INTERACTION OF AFLATOXIN B_1 WITH DNA *IN VIVO* IN THE RAT AND MOUSE

by

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B.S., Clarkson College of Technology
(1970)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

(MAY, 1979)

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May 4, 1979

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ABSTRACT

Methods have been developed for qualitative and quantitative analysis of the AFB_1-hydrolysis products from DNA isolated *in vivo* following exposure to AFB_1. After both chemical and enzymatic hydrolysis procedures, AFB_1 derivatives were analyzed using preparative and analytical reversed-phase liquid chromatography. Thirteen products could be identified and measured. All products which have been characterized so far result from attack of an activated AFB_1 species at the N-7 position of guanine in DNA. The principal product, representing 80 percent of the covalently bound AFB_1, was previously identified as 2,3-dihydro-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N^7-GUA: Essigmann et al., 1977; Lin et al., 1977; Croy et al., 1978). Evidence has been provided indicating that activation of AFB_1 metabolites which have an intact 2,3-vinyl ether bond also occurs. Double label experiments using G[^{3}H] AFB_1 and -O$^{14}CH_3$ AFB_1 indicated that the O-demethylated derivative aflatoxin P_1 is activated and also attacks the N-7 atom of guanine in DNA, forming a covalent product which was found to represent 5 to 7 percent of the covalently bound AFB_1.

The relationship between the formation of covalent products in DNA and species and tissue susceptibility to the acute toxicity of AFB_1 was investigated by the comparison of a highly susceptible species, the male Fischer rat, and a resistant one, the male Swiss mouse. Qualitative analysis revealed no apparent correlation between the spectrum of covalent products formed in DNA and tissue sensitivity to AFB_1. The major product formed in the kidneys and liver of both species was AFB_1-N^7-GUA. Differences in the spectrum of minor products probably reflect the contributions of pathways other than direct epoxidation of the 2,3-bond in the metabolism of AFB_1 by the various organs in either species. Quantitatively, a good correlation was found between the overall level of modification of DNA by AFB_1 and species and tissue susceptibility. After an LD_{50} dose in either species DNA in the liver, the target organ in the rat had the highest level of AFB_1 modification, one AFB_1 adduct per 8000 DNA bases. Kidney DNA was adducted at one-tenth this level. An opposite situation was found in these two organs in the mouse (liver, 1 AFB_1 adduct/440,000 DNA bases; kidney, 1 AFB_1

adduct/140,000 DNA bases), reflecting the different organotropism of AFB_1 in these two species.

Studies *in vitro* investigated the kinetics of activation of AFB_1 by microsomal fractions of the livers of the rat and mouse. These studies suggested that the mouse has a lower capacity to produce covalent AFB_1-DNA products which may be a result of the inactivation of microsomal enzymes by activated AFB_1 derivatives.

Removal of all covalent AFB_1 derivatives from rat liver DNA *in vivo* did not occur at the same rate. Primary N-7 lesions containing a positively charged imidazole ring were rapidly removed from DNA with half-lives of 7 to 11 hours. Secondary lesions, resulting from the hydrolysis of the imidazole ring in the N-7 substituted guanine forming an AFB_1-formamido-pyrimidine derivative, were presistent with half-lives of several days. Alkaline sucrose gradient studies did not reveal the presence of a large number of persistent alkaline labile apurinic sites or single-strand breaks, indicating that efficient repair of damaged sites takes place following the removal of AFB_1 lesions.

Accumulation of persistent secondary lesions was seen when multiple doses of AFB_1 were administered to male Fischer rats. Two hours after a single 25 µg dose of AFB_1, approximately 8.8×10^{-4} µmoles of AFB_1 (1% of initial dose) is covalently bound to liver DNA. Twenty-four hours later 88 percent of this material has been removed while approximately 8 percent of the original AFB_1-N^7-GUA derivatives have been converted to persistent formamido products. After ten 25 µg doses administered over a two week period, it is estimated that 2.7×10^5 persistent lesions were present in each liver cell genome. Most of the accumulation of the products took place during the first five day dosing period. This pattern suggests that induction of detoxifying enzymes reduces the amount of damage to DNA produced by succeeding doses of AFB_1. These observations are discussed in relation to current theories concerning chemical carcinogenesis.

Thesis Supervisor: Gerald N. Wogan
Title: Professor of Toxicology

ACKNOWLEDGEMENTS

The encouragement and assistance of many people helped in the completion of this work. I wish to especially thank Dr. Gerald Wogan for his critical advise and enthusiasm and my thesis comittee Dr.s William Thilly (chairman), Christopher Walsh, and Elliot Alpert for their contributions.

I would also like to thank my comrades in the laboratory for their help and friendship and their ability to withstand the constant harassment to which I have subjected them. The collaborations between John Essigmann and myself have been particularly important in the understanding the chemical relationships between the various AFB_1-DNA adducts.

Additional acknowledgements must be made to: the Whitaker Health Sciences Fund which has provided financial support during the past year; my parents, George and Emma for reasons too numerous to mention; James K. Selkirk with whom I worked at the NIH, for his advise and encouragement to continue my education; Sue Croy for her support and toleration.

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CHAPTER ONE

INTRODUCTION

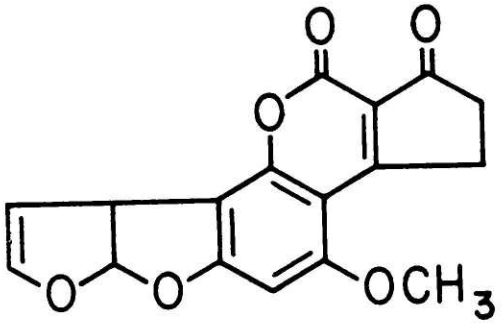
Aflatoxins are a group of toxic compounds produced as secondary metabolites of several species of the genus *Aspergillus*. This soil organism is widely distributed from arctic to tropic regions, and is capable of growth on a wide variety of substrates. These mycotoxins were discovered in contaminated peanut meal as causative agents of "turkey x" disease, which killed thousands of turkeys in England in 1960.

The aflatoxins (Fig. 1-1) are closely related chemically, having a bifurocumarin configuration. The names of these compounds are derived from their ultraviolet fluorescent properties. AFB_1 and AFB_2 fluoresce in the blue portion of the spectrum, while AFG_1 and AFG_2 fluoresce yellow-green. The minor change in chemical structure resulting from the reduction of the 2,3 bond of AFG_1 and AFB_1 to form AFG_2 and AFB_2 produces a dramatic change in biological properties.

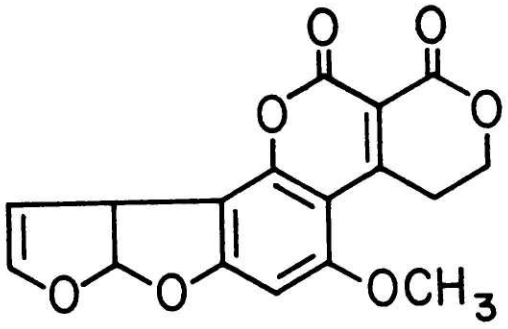
AFB_1 has been shown to be acutely toxic to most animal species although susceptibility varies widely. The duckling and trout are among the most sensitive and the mouse and hamster the least. The relative potency among the four different aflatoxins is illustrated by the oral 7-day LD_{50} values which were obtained in the 1 day old duckling: AFB_1, 18.2 µg; AFB_2 84.8 µg; AFG_1, 39.2 µg; AFB_2, 172.5 µg (Carnaghan et al., 1963). In addition to their acutely toxic effects AFB_1 and AFG_1 have been shown to have carcinogenic properties.

AFB_1, the most potent, has induced hepatocarcinomas in the rat, rainbow trout, ferret, guinea pig, and monkey (Wogan, 1973).

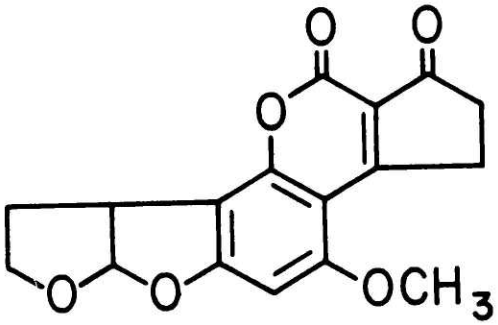
Figure 1-1. Naturally occurring aflatoxins.



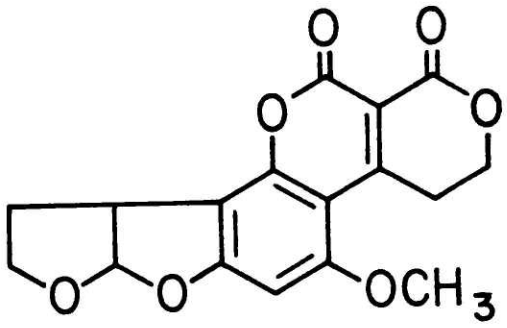
AFB_1



AFG_1



AFB_2



AFG_2

Epidemiological studies have implicated aflatoxins in the etiology of liver cancer in certain human populations (Wogan, 1976). The availability of animal models and association with human disease make the aflatoxins attractive compounds to study for investigations of morphological and biochemical changes associated with chemical carcinogenesis. Such investigations have primarily involved the rat, which is sensitive to both the toxic and carcinogenic effects of AFB_1.

PATHOGENESIS OF AFB_1 IN THE RAT AND MOUSE

In most animal species investigated, pathologic lesions caused by aflatoxin ingestion are primarily associated with the liver. The type and extent of these lesions are dependent upon both amount and schedule of exposure.

The histopathogenesis of acute and chronic AFB_1 administration to the rat has been described by several investigators (Newberne and Wogan, 1968; Butler, 1966; Clifford and Rees, 1967b; Newberne and Butler, 1969). An LD_{50} dose of AFB_1 produced a periportal zone of necrosis which was accompanied by marked biliary proliferation which developed during a three to five day period. Rapid regeneration of parenchymal cells, such as that which accompanies partial hepatectomy, is not seen. Two weeks after this dose, biliary proliferation was still present, along with increased mitotic activity in the parenchymal cells. Parenchymal cells with large hyperchromatic nuclei were present at this time and as late as one month later. They are a prominent feature of both acute and chronic AFB_1 poisoning (Butler, 1964).

The induction of hepatocellular carcinoma in the rat is strongly influenced by the dosage regimen employed. Prolonged and repeated administration of small amounts of AFB_1 are most effective (Wogan and Newberne, 1967). Table 1 lists several dosage regimens which have been evaluted. Further studies have indicated that male rats are more susceptible that female rats when AFB_1 is administered in multiple dosing regimens or at relatively high levels in the diet (e.g., 1.0 ppm). At 1.0 ppm AFB_1 intake per animal prior to tumor appearance was 2.9 mg for

TABLE 1-1

EFFECTIVE CARCINOGENIC REGIMENS FOR AFLATOXIN B_1 IN THE RAT[a]

DOSE OF AFB_1	METHOD OF ADMINISTRATION	PERIOD OF EXPOSURE	TIME OF EARLIEST TUMOR APPEARANCE (wks)	INCIDENCE OF LIVER CARCINOMA
1 ppb	diet	104 wks	104	2/22
50 ppb	diet	82 wks	82	20/25
100 ppb	diet	54 wks	54	28/28
1 ppm	drinking water	10 wks	90	3/10
1 ppm	drinking water	20 wks	90	19/30
1 ppm	diet	54 wks	54	11/11
25 µg	i.g.	40 doses over 8 weeks	--	18/18
32.5 µg	i.g.	40 doses over 8 weeks	--	9/9

[a]Source: Wogan, 1973.

males and 5.9 mg for females. However, at lower dietary levels (e.g., 15 ppb) total intake levels were similar in both (males, 95 μg; females, 115 μg) and equally effective in tumor induction (100%). It is interesting that the formation of preneoplastic lesions in the female at larger doses appeared at approximately the same rate and frequency as in the male. Delayed tumor appearance is apparently the result of slower progression of these lesions to neoplasms (Wogan and Newberne, 1967).

