Spontaneous Reactions of Aflatoxin B_1 Modified Deoxyribonucleic Acid in Vitro†

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ABSTRACT: The reactions of aflatoxin B_1 modified DNA (AFB_1–DNA) were investigated under physiological conditions in vitro. For these studies [^{3}H]AFB_1–DNA was prepared by the reaction of [^{3}H]aflatoxin B_1 ([^{3}H]AFB_1) with calf thymus DNA in the presence of rat liver microsomes, and [^{3}H]-AFB_1–DNA–[^{14}C]guanine was prepared by the microsome-mediated reaction of [^{3}H]AFB_1 with DNA selectively labeled with [^{14}C]guanine. DNA labeled by [^{14}C]guanine had been synthesized enzymatically by the polymerization of [^{14}C]-deoxyguanosine 5′-triphosphate and the three unlabeled deoxynucleoside 5′-triphosphates with *Escherichia coli* polymerase I. High-pressure liquid chromatography of acid hydrolysates of these AFB_1–DNA preparations revealed that approximately 90% of the covalent AFB_1 adducts were released in the form of 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N^7-Gua). The half-life times at 37 °C of the adducts in DNA which give rise to AFB_1-N^7-Gua upon treatment with acid were 19 h at pH 6.7, 12 h at pH 7.0, and 8 h at pH 7.3. Three major reactions were found to be responsible for the disappearance of these adducts from AFB_1–DNA under physiological conditions: (1) release from DNA of 2,3-dihydro-2,3-dihydroxyaflatoxin B_1 (AFB_1-dhd); (2) release from DNA of AFB_1-N^7-Gua; (3) appearance of the putative 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B_1 (AFB_1-triamino-Py) in the acid hydrolysates of AFB_1–DNA. Approximate rate constants were determined for these reactions at 37 °C at pH 6.7, 7.0, and 7.3. The release of AFB_1-dhd predominated at all pH values and presumably is accompanied by the reconstitution of an intact guanine moiety in the DNA. The release of AFB_1-N^7-Gua occurred at all pH values but was most rapid at pH 6.7. It is likely that aguaninic sites are formed in the DNA as a consequence of this reaction. The formation of adducts with increased chemical stability which can be released from the DNA by acid treatment in the form of the putative AFB_1-triamino-Py only was observed at pH 7.0 and pH 7.3. Our results have the following implications for the understanding of the biological effects of AFB_1. The primary adducts in AFB_1–DNA possess a short half-life time under physiological conditions. Nevertheless, they are present long enough to exert their effect on the processes of DNA metabolism in mammalian cells. At later times after exposure to AFB_1, the DNA is expected to contain mostly secondary lesions of increased chemical stability, in particular aguaninic sites and the adducts which are assayed as the putative AFB_1-triamino-Py in the acid hydrolysates of AFB_1–DNA.

Aflatoxin B_1 (AFB_1)[1] is a mycotoxin produced by certain strains of *Aspergillus flavus*. It is hepatotoxic and hepatocarcinogenic in several animal species (Campbell & Hayes, 1976; Wogan, 1973). Epidemiological studies suggest that it may also be an important factor in the etiology of human liver cancer (Shank et al., 1972; Peers & Linsell, 1973; Wogan, 1976).

Like many carcinogens, AFB_1 requires metabolic activation to exert its biological effects. It binds covalently to cellular macromolecules both in vivo (Garner & Wright, 1975; Lin et al., 1977; Croy et al., 1978) and after activation in vitro by liver microsomes (Swenson et al., 1977; Garner et al., 1972; Essigmann et al., 1977). The major covalent AFB_1–DNA adduct released after mild acid hydrolysis has been identified as AFB_1-N^7-Gua (Lin et al., 1977; Essigmann et al., 1977; Croy et al., 1978). Like other adducts formed at the N^7 position of deoxyguanosine (Lawley, 1975; Osborne et al., 1978), AFB_1-N^7-Gua in DNA is expected to be unstable because the imidazole ring of guanine carries a positive charge. We had observed previously that the AFB_1–DNA adducts which can be released as AFB_1-N^7-Gua by mild acid hydrolysis disappeared rapidly from DNA upon incubation under physiological conditions at pH 7 and 37 °C. However, only a fraction of the adducts was released in the form of AFB_1-N^7-Gua, and a major portion of the hydrolysis products remained unidentified (Wang & Cerutti, 1979).

We now report a detailed study of the reactions of AFB_1–DNA under physiological conditions in vitro. Our results allow the following conclusions based on the reasonable assumption that AFB_1-N^7-Gua is the structure of the primary adduct in DNA rather than a secondary product resulting from the mild acid treatment which is used for the analysis of AFB_1–DNA. The major reaction involves the hydrolytic cleavage of the linkage between the N^7 position of guanine and the C^2 position of AFB_1 and results in the reconstitution of an intact guanine moiety and the release of AFB_1-dhd. The second reaction occurring at neutrality and more rapidly under acidic conditions is the hydrolytic cleavage of the N-glycosylic bond of the AFB_1-deoxyguanosine adducts which results in the release of AFB_1-N^7-Gua and the formation of an aguaninic site in the DNA. A third reaction occurring under neutral and alkaline conditions leads to the formation of a secondary product which remains attached to the DNA and may possess the structure of AFB_1-triamino-Py as proposed by Lin et al. (1977).

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[1] Abbreviations used: AFB_1, aflatoxin B_1; AFB_1-N^7-Gua, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1; AFB_1-triamino-Py, 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B_1; AFB_1-dhd, 2,3-dihydro-2,3-dihydroxyaflatoxin B_1; AFB_1–DNA, DNA prepared by the reaction of AFB_1 with calf thymus DNA in the presence of rat liver microsomes; PSC, 0.01 M sodium phosphate–0.1 M sodium chloride–0.01 M sodium citrate; LC, high-pressure liquid chromatography.

