Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5445–5449, September 1981
Biochemistry

In vitro reactions of aflatoxin B_1-adducted DNA

(high-pressure liquid chromatography/carcinogen–nucleic acid interactions)

JOHN D. GROOPMAN*, ROBERT G. CROY†, AND GERALD N. WOGAN‡

Laboratory of Toxicology, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Contributed by Gerald N. Wogan, June 1, 1981

ABSTRACT The chemical stability of aflatoxin B_1 bound to calf thymus DNA was studied over a 48-hour exposure to phosphate buffers at pH 6.8–8.0 (37°C). During this time, aliquots of the aflatoxin B_1-modified DNA were acid-hydrolyzed and analyzed for the presence of 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyflatoxin B_1, 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1, and the tentatively identified, 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl-3-hydroxyflatoxin B_1 and 2,3-dihydro-2-(8,9-dihydro-8-hydroxy-N^7-guanyl)-3-hydroxyaflatoxin B_1. Initial experiments determined the stability of 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 in DNA at levels of modification of one residue per 60 and 1500 nucleotides. The acid-hydrolysis products obtained from these modified nucleic acids were qualitatively similar, but their proportional concentrations were different. These quantitative differences were dependent upon both pH and the initial level of modification of the DNA. During the first 6 hr of incubation, under all conditions examined, the formation of 2,3-dihydro-2,3-dihydroxyaflatoxin B_1 was responsible for the initial decrease of the 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 adduct in DNA. After 48 hr of incubation at pH 7.0, the major reaction of the modified DNA was depurination of the 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 adduct. However, at pH 8.0, the predominant reaction product formed in 48 hr was the putative 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxy-aflatoxin B_1. The putative DNA-bound products resulting from the elimination of the positive charge in the imidazole ring of the aflatoxin-N^7-guanine adduct [2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxy-aflatoxin B_1 and 2,3-dihydro-2-(8,9-dihydro-8-hydroxy-N^7-guanyl)-3-hydroxyaflatoxin B_1] were found to be stable in DNA for at least 24 hr at both pH 6.8 and 7.4. Taken together with observed patterns of stability of aflatoxin B_1 adducts *in vivo*, these observations strongly suggest the involvement of enzymatic repair processes in removal of the N^7-guanyl adduct and also emphasize the possible biological significance of the stable imidazole ring-opened adduct.

The aflatoxins are produced as secondary fungal metabolites by specific strains of *Aspergillus flavus* and *A. parasiticus* and are structurally a group of substituted coumarins containing a fused dihydrofurofuran moiety. Aflatoxin B_1 (AFB_1) is the most biologically potent of these compounds and is toxic, hepatocarcinogenic, and mutagenic in a wide range of organisms (1, 2). This mycotoxin is a consistent contaminant of the human food supply in many areas of the world (3) and is epidemiologically linked to increased incidences of human liver cancer in Asia and Africa (1, 2).

Experimental evidence indicates that many of the biological effects of AFB_1 are mediated through formation of covalent derivatives with cellular macromolecules. Covalent products that are formed in DNA have received particular attention. Modification of DNA by AFB_1 requires metabolic activation by microsomal mixed-function oxidases producing AFB_1-2,3-epoxide. This reactive electrophile attacks the N^7 atom of guanine in the DNA molecule forming an AFB_1-N^7-guanyl adduct. Addition at this position of the purine base in DNA labilizes both the imidazole ring and glycosyl bond. The products formed by the hydrolysis of both these bonds have been identified. Under acidic conditions, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1(AFB_1-N^7-Gua) was found to be released from AFB_1-modified DNA. Exposure of AFB_1-DNA to alkaline conditions prior to acid hydrolysis produced two products believed to be formed by hydrolysis of the imidazole ring of the N^7-substituted guanine moiety, thus producing a substituted pyrimidine in the DNA molecule still containing the AFB_1 moiety. Structures for these products have been proposed (4). Investigations in rat liver and cell culture have shown that these products are formed under physiological conditions and, in contrast to the principal AFB_1-N^7-Gua adduct, they are not readily removed from DNA (5, 6).

