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NMR-studies of carcinogen reactions with DNA: ethylene dibromide and aflatoxin B_1

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Abstract: Two examples are described of the use of NMR spectroscopy to study the modification of DNA structure by carcinogens. The reaction of ethylene dibromide involves initial conjugation with glutathione, catalysed by glutathione *S*-transferase. Reaction of this adduct with DNA occurs at N^7 of guanine. Through the use of stereospecifically 1,2-dideuteriated ethylene dibromide, the mechanism of reaction has been shown to involve an odd number, i.e. three, of S_N2 inversions. Correlation spectra (COSY) were employed to analyse reaction stereochemistry. The relative configuration of the deuterium atoms in the products was initially assigned by ^1H nuclear Overhauser effect (NOE) difference spectra and then confirmed by an independent synthesis of stereospecifically dideuteriated glutathione–guanine adducts. The second example involves reaction of the epoxide of aflatoxin B_1 with DNA to form covalent adducts at N^7 of guanine. Adduct formation was found to enhance duplex stability. Chemical shift changes for aflatoxin protons in the covalent adduct when compared with those for aflatoxin B_1 non-covalently associated with DNA suggest that covalently linked aflatoxin is intercalated. NOEs confirm that the aflatoxin moiety is intercalated and show that it is on the 5′ side of the guanine. This geometry leads to $d(ATCGAT)_2$ forming an adduct in which only one chain has been modified by aflatoxin, while $d(ATGCAT)_2$ forms a complex in which both chains have been modified.

Keywords: *NMR; xenobiotic metabolism; carcinogens; DNA adducts; ethylene dibromide; aflatoxin.*

NMR in Biomedical Research

NMR spectroscopy is now 40 years old. Although the value of NMR was quickly recognized by chemists, many years passed before it became a major research tool for investigators in the biological sciences. The slowness with which biochemists, pharmacologists, etc. took up this new technique was not because they were failing to keep abreast with new developments in analytical methodology but rather because biological problems often involve small samples of high structural complexity. Key developments which ultimately led to wide acceptance of NMR in the biomedical sciences include (1) introduction of Fourier transform (FT) spectrometers which permitted signal averaging of a large number of scans so that satisfactory signal-to-noise ratios could be obtained with small samples; (2) superconducting magnets, where the higher fields gave better dispersion of signals in complex spectra with an added bonus of increased sensitivity; and (3) most recently, two-dimensional spectral methods.

Two-dimensional Techniques in NMR

Two-dimensional methods permit spectra to be spread along two axes; then, depending upon details of the acquisition procedure, correlations can be made between the two spectra. Prominent examples of two-dimensional methods include COSY spectra in which protons can be identified which are coupled to one another, NOESY spectra in which protons can be identified which are not coupled but lie in proximity so that they have mutual relaxation, and heterocorrelation spectra in which connectivity between protons and carbon atoms can be identified. A seemingly endless array of two-dimensional methods have been and are being developed to examine structural relationships [1]. The rapid development of new pulse techniques reflects the fact that modern spectrometers are fully controlled by computers. Consequently, generation of most new pulse sequences requires one only to write software in order to achieve the desired pulse sequence.

Two-dimensional spectra are powerful tools

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for analysis of congested spectra, such as those of oligonucleotides, oligosaccharides and proteins [2]. They permit not only connectivities to be established but also three-dimensional structures. While the methodology does not give structural parameters as precisely as X-ray crystallography, the structure that is obtained is that of the substance in solution rather than in the solid state. Furthermore, the dynamics of conformational equilibria and substrate-receptor interactions can be studied by NMR. X-ray crystallography and NMR spectroscopy can be viewed as complementary tools for structural analysis.

NMR in Metabolism Studies

NMR spectroscopy has played a major rôle in the elucidation of metabolic processes, particularly the identification of metabolic products. Studies of metabolic products include both essential metabolites and those resulting from metabolism of xenobiotics including drugs, environmental pollutants, etc. NMR has also been used to elucidate biosynthetic pathways. In many cases this only involves viewing transient intermediates, but often stable isotopes, particularly ^{13}C and ^{2}H, have been used to establish the fate of individual atoms in transformations of precursors to metabolic products. The method provides an attractive alternative to radioisotopes in cases where incorporation efficiency is adequate. Moreover, the NMR method has a distinct advantage over mass spectroscopy for establishing the site and stereochemistry of isotopic substitution.

