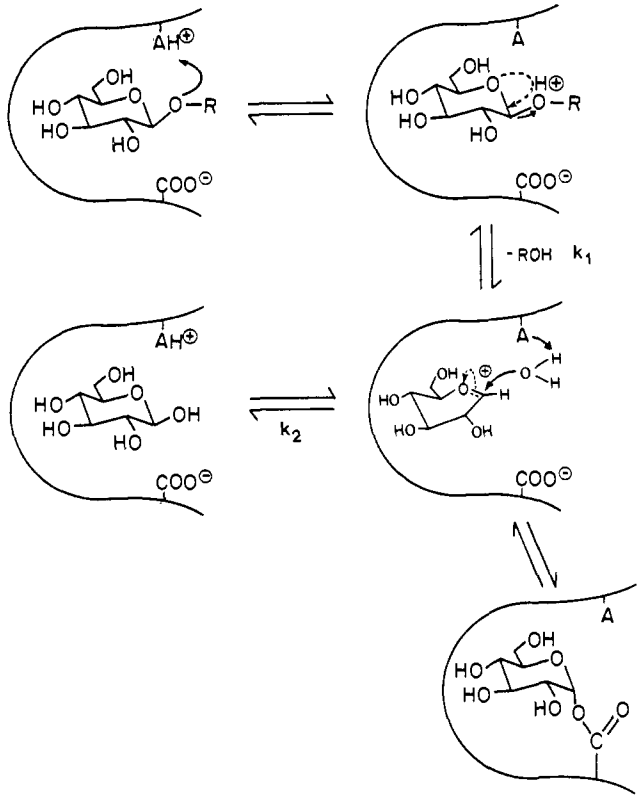
Scheme I



2-deoxy-2-fluoro-β-D-glucopyranoside (**1**).

Incubation of *A. faecalis* β-glucosidase[11,12] with **1** resulted in a rapid time-dependent loss of enzyme activity. Inactivation followed pseudo-first-order kinetics (Figure 1a) with a dissociation constant (K_i) of 0.05 mM and a rate constant (k_i) of 25 min^{-1}, measured at 37 °C, in 50 mM sodium phosphate buffer, pH 6.8 (Figure 1b). The inactivation rate was sufficiently high that measurements could not be made at concentrations approaching saturation since the half-life of this process, under saturating conditions, is 1.6 s. The value of K_i is therefore imprecise but interestingly quite similar to the K_m value of 0.03 mM measured previously[12] for 2,4-dinitrophenyl β-D-glucopyranoside suggesting that fluorine substitution at C-2 has negligible effects on binding.

Further proof that the observed inhibition is due to reaction at the active site is the protection against inactivation afforded by the competitive inhibitor isopropylthio β-D-glucopyranoside (K_i = 4 mM) (Figure 1c).

Since the inactivation process represents the first step of very slow substrate turnover, the inactivated enzyme should gradually hydrolyze liberating free enzyme which could be assayed under appropriate conditions. Inactivated β-glucosidase was therefore freed of excess inhibitor by gel filtration (Sephadex G10), incubated at 30 °C, and assayed for regain of activity. Surprisingly no reactivation was observed unless the enzyme was incubated in the presence of substrate, *p*-nitrophenyl β-D-glucopyranoside or other glucosides such as isopropylthio β-D-glucopyranoside. Under these conditions first-order reactivation kinetics were observed with a half life of ≈ 85 min (at 10 mM *p*-nitrophenyl β-D-glucopyranoside), and full activity was regained. In the absence of added glucoside the inactivated enzyme was stable for several weeks at 4 °C but could then be fully reactivated in the presence of substrate. This requirement for added glucosides was unexpected and is being investigated further but will be very useful in future attempts to identify the active site nucleophile and in planned ^{19}F NMR experiments to probe the 2-fluoroglucosyl enzyme intermediate.