In rat liver ≈20% of the AFB_1-N^7-Gua formed initially was converted to these persistent products whereas 70% was removed during a 24-hr period (6). The mechanism of removal of the principal adduct *in vivo* is not known. Recent experiments *in vitro* have indicated that the spontaneous hydrolysis of both the glycosyl bond, releasing AFB_1-N^7-Gua, and the aflatoxin-guanine bonds, forming 2,3-dihydro-2,3-dihydroxyaflatoxin B_1 (AFB_1-diol) and unmodified DNA, may play a predominent role in this process (7, 8). The pathways believed to be involved in the removal or transformation of AFB_1-N^7-Gua in DNA are shown in Fig. 1.

We report results of investigations *in vitro* on the stability of aflatoxin adducts in AFB_1-DNA. These studies indicate that the rate of adduct released and their distribution of products formed is dependent upon both the pH of the medium reported (7, 8) and upon the level of modification of the DNA.

MATERIALS AND METHODS

Preparation of AFB_1-Modified DNA. Calf thymus DNA type 1 (Sigma) was adducted by [^{14}C]AFB_1 [specific activity, 180 mCi/mmol, (1 Ci = 3.7×10^{10} becquerels) (Moravek Biochemicals, City of Industry, CA)] with phenobarbital-induced rat liver microsomes and was incubated in Hepes buffer (pH 7.0) as described by Essigmann *et al.* (9). [^{3}H]AFB_1 (specific activity, 20 Ci/mmol; Moravek Biochemicals) was bound to calf thymus DNA in 0.05 M sodium phosphate buffer (pH 6.0) with

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: AFB_1, aflatoxin B_1; AFB_1-N^7 Gua, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-aflatoxin B_1: AFB_1-FAPyr, 2,3-dihydro-2-(N^7-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B_1; AFB_1-diol, 2,3-dihydro-2,3-dihydroxyaflatoxin B_1; HPLC, high-pressure liquid chromatography.

* Present address: Boston University, School of Public Health, Boston, MA 01940.
† Present address: Sidney Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115.
‡ To whom reprint requests should be addressed.

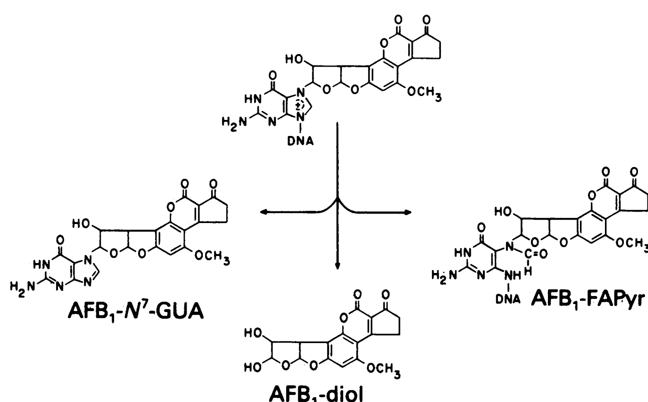

FIG. 1. Summary of the three major acid hydrolysis pathways of AFB_1-N^7-Gua-modified DNA.

m-chloroperoxybenzoic acid (Sigma) as a chemical catalyst; this procedure was adapted from that by Garner *et al.* (10). After either microsomal or *m*-chloroperoxybenzoic acid incubation, an equal volume of $CHCl_3$/isoamyl alcohol, 24:1 (vol/vol), was added, and the two phases were shaken vigorously for 20 min at room temperature. The aqueous and organic phases were separated by centrifugation at 7,000 × *g* for 10 min and the aqueous phase was isolated and extracted a second time. Nucleic acids were precipitated from the aqueous phase with three vol of cold ethanol, spun onto glass rods, washed twice in ethanol, and dried *in vacuo*.