NMR in the Study of Interactions of Xenobiotics and DNA

Compounds that interact with DNA represent one of the most important classes of xenobiotics. They include both compounds used in cancer chemotherapy and carcinogens. Although little structural information can be derived from spectra of solutions of very high polymers such as DNA, due to the complexity of the spectra and the broad lines resulting from short spin–spin relaxation times, useful studies can often be made with oligodeoxynucleotides [3]. Strategies have been developed for assignment of spectra of oligodeoxynucleotide duplexes which are dependent on the observation of NOEs between the base and deoxyribose of the individual nucleosides and between adjacent nucleosides along the individual chains [4]. Modification of a heterocyclic base by reaction with a drug or carcinogen alters the conformation of the duplex leading to changes in the network of NOEs that are observed. Additionally, NOEs will often be observed between the covalently linked substance and individual protons in the oligomer. As a consequence, changes which have occurred in the structure of the DNA can be determined, as well as the relative conformation of the modifying substance.

This paper addresses two specific cases of carcinogen interactions with DNA which have been investigated in this laboratory, namely ethylene dibromide and aflatoxin B_1.

Ethylene dibromide

Ethylene dibromide (EDB) has been used extensively as an intermediate in the chemical industry and as a pesticide in agriculture. It is carcinogenic in laboratory animals [5], and concern about the potential for human carcinogenicity has led to the recent ban of its use as a pesticide [6]. Genotoxicity is believed to result from glutathione *S*-transferase catalysed conjugation with glutathione (GSH) to yield *S*-(2-bromoethyl) glutathione; the conjugate reacts with DNA preferentially at N^7 to form *S*-[2-(N^7-guanyl) ethyl]glutathione (Fig. 1) [7]. Uncertainty concerning the mechanism of reaction of the conjugate with DNA led this group to investigate the stereochemistry of reaction [8]. The primary question was whether the DNA adduct is formed directly from the GSH–EDB conjugate or whether the conjugate first cyclizes to an episulphonium ion. If direct reaction occurs, two S_N2 processes are involved in proceeding from EDB to DNA adduct; if the episulphonium ion is involved, three S_N2 steps occur, as shown in Fig. 1. The reaction was studied with stereospecifically dideuteriated EDB, which was prepared in the *threo* and *erythro* forms by bromination of commercially available *cis*- and *trans*-1,2-dideuterioethylene [9]. The DNA adduct was formed on calf thymus DNA using liver cytosol from phenobarbital-treated rats as a source of glutathione *S*-transferase [7, 10]. Depurination by heating at 100°C for 30 min at pH 7.0 gave dideuteriated *S*-[2-(N^7-guanyl) ethyl]glutathione.

One-dimensional ^{1}H NMR spectra of the two samples were similar although not iden-

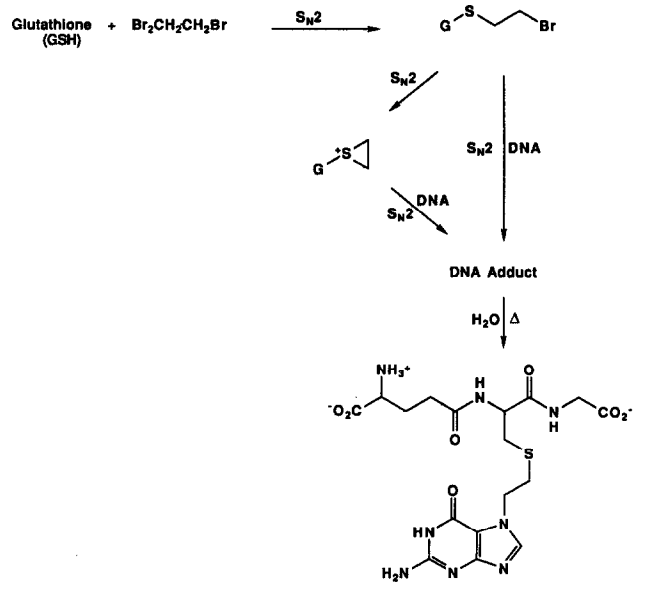

Figure 1
Two potential pathways for formation of the ternary adduct of glutathione and EDB with DNA.

tical, the differences lying in the signals for the ethylene bridge (Fig. 2AB). It should be noted that the pairs of protons of both bridge methylene groups are non-equivalent due to asymmetry in the glutathione fragment of the molecule, and thus they appear as an A,A′,X,X′ pattern. Major differences were apparent in the COSY spectra of the guanine adducts obtained from the two dideuteriated EDBs. The COSY spectrum of the *undeuteriated* guanine adduct contained correlation signals for each of the four bridge protons with the other three protons, i.e. six signals in all, two geminal correlations and four vicinal correlations; this is illustrated in Fig. 3. With the 1,2-dideuteriated adducts a stereochemical complication must be taken into account. The *erythro* form of dideuteriated EDB is *meso* and the *threo* is a racemic mixture. Irrespective of whether DNA adduct formation involves an even or an odd number of S_N2 processes, both dideuterio EDBs yield two diastereomeric adducts, as illustrated for the *erythro* form in Fig. 4. Each of these adducts will give a single vicinal correlation signal, as shown in Fig. 3, so that the *erythro* will give one pair of vicinal correlation peaks and the *threo* the other. Starting with [*erythro*-1,2-$^2H_2$]EDB, the guanine conjugate gave two vicinal correlation signals which were at a +45° angle to each other in the COSY spectrum; starting from the *threo* isomer the complementary correlation signals were seen, arranged at a −45° angle (Fig. 2AB) The fact that each sample yielded only two vicinal signals restricted the mechanistic possibilities, i.e. the reaction sequence must not involve a combination of processes having odd and even numbers of inversions, or go by S_N1 or other processes which cause loss of configuration. However, the COSY spectrum did not permit assignment of the relative configurations of the deuterium atoms in the two samples.