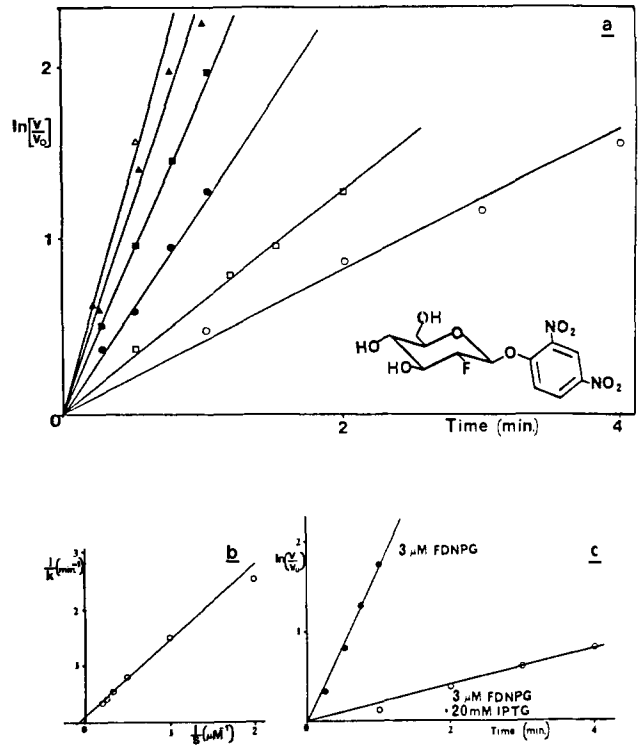


Figure 1. Inactivation of *A. faecalis* β-glucosidase with **1**. (a) β-glucosidase incubated with the following concentrations of **1** and aliquots assayed against *p*-nitrophenyl β-glucopyranoside at the times shown: ○ = 0.5 μM, □ = 1.0 μM, ● = 2.0 μM, ■ = 3.0 μM, ▲ = 4.0 μM, Δ = 5.0 μM). (b) Replot of first-order rate constants from **1a**. (c) Protection against inhibition given by isopropylthio β-D-glucopyranoside.

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada, the British Columbia Health Care Research Foundation and the B. C. Science Council & Forintek Canada Corp. for support of this work.

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HPLC Separation of DNA Adducts Based on Hydrogen Bonding[‡]

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Exposure of humans to genotoxic chemicals increases the risks of carcinogenesis and mutagenesis. Since these risks tend to correlate with the extent of chemical damage to DNA, i.e., with "DNA adducts", detection of such adducts in exposed humans is important.[1,2] However, extraordinary sensitivity is required: one adduct in 10^9 DNA base pairs or less may need to be determined, corresponding to about one adduct per cell. Highly sensitive techniques are therefore being developed for this purpose, e.g., radioenzymatic labeling with [^{32}P]phosphate[3] or derivatization

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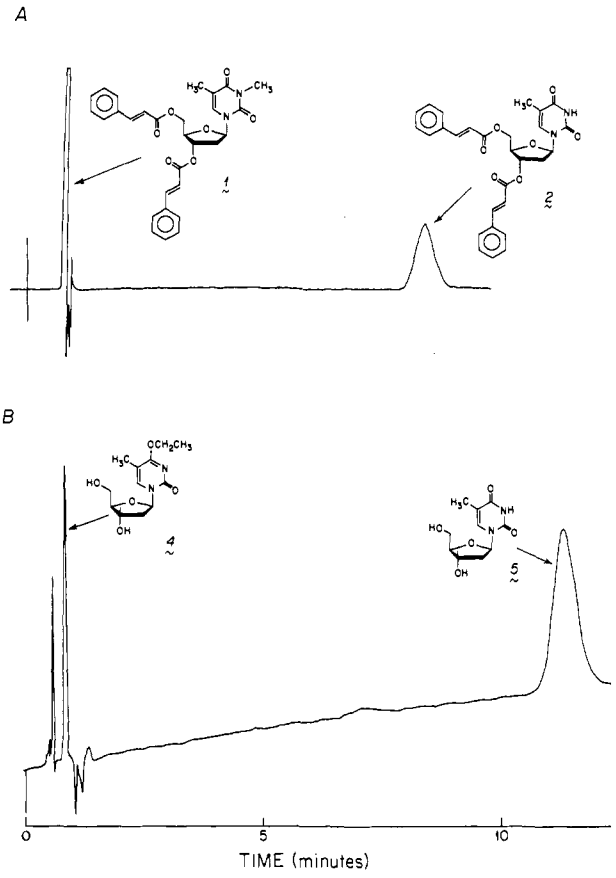
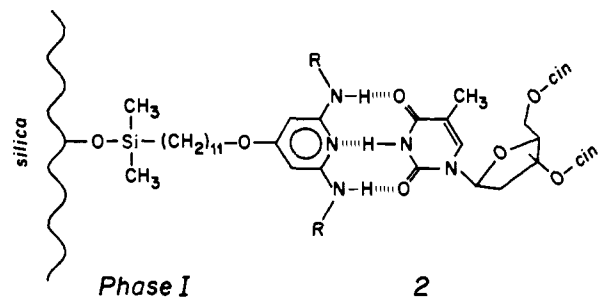