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Experimental Procedure

Chemicals. [^{3}H]Aflatoxin B_1 (sp act. 20 Ci/mmol) was purchased from Moravek Biochemicals, City of Industry, CA, and unlabeled aflatoxin B_1 was purchased from Calbiochem, San Diego, CA. [8-^{14}C]Deoxyguanosine 5′-triphosphate (sp act. 42 Ci/mol) was purchased from New England Nuclear, Zurich, Switzerland. *Escherichia coli* DNA polymerase I was obtained from Worthington, Basel, Switzerland. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were obtained from Sigma Chemical Co., Basel, Switzerland. A sample of authentic tritium-labeled 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N^7-Gua) was kindly supplied by Dr. J. Essigmann of the Massachusetts Institute of Technology, Cambridge, MA.

High-Pressure Liquid Chromatography. High-pressure liquid chromatography (LC) was accomplished with a μBondapak C_{18} reverse-phase column on a Waters ALC 200 liquid chromatograph equipped with a U6K injector system and a Model 660 solvent programmer. The following isocratic solvent systems were used: solvent I, 95% ethanol–1-propanol–water (10:5:85 v/v); solvent II, 95% ethanol–water–glacial acetic acid (5:15:0.001 v/v); solvent III, methanol–water–glacial acetic acid (30:70:0.01 v/v). The flow rate was 1 mL/min. Fractions of 0.5 mL were collected, and the radioactivity of each fraction was counted in a Beckman Model LS 8000 after the addition of 5 mL of Aquassure. Authentic [^{3}H]AFB_1-N^7-Gua and [^{3}H]-2,3-dihydro-2,3-dihydroxyaflatoxin B_1 (AFB_1-dhd) were used as markers for the calibration of the μBondapak C_{18} column used in the LC.

Preparation of Rat Liver Microsomes. Male Fischer rats weighing 190–210 g were given drinking water containing 0.1% sodium phenobarbital (Marshall & McLean, 1969) for 7 days. They were deprived of food for 24 h before killing by cervical dislocation. The two main lobes of the livers were used for preparation of microsomes as described by Kinoshita et al. (1973). The protein content of the microsomes was determined by the method of Lowry et al. (1951).

Preparation of ^{3}H-Labeled AFB_1-dhd. [^{3}H]AFB_1-dhd was prepared according to Swenson et al. (1975). AFB_1-dhd was purified by chromatography of the reaction mixture on a 0.9 × 25 cm column of Sephadex LH-20 with water as the eluant. The fractions containing AFB_1-dhd were flash-evaporated and redissolved in 30% methanol containing 0.01% acetic acid. AFB_1-dhd was further purified by LC on a μBondapak C_{18} column by using the conditions of Swenson et al. (1975). The purified AFB_1-dhd displayed the expected absorption spectrum with a bathochromic shift upon going from neutral conditions to 0.1 N NaOH (Swenson et al., 1973). It was stored in 50% methanol containing 0.01% acetic acid at −20 °C.

In Vitro Synthesis of [^{14}C]Guanine-Labeled DNA. The [^{14}C]guanine-labeled DNA was prepared enzymatically by polymerization of three unlabeled deoxyribonucleoside triphosphates, dATP, dCTP and dTTP, and [^{14}C]dGTP with *E. coli* DNA polymerase I using untreated calf thymus DNA as the template (Richardson et al., 1964). The reaction mixture (2.5 mL) contained 66 mM potassium phosphate buffer, pH 7.0, 6.6 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 μM each of dATP, dCTP, dTTP, and [^{14}C]dGTP, 20 μg/mL calf thymus DNA, and 8.5 units/mL DNA polymerase I. After incubation at 37 °C for 2 h, the reaction mixture was extracted with phenol–chloroform–isoamyl alcohol (50:50:1 v/v). The aqueous phase was removed, and 200 μg of calf thymus DNA was added as carrier before repeated precipitation with ethanol. The DNA was dissolved in 0.1 M sodium/potassium phosphate, pH 7.0, and stored at 4 °C.

Microsome-Mediated Binding of [^{3}H]AFB_1 to DNA. For the reaction of DNA with AFB_1, 4.4 mg of calf thymus DNA was reacted with 200 μCi of [^{3}H]AFB_1 (sp act. 20 Ci/mmol; 2 μM final concentration) in 5 mL of reaction mixture containing 0.1 M sodium/potassium phosphate buffer, pH 7.0, 0.07 M KCl, 3 mM $MgCl_2$, 5 mM glucose 6-phosphate, 0.8 mM NADP, 0.4 unit/mL glucose-6-phosphate dehydrogenase, and microsomes at 1 mg of microsomal protein per mL. For the modification of [^{14}C]Gua-labeled DNA (150 μg, 500 cpm/μg), 40 μCi of [^{3}H]AFB_1 (sp act. 0.2 Ci/mmol; 200 μM final concentration) was added and reacted at a final volume of 1 mL as described above. After incubation at 37 °C for 60 min, the DNA was extracted with phenol–chloroform–isoamyl alcohol (50:50:1 v/v) under slightly acidic conditions and precipitated 3 times with cold ethanol. The phenol extraction was completed within ~1 h. The [^{3}H]AFB_1 modified DNA preparations were divided into small aliquots, evaporated to dryness, and stored at −20 °C. Under these storage conditions, no change in the adduct distribution was observed over a period of 9 months.

Hydrolysis of [^{3}H]AFB_1–DNA. Samples of [^{3}H]AFB_1–DNA (100 μg/mL) were incubated at 37 °C in 0.01 M sodium phosphate–0.1 M sodium chloride–0.01 M sodium citrate (PSC), pH 6.7, pH 7.0, or pH 7.3. Samples were removed at different incubation times and cooled in ice, and the DNA was precipitated by the addition of 3 volumes of cold 95% ethanol. The ethanol-soluble material was separated from the precipitate by centrifugation. Aliquots of the ethanol-soluble fractions were counted directly in Aquassure, and the remainder of the samples were then flash-evaporated to dryness. The difference in the radioactivity content of the ethanol-soluble fractions before and after flash-evaporation is presumed to be due to [^{3}H]H_2O formed by the exchange of ^{3}H from [^{3}H]AFB_1. The radioactivity that was lost in this manner accounted for approximately 5% of total ^{3}H radioactivity for every 24 h of incubation at 37 °C. Both the ethanol-precipitated DNA and the ethanol-soluble material were stored at −20 °C before they were further analyzed.