Hepatic cell populations undergo sequential morphologic changes during administration of low levels of AFB_1 in the diet resulting in the development of cancerous lesions (Butler, 1966; Newberne and Wogan, 1968). In a typical response to administration of AFB_1 at a level of 1 ppm in the diet to male Fischer rats, proliferation of oval cells occurred within a few days after initial exposure. The first parenchymal cell changes noted were areas of focal hyperchromatic cells which developed after four weeks. Areas of vacuolated hypertrophic parenchymal cells are sometimes seen, characterizing a degenerative type of lesion which developed subsequently. This was followed by formation of nodules of hyperplastic parenchymal cells unrelated to proliferating bile duct cells and progression of these nodules to neoplasia. These changes are similar, but not idential, to the histopathogenesis of hepatocellular carcinoma induced by other liver carcinogens, such as the azo dyes dimethylaminoazobenzene and N-acetyl-2-aminofluorene (AAF) (Newberne and Wogan, 1968). Although chronic exposure to most other hepatotoxins and carcinogens results in a cirrhotic

response, AFB_1 produces only a slight fibrosis in the rat liver. Differences are also seen in the appearance of bile duct hyperplasia, which occurred within a few days after AFB_1 exposure, but required three to four weeks to appear in rats fed AAF (Reuber, 1965).

A sharp contrast to these responses is observed in the mouse. The LD_{50} dose of AFB_1 in Swiss mice is approximately ten times higher than in rats of the same age and sex (McGuire, 1969). Mice poisoned with AFB_1 show little gross or microscopic evidence of liver damage, but develop hemorrhagic lesions of the kidneys 48 to 72 hours after dosing (Akao et al., 1971). Lethal doses of the toxin fail to induce any biochemical changes in the liver, such as alterations in RNA metabolism as seen in other species (e.g., the rat), but strongly inhibit RNA polymerase activity in the kidney (Akao et al., 1971; Neal, 1972; Godoy and Neal, 1976). Adult Swiss mice are also refractory to the carcinogenic effects of AFB_1. No tumors were observed in mice fed AFB_1 at a level of 150 ppm in the diet for 20 months (Wogan, 1973). Hepatomas were induced in the hybrid progeny of C57 and CH3 mice by a single dose of AFB_1 administered to newbornes (Vesselinovitch et al., 1972).

INVESTIGATIONS OF AFLATOXIN-NUCLEIC ACID INTERACTIONS

The biochemical effects of AFB_1 have been described by investigators in several experimental systems. Nuclear DNA and RNA synthesis are the biochemical processes most sensitive and rapidly affected by AFB_1. Alterations in nucleic acid structure and functions are thought to play an important role in the toxic,

mutagenic, and carcinogenic responses of organisms to the aflatoxins.

Early investigations into the mechanisms of action of the aflatoxins concerned their non-covalent interactions with DNA. AFB_1 was shown to inhibit DNA dependent RNA polymerases and protein synthesis *in vivo* in a temporal sequence which was similar to that of Actinomycin D (Gelboin et al., 1966; Sporn et al., 1966; Clifford and Rees, 1966). This antibiotic is known to interact with the DNA template non-covalently, directly inhibiting DNA dependent RNA synthesis and protein synthesis secondarily. Alterations in the characteristic absorption spectrum of AFB_1 in the presence of calf thymus DNA were similar to the shifts in the Actinomycin D and acridine orange spectra under the same conditions (King and Nicholson, 1969). The weak aflatoxin-DNA complex did not alter the melting temperature of DNA and was dissociated on elution through a Sephadex G50 column (Clifford and Rees, 1967a). A comparison of AFB_1, AFG_1, and AFB_2 showed that the extent of their inhibitory action on RNA and protein synthesis by rat liver slices was proportional to the degree of spectral shift obtained by their interaction with DNA (Clifford et al., 1967). However, investigations with aflatoxin analogs showed that *in vitro* binding to calf thymus DNA did not accurately reflect the *in vivo* potency of these toxins. The analog 5,7-dimethoxycyclopentenone [2,3-C] coumarin was non-toxic to rats at levels more than 200 times higher than the LD_{50} of AFB_1 but had more than twice the affinity of AFB_1 for DNA (Wogan et al., 1971). These results suggested that

binding to DNA was necessary but not sufficient for these compounds to exert their biological effects.

A further lack of correlation between *in vivo* and *in vitro* results was seen during investigations concerning the inhibition of RNA polymerase activity. Incorporation of labeled precursors into RNA was inhibited when AFB_1 was administered *in vivo* or in isolated nuclei or nucleoli (Sporn et al., 1966; Gelboin et al., 1966; Edwards and Wogan, 1960; Moule and Frayssinet, 1968). However, AFB_1 was without effect on RNA synthesis when deoxyribonucleoprotein, isolated rat liver nuclei or nucleoli, or native DNA were exposed to AFB_1 *in vitro* (King and Nicholson, 1967; Clifford and Rees, 1967b; Edwards and Wogan, 1960). DNA synthesis *in vivo* was also rapidly inhibited while DNA polymerase activities assayed *in vitro* were unaffected (DeRecondo, 1966). These results led some investigators to suggest that AFB_1 might be converted to an active metabolite *in vivo* before it exerted its biological effects. DNA dependent RNA synthesis was found to be inhibited *in vitro* by AFB_1 if DNA was exposed to AFB_1 in the presence of a microsomal fraction with appropriate cofactors needed for AFB_1 metabolism (Neal, 1973).

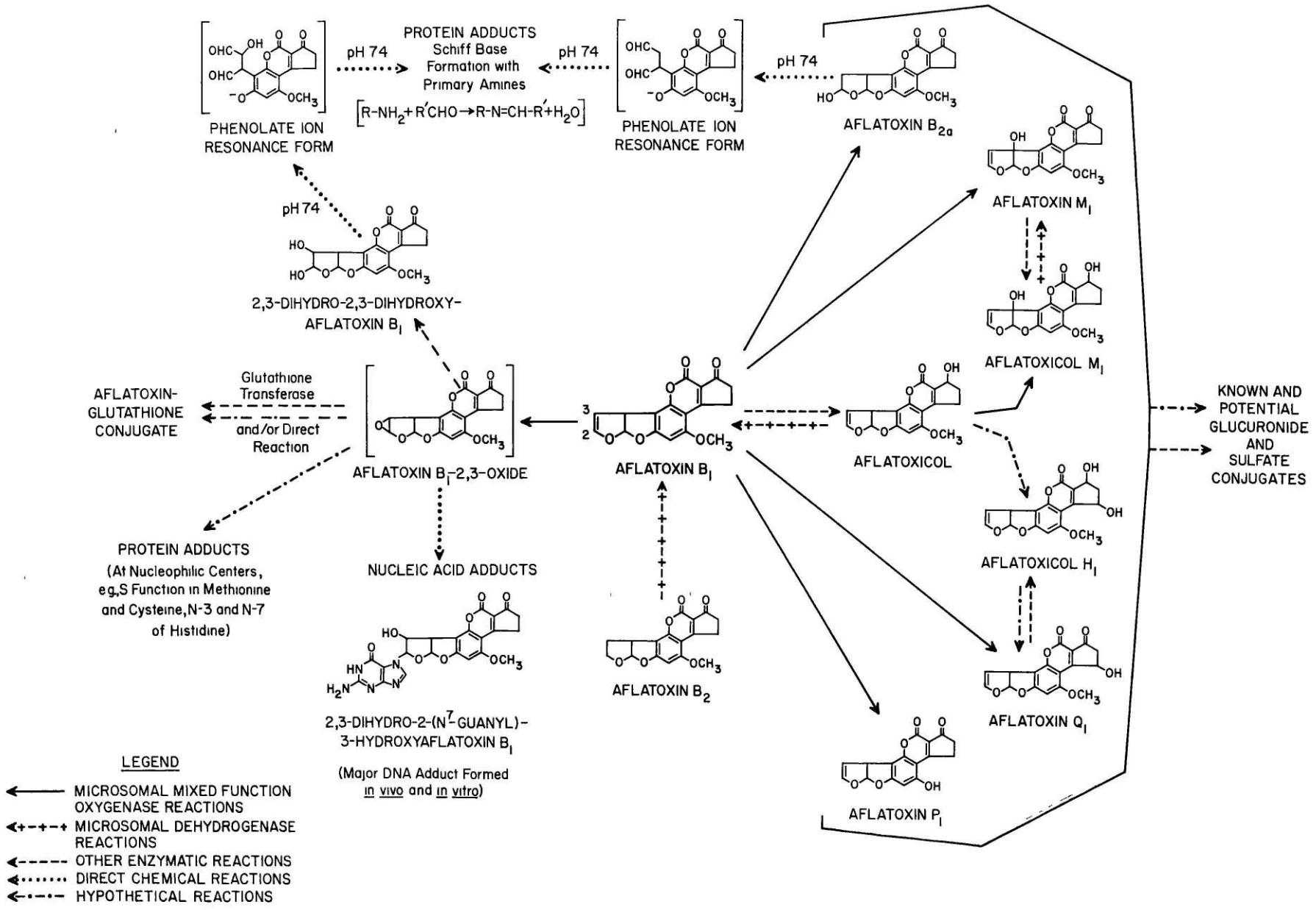
Microsomal metabolism of AFB_1 in the presence of certain strains of *Salmonella*, produced labile derivatives which were toxic and bound to DNA, RNA, and protein (Garner et al., 1972). The DNA-metabolite complex was not separable by gel chromatography. Other investigators demonstrated apparent covalent binding of AFB_1 to DNA when both were present along with a microsomal metabolizing system (Alexandrox et al., 1964; Gurtoo

et al., 1965). Covalent binding of AFB_1 to cellular macromolecules *in vivo* has been investigated using ^3H (Swenson et al., 1977) and ^{14}C (Garner and Wright, 1975) labeled AFB_1. The specific activity of DNA and rRNA in the latter study were found to be 15 and 20 times, respectively, that of protein, indicating a high selectivity of the activated AFB_1 species toward nucleic acids.

Thus, the initial discrepancy between *in vivo* and *in vitro* experiments was explained by the requirement for metabolic activation of the aflatoxins to reactive derivatives which bound covalently to susceptible cellular macromolecules. The variety of pathways by which AFB_1 is metabolized is shown in Figure 1-2. The molecule can undergo reduction (aflatoxicol), hydroxylation (AFM_1, AFQ_1, AFH_1), demethylation (AFP_1), hydration (AFB_{2a}) and epoxidation (AFB_1-2,3-oxide). Subsequent investigations concerning nucleic acid interactions have revealed that epoxidation of the 2,3-vinyl ether bond was the predominant pathway leading to the formation of covalently bound derivatives.

The reduced biological activity of aflatoxin B_2 indicated that the unsaturated 2,3-bond of the terminal furan ring of AFB_1 was important for biological activity. Additional support for this theory was obtained when 2,3-dihydro-2,3-dihydroxy-AFB_1 was identified as an acid hydrolysis product of the covalent derivative(s) formed in RNA by AFB_1 activated by microsomal enzymes (Swenson et al., 1973) and from DNA and RNA isolated from the livers of rats following exposure to AFB_1 (Swenson et al., 1974). This provided evidence that oxidation of the 2,3-bond,

Figure 1-2. Known metabolic pathways of aflatoxin B_1.


PHENOLATE ION RESONANCE FORM
pH 74
PROTEIN ADDUCTS
Schiff Base Formation with Primary Amines
[R-NH2+R'CHO→R-N=CH-R'+H2O]
pH 74
PHENOLATE ION RESONANCE FORM
pH 74
AFLATOXIN B2a
AFLATOXIN M1
AFLATOXICOL M1
AFLATOXICOL H1
AFLATOXIN Q1
AFLATOXIN P1
AFLATOXICOL
AFLATOXIN B1
AFLATOXIN B2
pH 74
2,3-DIHYDRO-2,3-DIHYDROXY-AFLATOXIN B1
AFLATOXIN-GLUTATHIONE CONJUGATE
Glutathione Transferase
and/or Direct Reaction
AFLATOXIN B1-2,3-OXIDE
PROTEIN ADDUCTS
(At Nucleophilic Centers, eg,S Function in Methionine and Cysteine,N-3 and N-7 of Histidine)
NUCLEIC ACID ADDUCTS
2,3-DIHYDRO-2-(N7-GUANYL)-3-HYDROXYAFLATOXIN B1
(Major DNA Adduct Formed in vivo and in vitro)
KNOWN AND POTENTIAL GLUCURONIDE AND SULFATE CONJUGATES
LEGEND
MICROSOMAL MIXED FUNCTION OXYGENASE REACTIONS
MICROSOMAL DEHYDROGENASE REACTIONS
OTHER ENZYMATIC REACTIONS
DIRECT CHEMICAL REACTIONS
HYPOTHETICAL REACTIONS

probably by epoxidation, was necessary for covalent bond formation between these molecules. Further investigations identified the principal adduct formed *in vitro* by AFB_1 in DNA. Acid hydrolysis of the modified DNA liberated a compound identified as 2,3-dihydro-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N^7-GUA). The *trans* configuration of the hydroxyl and guanine moieties attached to the 2,3-bond provided direct evidence that this compound was formed through attack of the nucleophilic N^7 atom of guanine on the β epoxide of AFB_1 (Essigmann et al., 1977; Lin et al., 1977). Further studies *in vivo* indicated that the principal product formed in rat liver DNA after exposure of the animal to AFB_1 was also AFB_1-N^7-GUA (Lin et al., 1977; Croy et al., 1978).