Hydrolysis of DNA. Nucleic acids were adjusted to 0.15 M HCl and treated for 15 min at 90–95°C as detailed by Lin *et al.* (4). This procedure releases >90% of the covalently bound radioactivity from the modified DNA. Hydrolysates were rapidly cooled on ice, neutralized with 1 M KOAc (pH 5.0) and 1 M KOH, and, after adding methanol to 10% (vol/vol), applied to a C_{18} Sep-Pak column (Waters Associates). The Sep-Pak was washed with 10% methanol to remove unhydrolyzed DNA and other polar compounds and then was eluted with 80% methanol to release the more lipophilic aflatoxin derivatives. The methanol was removed from these samples by rotary evaporation under reduced pressure, and the resulting mixture was adjusted to 20% (vol/vol) ethanol prior to high-pressure liquid chromatography (HPLC).

Chromatography. Nucleic acid hydrolysates were analyzed by HPLC with a μ Bondapak C_{18} column (Waters Associates) and a Waters Associates model 204 or a Beckman model 322 liquid chromatograph; both instruments were equipped with U6K injectors (Waters Associates) and model 440 detectors (254 and 365 nm; Waters Associates). Isocratic chromatography was performed at ambient temperature with an elution buffer of 18% redistilled ethanol/20 mM KOAc, pH 5.0, at 1.0 ml/min.

Quantitation of AFB_1 Bound to DNA. The level of AFB_1 modification of calf thymus DNA was determined by both radioactivity and UV spectroscopy. The ^3H and ^{14}C were measured by liquid scintillation with a Beckman model LS 8100 counter. AFB_1-N^7-Gua was quantitated from DNA hydrolysates by using a molar absorbtivity of 18,000 at 360 nm. (4) DNA was determined by UV absorbance at 260 nm and by colorimetry (11) as modified (12).

Conversion of AFB_1-N^7-Gua-Adducted DNA to 2,3-dihydro-2-(N^7-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl-3 hydroxyaflatoxin B_1 (AFB_1-FAPyr)-Modified DNA. AFB_1-N^7-Gua-adducted DNA solutions were adjusted to alkaline conditions by using either 0.1 M carbonate buffer (pH 9.6) or 1 M NaOH to a final concentration of 0.1 M. DNA was incubated in carbonate buffer for 1 hr at ambient temperature or in 0.1 M NaOH for 10 min at 37°C and was readjusted to acidic conditions.

Measurement of AFB_1-N^7-Gua Depurination. Aliquots of [^{14}C]AFB_1-modified DNA at a level of one AFB_1 residue per 1500 nucleotides were dissolved on 0.067 M Sorensen's phosphate buffer at pH 7.0 and 8.0 (DNA concentration, ≈0.5 mg/ml) and incubated for 0, 6, 12, 24, and 48 hr at 37°C. At each time point, two 0.5-ml samples were removed, and one aliquot was incubated in base to convert the bound AFB_1-N^7-Gua to bound AFB_1-FAPyr derivatives; the other sample remained untreated. Both were acid hydrolyzed and analyzed by HPLC.

Stability of AFB_1 Bound to DNA. [^3H]AFB_1-modified DNAs with one AFB_1 residue per 60 and 1500 nucleotides were dissolved in triplicate in 0.1 M sodium phosphate at pH 6.8 and 7.4 and incubated for 0, 2, 6, 16, and 24 hr. At each time point the samples were immediately acid hydrolyzed and submitted to HPLC analysis.

RESULTS

Stability of AFB_1-N^7-Gua in DNA. Calf thymus DNA was modified by AFB_1 to levels of either one adduct per 60 or 1500 nucleotide residues. Hydrolysis and HPLC analysis determined that 95% of the bound AFB_1 was present as AFB_1-N^7-Gua in DNA modified at both levels; small amounts of AFB_1-FAPyr derivatives were initially present.