For LC analysis of the AFB_1 adducts released from the DNA under the above hydrolysis conditions, the ethanol-soluble material was dissolved in 0.2 mL of 95% ethanol–1-propanol–water (10:5:85 v/v; solvent I).

For analysis of AFB_1 adducts in the ethanol-precipitable DNA, the samples were incubated in 0.2 mL of 0.15 N HCl at 100 °C for 15 min (Lin et al., 1977). The samples were then adjusted to pH 4 with 2 N NaOH before injecting 0.1-mL aliquots into the LC apparatus for analysis.

Results

Identification of Adducts in Acid Hydrolysates of AFB_1–DNA Prepared in Vitro. The rat liver microsome mediated binding of AFB_1 to DNA resulted in adduct levels of one AFB_1 moiety per 8000 and 32 nucleotides at 2 and 200 μM AFB_1, respectively. These levels of modification are comparable to those obtained by other investigators under similar conditions (Garner, 1973; Gurtoo & Dave, 1975; Essigmann et al., 1977).

For routine analysis the [^{3}H]AFB_1–DNA preparations were hydrolyzed with 0.15 N HCl, and the neutralized hydrolysates were chromatographed on a μBondapak C_{18} column by LC. Since mild acid treatment is being used for the release of AFB_1 adducts from DNA for LC analysis, it is conceivable that the released material contains secondary products with structures which differ from the primary adducts attached to DNA. Therefore, in the following we refer to the primary adducts in DNA as "precursors" to the products contained in the

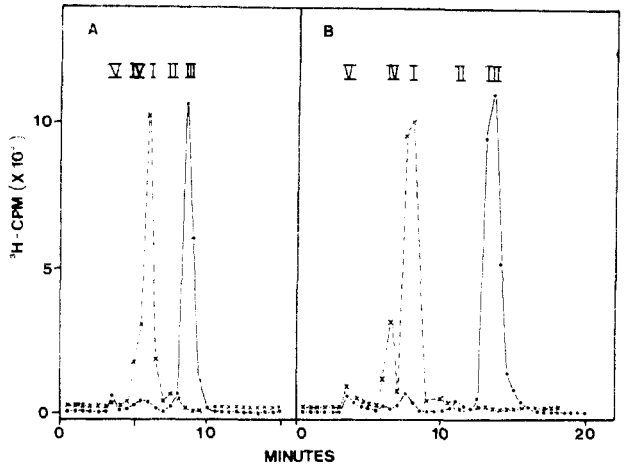

FIGURE 1: LC of acid hydrolysates of [^{3}H]AFB$_1$–DNA. The [^{3}H]AFB$_1$–DNA was prepared by the microsome-mediated reaction of [^{3}H]AFB$_1$ with calf thymus DNA. (A) LC with solvent II. (●) No preincubation before acid hydrolysis; (×) preincubation for 30 min at pH 9.6 at 22 °C before acid hydrolysis of the ethanol-precipitable fraction. (B) LC with solvent I. (●) No preincubation before acid hydrolysis; (×) preincubation for 30 min in a carbonate buffer, pH 9.6, at 22 °C before acid hydrolysis of the ethanol-precipitable fraction.

hydrolysis mixture. With this, we do not imply that the structures of the precursors and their hydrolysis products are necessarily different, however. Throughout this paper the numbering system of Lin et al. (1977) is adopted for the LC peaks in order to facilitate correlations. A series of chromatograms was developed with solvent II (Lin et al., 1977). As shown in Figure 1A (solid curve), ~90% of the radioactivity eluted with a retention time of 8.5 min (peak III), identical with that of the authentic marker AFB$_1$-N^7-Gua. The remaining 10% of radioactivity eluted at 3.5 min (peak V) and 5–6 min (peak I), respectively. Higher resolution was obtained with solvent I. As shown in Figure 1B (solid curve), five peaks were discernible. Comparison with authentic markers allowed the identification of peak II as AFB$_1$-dhd and of peak III as AFB$_1$-N^7-Gua. Incubation of AFB$_1$–DNA in a carbonate buffer, pH 9.6, for 30 min at room temperature before analysis of the acid hydrolysates of the ethanol-precipitable material lead to the disappearance of peak III and concomitant increases in the radioactivity content of peaks I and IV (see dashed curve in Figure 1B). Continuation of the incubation at pH 7.0 or 7.3 for 48 h did not lead to any change in the chromatographic profile. On the basis of this result and the observed retention times, it appears most likely that our peaks I and IV correspond to the peaks with the same numerals of Lin et al. (1977) for which these investigators have proposed the structures of 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B$_1$ (AFB$_1$-triamino-Py) and 2,3-dihydro-2-(8,9-dihydro-8-hydroxy-7-guanyl)-3-hydroxyaflatoxin B$_1$, respectively. It should be noted that our retention times for peaks I and IV were somewhat shorter than those of Lin et al. (1977). This is attributed to the known variability between individual μBondapak C$_{18}$ columns. Shorter retention times were apparently also obtained with two additional μBondapak C$_{18}$ columns by Lin et al. (1977). The identity of peak V material remains unknown.