Indirect evidence for the formation of an aflatoxin-adenine derivative of DNA *in vitro* has been obtained by the mapping of alkali-labile sites in partially depurinated DNA following adduction by aflatoxin B_1 (D'Andrea and Haseltine, 1978). However, no AFB_1-adenine derivative has been isolated.

EFFECTS ON NUCLEIC ACID METABOLISM

AFB_1 has been shown to inhibit DNA, RNA, and protein synthesis in susceptible cells. Investigations have indicated that these changes are reversible when non-lethal doses of the toxin are given. Rapid and marked inhibition of RNA polymerase activity occurred in the liver nuclei of rats treated with 1 mg AFB_1/kg body weight; "From 15 minutes to two hours after administration of AFB_1, there was a 35 to 70 percent inhibition of

DNA-directed RNA synthesis. The inhibition was reversed 12 and 24 hours later" (Gelboin et al., 1966). In partially hepatectomized rats nuclear and nucleolar hepatic RNA synthesis were inhibited maximally at two hours and returned to control values approximately 30 hours after a 1 mg/kg dose (Lafarge and Frayssinet, 1970). DNA synthesis was inhibited maximally for 48 hours and returned to normal after 72 hours. Ultrastructural investigations revealed a temporal association between RNA polymerase inhibition and nucleolar segregation induced by AFB_1 (Pong and Wogan, 1970). Nucleolar alterations occurred simultaneously with maximal inhibition of DNA-dependent RNA polymerase activity. Within one hour after dosing with 1 mg/kg AFB_1, nucleolar capping and macrosegregation were observed. Thirty-six hours after dosing, fibrillar and granular components were well integrated and RNA polymerase values had returned to normal. Inhibition of RNA synthesis was also accompanied by a decrease in the nuclear RNA/DNA ratio.

The inhibition of RNA synthesis by AFB_1 is primarily the result of template alteration. Inactivation of the DNA template has been investigated both in DNA modified by AFB_1 activated by microsomes *in vitro*, and in DNA isolated from *in vivo* following exposure of animals to AFB_1. The ability of bacterial RNA polymerases to transcribe AFB_1-modified DNA was unimpaired (King and Nicholson, 1967; Edwards and Wogan, 1970). However, when mammalian polymerases were assayed for transcriptional activity on AFB_1-modified templates, inhibition was marked (Edwards and Wogan, 1970). These differences may reflect the

less stringent requirements of the bacterial enzymes for initiation. Inhibition of RNA polymerase enzymes within the nucleus has also been reported (Yu, 1977). Nucleoplasmic α amanitin sensitive activity was inhibited 50 to 70 percent in the liver, two hours after administration of a 3 mg/kg body weight dose of AFB_1 to a rat. The nucleolar, α amanitin insensitive activity was not affected. Interpretation of these results in relation to the contributions which enzyme inactivation makes to the inhibition of RNA synthesis *in vivo* is difficult since the results of these experiments depend upon quantitative recoveries of these enzymes from control and treated animals.

DNA synthesis in cell populations of the rat liver after AFB_1 administration has been studied by autoradiographic methods (Rogers and Newberne, 1967). Following an acute dose in weanling rats, the mitotic index decreased in parenchymal cells. The maximum effect, seen at three hours, produced significant reduction of mitoses which persisted through 50 hours after dosing. The effect on the Kupffer cell populations was variable and the oval cells showed increased labeling over controls at 50 hours after dosing. Selective inhibition of DNA synthesis in mammalian cells in culture was seen with low doses of AFB_1 (e.g., 0.01 and 0.1 µg/ml; Meneghini and Schumacher, 1977). This inhibition persisted up to eight hours after removal of AFB_1. Increasing the concentration of AFB_1 to 0.5 µg/ml produced comparable levels of inhibition in both RNA and DNA

synthesis. These results indicate that DNA replication is most sensitive to AFB_1 inhibition.

Repair synthesis of DNA following exposure to AFB_1 has been examined in several *in vitro* cell culture models. Fibroblasts from normal and repair deficient xeroderma pigmentosum (XP) patients were exposed to AFB_1 activated by incubation with microsomes from liver preparations (Stich and Laishes, 1975). XP cells were much more sensitive to the chromosome-damaging and lethal effects of AFB_1 and had reduced levels (20-25%) of repair synthesis compared to controls. Levels of repair synthesis in these cells following exposure to activating systems containing equimolar concentrations of aflatoxin B_1, G_1, B_2, and G_2 paralleled their cytotoxicity and oncogenicity. Repair synthesis was also detected in WI38 cells exposed to AFB_1 in the presence of a microsomal activating system (Sarasin et al., 1977). The extent of repair was proportional to aflatoxin concentration from 40 to 60 µg/ml.

MUTAGENIC EFFECTS OF THE AFLATOXINS

Interactions of the aflatoxins with cellular macromolecules can cause permanent, heritable changes in cell structure and function, in addition to interfering with replication, transcription, and translation. Eukaryotic and prokaryotic cells which are either capable of metabolizing aflatoxins or are exposed to an active metabolite generated *in situ* are susceptible to their mutagenic effects (Ong, 1975).

Aflatoxins B_1 and G_1 induced mutations in the ad-3 region of *Neurospora crassa* (Ong, 1971). Genetic analysis of these

mutants detected base-pair, frameshift, and deletion mutations. Mutations were not induced in non-growing conidia exposed to AFB_1. However, when conidia were treated with AFB_1 in the presence of a homogenate prepared from hamster liver, a significant mutant fraction was induced (Matzinger and Ong, 1976).

AFB_1 and AFG_1 are also toxic and mutagenic to bacteria. AFB_1 reverted frameshift and base-pair mutations in strains of *Salmonella typhimurium* when activated by the postmitochondrial fraction of rodent liver (Garner and Wright, 1973; Ames et al., 1973; Wong and Hsieh, 1976). The relative mutagenic potency observed using the Ames *Salmonella* mutagen assay showed a positive correlation with *in vivo* carcinogenic data for a series of aflatoxins and their metabolites (Wong and Hsieh, 1976). Another study has shown a relationship between the number of AFB_1 lesions produced in the *Salmonella* genome and the observed forward mutation rate to 8-azaguanine resistance. Approximately one mutation per 32 AFB_1 lesions was observed (Stark et al., 1979).

DNA DAMAGE AND MOLECULAR MECHANISMS OF CARCINOGENESIS

Theories concerning the molecular mechanisms of chemical carcinogenesis all postulate the interaction of the carcinogenic agent with some cellular macromolecule(s) producing a heritable, phenotypic and/or genotypic change in the cell, resulting in its transformation. Investigations have revealed that the ultimately reactive forms of most if not all chemical carcinogens are electrophilic derivatives produced either spontaneously or through metabolic activation, primarily by the microsomal

primarily from studies on DNA repair deficient syndromes in human cells (for review see Setlow, 1978). Xeroderma pigmentosum (XP), ataxia telangiectasea (AT), and Franconi's anemia (FA) are recessively inherited human disorders associated with defects in the ability of cells to repair certain kinds of physical and chemical damage to their DNA. Homozygous individuals affected by these disorders have a significantly higher risk of developing cancer (up to 1,000 times) than the general population (Scott and Straf, 1977).

Cells from XP patients have been studied most extensively (for review see Cleaver, 1975). Six complementation groups have been identified. Five are defective in the excision repair pathway (Kraemer et al., 1975). The additional one is defective in the post replication repair process (Lehmann et al., 1975). These cell lines have a greatly impaired capacity for removal of lesions in DNA induced by UV light and certain chemicals (Setlow, 1978). The toxic and mutagenic effects of carcinogens in these cells occur at lower doses than in normal cells (Stitch and Laishes, 1975). While this information does not establish a cause and effect relationship between DNA damage and neoplastic transformation, it does suggest a relationship between damange to DNA, genomic alteration, and carcinogenesis.

This relationship is further strengthened by the correlation between the carcinogenic and mutagenic properties of many carcinogens in bacteria and mammalian cells (Purchase et al., 1976; Stoltz et al., 1975; McCann et al., 1975). Several

mixed function oxidases (Miller, 1970). These reactive derivatives attack nucleophilic atoms in cellular macromolecules, resulting in their covalent modification. Proteins, RNA, and DNA are attacked indiscriminately; however, the relative number of nucleophilic atoms present in these macromolecules as well as their nucleophilicity and stereochemical availability, may determine the qualitative and quantitative features of their modification (Brown, 1975).

Two general types of mechanisms have been proposed to explain how these macromolecular interactions induce neoplastic changes (Foulds, 1969a). Epigenetic mechanisms assume that neoplasia is the result of a change in the expression of the genetic compliment of a cell without alteration of its genotype. Genetic mechanisms assume that neoplastic changes are dependent upon alteration of the information contained in the cell genome. The critical macromolecular targets for reactive electrophiles in either mechanism are not distinguishable *a priori*. Genotypic changes resulting from mutation may result from direct alteration of the DNA base sequence by formation of covalent DNA derivatives, or through errors in replication or repair of DNA made by altered enzymes. Likewise, changes in the expression of genetic information could be induced by the alteration of interactions of regulatory molecules such as RNA or protein with DNA through covalent modification of these molecules or their sites of interactions.

Evidence that interactions of physical and chemical agents with DNA are important in the carcinogenic and mutagenic processes comes

studies have shown a positive correlation between the carcinogenic potency *in vivo* and mutagenic potency *in vitro* for a series of related compounds (Meselson and Russell, 1977). The identification of stable, temperature-sensitive transformants of BHK cells produced by 4-nitroquinoline-N-oxide and N-nitrosomethylurea may also be viewed as evidence for a direct link between mutation and cancer (Bouck, N. and de Mayorca, 1976).

The relationships between mutation and cancer, however, are clouded by the complex nature of both processes. Most of our knowledge concerning mutagenic mechanisms at the molecular level is derived from studies on repair deficient bacterial strains. Relatively little is known concerning the type and origin of somatic mutations in mammalian cells. Studies in bacteria indicate that lesions in the DNA molecule are often merely initiating signals and the presence or absence of various cellular enzymes responsible for repair or replication of DNA are the ultimate determinants of the type and extent of damage which is produced (Kondo et al., 1970; Ishii and Kondo, 1975).

In addition carcinogenesis is believed to involve a two-step process (Foulds, 1969b): *initiation*, in which the potentiality of a neoplastic cells is determined either by mutation or temporary changes in the regulation of its genome; and *promotion*, in which the expression of the neoplastic state occurs. Experiments in which malignant cells or their nuclei were transplanted into normal developing embryos illustrate the complex relationships among mutation, genomic alteration, and expression of the neoplastic state. Apparently normal

individuals were produced when nuclei from frog renal carcinoma cells were transplanted into enucleated, fertilized eggs (McKinnell et al., 1969), or when mouse terotocarcinoma were implanted into mouse blastulas (Mintz and Illnensee, 1965). The chimeric mice possessed a variety of somatic organs which developed from the carcinoma cells. Thus, although differentiation or expression of neoplasia is ultimately under nuclear control, in metazoan organisms nuclear differentiation must be controlled by extracellular signals. This imposes further obstacles to the establishment of a cause and effect relationship which can distort or obscure the contributions which genetic, epigenetic, or both types of mechanisms make to the initiation and expression of cellular transformation.

In summary, current evidence supports the hypothesis that interactions of chemical carcinogens with DNA are important in the initiation of neoplastic development. However, they do not rule out the importance of interactions with other cellular macromolecules such as RNA and protein. Elucidation of the interactions of electrophilic derivatives with DNA may provide important clues to the types of macromolecular interactions which are initiating events in carcinogenesis and enable the study of the cellular mechanisms which control this process.