The AFB_1-modified DNAs were incubated in 0.1 M phosphate buffers representing a four-fold difference in acidity (at pH 6.8 and 7.4) for up to 24 hr at 37°C. At each time point, aliquots were analyzed for AFB_1-N^7-Gua, AFB_1-diol, and AFB_1-FAPyr derivatives. The products formed at pH 6.8 from the highly adducted and less-adducted AFB_1-DNAs are qualitatively identical, but quantitative differences are apparent in their distribution (Figs. 2*A* and 3*A*). Small amounts of AFB_1-FAPyr derivatives were formed in both experiments; however, the appearance of AF-diol and the disappearance of AFB_1-N^7-Gua is greater in the more extensively modified DNA; approximately twice as much AFB_1-N^7-Gua was lost in the DNA modified at a level of one AFB_1 derivative per 60 nucleotides (Fig. 2*A*) as in the less-modified DNA (Fig. 3*A*). The AFB_1 products and their distributions formed after incubation of AFB_1-DNAs at pH 7.4 are shown in Figs. 2*B* and 3*B*. Comparison of the kinetics of formation of AFB_1 products from the DNAs reveals the similar relationship to the one seen at pH 6.8; greater lability of AFB_1-N^7-Gua in the more highly adducted DNA. The major product formed from AFB_1-N^7-Gua under these conditions was AFB_1-diol, which reached a maximum level at 16 hr. The decrease in the amount of AFB_1-diol after this time is attributed to its sensitivity to basic conditions, resulting in the formation of a series of uncharacterized aldehydic derivatives represented by the increasing amount of unknown products (4). By 24 hr at pH 7.4, twice as much AFB_1-N^7-Gua disappeared from the DNA with one AFB_1 per 60 bases (Fig. 2*B*) as from DNA with one AFB_1 per 1500 bases (Fig. 3*B*). Approximately identical amounts of AFB_1-FAPyr derivatives were formed in either DNA at pH 7.4.

Rate of Spontaneous Depurination of AFB_1-N^7-Gua. The results reported above indicate the chemical stability of AFB_1-N^7-Gua in relation to its transformation to other chromatographically separable products. However, they do not reveal the amount of nucleic acid-bound AFB_1-N^7-Gua that is removed from the DNA molecule during the incubation. We took advantage of the differential reactivity of covalently bound and free AFB_1-N^7-Gua adducts to alkaline conditions to estimate its rate of loss from DNA by depurination. DNA-bound AFB_1-N^7-Gua due to the positive charge on the imidazole ring of guanine is rapidly converted to AFB_1-FAPyr derivatives in the presence