An attempt was made to make the assignments based on the magnitude of ^1H–^1H vicinal coupling constants using the Karplus relationship [11] on the assumption that the ethylene bridge of the guanine–GSH adduct would have a preferred antiperiplanar geometry. The configurations of the 1,2-dideuterio EDBs were originally established by IR spectroscopy [9]. It was possible to confirm the assignments by NMR on the premise that the dominant conformation was antiperiplanar (Fig. 5), although the ^1H–^1H vicinal coupling constants could only be observed indirectly via the ^{13}C satellites of the major signal, since the two protons are equivalent. With the guanine–GSH adducts, non-equivalence of the two methylene groups meant that coupling constants could be viewed in the major signals; however, internally consistent values could not be obtained for the two pairs of diastereomers. The failure may be due to the antiperiplanar assumption being unwarranted in this case, internal motion in the adduct, or use of an

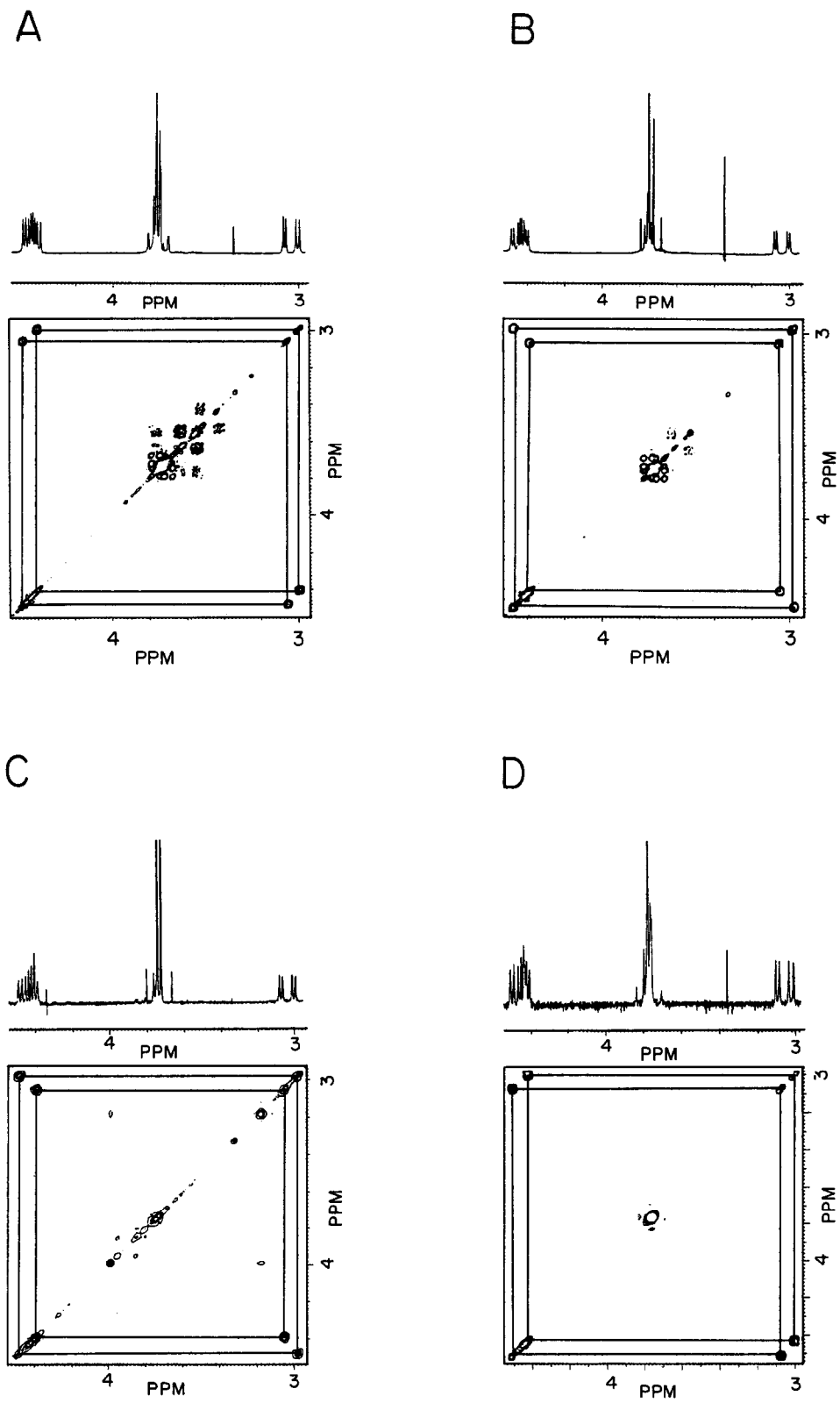

Figure 2
One-dimensional and COSY spectra of the ternary adduct. Panels A and B generated *in vitro* by the procedure of Fig. 1 and panels C and D by independent synthesis (see text) from [*erythro*-1,2-$^2H_2$]EDB (A and C) and [*threo*-1,2-$^2H_2$]EDB (B and D). Only the region of the ethylene bridge is shown. Lines are drawn indicating vicinal correlation.

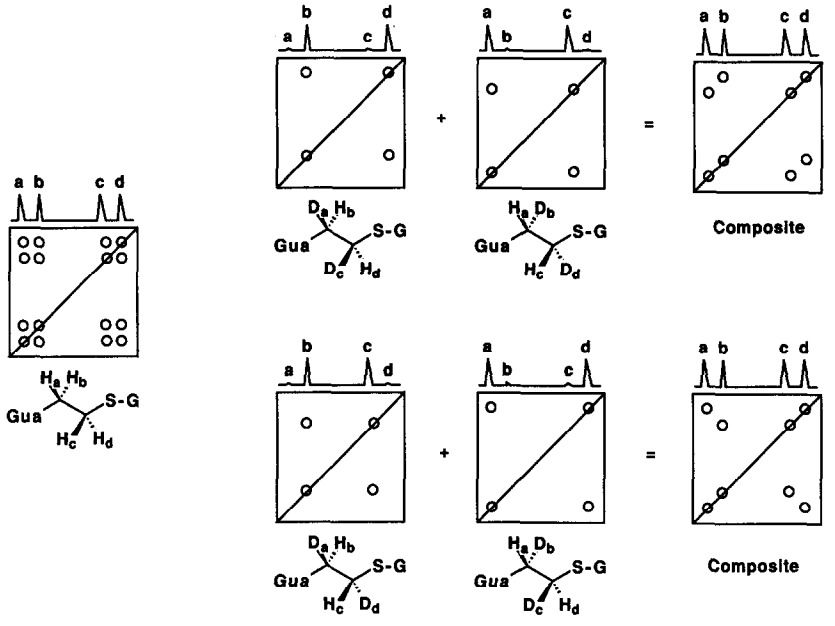

Figure 3
Simplified COSY spectra (ethylene bridge region) of undeuteriated (left), and vicinally deuteriated (right) ternary adduct. The upper row shows one pair of diastereomers and the lower row the other. The chemical shifts for the two pairs of protons are chosen arbitrarily and do not represent assignments of the individual diastereomers.

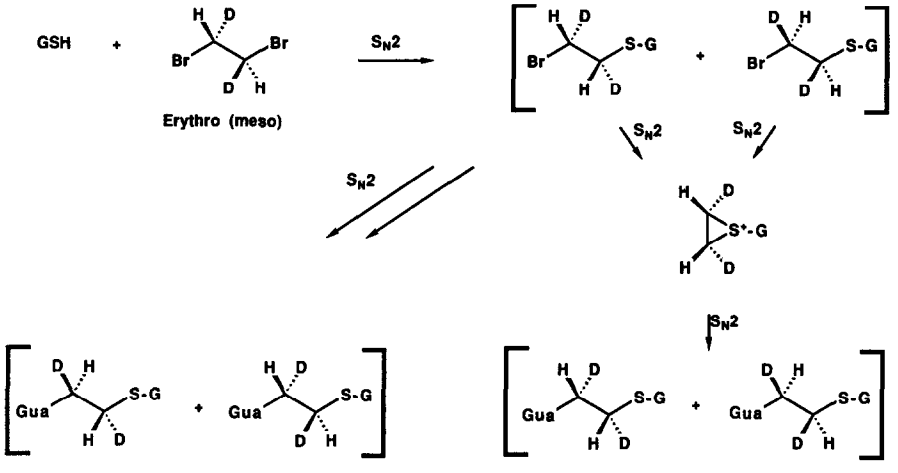

Figure 4
Stereochemical consequences of two versus three S_N2 displacements in the conversion of [*erythro*-1,2-$^2H_2$]EDB to ternary adduct showing the manner in which two diastereomers arise in each case.