STATEMENT OF THE PROBLEM AND OBJECTIVES

Compelling evidence has been accumulated from comparative experimental studies with toxic, mutagenic, and carcinogenic chemicals which indicates the necessity for their covalent interactions with cellular macromolecules to produce their biological effects. These interactions can occur between nucleophilic centers in proteins, RNA and DNA, and activated electrophiles produced spontaneously or enzymatically. Modification of essential cellular components may lead to their inactivation and destruction, resulting in cell death. Survival of a cell after exposure to such xenobiotics will depend upon its ability to replace or repair damaged components. Synthesis of new components or restoration of damaged ones to their initial state will restore the cell to its previous relationship with the environment. Improper or incomplete repair may induce permanent changes in the functioning of the cell if it survives. Such permanent changes may result in the transformation of a cell to the neoplastic state.

The heritability of neoplasia is most readily explained by permanent change(s) in the expression or content of information in the genetic material of the cells. These alterations may be effected by direct covalent modification of DNA or other molecules which are responsible for regulation of its expression and metabolism. These interactions may also play a role in the acute toxic effects of various chemicals.

The molecular mechanisms responsible for the biological effects of AFB_1 are not known. This xenobiotic is a potent

toxin and carcinogen in several animal species and is a potent mutagen in both prokaryotic and eukaryotic cells. Although there is no evidence which directly implicates the covalent interactions of AFB_1 with DNA in the production of these responses, functional changes in this macromolecule as a result of AFB_1 modification have been discovered, and the principal covalent AFB_1-DNA product identified. The purpose of this work is to examine further the covalent interactions of AFB_1 and DNA *in vivo* in relation to their toxic and carcinogenic effects. Further identification of minor interactions and a description of the fate of these interactions following an acute insult or during chronic exposure to AFB_1 may provide a more complete understanding of the mechanisms by which AFB_1 produces its biological effects, and provide further information upon which to base hypotheses concerning its carcinogenic properties.

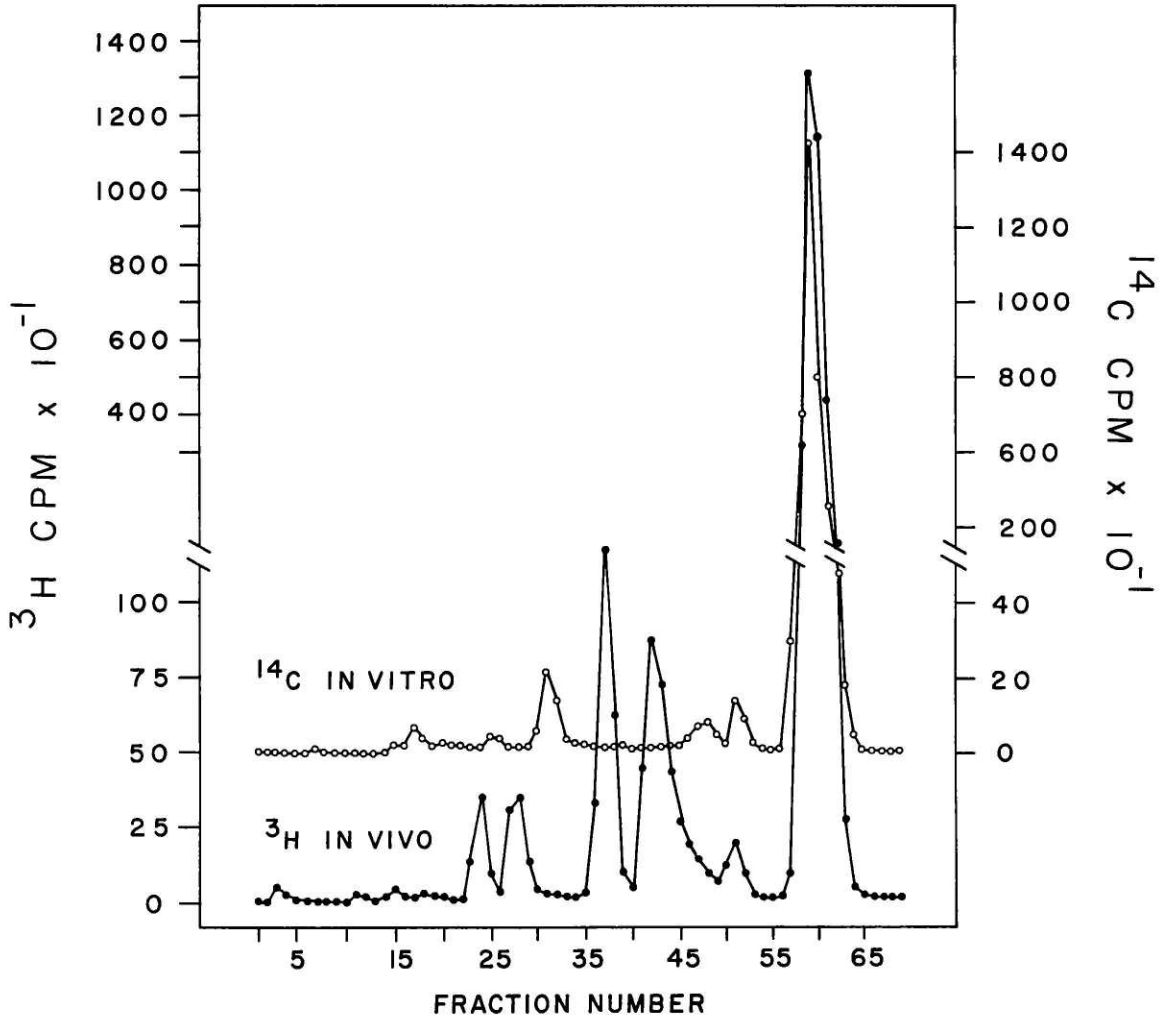
CHAPTER TWO

ISOLATION AND CHARACTERIZATION OF AFLATOXIN B_1-DNA HYDROLYSIS PRODUCTS

INTRODUCTION

Previous studies investigating the nature of covalent AFB_1-DNA interactions have used the rat liver microsomal system to activate AFB_1 in the presence of calf thymus DNA (Swenson et al., 1973, 1974; Lin et al., 1977; Essigmann et al., 1977). This method provided large amounts of covalently bound products for use in the development of techniques for their hydrolysis, isolation, and identification. The N-7 atom of guanine was identified as the principal site of covalent modification both *in vitro* (Lin et al., 1977; Essigmann et al., 1977) and *in vivo* (Lin et al., 1977; Croy et al., 1978). Quantitative analysis revealed that this derivative, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1, represented greater than 95 percent of the hydrolyzed products from *in vitro* adducted DNA but only 70 to 80 percent in DNA isolated from rat liver two hours after administration of AFB_1. Chromatographic analysis of the hydrolysis products from *in vivo* adducted DNA revealed a greater proportion of material eluting in several peaks at shorter retention times. A comparison of the patterns of AFB_1 hydrolysis products obtained from DNA isolated from rat liver *in vivo* and adducted with AFB_1 activated by rat liver microsomes *in vitro* is presented in Figure 2-1. Several peaks, notably E and H, are products from AFB_1 adducts DNA *in vivo* but not *in vitro*. The low concentration of these products relative to the N-7 guanine derivative precludes their rigorous structural identification at this time. However, several techniques have provided information concerning their identity.

Figure 2-1. Chromatographic comparison between the patterns of acid hydrolysis products obtained from [^{3}H]AFB$_{1}$-DNA isolated from rat liver two hours after administration of 1 mg/kg AFB$_{1}$ and those obtained from [^{14}C]-AFB$_{1}$ calf thymus DNA modified by [^{14}C]AFB$_{1}$ activated by rat liver microsomes *in vitro*. Chromatography was performed using a μ Bondapak C$_{18}$ column (Waters Associates) eluted with a linear, 25 minute, 14-18 percent ethanol gradient at ambient temperature. Flow rate was 1.0 ml/min. The eluant contained 0.02 M KAc, pH 5.0. Thirty drop fractions were collected for the determination of ^{14}C and ^{3}H activity.


1400
1200
1000
800
600
400
100
75
50
25
0
^{3}H CPM x 10^{-1}
^{14}C IN VITRO
^{3}H IN VIVO
1400
1200
1000
800
600
200
40
20
0
^{14}C CPM x 10^{-1}
5
15
25
35
45
55
65
FRACTION NUMBER

Purine and pyrimidine bases, and some of their methylated derivatives, are stable to treatment with 70 percent $HClO_4$ at 100°C for one hour. Under these conditions the aflatoxin B_1 molecule is readily hydrolyzed. This procedure thus enables the isolation of the nucleic acid base portion of an adduct molecule. The identity of the base can subsequently be established by analysis of the hydrolysate using ion-exchange liquid chromatography. Additional structural information can be obtained if the adduct molecule is methylated before $HClO_4$ treatment. Reaction with dimethylsulfate under conditions which selectively methylate the imidazole ring nitrogens of purine bases results in the methylation of positions in the purine moiety not occupied by the AFB_1 molecule. The position of attachment of the AFB_1 molecule to the base can be inferred by analysis of the methylated purine bases which are produced by $HClO_4$ hydrolysis. The absence of a specific methylated product indicates which position was occupied by the AFB_1 molecule. These techniques are discussed in detail in Appendix II and papers by Essigmann et al. (1977) and Croy et al. (1978).

The UV-VIS spectrum of hydrolysis products has provided information which is useful in differentiating aflatoxin B_1 metabolites or non-adducted derivatives from products which are likely to be covalently associated with nucleic acid components. 2-Hydroxy substituted AFB_1 molecules isomerize to a phenoxide in alkaline conditions resulting in a bathochromic shift of their UV spectrum (Büchi and Rae, 1969). These derivatives may be produced by hydration of aflatoxin B_1-2,3-oxide (2,3-dihydro-2,3-dihydroxy AFB_1), acid catalyzed hydration (2,3,3-dihydro-

2-hydroxy AFB_1), or the hydrolysis of a bond between the aflatoxin and base moieties of an adduct (2,3-dihydro-2,3-dihydroxy AFB_1). A method of identifying 2-hydroxylated derivatives is important because of the variety of isomeric forms to which these hemiacetals are readily converted, increasing the apparent number of unidentified compounds and forming other isomeric products during attempts at their isolation and purification.

In order to provide information as to the identity of some of the minor hydrolysis products isolated from the liver DNA of rats after exposure to AFB_1, a preparative scale isolation and hydrolysis of adducted DNA from rat liver was performed.

TREATMENT OF ANIMALS; ISOLATION AND HYDROLYSIS OF DNA

Twenty-six male, Fischer rats weighing 170-180 g were administered a dose of 7 mg AFB_1/kg body weight i.p. in 50 µl DMSO. The animals were sacrificed 2 hrs later and nucleic acids isolated from a crude preparation of liver cell nuclei. Approximately 200 mg of DNA was obtained.

