data obtained on **3b** at 19 °C[9b] (Table I) gave the following conformational parameters: $P(N) = 335°$ (E_2), $\tau_m(N) = 45°$, $P(S) = 234°$ (^4E), $\tau_m(S) = 42°$, and $X_n = 0.52$, where $P(N)$ and $P(S)$ are the phase angles of pseudorotation[10] for north (N) and south (S) conformers, respectively, $\tau_m(N)$ and $\tau_m(S)$ are the puckering amplitudes of the N and S conformers, respectively, and X_S is the mole fraction of S conformers. Thus, the preferred N and S conformers of **3b** were found to be E_2 and ^4E, respectively, and both conformers are puckered ~43° and have comparable stabilities. However, are **3a** and **3b** reasonable model structures of **1** in abasic DNA? We have addressed this question by evaluating the conformational behavior of the furanose anomers of 5-*O*-methyl-2-deoxy-D-*erythro*-pentose **2**[11] (Scheme I). In contrast to the glycosides **3a** and **3b**, **2** anomerizes spontaneously in aqueous solution to give α-furanose **2a** and β-furanose **2b** (Scheme I), thus mimicking the behavior of **1** in abasic DNA more closely than **3a** and **3b**.

In opposition to arguments made by Raap and co-workers,[8] the 620-MHz ^{1}H NMR spectrum of **2** (Figure 1A) is disperse enough to permit signal assignments to each furanose anomer with the assistance of ^{1}H-^{1}H COSY data (Figure 1B). A least-squares treatment[9a] of $^3J_{HH}$ in the predominant β-anomer **2b** (Table I) gave $P(N) = 334°$ (E_2), $P(S) = 154°$ (^2E), and $X_n = 0.58$, with $\tau_m(N)$ and $\tau_m(S) \approx 40°$. Thus, the conformational behavior of **2b** differs significantly from that of **3b** with respect to the preferred S conformer (in **3b**, ^4E is preferred, whereas in **2b**, ^2E is preferred), although the mole fractions of N and S conformers for **2b** and **3b** are similar. Computations conducted with $^3J_{HH}$ values observed in **2a** (Table I) gave $P(S)$, $\tau_m(S)$, and X_n similar to those found for **3a**,[8] but different values of $P(N)$ and $\tau_m(N)$. In **2a**, $P(N) = 0°$ and $\tau_m(N) = 43°$, whereas values of 29° and 26°, respectively, were reported for **3a** at 19°.[8]

These results show that methyl glycosidation affects the conformational behavior of the 2-deoxy-D-*erythro*-pentofuranosyl ring and that its effect is not identical in both anomers. Hence, **3b** and, to some extent, **3a** are probably not good conformational models of **1** in abasic DNA. In aqueous solution, the OCH_3 functionality at C1 of **3** appears to provide preferential stability to furanose conformers having the C1–O1 bond quasi-axial;[12] this is not unexpected, since the "anomeric effect"[13] is likely to be more pronounced in methyl furanosides than in the corresponding furanoses. These expectations are consistent with the observation that the ^4E conformer of the glycoside **3b** (its preferred S conformer) orients the C1–O1 bond near quasi-axial, whereas the ^2E conformer of the reducing sugar **2b** (its preferred S conformer) orients this bond near quasi-equatorial. In contrast, since the E_1 conformer having C1–O1 quasi-axial is already highly preferred (~80%) by **3a**, little change is expected, and is observed, upon methyl glycosidation.

Thus, conformational extrapolations based on the behavior of model compounds must be viewed with caution, especially when conformationally flexible structures such as aldofuranose rings are the focus of attention. An understanding of the conformational properties of **1** in abasic DNA, and the role these properties may play in mediating biological recognition during DNA repair, will more likely derive from direct studies of the 2-deoxy-D-*erythro*-pentofuranose anomers at AP sites in small DNA oligomers, rather than from studies of simple monosaccharide models.