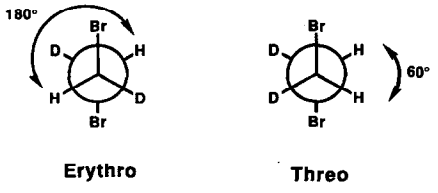

Figure 5
Newman projections of *erythro* and *threo* [1,2-$^2H_2$]EDB showing the torsional angle between vicinal protons when the bromine atoms are antiperiplanar.

inappropriate Karplus function [11]. Attempts to slow rotation or alter the conformation by lowering the temperature and by converting the sulphide to the sulphone were unproductive. NOEs, which are a function of interproton distances, were determined for the vicinal protons in the ethylene bridge. The guanine–GSH adduct derived from *erythro* dideuterio EDB showed a 4% NOE, and the adduct derived from *threo* an 0.8% effect. On

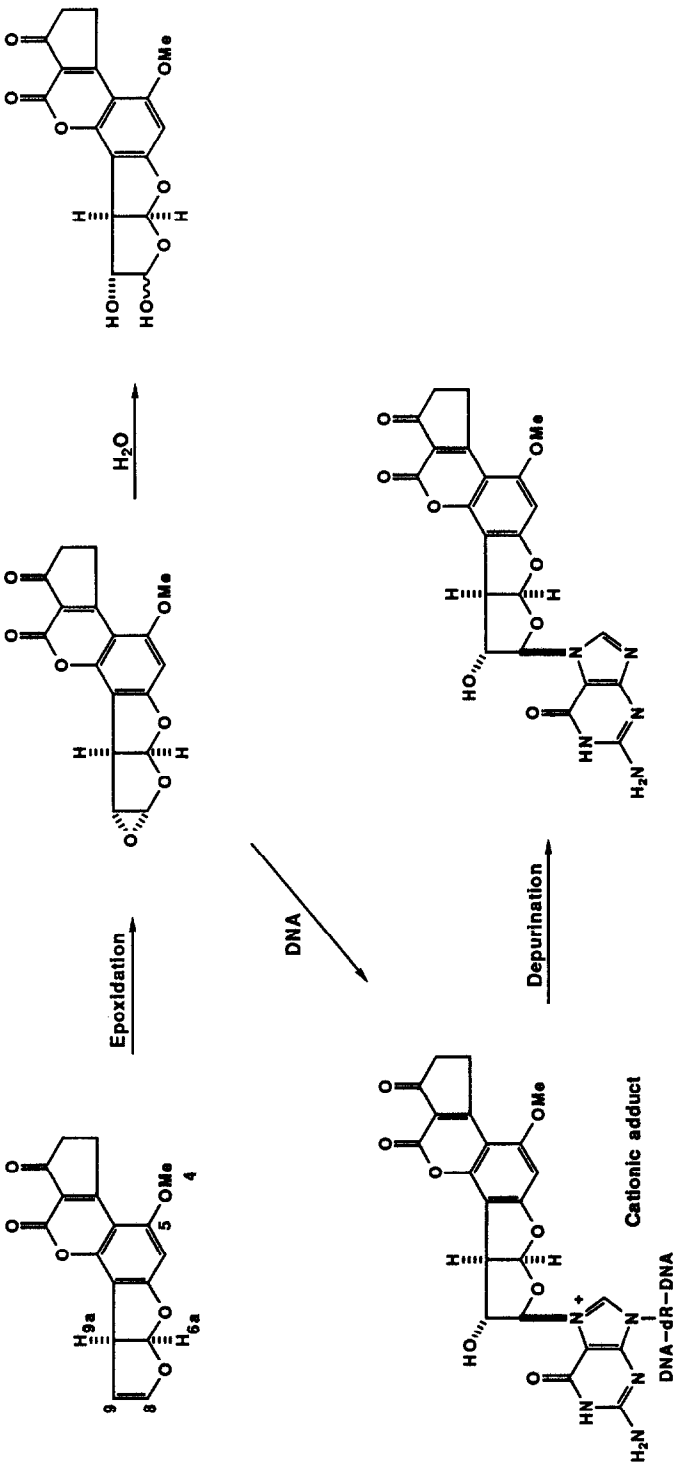

Figure 6
Transformation of aflatoxin B_1 to guanine N^7 adduct and to the 8,9-dihydro-8,9-diol via the *exo*-8,9-epoxide.

this basis, the protons are assigned anti in the first case and gauche in the second indicating that the reactions had occurred with net inversion of configuration. This assignment supports the mechanism involving the episulphonium ion.