Figure 1. HPLC separation on phase I of (A) 3′,5′-dicinnamyl nucleosides in isopropyl alcohol/1,2-dichloroethane, 2.5:97.5, v/v, and (B) nucleosides in methanol/1,2-dichloroethane, 5:95, v/v, with UV detection.

with an electrophore followed by gas chromatography.[4]

High specificity is also needed, since the DNA adduct is a "needle" in a "haystack" of normal DNA or DNA hydrolysate. Because a significant adduct perturbs the normal structure and function of DNA, it is logical to employ some facet of this event for separation purposes. Our strategy is to use a liquid chromatographic column that mimics the multiple hydrogen bonding between complementary bases in double-stranded DNA. While the "haystack" of a normal monomer from a DNA hydrolysate should be highly retained on such a column, adducts of this monomer will tend to hydrogen bond abnormally and thereby elute rapidly from the column.

We selected a silica gel bonded with *N,N′*-[4-(11-(dimethylchlorosilyl)undecyloxy)-2,6-pyridinediyl]*bis*[2-(*S*)-phenylbutanamide][5] (phase I) to separate the 3′,5′-di-*O*-cinnamyl derivatives

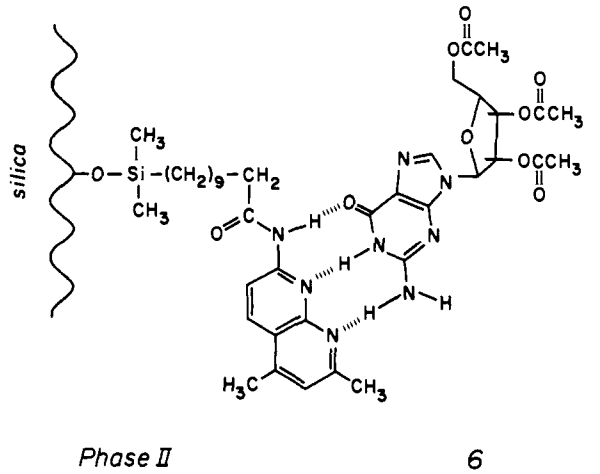


of 3-methylthymidine (**1**) and thymidine (**2**).[6] Phase I was intended to function as a hydrogen-bonded donor–acceptor–donor (101) complementary to the hydrogen bond structure of **2** (010).

No such triple H bond interaction should develop between phase I and **1**. Consistent with this expectation, **1** elutes unretained ($k' = 0$) on this phase, whereas **2** is highly retained ($k = 9.8$) as shown in Figure 1A. The formation of a triple H bond complex between **2** but not **1** with phase I was confirmed by observing characteristic shifts for the protons involved in hydrogen bonding by NMR[7] when 2′,3′,5′-tri-*O*-acetyluridine (**3**), an analogue of **2**, was combined with *N,N′*-2,6-pyridinediyl*bis*[butanamide] (**7**),[5] a soluble analogue of the phase I ligand. For **3** on phase I, k' is 9.6, essentially the same as that of **2**, consistent with the remoteness of the sugar from the triple hydrogen bond.

We also resolved the DNA adduct O^4-ethylthymidine (**4**, $k' = 0.3$) from thymidine (**5**, $k' = 8.0$) on phase I, as shown in Figure 1B. Thus, it is not necessary to derivatize the sugar for solubility purposes or to reduce competing hydrogen bonding. Potentially this makes the technique directly applicable to samples derived from enzymatic hydrolysis of DNA.