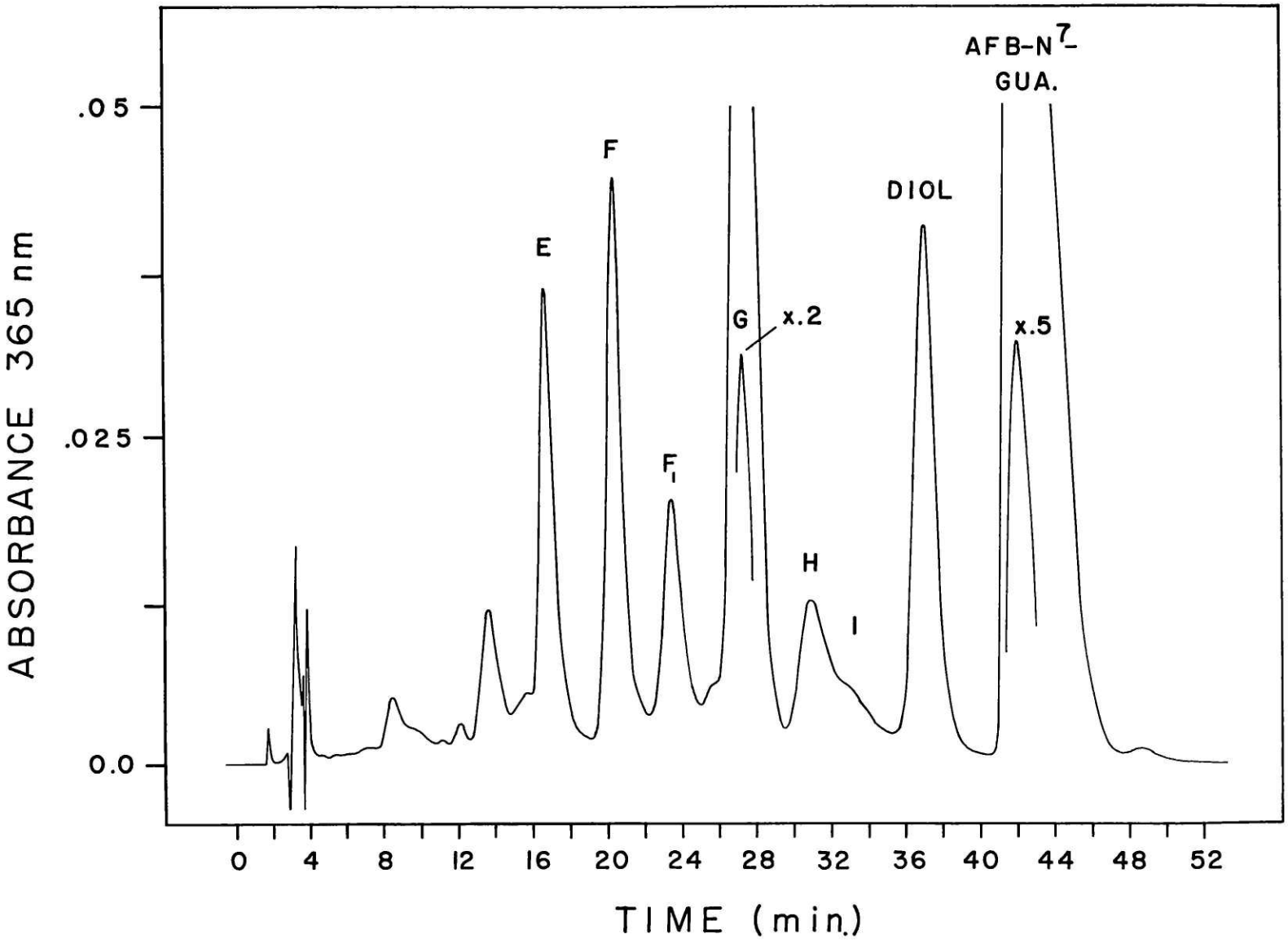
The hydrolysis of this material involved both chemical and enzymatic steps. It was dissolved in deinonized H_2O and the solution adjusted to 0.1 N HCl by the slow addition of 1.0 N HCl. A flocculent white precipitate formed which redissolved when the solution was incubated at 95°C for 10 min. These conditions have been shown to liberate substituted purine derivatives from DNA (Lawley, 1976). In addition, these denaturing conditions render the partially depurinated DNA susceptible to digestion with nuclease P_1 and efficient, single-strand specific nuclease. This enzymatic treatment facilitated subsequent chromatographic analysis. A total volume of 250 ml

of hydrolysate was obtained and analyzed chromatographically in 50 ml aliquots.

CHROMATOGRAPHIC ANALYSIS

Preparative chromatographic methods were used to initially separate AFB_1 derivatives from unmodified bases and nucleotides. The hydrolysate was eluted through a small, preparative reversed-phase column which was washed with 10 percent methanol. Polar, water soluble molecules, such as nucleic acid bases and nucleotides, were not retained by this column under these conditions while the relatively nonpolar, hydrophobic, AFB_1-containing components were. The retained fraction was subsequently eluted with a small volume of 80 percent methanol, which was reduced to 0.2-0.5 ml. This fraction of AFB_1 derivatives was analyzed by analytical high-pressure liquid chromatography using a reversed-phase micro C_{18} column eluted with a linear ethanol/H_2O gradient. The chromatographic profile of 365 nm absorbing material, characteristic of the aflatoxin B_1 chromophore, from one 50 ml aliquot of the hydrolysate is shown in Figure 2-2. The labeled peaks were collected and pooled from four identical chromatographic runs and used for further analysis. The volumes of these pooled fractions were reduced and each peak was reisolated using conditions identical to those in Figure 2-2. This reisolated material was then subjected to further analysis.

Figure 2-2. The pattern of AFB_1 acid hydrolysis products from approximately 40 mg of liver DNA isolated from rats two hours after the administration of 7 mg/kg AFB_1. 0.4 ml of material was injected on a μ Bondapak C_{18} column (Waters Associates) which was subsequently eluted with a linear, 65 minute gradient of 12 to 18 percent ethanol at ambient temperature. Flow rate was 1.0 ml/min. The eluant contained 0.02 M KAc pH 5.0 and was monitored at 254 (not shown) and 365 nm.


ABSORBANCE 365 nm
.05
.025
0.0
E
F
F1
G
x.2
H
I
DIOL
AFB-N7-GUA.
x.5
0
4
8
12
16
20
24
28
32
36
40
44
48
52
TIME (min.)

CHARACTERIZATION OF ISOLATED PEAKS

PEAK E

0.2 absorbance units (365 nm) of material was recovered during the second isolation detailed above. This material was analyzed using isocratic conditions of 12 percent ethanol/H_2O (other conditions were the same as in Figure 2-2). These conditions revealed the presence of two compounds. The major one eluted at 20.2 min and absorbed both at 365 and 254 nm. A minor one eluted at 19 min and absorbed only at 254 nm. Because of peak spreading only a small amount of material could be applied to the column using these conditions while still maintaining adequate resolution. Effective separation of these components was obtained by eluting them from the reversed phase column with a linear 130 min gradient of 11 to 18 percent ethanol/H_2O containing 8 drops of glacial acetic acid per liter. The two peaks, minor and major, eluted at 23.5 and 24.3 minutes respectively. Approximately 0.15 absorbance units (365 nm) of the major component was recovered in 2 ml of eluant.

The UV-VIS spectrum of this peak obtained in the acidic ethanol/H_2O eluant revealed absorbance maxima at 361, 296, 264, and 216 nm. These were unchanged by the addition of 50 µl, 0.1 N NaOH to approximately 1 ml of solution. After neutralization by the addition of 50 µl 0.1 N HCl the aqueous-organic phase was removed by lypophilization. The white residue was hydrolyzed with 70 percent $HClO_4$ at 100°C for 1 hour. Following neutralization analysis of the hydrolysate by cation exchange, chromatography revealed the presence of guanine.

Peak E has thus been characterized as an aflatoxin B_1 derivative attached to guanine. Adequate material with which to perform a methylation experiment and provide information on the position of the guanine molecule substituted by the AFB_1 derivative was not available.

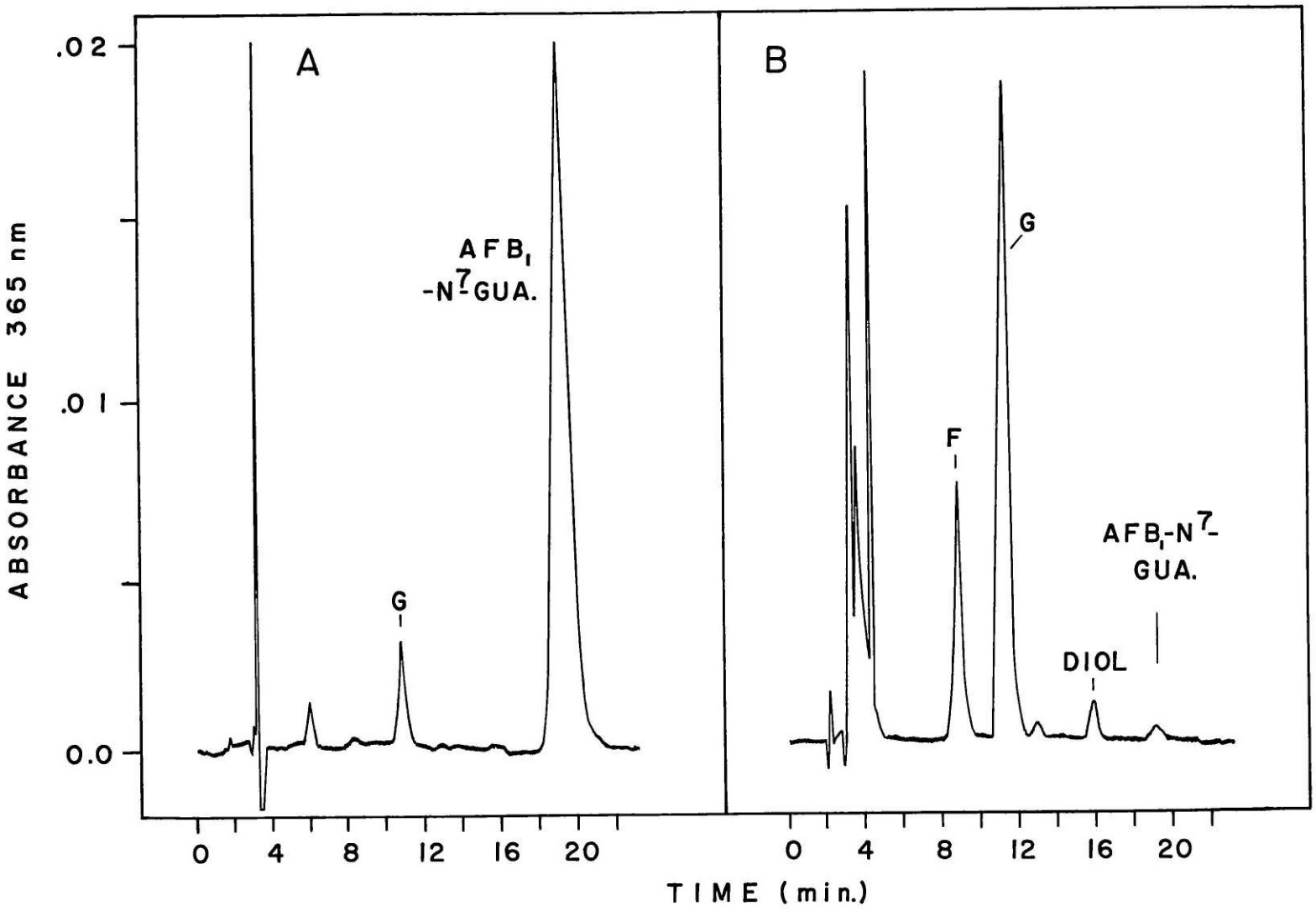
PEAKS F AND G

Peaks F and G have been investigated previously with DNA adducted *in vitro* by AFB_1 activated by rat liver microsomes (Lin et al., 1977). These products are readily formed by exposure of AFB_1 adducted DNA to alkaline conditions. Figure 2-3 shows the chromatographic profile of hydrolyzed products obtained from AFB_1-DNA before and after treatment with 0.1 N NaOH for 15 min at 37°C. Quantitative conversion of the AFB_1-N^7-GUA derivative (2,3-dihydro-(N^7-guanyl)-3-hydroxyaflatoxin B_1 to peaks F and G has occurred.

Several investigators have speculated as to the structures of F and G (Lin et al., 1977). They are thought to be the products of the alkaline-catalyzed hydrolysis of the positively charged imidazole ring, present in the 7,9 disubstituted guanine moiety of DNA.

The UV-VIS specturm of F had maxima at 367, 333, and 263 nm which remained unchanged when the solution was adjusted to pH 10 with 0.1 N NaOH. Analysis of the $HClO_4$ hydrolysate of approximately 0.5 µg identified guanine. Methylation of F prior to $HClO_4$ hydrolysis and chromatographic analysis produced N-9 methyl guanine as the only base present. These results imply that the guanine molecule or its derivative is substituted at

Figure 2-3. Effect of the exposure of AFB_1-DNA to alkaline conditions. AFB_1 adducted calf thymus DNA was treated with 0.1 N NaOH for 15 min at 25°C. Following neutralization, AFB_1 derivatives were hydrolyzed by treatment with 0.1 N HCl, 10 min, 100°C.
A: control AFB_1-DNA not treated with NaOH;
B: after NaOH exposure. Hydrolyzed products were separated using a μ Bondapak C_{18} column (Waters Associates) eluted at ambient temperature with 18 percent ethanol/H_2O containing 0.02 M KAc pH 5.0, at a flow rate of 1.0 ml/min.


