochim. Biol.*, **65**, 1889 (1968).

(4) I. C. Lewis and L. S. Singer, *J. Chem. Phys.*, **43**, 2712 (1965).

(5) O. W. Howarth and G. K. Fraenkel, *J. Amer. Chem. Soc.*, **88**, 4514 (1968).

(6) B. Badger and B. Brocklehurst, *Nature (London)*, **219**, 263 (1968).

(7) M. J. Aroney, G. M. Hoskins, and R. J. W. LeFévre, *J. Chem. Soc. B*, 1206 (1968).