Hydrolysis of AFB$_1$–DNA at pH 7. According to LC and Sephadex LH-20 analysis, incubation of [^{3}H]AFB$_1$–DNA in PSC buffer, pH 7.0, resulted in a rapid loss of the adducts which are released by acid treatment in the form of AFB$_1$-N^7-Gua (Wang & Cerutti, 1979). In the following these primary adducts in DNA are referred to as "precursors to

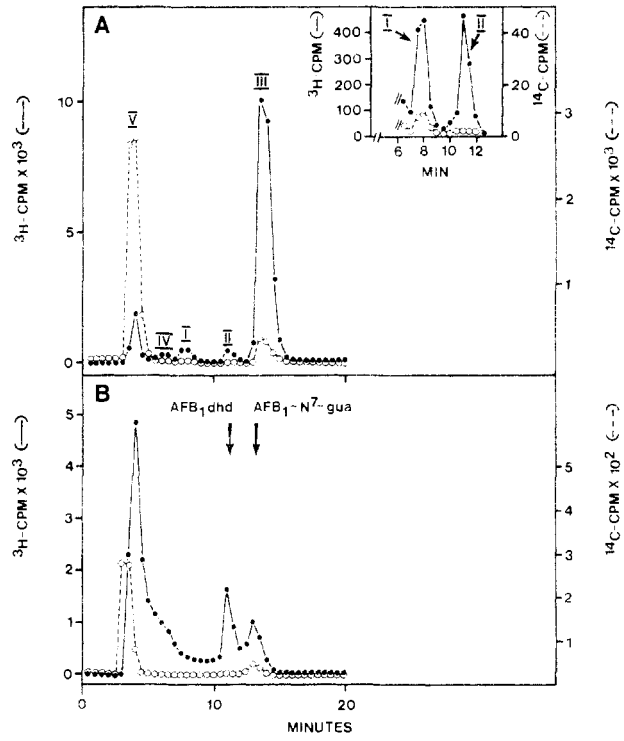

FIGURE 2: Analysis of [^{3}H]AFB$_1$–DNA–[^{14}C]guanine, i.e., DNA selectively labeled in [^{14}C]guanine and reacted with [^{3}H]AFB$_1$ in the presence of microsomes by LC. (A) Acid hydrolysates of the ethanol-precipitable material. Inset: expanded scale of the same chromatogram from 6- to 12-min retention time. (B) Ethanol-soluble fraction from [^{3}H]AFB$_1$–DNA–[^{14}C]guanine after incubation in PSC buffer, pH 7.0, at 37 °C for 6 h.

AFB$_1$-N^7-Gua". LC analysis revealed that the disappearance of precursors to AFB$_1$-N^7-Gua from the DNA was accompanied by small increases of peak I and peak IV in the acid hydrolysates of the alcohol-precipitable DNA, but the majority of ^{3}H radioactivity appeared in the ethanol-soluble fraction. When the ethanol-soluble material was analyzed by LC, only a small fraction of the ethanol-soluble radioactivity was AFB$_1$-N^7-Gua and a major portion of the radioactivity remained unidentified (Wang & Cerutti, 1979).

In order to further characterize the reactions of AFB$_1$–DNA in vitro, we reacted DNA selectively labeled by [8-^{14}C]guanine with [^{3}H]AFB$_1$ in the presence of rat liver microsomes. Covalent adducts between guanine and AFB$_1$ in such a preparation are labeled with ^{14}C and ^{3}H. Figure 2A and the inset to Figure 2A show the LC profiles of the acid hydrolysates of a [^{3}H]AFB$_1$–DNA–[^{14}C]guanine preparation which contained one AFB$_1$ residue for every 32 deoxynucleotides. As expected, the ^{3}H profile corresponds to that shown in Figure 1B and contains five peaks. Only peaks V, I, and III also contain significant amounts of ^{14}C radioactivity. From the specific activities of the starting materials, it follows that peaks I and III contain approximately equal amounts of the AFB$_1$ moiety and of guanine. These results further support the assignment of these peaks to AFB$_1$–guanine adducts, i.e., peak I to the putative AFB$_1$-triamino-Py and peak III to AFB$_1$-N^7-Gua. Peak V contains all the materials which are not retained on the μBondapak C$_{18}$ column, most importantly [^{14}C]guanine. The radioactivity in peak IV was insufficient to allow conclusions about its identity.

Incubation of the same preparation of [^{3}H]AFB$_1$–DNA–[^{14}C]guanine in a PSC buffer, pH 7.0, at 37 °C for 6, 12, and 24 h resulted in the release of 35, 52, and 69% of ^{3}H radioactivity into the ethanol-soluble fraction. Approximately

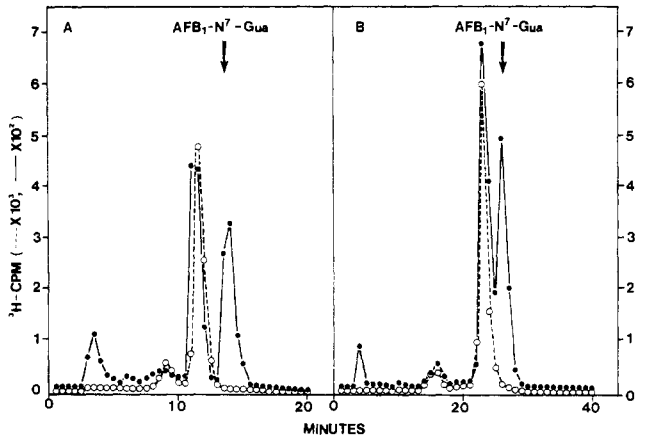

FIGURE 3: LC analysis of [^{3}H]AFB_1-dhd and the ethanol-soluble fraction of [^{3}H]AFB_1–DNA following incubation at pH 7 for 30 min at 37 °C. Before chromatography, the samples were kept for 1 h at pH 5 at 22 °C. (A) LC with solvent I. (○) [^{3}H]AFB_1-dhd; (●) [^{3}H]AFB_1–DNA. (B) LC with solvent III. (○) [^{3}H]AFB_1-dhd; (●) [^{3}H]AFB_1–DNA.

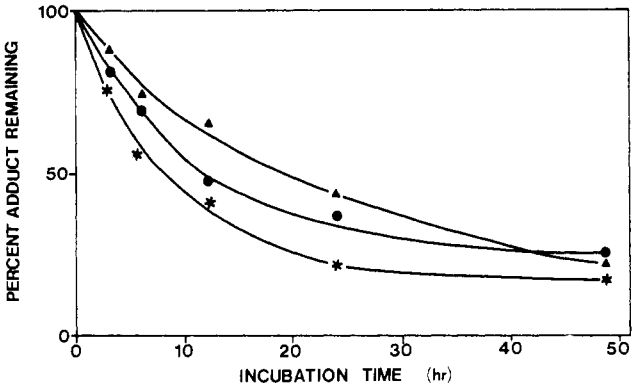

FIGURE 4: Kinetics of disappearance of total adducts from AFB_1–DNA. [^{3}H]AFB_1–DNA was incubated in PSC buffer, pH 6.7, 7.0, and 7.3, at 37 °C. Aliquots were withdrawn after different lengths of incubation, and the ^{3}H content was determined in the ethanol-precipitable fractions. The data are plotted as percent radioactivity remaining in alcohol-precipitable form as a function of incubation time. (▲) pH 6.7; (●) pH 7.0; (*) pH 7.3.