A
B
.02
.01
0.0
ABSORBANCE 365 nm
AFB1
-N7GUA.
G
F
G
AFB1-N7-
GUA.
DIOL
0
4
8
12
16
20
TIME (min.)

the N-7' position as would be expected since F is derived from AFB_1-N^7-GUA.

The UV-VIS spectrum of G had maxima at 264, 335, and 363, none of which exhibited a bathochromic shift under alkaline conditions (pH 10). $HClO_4$ hydrolysis of approximately 1 µg of G failed to detect the presence of guanine. These results are consistent with speculation that G is a formamido-pyrimidine derivative of the N-7 guanine adduct.

Neither F nor G exhibited any fluorescent properties when excited at 362 nm. These is evidence that F is formed from G; when G is isolated, increasing amounts of F are seen in the originally pure isolate during subsequent analyses. Since guanine is produced with $HClO_4$ hydrolysis of F but not G, F may be formed by the closure of the imidazole ring, possibly forming a hydrated derivative. The evidence as to the structure of these molecules is still tentative.

PEAK F_1

The peak designated F_1 does not appear to contain any nucleic acid moiety. As shown in Figure 2-2 this compound originally had a retention time of 22 min. When reisolated using identical chromatographic conditions, the retention time of the major peak, containing a shoulder, was 37 min. Minor peaks were present at 22, 24, 26, and 43 min. The major peak was collected and chromatographed using isocractic conditions of 15.5 percent ethanol/H_2O (other conditions identical to those in Figure 2-2). A single peak was present with a retention time of 23 min, which was intensely fluorescent when excited at 362 nm.

The UV-VIS spectrum revealed maxima at 363, 260, and 217 nm at pH 4 to 5. Increasing the pH to 10 by addition of 0.1 N NaOH resulted in a bathochromic shift of the 363 and 260 absorbance maxima to 388 and 262 nm respectively.

$HClO_4$ hydrolysis of approximately 0.05 absorbance units (365 nm) of this material failed to reveal the presence of guanine or adenine upon chromatographic analysis.

This evidence indicates that F_1 is probably a hydrolysis product of other covalent AFB_1 derivatives. The fact that it is readily converted to a number of products and that its UV maximum at 363 nm exhibits a 25 nm bathochromic shift in alkaline solution, and the change in retention time during reisolation to 37 min, identical to that of 2,3-dihydro-2,3-dihydroxyaflatoxin B_1 (diol), suggest that F_1 is an isomeric form of this compound.

PEAK H

The results of an experiment in which the aflatoxin B_1 molecule was labeled with ^{14}C or ^{3}H provided evidence as to the identity of this peak. Approximately 25 percent of the AFB_1 administered i.p. to a rat has been shown to be metabolized to the demethylated derivative, aflatoxin P_1 (AFP_1) (Wogan et al., 1967). Although this molecule is readily conjugated and excreted in the form of glucuronide and sulfate derivatives (Dalezios and Wogan, 1972), the possibility for activation via epoxidation of the 2,3-vinyl ether bond forming an electrophilic species, capable of reacting with cellular macromolecules, also exists. In this experiment AFB_1 was labeled nonspecifically with ^{3}H or specifically with ^{14}C in the methoxycarbon atom. An

AFP_1 derivative in the DNA hydrolysate from both ^3H and ^{14}C labeled compounds would be evidenced by a peak containing only ^3H label.

^{14}C-methoxy labeled AFB_1 was produced by providing an AFB_1 producing strain of *Aspergillus flavus* with L-methyl-^{14}C labeled methionine (Adye and Mateles, 1964). This material had been previously isolated in our laboratory. It was repurified using reversed-phase HPLC by the methods outlined in Appendix II. 312 µg of $-O^{14}CH_3AFB_1$ was obtained. This material had a specific activity of 9.65 mCi/mM.

A single male Fischer rat weighing 150 grams was injected i.p. with 156 µg of radiolabeled AFB_1 in 50 µl DMSO: 48 µCi [^3H]AFB_1 (specific activity 20 Ci/mM) and 4.8 µCi $-O^{14}CH_3AFB_1$ (specific activity 9.65 mCi/mM). The animal was then placed in a metabolic cage which was connected to two CO_2 traps containing 100 ml and 50 ml of 8 N KOH. After two hours the animal was sacrificed. Eleven percent of the ^{14}C activity administered to the rat was recovered in the two CO_2 traps during the two hour period before sacrifice. This was approximately the same as previously reported (Wogan et al., 1967). DNA was isolated from the liver, hydrolyzed, and the AFB_1-containing products analyzed chromatographically.

During all chromatography procedures the pH of the eluants was maintained at 4-5 either by using 0.02 M KAc pH 5.0 in the aqueous portion, or by the addition of 8 drops of glacial HAc per 1000 ml of eluant. Without these pH adjustments the AFP_1 derivative appeared as a very broad non-resolved peak and quantitative recoveries were impossible. The chromatographic

profile of the ^{3}H and ^{14}C labeled hydrolysis products is shown in Figure 2-4. The peak designated H contains only ^{3}H label implying that it is a demethylated derivative of AFB_1.

Further characterization of this peak was performed using material obtained from the large scale *in vivo* experiments. This material was isolated a third time using the same reversed-phase system as in Figure 2-4, except that the column was eluted with a 65 min gradient of 12.4 to 18 percent methanol/H_2O (8 drops HAc/l). A single tailing peak absorbing at 365 and 254 nm with a retention time of 34 min was present. Approximately 0.05 absorbance units (365 nm) of material was recovered.

The UV-VIS spectrum in acidic eluant had absorbance maxima at 362, 333, 267, and 227 nm. The addition of 25 µl, 0.1 N NaOH to approximately 0.8 ml of solution produced absorbance maxima of 420, 333, 289, and 227 nm. The bathochromic shifts of 58 and 22 nm of the 362 and 267 nm abosrbances in alkaline conditions are characteristic of a 5-hydroxycoumarin derivative, e.g., AFP_1 (Büchi et al., 1967).

Ion-exchange chromatographic analysis of the $HClO_4$ hydrolysate of a portion of the isolated compound revealed the presence of guanine. Similar analysis of products produced after $HClO_4$ hydrolysis of the methylated derivative found N-9 methylguanine to be the sole base present, indicating that the AFP_1 molecule is most likely attached to the guanine moiety at the 7 position. The proposed structure of this compound and the pathway leading to its formation is shown in Figure 2-5.

Figure 2-4. Comparison of the AFB_1 hydrolysis products obtained from rat liver DNA 2 hours after administration of 48 μCi G[^{3}H]AFB_1 and 4.8 μCi -O$^{14}CH_3AFB_1$. Acid hydrolysis products of isolated liver DNA were separated by reversed-phase HPLC using a μ Bondapak C_{18} column (Waters Associates) eluted with a 25 min linear gradient of 14 to 18 percent ethanol/H_2O at ambient temperature. The eluant contained 0.02 M KAc pH 5.0. 30-drop fractions were collected for the determination of ^3H and ^{14}C activity.

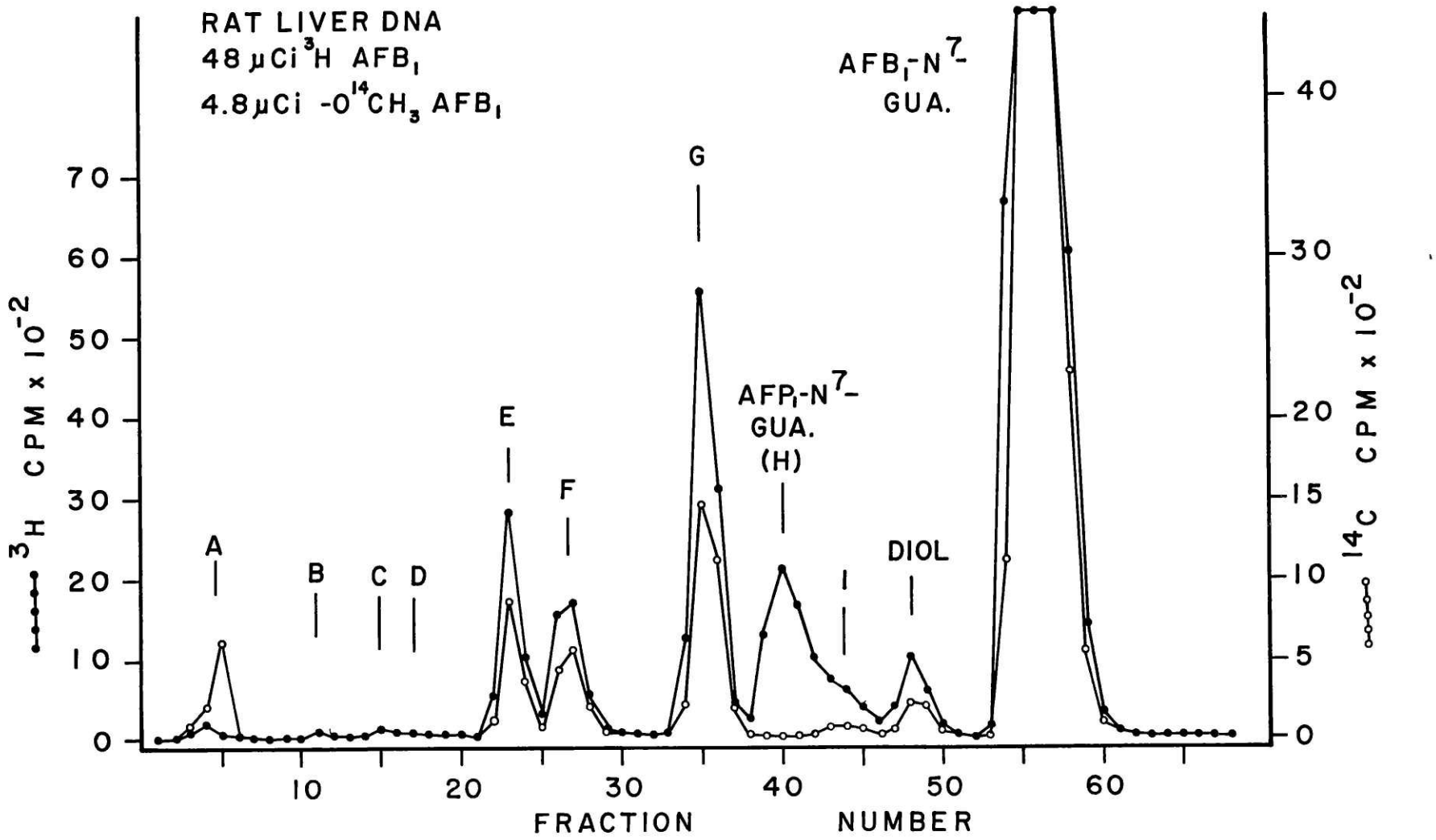
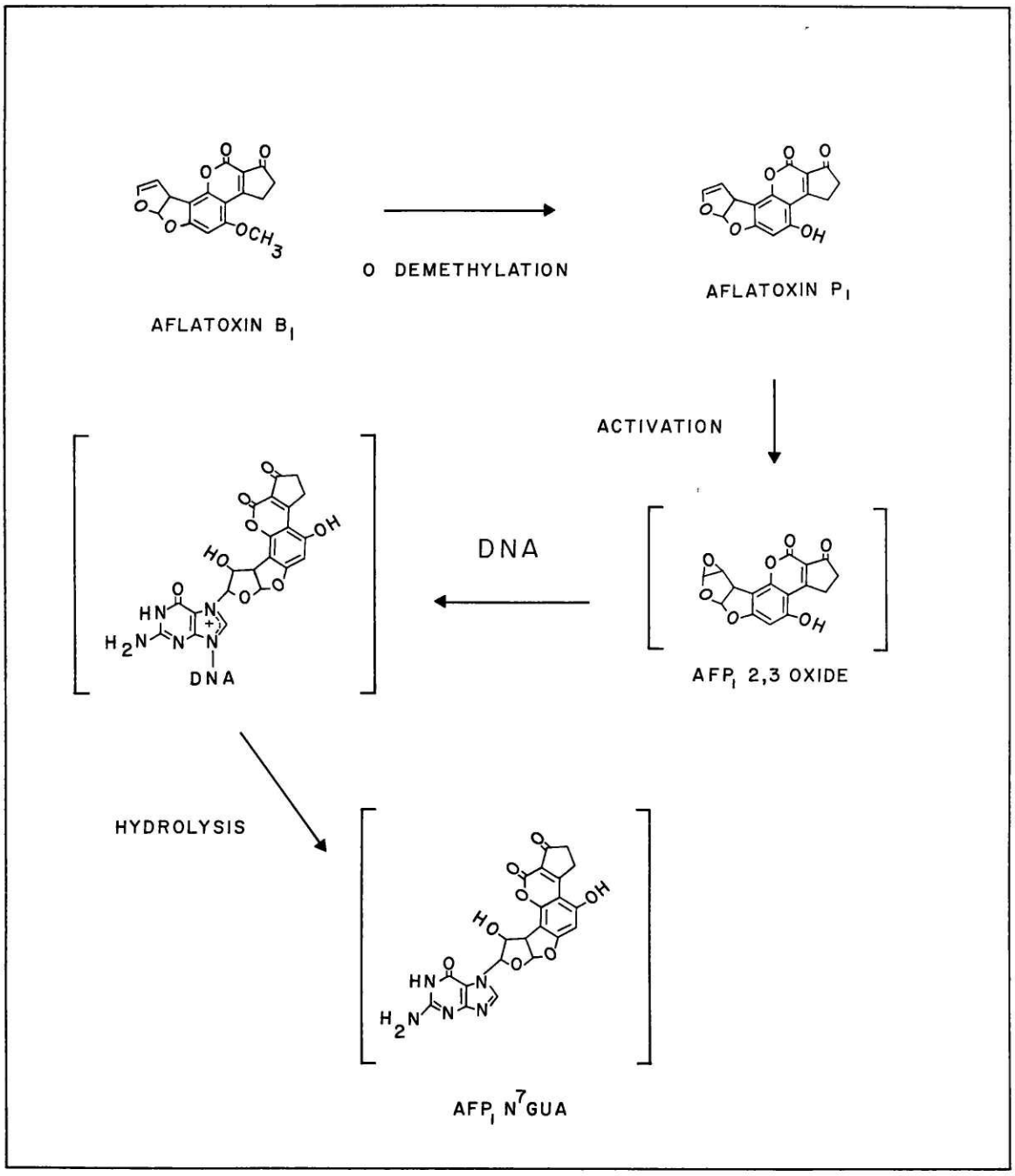