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Unequivocal Demonstration of the Involvement of a Glutamate Residue as a Nucleophile in the Mechanism of a "Retaining" Glycosidase

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The exact nature of the involvement of aspartic acid and glutamic acid residues in catalysis by glycosidases has been a matter of considerable debate for some years.[1,2] Evidence has accrued for the involvement of a carboxylic acid as the acid catalyst in several glycosidases, such evidence resting primarily upon X-ray crystallographic studies,[3] as well as more recent site-directed mutagenesis experiments.[4,5] A carboxylate residue has also been implicated in stabilizing the positive charge of an oxocarbonium ion transition state or intermediate, but the question of whether it forms a covalent intermediate has not been fully answered.[1,2] The case for the stabilized ion-pair intermediate has rested upon X-ray crystallographic studies of hen egg white lysozyme,[3] where an aspartic acid residue (Asp 52) was found to be suitably positioned to stabilize such an intermediate. Labeling studies with mechanism-based inhibitors and affinity labels have shown that a carboxylate group might be similarly disposed in a number of other glycosidases.[6] In several other cases,[7-9] a slow substrate (either a D-glycal or an aryl 2-deoxyglycoside) has been used to generate a steady-state concentration of the corresponding glycosyl enzyme intermediate which was stabilized by denaturation trapping and digested with proteases and the purified, labeled peptide then sequenced. All these experiments suggest some involvement of a carboxylate side chain in stabilizing an intermediate, but as pointed out recently,[10,11] none have directly proven the involvement of a carboxyl group in a covalent linkage with a catalytically competent intermediate since the inactivator studies did not involve catalytically competent species, and since denaturation trapping does not allow distinction between covalent and ion-pair intermediates. In this paper we demonstrate that the covalent 2-deoxy-2-fluoro-α-D-glucopyranosyl enzyme intermediate generated by reaction of a β-glucosidase with the corresponding glycosyl fluoride or 2,4-dinitrophenyl glycoside[12,13] is catalytically competent since addition of a second sugar to this species promotes turnover and generation of a disaccharide glycoside product. In addition we demonstrate that a glutamate residue (Glu 358) is the amino acid through which this intermediate is attached.

We have recently[12-14] described the use of 2-deoxy-2-fluoroglucosides with reactive leaving groups (dinitrophenolate or fluoride) in trapping a covalent intermediate involved in the normal

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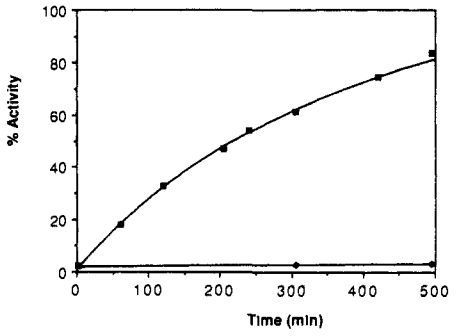
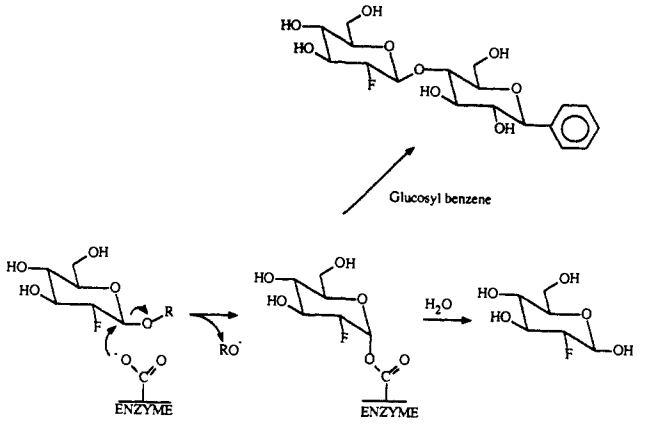


Figure 1. Reactivation of 2-deoxy-2-fluoro-α-D-glucosyl-β-glucosidase in the presence (■) and absence (◆) of β-glucosylbenzene (200 mM). Conditions are as described in text.

Scheme I



catalytic mechanism of a β-glucosidase and thereby effectively inactivating the enzyme. The rationale for such trapping is that the fluorine at C-2 destabilizes the transition states for glycosyl enzyme formation and hydrolysis, thus slowing *both* steps, while use of a reactive leaving group accelerates the glycosylation step sufficiently that the enzyme accumulates as its glycosyl enzyme adduct. This intermediate is sufficiently stable to allow ^{19}F NMR investigations, which have demonstrated[13] that the sugar is linked to the enzyme via an α-anomeric linkage, as shown in Scheme I. We have now investigated the stability and catalytic competence of this intermediate through measurement of rates of reactivation of such inactivated enzyme.