4% of the total ^{14}C radioactivity was also recovered in the ethanol-soluble fraction and stayed constant with increasing incubation times. Figure 2B shows the LC profile of the ethanol-soluble fraction after 6-h incubation. Two minor peaks and one major peak are discernible. The minor ^{3}H peak with a retention time of 13 min contains also ^{14}C radioactivity and corresponds to AFB_1-N^7-Gua. The ^{3}H peak eluting with a retention time of 11 min possesses the chromatographic mobility of AFB_1-dhd. The bulk of the remaining ^{3}H radioactivity was eluted earlier than 8 min as a broad heterogeneous band. The trailing edge of this band in part overlaps with ^{14}C radioactivity eluting from 3 to 3.5 min but for the most part it contains only ^{3}H radioactivity. It is concluded that the AFB_1 moiety which appears in alcohol-soluble form upon incubation of AFB_1–DNA at neutrality is mostly released in free form; i.e., it is no longer attached to guanine. As shown below, the bulk of this ^{3}H radioactivity consists of degradation products of AFB_1-dhd. The ^{14}C radioactivity eluting from 3 to 3.5 min most likely corresponds to alcohol-soluble oligonucleotides containing guanine.

Identification of AFB_1-dhd as the Major Hydrolysis Product of AFB_1–DNA at pH 7.0. Kinetic studies of the hydrolysis of [^{3}H]AFB_1–DNA at neutral pH revealed that AFB_1-dhd appeared transiently in the alcohol-soluble fraction with a maximal yield from 6 to 12 h of incubation at 37 °C. No AFB_1-dhd was detected in the ethanol-soluble fraction after 48 h or longer incubation. Therefore, it was suspected that the majority of the ^{3}H radioactivity containing the free AFB_1 moiety in the ethanol-soluble fraction might consist of polar degradation products of AFB_1-dhd.

AFB_1-dhd is known to be unstable at pH 7.0 or greater (Lin et al., 1978). It has been suggested that AFB_1-dhd may form a dialdehydic phenolate ion (Swenson et al., 1975). In the absence of acceptor molecules such as amines in the incubation milieu, it was possible to re-form AFB_1-dhd from its secondary derivatives by lowering the pH (Swenson et al., 1975; Neal & Colley, 1979). These results were corroborated by the following experiments. When authentic [^{3}H]AFB_1-dhd was incubated in PSC buffer, pH 7, at 37 °C for 30 min, followed by addition of 3 volumes of ethanol and flash-evaporation, i.e., our standard conditions for the analysis of the alcohol-soluble products released from [^{3}H]AFB_1–DNA, ~40% [^{3}H]-AFB_1-dhd remained while 60% of the radioactivity was eluted as a broad peak with short retention times. However, when the samples were adjusted to pH 5 and allowed to stand at room temperature for 1 h in the dark before addition of ethanol and flash-evaporation, 90% of the ^{3}H radioactivity possessed the chromatographic mobility of AFB_1-dhd. The remaining 10% of ^{3}H radioactivity eluted as a single peak with a retention time of 9 min in solvent I and of 16 min in solvent II. These results are shown by the dashed curves in parts A and B of Figure 3. Prolonged incubation of AFB_1-dhd at pH 7.0 resulted in a decrease of the materials which could be reverted to AFB_1-dhd at pH 5. Presumably, the dialdehydic phenolate ion further degraded to compounds that were no longer reversible to AFB_1-dhd.

On the basis of these results with authentic AFB_1-dhd, the following experiments were carried out to test the hypothesis that a major portion of the AFB_1 moiety of AFB_1–DNA was released in the form of AFB_1-dhd at neutrality. [^{3}H]-AFB_1–DNA was incubated in PSC buffer, pH 7, for 30 min at 37 °C and then precipitated with ethanol. The ethanol-soluble fraction was evaporated, and the residue was taken up in pH 5 buffer and incubated for 1 h at room temperature before analysis by LC in solvent systems I and III. The results are given by the solid curves in parts A and B of Figure 3. It is evident that ~60% of the radioactivity now eluted as AFB_1-dhd and 30% eluted as AFB_1-N^7-Gua. The remaining radioactivity possessed a shorter retention time. It is concluded that the disappearance of the precursors to AFB_1-N^7-Gua from DNA under physiological conditions in vitro is mostly due to the release of AFB_1-dhd with concomitant reconstitution in the DNA of an unaltered deoxyguanosine residue and the release of AFB_1-N^7-Gua which results in the formation of an aguaninic site.

Kinetics of the Reactions of AFB_1–DNA from pH 6.7 to pH 7.3. A kinetic study was carried out of the reactions of [^{3}H]AFB_1–DNA in the range from pH 6.7 to pH 7.3 at 37 °C. The [^{3}H]AFB_1–DNA used in these experiments had been prepared by the rat liver microsome mediated reaction of [^{3}H]AFB_1 with calf thymus DNA and contained one covalent AFB_1 adduct per 8000 deoxynucleotides. Of the adducts, 91% were precursors to AFB_1-N^7-Gua; i.e., they could be released in the form of AFB_1-N^7-Gua by mild acid treatment. The following reactions were monitored: (1) loss of total [^{3}H]AFB_1 radioactivity from ethanol-precipitable DNA; (2) disappearance of precursors to AFB_1-N^7-Gua from the ethanol-precipitable DNA; (3) appearance of the putative AFB_1-triamino-Py, i.e., LC peak I, in the acid hydrolysates of the