RAT LIVER DNA
48 µCi ^{3}H AFB$_1$
4.8µCi -O$^{14}CH_3$ AFB$_1$
AFB$_1$-N^7-GUA.
AFP$_1$-N^7-GUA. (H)
DIOL
A
B
C
D
E
F
G
I
^{3}H CPM x 10^{-2}
^{14}C CPM x 10^{-2}
FRACTION NUMBER

Figure 2-5. Pathways of formation and activation of aflatoxin P_1 and subsequent production of a covalent guanine derivative in DNA. Brackets [] identify proposed structures.


AFLATOXIN B1
O DEMETHYLATION
AFLATOXIN P1
ACTIVATION
DNA
AFP1 2,3 OXIDE
HYDROLYSIS
AFP1 N7GUA

DIOL

This peak was characterized as 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 (AFB_1 diol) by chromatographic comparison with authentic material synthesized photochemically and by its UV-VIS spectral properties. The compound isolated from the hydrolysis solution had absorbance maxima of 362, 266, and 219 nm. Addition of 0.1 N NaOH produced a shift in the maxima to 396, 285, and 247 nm, identical to the authentic AFB_1 diol.

AFB_1-N^7-GUA

AFB_1-N^7-GUA has been identified *in vitro* and *in vivo* as 2,3-dihydro-(N^7-guanyl)-3-hydroxyaflatoxin B_1 by several groups of investigators (Essigmann et al., 1977; Lin et al., 1977; Croy et al., 1978). It is the principal covalent AFB_1-DNA product.

DISCUSSION

Investigation of the products of the chemical and enzymatic hydrolysis of AFB_1 adducted DNA isolated from the livers of rats after exposure to AFB_1 has revealed the presence of two N-7 substituted guanine derivatives. The principal product is formed by direct activation of the AFB_1 molecule. Two other products were found to be derived from the principal AFB_1-N^7-GUA adducts, most probably through hydrolysis of the positively charged imidazole ring of the 7,9-disubstituted guanine moiety in DNA. Thus the products formed from attack of the aflatoxin B_1-2,3-oxide on the N-7 position of guanine account for approximately 90 percent of the covalently bound material.

Activation of the demethylated metabolite of AFB_1, aflatoxin P_1, and formation of a covalent N-7 guanine derivative have also been demonstrated. This adduct may also be susceptible to hydrolytic cleavage of the imidazole ring while bound to the DNA sugar-phosphate backbone through a glycosidic linkage, theoretically producing two more unidentified products by analogy to the principal AFB_1-N^7-GUA adduct. There is also evidence that peak E may be produced by the attack of another activated AFB_1 metabolite on the guanine moiety in DNA. Hydroxlyation at the 4 position of the AFB_1 molecule is known to be another prominent metabolic pathway in the rat, forming aflatoxin M_1 (AFM_1). Oxidation at this position would not seem to preclude further metabolism of the molecule at the 2,3-vinyl ether bond, forming an activated electrophile. The fact that this peak contains guanine and its kinetics of formation and removal from DNA *in vivo* are similar to those of the AFP_1 derivative (see Chapter Four) provide support for these speculations.

The identities and characteristics of compounds which elute at earlier retention times than peak E have not been investigated. These peaks are designated A, B, C, D, and E_1 in subsequent chapters. These products each represent less than 0.1 percent of the covalently bound AFB_1 material *in vivo* and are at too low a concentration to facilitate their isolation and identification. However, it is not difficult to speculate as to their probable identity. By analogy with the known chemistry of the principal AFB_1-N^7-GUA adduct, the minor

N-7 guanine adducts formed from AFP_1 and possibly AFM_1 may undergo hydrolysis of their positively charged imidazole rings, resulting in the formation of two additional products each, corresponding to F and G produced from AFB_1-N^7-GUA. These products would be expected to elute at short retention times in the reversed phase chromatographic system.

Two products, AFB_1-dihydrodiol (diol) and F_1, are not covalently associated with any nucleic acid components and are most probably artifacts of the hydrolysis procedure. Peak I has not been investigated because the small amount of material present in this peak could not be completely resolved from the adjacent tailing peak H without considerable loss of material.

These investigations have not identified any bases other than guanine as sites of covalent modification; however, evidence has been provided indicating a low level of modification of adenine residues *in vitro* by AFB_1 (D'Andrea and Haseltine, 1978). Efforts to identify this product have been unsuccessful primarily because of a lack of knowledge of its chemical and chromatographic properties. Investigations of these minor products will be much facilitated by manipulation of microsomal activation systems or development of synthetic chemical methods to enable the isolation of adequate quantities of material for analysis.

CHAPTER THREE

STUDIES ON THE RELATIONSHIP BETWEEN THE FORMATION OF COVALENT AFB_1-DNA PRODUCTS, SPECIES SUSCEPTIBILITY, AND ORGANOTROPISM OF AFB_1 IN THE RAT AND MOUSE

INTRODUCTION

The purpose of this experiment is to compare qualitatively and quantitatively the covalent products formed in DNA by aflatoxin B_1 (AFB_1) isolated from the liver and kidneys of the mouse and rat following equitoxic doses of AFB_1. The adult Swiss mouse is resistant to the toxic effects of AFB_1 and completely refractory to its carcinogenic properties (Wogan, 1973). Studies on the acute toxicity of AFB_1 in this species show that the kidney is the primary site of histopathologic and biochemical damage, including hemorrhagic necrosis and rapid inhibition of nuclear RNA synthesis (Akao et al., 1971). By contrast the male Fischer rat is highly susceptible to both the toxic and carcinogenic properties of AFB_1 (Wogan, 1973). The organotropism of AFB_1 in the rat is different than in the mouse, the liver being the primary site of both toxic and carcinogenic responses.

The mechanism of resistance of the mouse liver to AFB_1 has been investigated. Studies have shown that mouse liver RNA polymerases are resistant to inhibition by AFB_1 *in vivo* (Akao et al., 1971; Neal, 1972). However, [^{14}C] orotic acid incorporation into RNA was inhibited *in vitro* when liver slices or isolated nuclei in the presence of a mouse liver microsomal fraction were incubated with AFB_1. The microsomal fraction was also capable of activating [^{14}C]AFB_1 to a reactive intermediate which bound to DNA *in vitro* (Godoy and Neal, 1976).

The kinetics of activation or detoxification of AFB_1 and/or the resistance of the transcriptional apparatus to covalent

modification have been suggested as possible explanations for the resistance of RNA synthesis in the mouse liver to inhibition by AFB_1 (Neal, 1972; Godoy and Neal, 1976). Knowledge of the quantitative and qualitative nature of the covalent AFB_1-DNA products in the livers and kidneys will enable their comparison and provide further insight into the mechanisms governing the different responses of these animals to AFB_1.

EXPERIMENTAL DESIGN

The susceptibility of both the rat and mouse to the acute toxicity of AFB_1 is age dependent (McGuire, 1969). Fischer rats are least sensitive approximately 14 days after birth. At this time the LD_{50} is approximately 14 mg/kg body weight. This values declines exponentially until around 65 days of age when it reaches a value of approximately 1 mg/kg body weight.

Swiss mice are highly resistant to AFB_1 toxicity. The LD_{50} is greater than 150 mg/kg body weight at 30 days of age and declines exponentially to a value of 12-13 mg/kg body weight at 100 days of age. Rats and mice used in this study were 75 and 110 days old respectively. At these ages both species have reached their most sensitive period and comparisons are not complicated by the rapidly changing susceptibilities of younger animals.

MATERIALS AND METHODS

Male Fischer rats were obtained as weanlings from Charles River Laboratories (N. Wilmington, MA), housed in suspended, wire-bottom cages, and fed a semi-synthetic agar gel diet

(Wogan and Newberne, 1967) *ad libitum*. Male Swiss mice were obtained from the same supplier at the age of 100 days, housed in plastic cages, and fed a standard lab chow diet *ad libitum*.

Three rats weighing approximately 150 g each were injected i.p. with 1.0 mg/kg [^{3}H] AFB_1 in 50 µl DMSO. DNA was isolated from individual livers; however, the six kidneys obtained from the three animals were divided into two groups of three to obtain adequate material for analysis. Fifteen mice weighing approximately 35 g each were injected i.p. with 12 mg/kg [^{3}H] AFB_1 in 50 µl DMSO. DNA was isolated from pooled groups of 5 livers and 15 kidneys.

AFB_1 (Makor Biochemicals, Jerusalem, Israel) and [^{3}H] AFB_1 (20 Ci/mM, Moravek Biochemicals, CA) were combined to obtain a specific activity of 100 mCi/nm. Animals were sacrificed two hours after AFB_1 administration and DNA isolated from a crude preparation of liver and kidney cell nuclei. The isolated DNA was then hydrolyzed by chemical and enzymatic methods and the hydrolysate subjected to chromatographic analysis. These procedures are detailed in Appendix I.

RESULTS AND DISCUSSION

Table 3-1 shows the total levels of modification of DNA by AFB_1 in the liver and kidneys of the rat and mouse. Rat liver is modified to the greatest extent at a level of 1.25×10^{-4} AFB_1 modifications per nucleotide. Rat kidney DNA is modified approximately one-tenth as much as the liver. By contrast, a higher level of modification is present in the

TABLE 3-1

MODIFICATION OF DNA BY AFLATOXIN B_1 *IN VIVO*[a]

	AFB_1 MODIFICATIONS/NUCLEOTIDE x 10^7 (NUCLEOTIDES/MODIFICATION x 10^3)
RAT (1 mg/kg)	
Liver	1250 (8.0)
Kidney	125 (80)
MOUSE (12 mg/kg)	
Liver	23 (440)
Kidney	71 (140)

[a]Calculations are detailed in Appendix II.

kidney than in the liver of the mouse: 7.1×10^{-6} and 2.3×10^{-6} AFB_1 modifications per nucleotide respectively.