Confirmation of this conclusion was obtained by preparation of the guanine adducts by an independent route with unambiguous overall stereochemistry involving two S_N2 displacements. Guanosine was converted to N^7-(2-bromo[1,2-$^2H_2$]ethyl) derivatives by reaction with the dideuteriated EDBs in the presence of silver nitrate to suppress Finkelstein reactions. Subsequent condensation with the anion of glutathione followed by removal of the ribose gave the two guanine–GSH adducts. COSY spectra showed that the one derived from *erythro* dideuteriated EDB gave correlation peaks arranged at a −45° angle and the *threo* isomer gave correlation peaks at a +45° angle, confirming that the original procedure for forming ternary adduct had involved an odd number of displacements, i.e. that it had involved an episulphonium ion intermediate.

Aflatoxin B_1

The second example of carcinogen–DNA interaction to be described involves aflatoxin B_1 (AFB1). AFB1 is a potent mutagen and carcinogen produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi that grow on seeds including corn and peanuts [12]. Contamination of food supplies is of world concern; aflatoxin contamination presents a serious problem in this country and elsewhere throughout the world with the problem being particularly severe in humid regions of China, India and Africa. Oxidative activation is required for reaction of AFB1 to occur with DNA [13]. Reaction of the activated species occurs at N^7 of guanine (Fig. 6) [14]. The ultimate carcinogen, inferred to be the *exo*-8,9-epoxide from the structure of the guanine adduct, has never been detected in biological systems. However, a synthesis of the epoxide was recently achieved in this laboratory using dimethyldioxirane as the oxidant [15]. The epoxide of AFB1 is stable in aprotic, non-nucleophilic media but undergoes rapid hydrolysis ($t_{1/2}$ <5 s) in water. Reaction with aqueous solutions of DNA is rapid and efficient and can give yields of adduct in excess of 70% [16]. Properties of the epoxide are fully concordant with it being the ultimate carcinogen of AFB1.

In a project aimed at discovering the structural basis for the exceptionally efficient reaction of AFB epoxide with DNA, investigations have been made of (1) equilibrium binding of AFB1 and related species with DNA (since the epoxide itself is too reactive to study equilibrium binding); and (2) the structure/conformation of the covalent adduct of AFB epoxide with DNA.

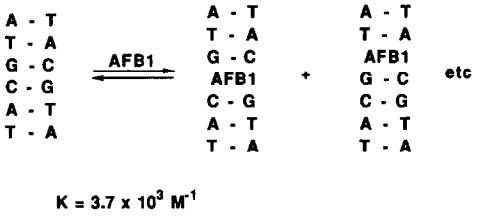

Figure 7
Equilibrium binding of AFB1 with d(ATGCAT)$_2$.

Table 1
Chemical shifts of selected protons in AFB_1, AFB covalently linked to guanine N7 of d(ATCGAT)$_2$, and AFB_1 associated non-covalently with d(ATCGAT)$_2$ in D_2O at 5°C

Proton	δFree*	δCovalent†	ΔδCovalent-free	δNon-covalent‡	ΔδNon-covalent-free
H6a	6.93	6.75	−0.18	6.72	−0.21
H5	6.69	5.75	−0.94	5.87	−0.82
H9a	4.8§	3.95	−0.85	4.47	−0.33
4-OCH_3	3.98	3.65	−0.33	3.62	−0.36
H8	6.59	6.38	−0.21‖	6.49	−0.10
H9	5.57	6.08	+0.51‖	5.37	−0.20

*Saturated solution of AFB1 dissolved in buffered D_2O.
†2.0 mM.
‡Chemical shifts measured from an equilibrium mixture containing 2.0 mM d(ATCGAT)$_2$ in a saturated solution of AFB1. Equilibrium is rapid on the NMR time scale; chemical shifts represent weighted averages of free and bound AFB1.
§Estimated chemical shift; this signal is located beneath the solvent resonance in D_2O. In DMSO-d_6 the H9a signal is observed at 4.75 ppm.
‖C8 and C9 of AFB1 change hybridization upon formation of adduct.

Equilibrium binding was studied by ^1H NMR spectroscopy and other methods [17, 18]. By NMR, a binding constant of $3.7 \times 10^3\ M^{-1}$ was obtained with the double-stranded hexamer d(ATGCAT)$_2$ (Fig. 7). Equilibrium was fast on the NMR time scale with the observed spectrum being a population weighted average of chemical shifts arising from free and bound states. The AFB1 signals were shifted upfield, consistent with intercalation of the AFB1 between base pairs (Table 1). One equivalent of actinomycin D or four equivalents of ethidium bromide, both intercalators with high binding affinities, displaced AFB1 from the oligomer but spermidine, a groove binder, did not. AFB1 binding to DNA apparently requires duplex structure; at increased temperature AFB1 is released concurrent with dissociation of the duplex. Supporting evidence for intercalative association by AFB1 was obtained from AFB1 titration of negatively supercoiled pBR322 plasmid DNA which caused unwinding, while titration of relaxed closed circular plasmid caused rewinding.