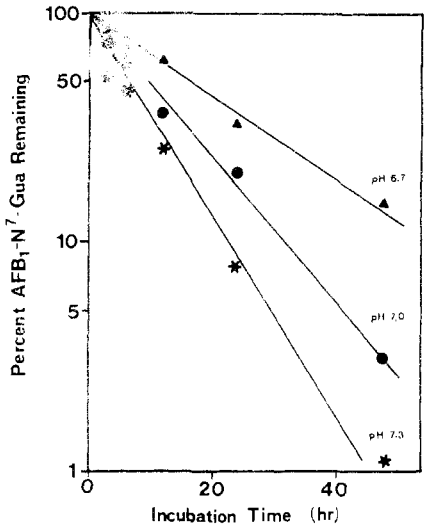

FIGURE 5: Kinetics of disappearance of [^{3}H]AFB$_1$-N^7-Gua from acid hydrolysates of ethanol-precipitable [^{3}H]AFB$_1$–DNA which had been incubated at 37 °C in the range from pH 6.7 to pH 7.3. The incubation conditions were as described in the legend to Figure 4. The acid hydrolysates of the ethanol-precipitable fractions were analyzed by LC for their content in [^{3}H]AFB$_1$-N^7-Gua. The data are plotted as percent [^{3}H]AFB$_1$-N^7-Gua remaining in the acid hydrolysates relative to the amount of [^{3}H]AFB$_1$-N^7-Gua released by acid treatment at 0-h incubation.

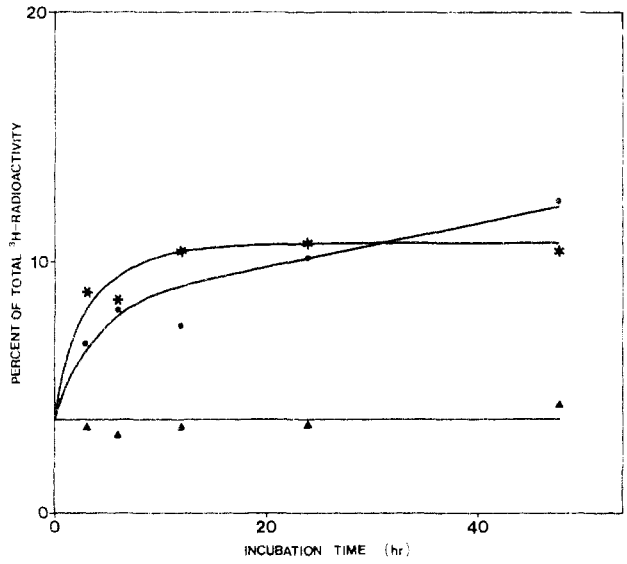

FIGURE 6: Kinetics of appearance of the putative [^{3}H]AFB$_1$-triamino-Py in the acid hydrolysates of ethanol-precipitable [^{3}H]-AFB$_1$–DNA which had been incubated at 37 °C in the range from pH 6.7 to pH 7.3. The incubation conditions were as described in the legend to Figure 4. The acid hydrolysates of the ethanol-precipitable fractions were analyzed by LC for their content in [^{3}H]-AFB$_1$-triamino-Py, i.e., LC peak I. The data are plotted as percent [^{3}H]AFB$_1$-triamino-Py radioactivity present in the acid hydrolysates relative to the total radioactivity in [^{3}H]AFB$_1$–DNA at 0-h incubation. (▲) pH 6.7; (●) pH 7.0; (*) pH 7.3.

ethanol-precipitable DNA; (4) appearance of AFB$_1$-N^7-Gua in the ethanol-soluble fraction; (5) appearance of AFB$_1$-dhd plus its degradation products in the ethanol-soluble fraction. The results are given in Figures 4–7. Figure 4 shows that the initial rates of the loss of the AFB$_1$ moiety from DNA increase from pH 6.7 to pH 7.3. While the kinetics are complex at pH 7.0 and 7.3, they are of pseudo first order at pH 6.7. Similarly, the rates of the disappearance from DNA of precursors to AFB$_1$-N^7-Gua increase from pH 6.7 to pH 7.3. As shown in Figure 5, pseudo-first-order kinetics were

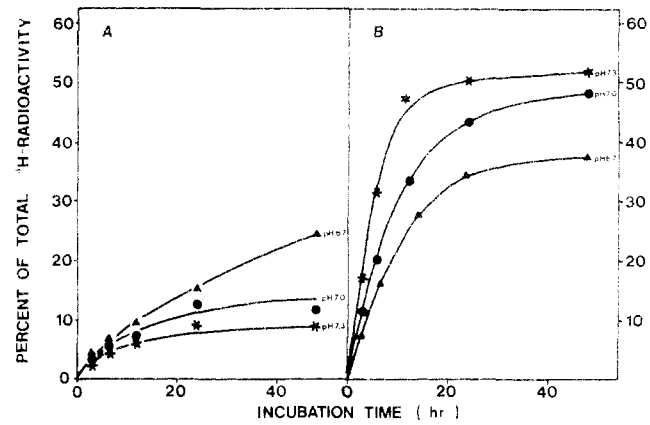

FIGURE 7: Kinetics of appearance of [^{3}H]AFB$_1$-N^7-Gua and [^{3}H]-AFB$_1$-dhd plus its degradation products in the alcohol-soluble fraction upon incubation of [^{3}H]AFB$_1$–DNA at 37 °C in the range from pH 6.7 to pH 7.3. The incubation conditions were as described in the legend to Figure 4. The ethanol-soluble material released from [^{3}H]AFB$_1$–DNA was analyzed by LC. (A) Appearance of [^{3}H]-AFB$_1$-N^7-Gua. The data are plotted as percent [^{3}H]AFB$_1$-N^7-Gua radioactivity present in the alcohol-soluble fraction relative to the total radioactivity in [^{3}H]AFB$_1$–DNA at 0-h incubation. (▲) pH 6.7; (●) pH 7.0; (*) pH 7.3. (B) Appearance of [^{3}H]AFB$_1$-dhd plus its degradation products. The data are plotted as percent [^{3}H]AFB$_1$-dhd plus degradation products radioactivity present in the alcohol-soluble fraction relative to the total radioactivity in [^{3}H]AFB$_1$–DNA at 0-h incubation. (▲) pH 6.7; (●) pH 7.0; (*) pH 7.3.