Inactivated enzyme [generated by treating β-glucosidase (1 mg in 1.2 mL of sodium phosphate buffer, pH 6.8) with 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside (50 μM)] was freed of excess inactivator by gel filtration, incubated at 30 °C in the same buffer containing BSA (1 mg mL^{-1}), and assayed for return of enzyme activity associated with hydrolytic turnover of the intermediate. Reactivation was found to occur extremely slowly, with a half-life greater than 500 h. However, inclusion of a simple glucoside such as *p*-nitrophenyl β-D-glucoside, or preferably the C-linked glucoside β-D-glucopyranosylbenzene (which is resistant to enzymic hydrolysis), in the mixture resulted in relatively rapid reactivation of the enzyme. Indeed, upon incubation of the isolated 2-deoxy-2-fluoroglucosyl enzyme intermediate with high concentrations of β-D-glucosylbenzene (200 mM), 80–90% of the expected enzyme activity had returned to the enzyme within a few hours (Figure 1). The rate of reactivation was shown to be dependent, in a saturable fashion, on the concentration of β-glucosylbenzene. Kinetic analysis of this process yielded values for the dissociation constant of β-glucosylbenzene and the maximal reactivation rate of K_d = 59 mM and k_{react} = 5.3 × 10^{-3} min^{-1}. This relatively rapid turnover of the glycosyl enzyme intermediate was shown to be due to an efficient transglycosylation reaction through which the 2-deoxy-2-fluoro-D-glucose moiety was ultimately incorporated into a disaccharide derivative (Scheme I), as follows. β-Glucosidase (0.5 mL, 0.6 mM) was incubated with 2-deoxy-2-fluoro-β-D-glucosyl fluoride

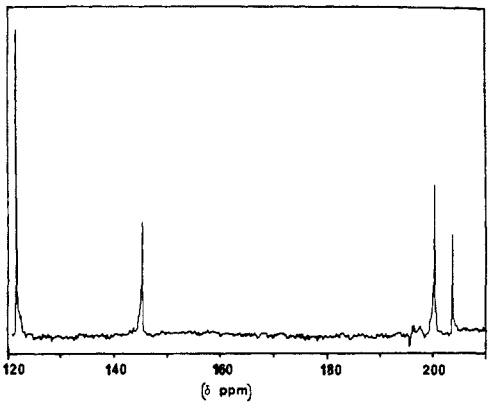


Figure 2. Proton decoupled ^{19}F NMR spectrum of *Agrobacterium* β-glucosidase (0.6 mM), 2-deoxy-2-fluoro-β-glucosyl fluoride (2.3 mM), and β-glucosylbenzene (35 mM) after incubation for 72 h at 30 °C in 50 mM sodium phosphate buffer, pH 6.8. Spectra were recorded by using gated proton decoupling (decoupler on during acquisition only) and a 90° pulse angle with a repetition delay of 2 s for a total of 10000 transients.

(2.3 mM) and glucosylbenzene (35 mM) in 50 mM sodium phosphate buffer, pH 6.8 at 30 °C, and reaction followed by ^{19}F NMR. Over a period of 72 h a slow decrease in the intensity of the resonances due to the free inhibitor (δ 203.4 and 144.8 ppm) was observed, concomitant with an increase in the intensity of the resonance due to inorganic fluoride (δ 121.4 ppm) and the appearance of a new peak at δ 200.0 ppm (Figure 2). Purification of this fluoro sugar product involved ultrafiltration to remove protein, chromatography of the filtrate on Biogel P-2, acetylation of the fluorine-containing product, and chromatography of this on silica gel (ethyl acetate–hexane, 1:1). ^{1}H and ^{19}F NMR[15] and UV/vis spectroscopy of this material (50 μg) demonstrated that it was a disaccharide in which the 2-deoxy-2-fluoro-D-glucose is β-linked to glucosylbenzene, most likely via its 4-hydroxyl as shown in Scheme I. Since such transglycosylations are normal reactions catalyzed by glycosidases,[1,2,16] this experiment clearly demonstrates that the 2-deoxy-2-fluoro-D-glucosyl enzyme intermediate is catalytically competent. While it is always possible that this substrate has perturbed the normal mechanism, these results suggest that such perturbation only involves a slowing of the normal glycosylation and deglycosylation steps.