Covalent DNA adducts of AFB1 are readily prepared with the epoxide. Earlier procedures for the preparation of DNA adducts had involved *in situ* generation of the epoxide by oxidation with *m*-chloroperbenzoic acid (MCPBA), with microsomes or photochemically and by solvolysis of 8-acyloxy-9-hydroxy derivatives of AFB1 [14, 19–21]. Initial attempts to produce high purity adducts on oligodeoxynucleotides which would be suitable for NMR studies were disappointing using MCPBA, because of difficulties in purification and reproducibility. Using the epoxide these problems could be largely circumvented [22]. With d(ATCGAT)$_2$, reaction occurred smoothly until one guanine per duplex had been modified (Fig. 8A). Attempts to drive the reaction beyond this stage by further treatment with AFB epoxide were unsuccessful. Purification could be achieved by reversed-phase C-18 high-performance liquid chromatography, the modified and unmodified strands eluted separately and could then be recombined. The modified duplex had a slightly increased T_m.

The ^1H NMR spectrum of the duplex adduct in D_2O at 0–5°C showed that the pseudo-dyad symmetry of the unmodified duplex had been lost producing a doubling of the number of DNA signals (Fig. 9). The H8 signal for the modified guanine could not be detected; the cationic charge on the AFB adduct increases the acidity of H8 so that deuterium exchange occurs readily. In spectra obtained in H_2O instead of D_2O, two types of G:C imino signals were present in the imino region. In addition, a new signal was observed at δ9.75, tentatively assigned to H8 of the modified guanine. ^{31}P

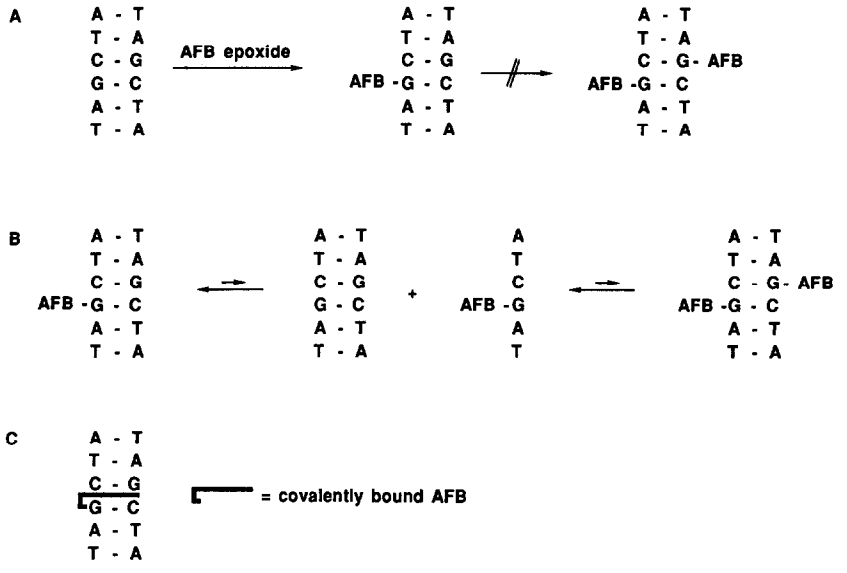

Figure 8
(A) Covalent reaction of aflatoxin B_1 epoxide with d(ATCGAT)$_2$ leading to singly modified duplex. (B) Potential disproportionation of singly modified duplex to unmodified duplex and modified single strand or doubly modified duplex. (C) Intercalation of AFB on the 5′ side of guanine precludes a second intercalation if AFB were to be bound also to the guanine in the second strand. NOEs are observed between AFB and cytosine.

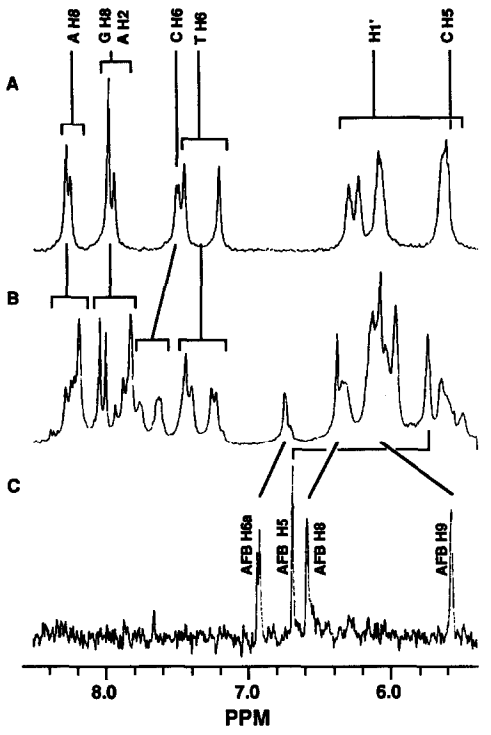

Figure 9
A partial spectrum of (A) $d(ATCGAT)_2$, (B) covalent adduct of AFB epoxide with $d(ATCGAT)_2$, and (C) AFB1. Assignments of selected DNA protons are shown at the top of the figure and four of the aflatoxin protons at the bottom. Increased complexity should be noted in the spectrum of the covalent adduct reflecting loss of dyad symmetry. AFB proton H5 is strongly shielded in the adduct, H6a to a lesser extent. The changes in AFB H8 and H9 reflect structural changes in that region.