obtained in this pH range. It should be noted that the kinetics at pH 6.7 are virtually identical with those for the release of the total AFB$_1$ moiety from DNA at the same pH shown in Figure 4. Figure 6 gives the kinetics of the formation of the putative AFB$_1$-triamino-Py in the acid hydrolysates of the ethanol-precipitable DNA as a function of pH. It is evident that the adducts which are released by acid treatment in the form of AFB$_1$-triamino-Py are formed at pH 7.0 and pH 7.3 but not at pH 6.7. These adducts are chemically considerably more stable than the precursors to AFB$_1$-N^7-Gua from which they derive and remain on the DNA over an extended period at pH 7.0 or 7.3 at 37 °C. In Figure 7A the kinetics of the appearance of AFB$_1$-N^7-Gua in the ethanol-soluble fraction are shown. The rate and yield of formation of AFB$_1$-N^7-Gua are highest at pH 6.7. Finally, Figure 7B presents the kinetics of the appearance of AFB$_1$-dhd plus its degradation products in the alcohol-soluble fraction. The rates and final yields of formation increase with increasing pH; i.e., the influence of the pH is the opposite to that for the production of free AFB$_1$-N^7-Gua.

Discussion

Figure 8 gives a synopsis of the three major reactions of AFB$_1$-N^7-Gua in DNA or, more strictly, of "precursor to AFB$_1$-N^7-Gua". This synopsis is based on the assumption that AFB$_1$-N^7-Gua represents the structure of the primary AFB$_1$ adduct in DNA rather than a secondary product of the acid treatment which is used routinely for the analysis of AFB$_1$–DNA. This assumption is reasonable since AFB$_1$-N^7-Gua is also released from AFB$_1$–DNA under very mild physiological conditions at pH 7 and 37 °C (Figure 7A), albeit at a slower rate and in lower yield than under acidic conditions. As depicted in Figure 8, all three reactions lead to the disappearance of the positive charge which is located in the five-membered ring of guanine in AFB$_1$-N^7-deoxyguanylic acid and which appears to be the major reason for the chemical instability of the adduct in DNA. In reaction a the AFB$_1$ moiety is removed in the form of AFB$_1$-dhd by the hydrolytic cleavage of the bond between N^7 of guanine and C^2 of AFB$_1$ and leads to the

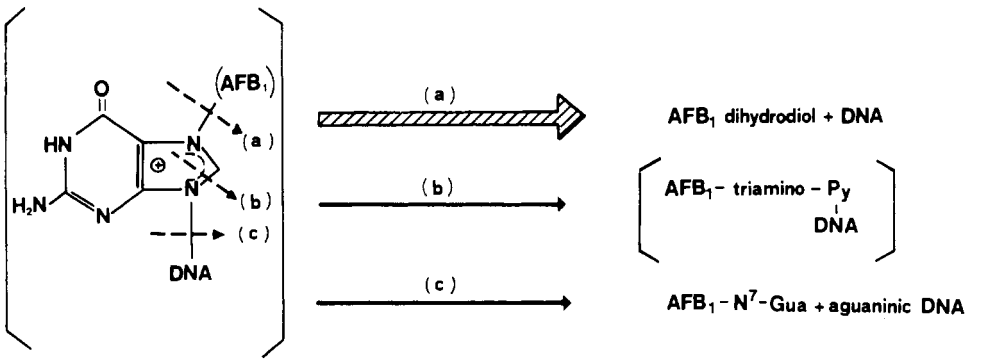

FIGURE 8: Major reactions of AFB_1–DNA under physiological conditions in vitro. It is assumed that the major primary adduct formed by the microsome-mediated reaction of AFB_1 with DNA in vitro possesses the structure of AFB_1–N^7-deoxyguanylic acid.

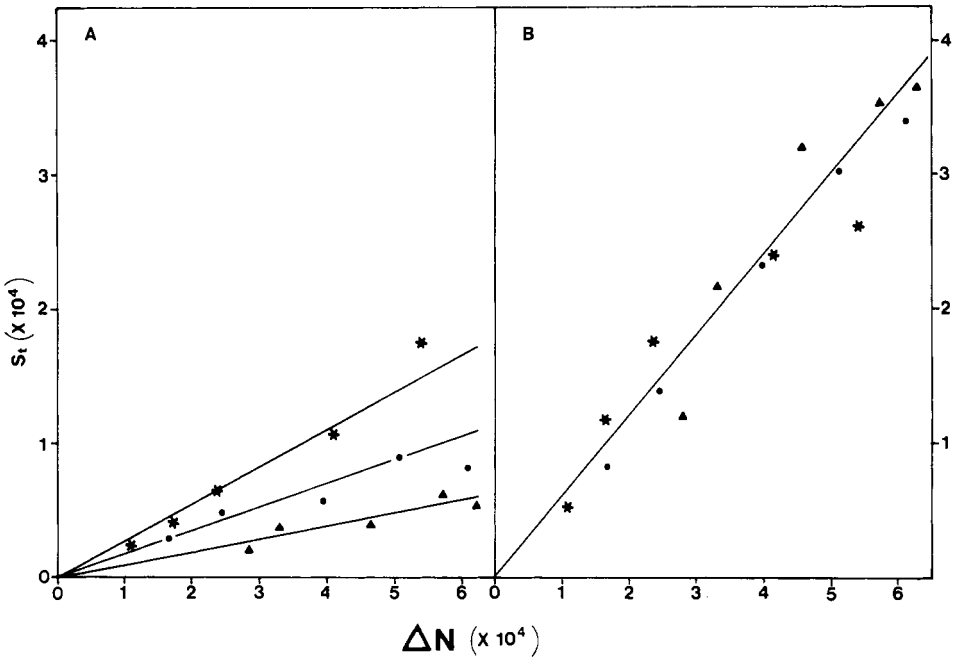

FIGURE 9: Kinetic analysis of the appearance of [^{3}H]AFB_1-N^7-Gua and [^{3}H]AFB_1-dhd plus its degradation products in the alcohol-soluble fraction upon incubation of [^{3}H]AFB_1–DNA at 37 °C in the range from pH 6.7 to pH 7.3. The data given in Figure 7 were transformed according to the equation $S_t = (k/c)\Delta N$ as described in the text. (A) Appearance of [^{3}H]AFB_1-N^7-Gua in the alcohol-soluble fraction. (▲) pH 6.7; (●) pH 7.0; (*) pH 7.3. (B) Appearance of [^{3}H]AFB_1-dhd plus its degradation products in the alcohol-soluble fraction. (▲) pH 6.7; (●) pH 7.0; (*) pH 7.3.