To demonstrate the generality of our method, we prepared *N*-[7-(2,4-dimethyl-1,8-naphthyridyl)]-10-undecenamide (**8**), a ligand with a 100 pattern of hydrogen bonding. ^{1}H NMR shows that **8** can form a triple hydrogen bond complex with 2′,3′,5′-tri-*O*-acetylguanosine (**6**).[8] By using the same types of reactions that we used before to prepare phase I, we bonded **8** to silica giving phase II. A high retention ($k' = 32.4$) was then observed for **6** on this column, whereas **6** elutes essentially unretained ($k' = 0.6$) on phase I (mobile phase: same as for Figure 1A). Assuming that the amide bond of phase II is trans when its ligand complexes with **6**, then the high retention of **6** on this column is postulated to arise as follows.



Thus, hydrogen bond chromatography should contribute significantly to the problem of assessing chemical damage to human DNA. It will be particularly useful as a tool to select unknown trace damage to DNA for further characterization by other techniques. Such selection is important and difficult to achieve by other methods. Correlations between the retention properties of DNA adducts and their function in DNA may also develop

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(7) The chemical shifts (^{1}H NMR) in $CDCl_3$ of the critical protons for H bonding are as follows: 2′,3′,5′-tri-*O*-acetyluridine (**3**), an analogue of **2**, δ 9.45 (1 H, s, ring NH); *N,N′*-2,6-pyridinediylbis[butanamide] (**7**), a soluble analogue of phase I, δ 7.9 (2 H, s, 2 NH, overlaps with a pyridine ring proton); and the complex of **3** and **7**, δ 11.85 (1 H, s, NH of **3**) and δ 9.65 (2 H, s, 2 NH of **7**).
(8) The chemical shifts (^{1}H NMR) in DMSO-d_6 of the critical protons are as follows: **6**, δ 10.95 (1 H, s, ring NH) and δ 6.37 (2 H, s, NH_2). For **8**, a soluble analogue (and synthetic precursor) of the phase II ligand, the corresponding chemical shift in $CDCl_3$ is δ 8.69 (1 H, s, NH). While **6** is insoluble in $CDCl_3$, it dissolves upon addition of **8** due to complex formation, giving δ 11.62 (1 H, s, tentatively NH of **8**) and δ 13.15 (1 H, s, tentatively ring NH of **6**). Due to broadening and/or overlap with other proton absorptions, the NH_2 protons of **6** are not apparent in the room temperature spectrum of the **6**:**8** complex. However, at −20 °C, a broad peak for one proton of this pair appears at δ 10.20 which sharpens further at lower temperatures (lowest −80 °C) without any change in its chemical shift.

since hydrogen bond chromatography can mimic various modes of DNA base pairing.

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Registry No. 4, 59495-22-6; 2',3',5'-tri-*O*-acetylguanosine, 6979-94-8; 3-methylthymidine, 958-74-7; thymidine, 50-89-5.

Porphyrin Vinyl Groups Act as Antennae for Electron Transfer within [Fe,Zn] Hemoglobin Hybrids

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Long-range electron transfer[1] at variable distance[2,3] between a donor and acceptor that are *not* small compared to their separation raises the question of the proper measure of intersite distance. If in fact a single value can be assigned,[4] when the process involves a metalloprotein should one consider the distance to the metal or to the atom(s) at the nearest edge of a conjugated ligand? The latter hypothesis would suggest that a heme vinyl group conjugated with the ring might act as an "antenna" and thereby increase the transfer rate.[5]

In most hemoprotein electron-transfer complexes[6] the vinyl groups are directed away from the redox partner (e.g., hemoglobin, cytochrome b_5)[7] and/or are reduced (e.g., cytochrome *c* peroxidase, cytochrome *c*)[8] and thus only influence electron transfer indirectly.[9] However, in the $[\alpha_1, \beta_2]$ electron-transfer complex of [Fe^{III}P, ZnP][10] hemoglobin hybrids,[11] vinyl groups of the $\alpha_1(Fe^{III}P)$ and β_2(ZnP) point toward each other and are involved in the distances of closest approach (Figure 1).[12] Use of hybrid prepared with ZnD,[10] which lacks vinyl groups, rather than ZnP, lengthens the minimum distances between unsaturated carbons by 1 Å without significantly perturbing either the electron distribution of the zinc-porphyrin core[13] or, as shown by protein X-ray diffraction