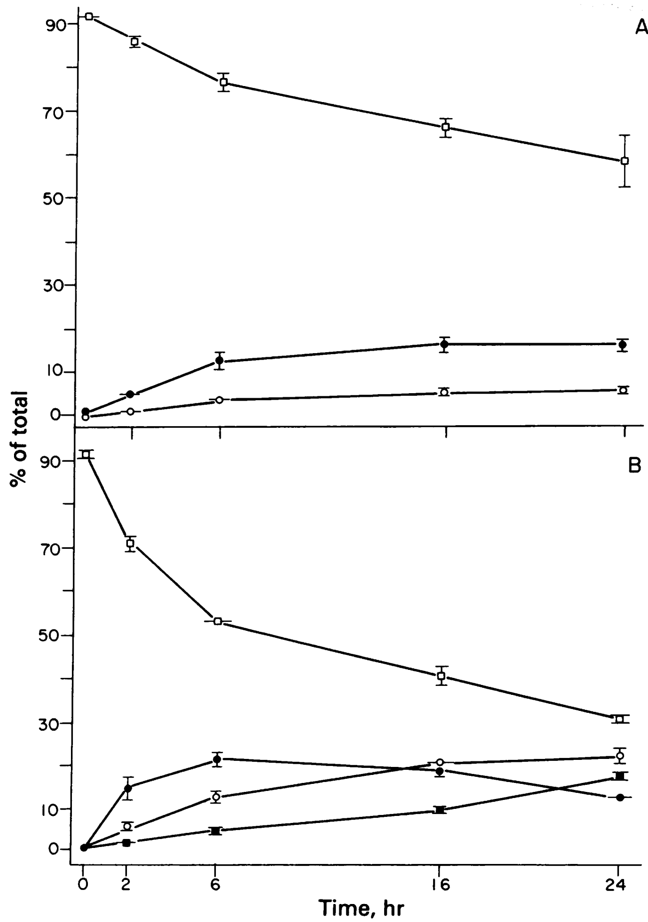

FIG. 2. Time course of the AFB$_1$-N^7-Gua-DNA derivatives produced from DNA adducted at a level of one AFB$_1$ residue per 60 nucleotides and incubated at pH 6.8 (*A*) and pH 7.4 (*B*). □, AFB$_1$-N^7-Gua; ●, AFB$_1$-diol; ○, AFB$_1$-FAPyr; ■, unknown.

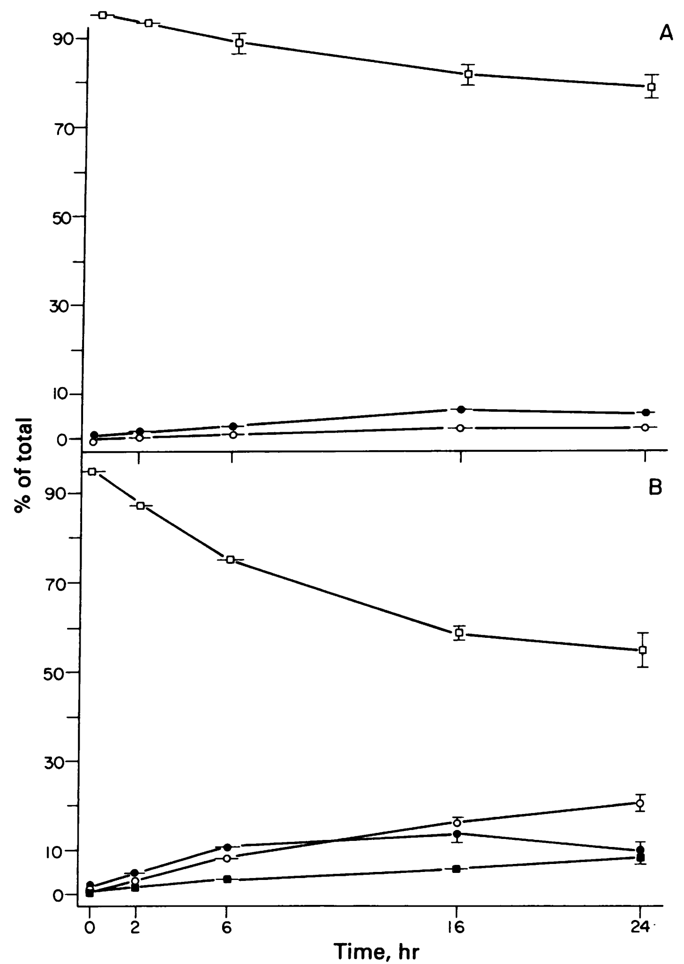

FIG. 3. Time course of the AFB$_1$-N^7-Gua-DNA derivatives produced from DNA modified at a level of the AFB$_1$ residue per 1500 nucleotides and incubated at pH 6.8 (*A*) and pH 7.4 (*B*). □, AFB$_1$-N^7-Gua; ●, AFB$_1$-diol; ○, AFB$_1$-FAPyr; ■, unknown.

of base. Free AFB$_1$-N^7-Gua is not affected by this treatment. In these experiments, one aliquot of the AFB$_1$-DNA incubation mixture was treated with base prior to acid hydrolysis, changing all bound AFB$_1$-N^7-Gua to AFB$_1$-FAPyr products. Acid hydrolysis of another aliquot without prior treatment released any bound AFB$_1$-N^7-Gua and AFB$_1$-FAPyr derivatives that had formed spontaneously during incubation. Therefore, the amount of AFB$_1$-N^7-Gua in the base-treated sample indicated the quantity of AFB$_1$-N^7-Gua released from the DNA molecule by spontaneous hydrolysis of the glycosyl bond, and the amount of AFB$_1$-FAPyr derivatives in the acid-hydrolyzed sample represented the amount of AFB$_1$-N^7-Gua converted to these products during the incubation period. The experiments were performed only with DNA modified at the lower level (one AFB$_1$ per 1500 bases). [^{14}C]AFB$_1$-DNA was incubated at pH 7.0 or 8.0 in 0.067 M phosphate buffer for the times indicated in Fig. 4.