reconstitution of an undamaged guanine residue. In reaction b hydrolytic cleavage of the N^9–C^8 bond of the five-membered guanine ring of AFB_1–N^7-deoxyguanylic acid eliminates the positive charge and generates a secondary lesion with increased chemical stability. This lesion can be released from DNA by acid treatment in the form of the putative AFB_1-triamino-Py of Lin et al. (1977). If AFB_1-triamino-Py indeed turns out to be the correct structure for the material in LC peak I of acid hydrolysates, the possibility still remains that it is produced by the acid treatment from a structurally different precursor adduct in the DNA. Reaction c is the hydrolytic cleavage of the N-glycosylic bond in AFB_1–N^7-deoxyguanylic acid which generates an aguaninic site in DNA. Our results show that reaction a predominates from pH 6.7 to pH 7.3 and that reactions b and c are of comparable importance at pH 7.0 and 7.3. At pH 6.7, reaction b is not observed [cf. Lin et al. (1977)] while reaction c gains in importance. Together, these three reactions account for ~85% of the disappearance of precursor to AFB_1-N^7-Gua from DNA under physiological conditions in vitro. Since some tritium exchange between the [^{3}H]AFB_1 moiety and water takes place under the incubation conditions used in the present work, the above value is a minimal estimate. After incubation of AFB_1–DNA at pH 7.0 and 7.3 for 30–40 h, the DNA mainly contains two secondary lesions of increased chemical stability, namely, the precursors to AFB_1-triamino-Py and aguaninic sites.

From the kinetic data shown in Figure 5, approximate rate constants were derived for the decay of precursor to AFB_1-N^7-Gua in DNA at 37 °C. From pH 6.7 to pH 7.3, the decay is exponential and the pseudo-first-order decay constants c were calculated from the equation $c = \ln (N_t/N_0)/t$, where t is time, N_0 is the initial number of precursors to AFB_1-N^7-Gua in DNA at time 0, and N_t is the number of precursors to AFB_1-N^7-Gua in DNA at time t. The decay constants are 0.045 h^{-1} at pH 6.7, 0.072 h^{-1} at pH 7.0, and 0.10 h^{-1} at pH 7.3. Correspondingly, the half-life times of precursor to AFB_1-N^7-Gua on DNA are ~19, ~12, and ~8 h at pH 6.7, 7.0, and 7.3 at 37 °C. It is evident that AFB_1-N^7-Gua on DNA is considerably less stable than 7-methylguanine with a half-life time of 150 h at pH 7.0 and 37 °C (Lawley, 1975), but it is more stable than the N^7-guanine adduct formed by 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]-pyrene in DNA (Osborne et al., 1978).

From the decay constants for precursor to AFB_1-N^7-Gua

Table I: Approximate Rate Constants of the Major Reactions of AFB_1-DNA under Physiological Conditions in Vitro[a]

formation of	k (h^{-1})		
	pH 6.7	pH 7.0	pH 7.3
AFB_1-N^7-Gua[b]	0.012	0.011	0.009
AFB_1-dhd plus degrad prod[b]	0.027	0.043	0.060
AFB_1-triamino-Py[c]	0.000	0.007	0.01

[a] AFB_1-DNA was prepared by the rat liver microsome mediated reaction of AFB_1 with calf thymus DNA and contained approximately one AFB_1 adduct per 8000 deoxynucleotides. 91% of the adducts could initially be released from the DNA by acid treatment in the form of AFB_1-N^7-Gua. Incubation was in PSC buffers of specified pH at 37 °C. [b] Appearance in the alcohol-soluble fraction. [c] Appearance in acid hydrolysates of the alcohol-precipitable fraction. Approximate initial rates are listed.

on DNA given above and the data contained in Figure 7, approximate rate constants were determined for the release of free AFB_1-N^7-Gua and AFB_1-dhd plus degradation products into the alcohol-soluble fraction. The equation $S_t = (k/c)\Delta N$ was used, in which S_t is the number of products released at time t, k is the rate constant for the formation of S, and $\Delta N = N_0 - N_t$. As shown in Figure 9, linear curves were obtained for plots of S_t as a function of ΔN from pH 6.7 to pH 7.3. From the slopes of the curves, k/c, and the values for c given above, the rate constants k were calculated. The values are given in Table I. Analogous treatment of the data for the formation of the precursor lesions on DNA which yield the putative AFB_1-triamino-Py upon acid hydrolysis (Figure 6) yielded close to linear curves at pH 7.0 and 7.3, but there was considerable variation between individual experiments. The rate constants for this reaction listed in Table I have to be considered as rough estimates, therefore.

Although the half-life time of precursor to AFB_1-N^7-Gua on DNA at neutrality is short, it is long enough relative to the processes of DNA metabolism in mammalian cells that these primary lesions cannot be ignored for the understanding of the biological effects of AFB_1. At later times after AFB_1 exposure, secondary lesions with increased chemical stability, in particular aguaninic sites and precursors to AFB_1-triamino-Py, predominate in the DNA. The relative abundance of these secondary lesions may vary for different tissues, depending on local pH and on the action of enzymes which may catalyze the reactions of the primary lesions on DNA. In addition, repair enzymes may act on both secondary lesions. Evidence has recently been obtained for the active removal of precursors to AFB_1-triamino-Py in human lung cells A549 (Wang & Cerutti, 1979) but not in mouse embryo fibroblasts $10T_{1/2}$ (Wang and Cerutti, unpublished experiments), and apurinic site endonucleases have been isolated from a large variety of tissues.

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