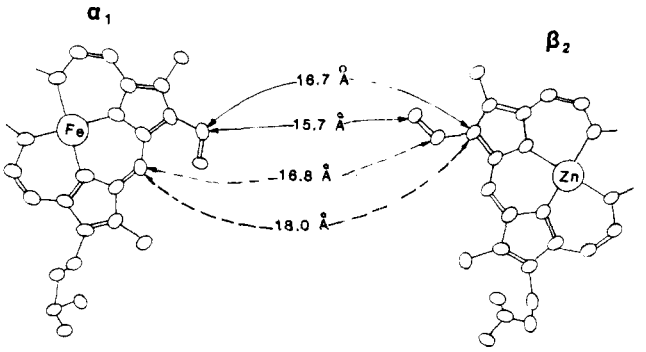

Figure 1. Schematic representation of distances between the α_1 and β_2 hemes within the T-state hemoglobin tetramer.[12] Preparation of the [α(Fe),β(Zn)] hybrid with ZnD eliminates the vinyl group from the β-chain. The solid arrows indicate the shortest interporphyrin separations in the presence and absence of the vinyl group of the β-chain. The dashed arrows indicate the analogous distances for a ring-edge carbon of the α-chain heme.

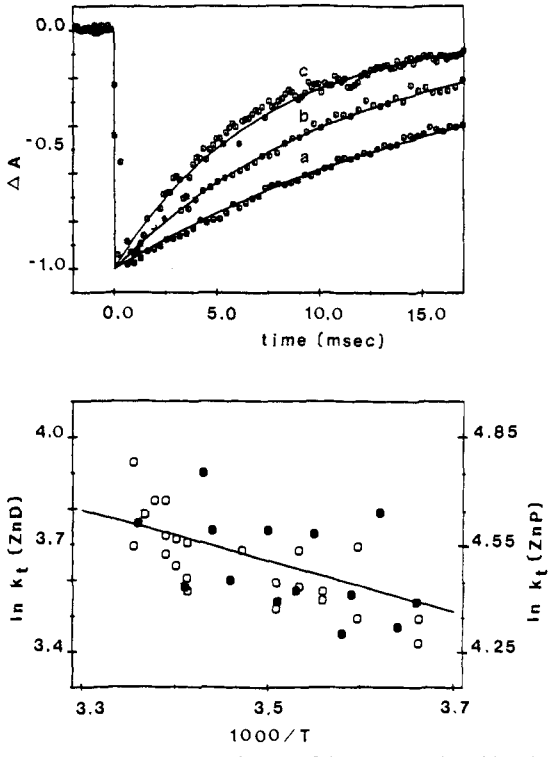

Figure 2. Kinetic properties of [Fe,Zn] hemoglobin hybrids. (upper) Normalized transient absorbance decay of ^{3}Zn porphyrins within (a) [Fe^{II}P,ZnD]; (b) [$Fe^{III}(H_2O)$P,ZnD]; and (c) [$Fe^{II}(H_2O)$P,ZnP]. Conditions: 415 nm; 25 °C; 10 mM KP_i buffer (pH 7.0). (lower) Temperature dependence of electron transfer rates: k_t(ZnD) for the [$Fe^{III}(H_2O)$P,ZnD] hybrid (left ordinate, ●); k_t(ZnP) for the [$Fe^{III}(H_2O)$P,ZnP] hybrid (right ordinate, ○). The right ordinate scale and the k_t(ZnP) points have been shifted downward by 0.85 to facilitate comparison of the two data sets. The solid line corresponds to the best fit line for the [$Fe^{III}(H_2O)$P,ZnD] hybrid; ΔE^*(ZnD) = 0.06 eV.

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(10) Abbreviations: Hb, hemoglobin; P, protoporphyrin IX; D, deuteroporphyrin; [α(Fe),β(Zn)], hybrid hemoglobin in which the α chains contain an Fe porphyrin and the β chains a Zn porphyrin. When the type of porphyrin is significant, it is indicated.

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