spectra showed doubling of the number of phosphorus signals consistent with the loss of symmetry; one ^{31}P signal was shifted downfield from the others.

Slow exchange on the NMR time scale was observed between unmodified and singly modified duplexes when the two were mixed; i.e. two distinct spectra were seen, one for each duplex. The spectrum of singly modified duplex alone indicated that the potential equilibrium of it with unmodified and doubly modified duplex (or with modified single strands) lay so far on the side of singly modified duplex that characteristic signals for the unmodified duplex could not be detected (Fig. 8B).

Partial assignments have been made on the NMR spectrum of the modified duplex. Of particular interest, the signals for the AFB 4-OCH_3, H5, H6a and H9a signals all move upfield. A similar effect had been seen for AFB1 non-covalently associated with an oligomer. Nuclear Overhauser difference spectra showed NOEs between AFB protons at positions 6a and 9a, and cytosine protons H5 and H6. In addition, NOEs were absent between the guanine H1's in the two strands and between guanine H8 and cytosine H1′ in the unmodified strand. These data support the hypothesis that covalently bound AFB is intercalated on the 5′ side of the guanine of attachment (Fig. 8C).

As a test of this model, the hexamer $d(ATGCAT)_2$ was treated with AFB epoxide. In this case, reaction proceeded smoothly to doubly modified duplex (Fig. 10A). NMR spectra indicated that symmetry had been retained. NOE difference spectra showed the AFB 6a and 9a were in proximity to thymine, consistent with intercalation again occurring on the 5′ side of guanine (Fig. 10B). In the case of

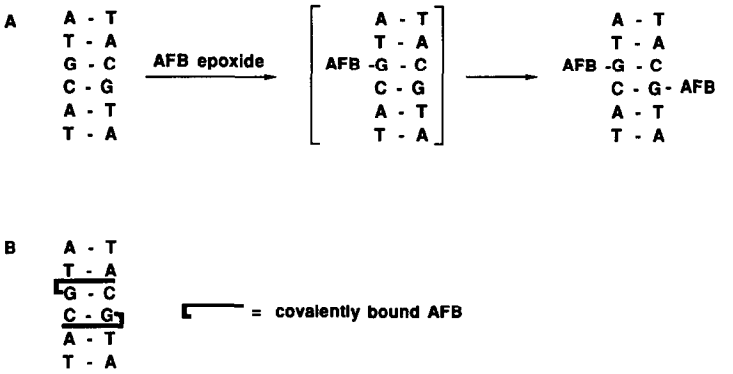

Figure 10
(A) Covalent reaction of AFB epoxide with $d(ATGCAT)_2$ leading to doubly modified duplex. Singly modified duplex was not observed during the reaction. (B) Intercalation of AFB on the 5′ side of guanine permits AFB attached to the guanine in the other strand to intercalate. NOEs are observed between AFB and thymine.

d(ATCGAT)$_2$, intercalation on the 5′ side of guanine placed the AFB between the G:C and the C:G pairs precluding intercalation of an AFB molecule linked to the guanine of the antiparallel chain. With d(ATGCAT)$_2$, intercalation occurs between G:C and A:T so that filling one site does not block intercalation of an AFB residue attached to the second guanine.

A central issue in these studies was the conformation of AFB epoxide in the transition state of the alkylation reaction. There is no requirement that the structure of the transition state for this reaction resemble that of the associative complex of AFB1 with DNA or that of the covalently modified duplex. However, the stereochemistry of opening of the epoxide ring is rigorously antiperiplanar, i.e. involving endo attack with a trajectory roughly parallel to the face of the aflatoxin ring system. By contrast, solvolysis of AFB epoxide is not stereospecific. These facts in conjunction with the structural data on the DNA–AFB1 complex and DNA–AFB adduct support an attractive but as yet unproven hypothesis that intercalated complexes of AFB epoxide, formed to varying degrees at all sites along the duplex, when properly oriented on the 5′ side of guanine lead to nucleophilic attack by guanine N^7 on C8 of the epoxide to give the observed endo product. Therefore only relatively minor changes in conformation may be involved in proceeding from non-covalent complex to transition state to covalent adduct.

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