The identity of the active-site nucleophile was determined by inactivation of the enzyme with [1-^{3}H]-2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside,[17] cleavage of the resultant inactivated enzyme into peptides using pepsin (50 mM phosphate buffer, pH 2.1, 1:100, 2h), separation of the resultant peptides by microbore reverse-phase HPLC, and Edman sequencing of the two radiolabeled peptides so obtained. The two sequences obtained in this way were I·T·E·N·G·A and Y·I·T·E·N·G·A. The data are consistent with the attachment of the sugar moiety to the glutamic acid residue since a "burst" of radioactivity was released upon cleavage of the glutamic acid residue, and since the quantity of glutamic acid derivative detected was extremely low compared to neighboring residues. This would be expected if the glutamic

(15) Full NMR data will be provided elsewhere, but pertinent data include the following: ^{19}F NMR (254 MHz, $CDCl_3$) δ 199.3 ppm (br dd, $J_{F,2'}$ = 50 Hz, $J_{F,3'}$ = 14 Hz); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.28 (5 H, aryl), 4.65 (dd, 1 H, $J_{1',2'}$ = 7.8 Hz, $J_{1',F}$ = 3.1 Hz, H-1), 2.09, 2.07, 2.05, 2.04, 2.00, 1.79 (6 s, 18 H, 6 × OAc).

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acid residue is present as its 2-deoxy-2-fluoroglucosyl ester since this would not extract well into the organic solvent. This sequence aligns perfectly with residues 355-361 of the previously published[20] sequence of *Agrobacterium* β-glucosidase, thus identifying glutamic acid residue 358 as the nucleophilic residue. Experiments are currently underway to further probe the function of this and nearby amino acid residues by mutagenesis analyses.

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A Polyarylmethyl Carbotetraanion

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π-Conjugated polyradicals such as meta-connected triarylmethyls have been proposed as candidates for ferromagnetic polymers (Figure 1).[1] Such molecules and polymers possess unique half-filled, multifold near-degenerate, nonbonding, and nondisjoint HOMOs,[2] These MO theory properties are associated with high-spin π-conjugated molecules.[2,3] Furthermore, calculations suggest uniform charge delocalization over the molecular fragments for such systems.[4] For typical π-conjugated systems such as polyenes and quinone-type structures, an excess of negative charge is localized in one part of the molecule.[5]

In the case of odd-alternant π-conjugated polyradicals, the related polyanions, which possess fully occupied HOMOs, should have similar electron distributions. Therefore, such diamagnetic polyanions can be used to probe the electron distribution in the corresponding polyradicals. We report preparation of the carbotetraanion **1**$^{4-}$,**4Li**$^{+}$ and the reference carbodianion **2**$^{2-}$,**2Li**$^{+}$ and carbanion **3**$^{-}$,**Li**$^{+}$ (Figure 2). The synthesis of the precursor **1-(OEt)$_4$** for the tetraanion is outlined in Scheme I.[6]

The carbanions are generated from the ether precursors by using lithium metal in tetrahydrofuran-d_8 (THF-d_8).[7] Solutions of carbanions in THF-d_8, which also contain equivalent amounts of EtOLi, are examined by ^{1}H, ^{13}C, ^{13}C DEPT, and ^{7}Li NMR.[8] At a typical concentration of 0.04 M, there are less than 5% impurities and all spectral data corroborate the structures of carbanions.

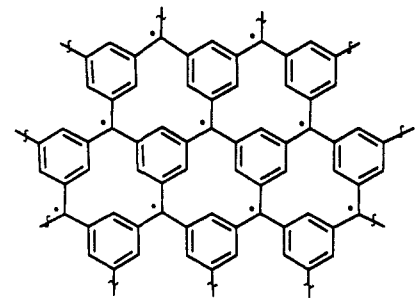

Figure 1.