As shown by the previous experiments, the distribution of products and their kinetics of formation are dependent upon pH. At pH 7.0 (Fig. 4*A*) the half-life for the hydrolysis of the glycosyl bond, releasing the modified base, was approximately 60 hr. The rate of the competing reaction, scission of the imidazole ring was much lower; only 4% of the AFB$_1$-N^7-Gua adduct was converted to AFB$_1$-FAPyr derivatives during the 48-hr period. In contrast, at pH 8.0 (Fig. 4*B*) formation of AFB$_1$-FAPyr derivatives was rapid, and little hydrolysis of the glycosyl bond occurred after 6 hr. At no time during the incubation under either condition was AFB$_1$-diol or other unidentified products determined to account for 5–10% of the total radioactivity present in the incubation mixture. Recovery of ^{14}C activity from the hydrolyzed incubation mixtures was ≥95% in all cases.

Stability of "AFB$_1$-FAPyr" Derivatives in DNA. The stability of the products of scission of the imidazole ring of the 7-substituted guanine moiety in DNA was also examined. [^{3}H]AFB$_1$-DNA with either one adduct per 60 or one adduct per 1500 nucleotides was exposed to alkaline conditions to convert bound AFB$_1$-N^7-Gua to AFB$_1$-FAPyr products that were still covalently bound to the DNA molecule. The modified nucleic acids were then incubated at either pH 7.4 or 6.8 in 0.1 M phosphate buffer at 37°C (Table 1). These data suggest that elimination of the positive charge on the imidazole ring of the 7-substituted guanine moiety in the DNA molecule results in the formation of a product in DNA that is stable to these incubation conditions. At each point, 85% of the acid-hydrolyzed products were the AFB$_1$-FAPyr derivatives. AFB$_1$-diol was present and represented 2–6% of total radioactivity. The principal product, believed to be AFB$_1$-FAPyr represented 68–79% of the total AFB$_1$ derivatives. A minor product, which is believed to be derived from this adduct, represented 14–17%. It is significant that there was no change in the relative amounts of these derivatives during the course of this experiment. It has not been determined whether the formation of the minor product occurs in