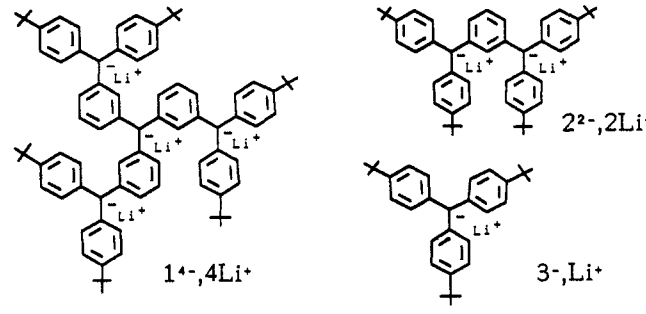

Figure 2. Carbanions **1**$^{4-}$,**4Li**$^{+}$, **2**$^{2-}$,**2Li**$^{+}$, and **3**$^{-}$,**Li**$^{+}$.

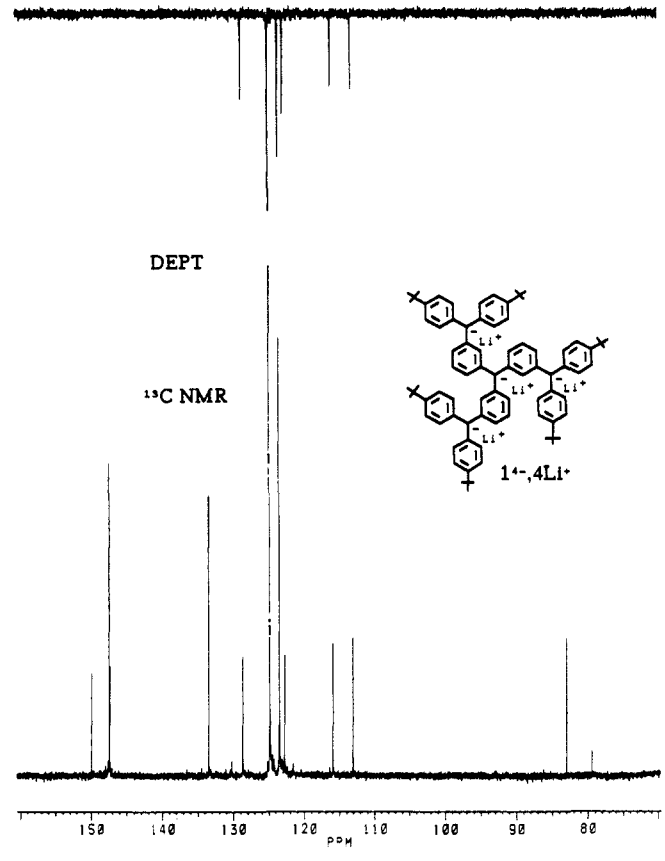

Figure 3. ^{13}C NMR spectra of **1**$^{4-}$,**4Li**$^{+}$ in THF-d_8 at 303 K. The apparent single resonance at 147 ppm consists of two well-resolved resonances at 147.3 and 147.2 ppm.

Table I. Selected ^{13}C and ^{7}Li Chemical Shifts for Carbanions[a]

	peripheral[a]	central[a]	^{7}Li
1^{4-},**4Li**$^{+}$	82.9	79.3	-1.31
2^{2-},**2Li**$^{+}$	83.0		-1.08
3^{-},**Li**$^{+}$	87.2		-0.85

[a] The corresponding chemical shifts for **1-(OEt)$_4$** and **2-(OEt)$_2$** are 86.2-86.6 ppm and for **1-H$_4$** and **2-H$_2$** 56.0-56.5 ppm.

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(6) (a) For a comparable synthesis, see: Hellwinkel, D.; Stahl, H.; Gaa, H. G. *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 794. (b) **1-(OEt)$_4$**: FABMS, M$^+$ - OEt + H = 1210. Anal. Calcd for $C_{90}H_{110}O_4$: C, 86.08; H, 8.83. Found: C, 86.29; H, 8.74. (c) The synthesis of **2-(OEt)$_2$** required a simple modification in Scheme I; that is, dimethyl carbonate was replaced with 4,4'-di-*tert*-butylbenzophenone. **3-OEt** was similarly prepared by using (4-*tert*-butylphenyl)lithium; (d) **4-OEt**: Anal. Calcd for $C_{29}H_{35}BrO$: C, 72.64; H, 7.36. Found: C, 72.68; H, 7.18.

(7) Lithium (98+%, high in sodium, Aldrich) that was freshly cut under argon was used.

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