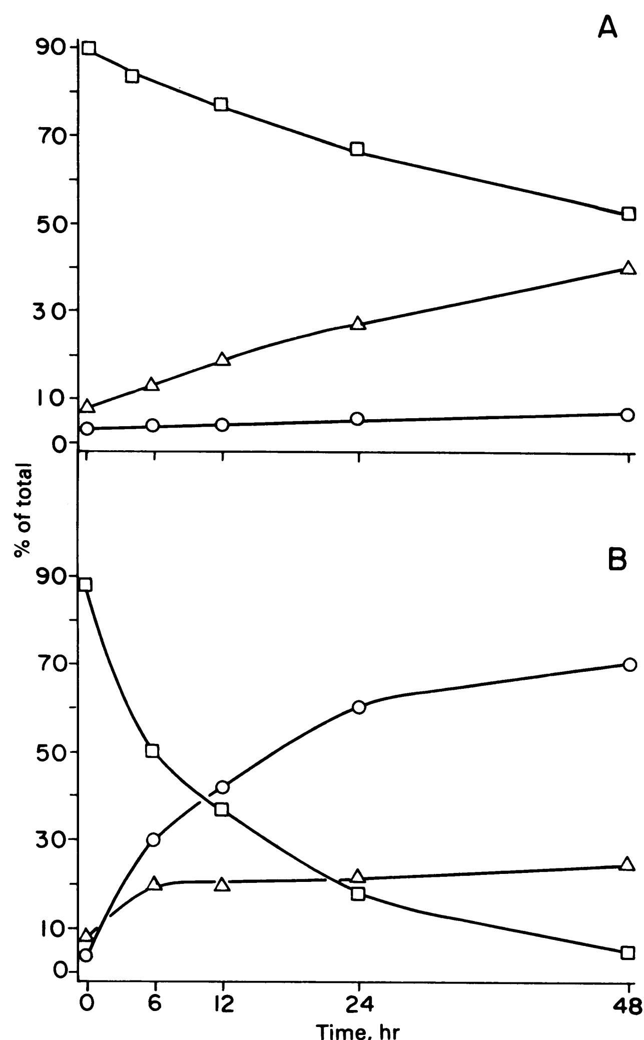

FIG. 4. Time course of the reaction products formed by AFB$_1$-N^7-Gua-adducted DNA (1 adduct per 1500 bases) and incubated at pH 7.0 (*A*) and pH 8.0 (*B*), for up to 48 hr at 37°C. □, Bound AFB$_1$-N^7-Gua; △, free AFB$_1$-N^7-Gua; ○, AFB$_1$-FAPyr.

DISCUSSION

Examination of the acid hydrolysis products of AFB$_1$-modified calf thymus DNA exposed to mild chemical conditions reveals that the quantitative distribution of the resulting products is dependent upon the pH of the aqueous environment and the level of modification of the DNA.

At neutral or slightly acidic pHs and at low levels of modification (one adduct per 1500 bases), the AFB$_1$-N^7-Gua lesion was quite stable. Its half-life was approximately 100 hr. Increasing the level of DNA modification increased the adduct lability; at one adduct lesion per 60 nucleotides, the half-life was about 48 hr. Alkalinity increased lability and also rates of formation of AFB$_1$-diol and AFB$_1$-FAPyr derivatives to the same extent in the DNAs modified once per 60 bases and per 1500 bases. At pH 7.4 the half-life of AFB$_1$-N^7-Gua in the DNAs of low and high modifications was 60 and 6 hr, respectively. AFB$_1$-diol and AFB$_1$-FAPyr derivatives were the major products formed from AFB$_1$-N^7-Gua in DNA in all environments. The greatest amount of AFB$_1$-diol was produced from highly adducted DNA exposed to slightly alkaline pH. The spontaneous depurination rate of AFB$_1$-N^7-Gua at neutral pH was determined to have a half-life of approximately 50 hrs. The three competing reactions responsible for the removal or transformation of this adduct in DNA is shown in Fig. 1.

These findings are of obvious importance in relation to possible functional effects *in vivo*. Hydrolysis of the glycosidic bond, releasing AFB$_1$-N^7-Gua would produce an apurinic site in the DNA molecule. Transformation of the initial adduct to AFB$_1$-FAPyr by imidazole-ring scission has been shown to produce a persistent lesion in DNA *in vivo* (6). Formation of AFB$_1$-diol would restore the DNA to its original unmodified, undamaged state. Thus, the relative contribution of each of these pathways to ultimate disposition of the initial lesion may play a role in determining the biological consequences of the initial DNA damage by AFB$_1$. At the present time, however, lack of knowledge of the specific localization of AFB$_1$ lesions in DNA in the nucleus and the local environmental conditions to which these lesions are exposed limit one's ability to extrapolate the data reported here to the *in vivo* situation. These data suggest that if AFB$_1$-adduct distribution is nonrandom, as some evidence suggests (13), then the fate of individual adducts will depend upon the proximity of other modified bases. For instance, formation of AFB$_1$-diol in highly modified regions of DNA would presumably restore a greater proportion of modified guanines directly to their original state. However, we must also note that the lower level of modification of DNA investigated here (i.e., one adduct per 1500 bases) is approximately 5 times higher than that produced in rat liver DNA by a sub-acutely toxic dose of AFB$_1$ (14) and 50 times higher than seen in rat liver DNA from animals receiving a carcinogenic dose of AFB$_1$ (6). Therefore, quantitative estimates of the relative importance of the processes investigated here in somatic cells is difficult.

The kinetics of disappearance of AFB$_1$-DNA lesions have been investigated in rat liver (6), and epithieloid human lung cells (5). The half-life of the principal lesion, AFB$_1$-N^7-Gua, from rat liver DNA was determined to be 7.5 hours. Its disappearence resulted from both removal from DNA and transformation to AFB$_1$-FAPyr derivatives. The removal of the principal lesion from rat liver DNA was also found to parallel its excretion into urine as AFB$_1$-N^7-Gua. Approximately 70% of the adduct initially formed in the liver was recovered in the urine 48 hr later (15). These data indicate that spontaneous depurination or enzymatic excision are primarily responsible for adduct removal from DNA. However, because the former process is relatively slow above neutrality (see Fig. 4), then enzymatic excision is considered likely.

Table 1

	Percentage of AFB$_1$-FAPyr in DNA			
	1 adduct/60 bases*		1 adduct/1500 bases*	
Hr	pH 6.8	pH 7.4	pH 6.8	pH 7.4
0	70	72	75	74
2	72	72	75	72
6	73	69	76	76
16	71	68	79	75
24	70	69	79	77
	Percentage of AFB$_1$-8-Hydroxy-N^7-Gua in DNA			
0	15	15	17	15
2	16	15	16	14
6	16	15	16	16
16	16	16	16	15
24	16	16	15	17

AFB$_1$-8-hydroxy-N^7-Gua, 2,3-dihydro-2-(8,9-dihydro-8-hydroxy-N^7-guanyl)-3-hydroxyaflatoxin B$_1$.
* Level of DNA modification.

The physiochemical basis of the phenomenon reported here is not understood at the present time. Increasing levels of DNA modification by the hydrophobic AFB_1 moiety most likely alters DNA conformation, changing the relative accessibility of functional groups or atoms to the aqueous environment. Further investigations are needed to determine the influence of different counter ions and nuclear proteins on the kinetics of these reactions, and their relative importance in the *in vivo* situation.

1. Busby, W. F. & Wogan, G. N. (1979) in *Food Borne Infections and Intoxications*, eds. Reimann, H. P. & Bryan, F. L. (Academic, New York), 2nd ed., pp. 519–610.
2. Wogan, G. N. (1973) in *Methods in Cancer Research*, ed. Busch, H. (Academic, New York), Vol. 7, pp 309–344.
3. Stoloff, L. (1976) in *Mycotoxins and Other Fungal Related Food Problems*, ed. Rodricks, J. V. (American Chemical Society, Washington, DC), pp. 23–50.
4. Lin, J. K., Miller, J. A. & Miller, E. C. (1977) *Cancer Res.* **37**, 4430–4438.
5. Wang, T. V. & Cerutti, P. A. (1979) *Cancer Res.* **39**, 5165–5170.
6. Croy, R. G. & Wogan, G. N. (1981) *Cancer Res.* **41**, 197–203.
7. Wang, T. C. & Cerutti, P. (1980) *Biochemistry* **19**, 1692–1698.
8. Hertzog, P. J., Lindsay-Smith, J. R. & Garner, R. C. (1980) *Carcinogenesis* **1**, 787–794.
9. Essigmann, J. M., Croy, R. G., Nadzan, A. M., Busby, W. F., Reinhold, V. N., Buchi, G. & Wogan, G. N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1870–1874.
10. Garner, R. C., Martin, C. N., Lindsey-Smith, J. R., Coles, B. F. & Tolson, M. K. (1979) *Chem-Biol Inter.* **26**, 57–73.
11. Burton, K. (1956) *Biochem. J.* **62**, 315–323.
12. Giles, K. W. & Meyers, A. (1965) *Nature (London)* **206**, 93.
13. D'Andrea, A. D. & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4120–4124.
14. Groopman, J. D., Busby, W. F. & Wogan, G. N. (1980) *Cancer Res.* **40**, 4343–4351.
15. Bennett, R. A., Essigmann, J. M. & Wogan, G. N. (1981) *Cancer Res.* **41**, 650–654.