HPLC analysis of the AFB_1 products obtained after hydrolysis of the DNA isolated from the livers and kidneys of these two species are shown in Figures 3-1 and 3-2. Eleven peaks can be identified in the rat liver DNA hydrolysate (Fig. 3-1), one of which, I, is not completely resolved from its neighboring peak, H. For purposes of quantification H and I have been combined in the following analyses. Quantitative data on individual peaks from the rat are presented in Table 3-2. Ten distinct peaks can be measured, six of which are present in the kidney at lower levels. The N-7 guanine adduct is predominant in both organs; however, other peaks account for a greater percentage of the hydrolyzed products in the kidney (30%) than in the liver (20%). The greater percentage of peak H, the aflatoxin P_1 derivative, in the kidney may indicate a greater relative level of O-demethylase activity or a lower level of glucuronide or sulfate conjugating enzymes in this organ than are present in the liver.

Figure 3-2 presents the chromatographic analysis of AFB_1-adducted DNA obtained from mouse liver and kidney. Although the mouse liver produces a more complicated spectrum of products, the kidney DNA is more highly modified. Quantitative data for mouse liver and kidney DNA are presented in Table 3-3. Eight peaks can be identified in the mouse liver and four in the kidney. Three of these, F, G, and AFB_1-N^7-GUA, are present at higher levels in the kidney (the diol being at a lower level).

Figure 3-1. HPLC reversed-phase separation of acid hydrolysis products obtained from [^{3}H] AFB_1-DNA isolated from rat liver and kidney two hours after administration of 1 mg/kg AFB_1. This separation was obtained using a μ Bondapak C_{18} column (Waters Associates) eluted with a 25 min, linear, 14 to 18 percent ethanol gradient run at ambient temperature. Flow rate was 1 ml/min. The eluant contained 0.02 M KAc pH 5.0. 30-drop fractions were collected for the determination of ^{3}H activity.

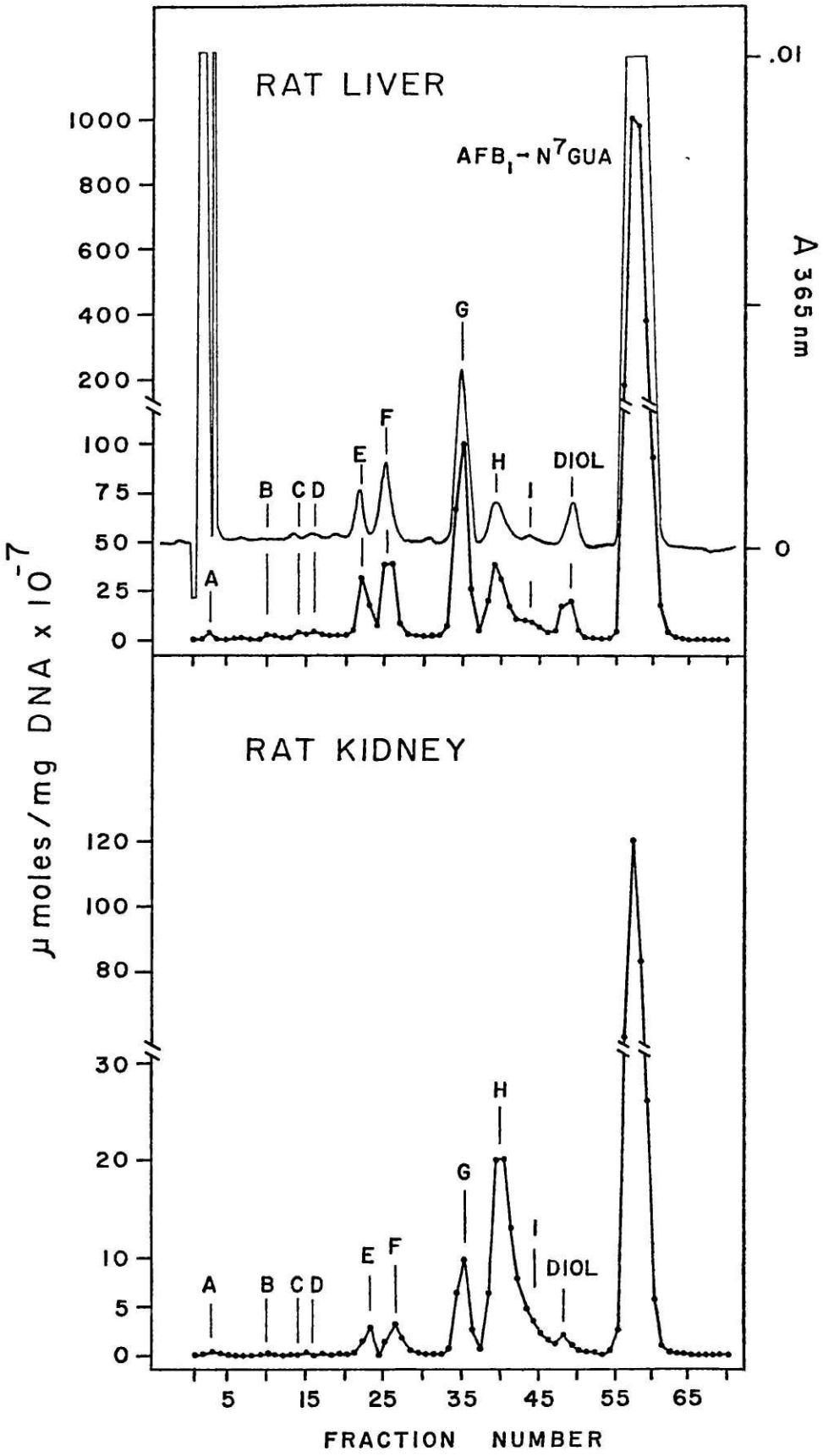

RAT LIVER
AFB1-N7GUA
RAT KIDNEY
μ moles / mg DNA x 10-7
A 365 nm
.01
0
1000
800
600
400
200
100
75
50
25
0
120
100
80
30
20
10
5
0
A
B
C
D
E
F
G
H
I
DIOL
5
15
25
35
45
55
65
FRACTION NUMBER

Figure 3-2. HPLC reversed-phase separation of acid hydrolysis products obtained from [^{3}H] AFB_1-DNA isolated from mouse liver and kidney 2 hours after administration of 12 mg/kg AFB_1. This separation was obtained using a μ Bondapak C_{18} column (Waters Associates) eluted with a linear, 25 min, 14 to 18 percent ethanol gradient run at ambient temperature. Flow rate was 1.0 ml/min. The eluant contained 0.02 M KAc pH 5.0. 30-drop fractions were collected for the determination of ^{3}H activity.

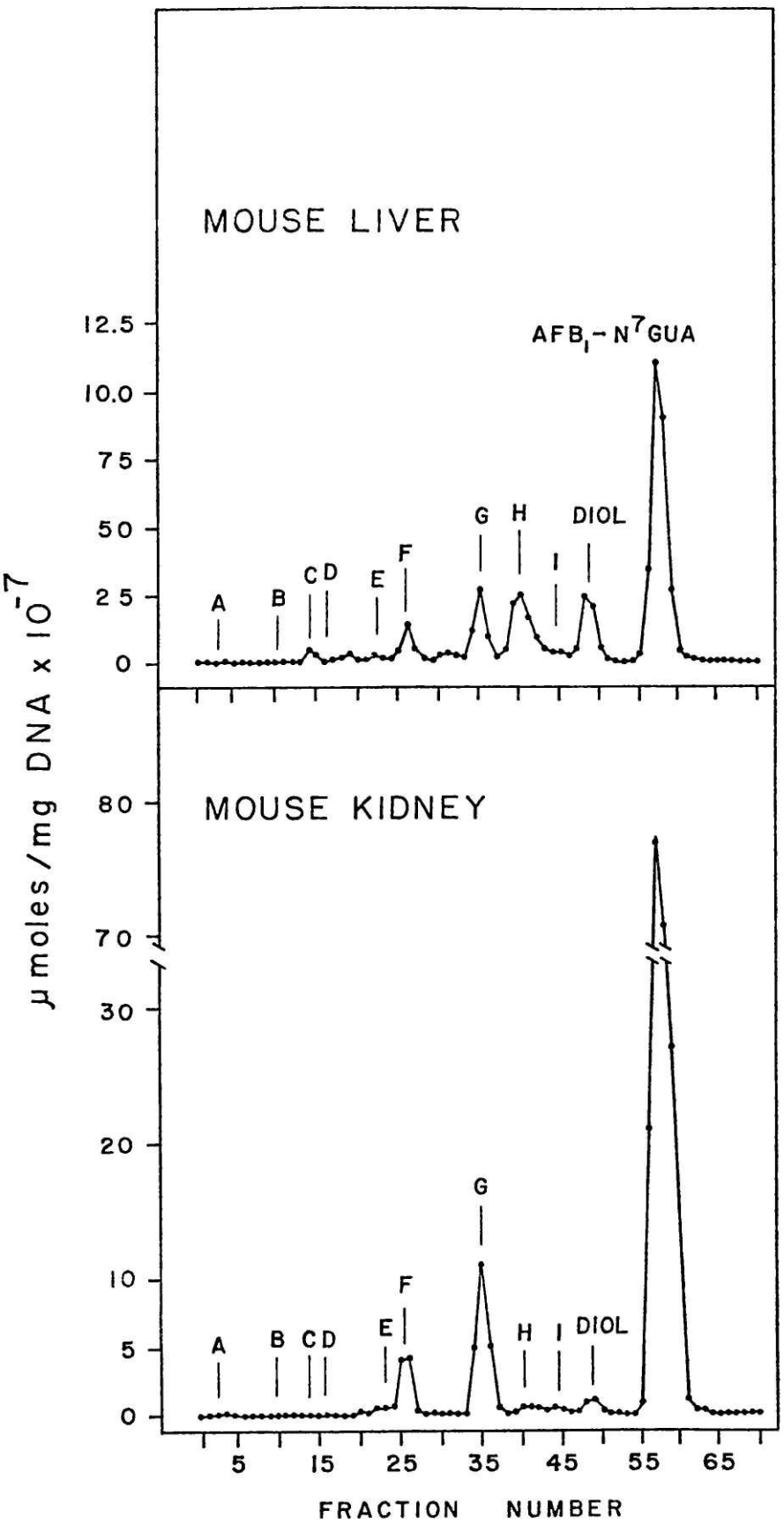

MOUSE LIVER
AFB1-N7GUA
12.5
10.0
7.5
5.0
2.5
0
A
B
C
D
E
F
G
H
I
DIOL
MOUSE KIDNEY
80
70
30
20
10
5
0
A
B
C
D
E
F
G
H
I
DIOL
5
15
25
35
45
55
65
FRACTION NUMBER
μmoles/mg DNA x 10-7

TABLE 3-2

QUANTIFICATION OF ACID HYDROLYSIS PRODUCTS[c] OF AFB_1-MODIFIED DNA

(AFB_1 Modifications/Nucleotide x 10^8)

PEAK DESIGNATION	RAT[a] LIVER	RAT[b] KIDNEY
A	22 ± 6	--
B	18 ± 3	--
C	24 ± 7	--
D	34 ± 8	--
E	230 ± 30	19 (22-16)
F	370 ± 60	22 (23-21)
G	780 ± 90	62 (63-61)
H,I	680 ± 150	240 (220-250)
DIOL	160 ± 10	23 (23-22)
AFB_1-N^7-GUA	9500 ± 600	880 (830-920)

[a]Average ± SD of three determinations.

[b]Mean and range of duplicate determinations.

[c]Calculations are detailed in Appendix II.

TABLE 3-3

QUANTIFICATION OF ACID HYDROLYSIS PRODUCTS[c] OF AFB_1-MODIFIED DNA

(AFB_1 Modifications/Nucleotide x 10^9)

PEAK DESIGNATION	MOUSE[a] LIVER	MOUSE[b] KIDNEY
A	--	--
B	--	--
C	96 ± 59	--
D	36 ± 8	--
E	29 ± 3	--
F	99 ± 2	290 (270-320)
G	230 ± 50	670 (650-680)
H,I	350 ± 50	--
DIOL	210 ± 10	75 (66-83)
AFB_1-N^7-GUA	1100 ± 200	6600 (6400-6700)

[a]Average ± SD of three determinations.

[b]Mean and range of duplicate determinations.

[c]Calculations are detailed in Appendix II.

The N-7 guanine adduct is again predominant in both organs, but a greater percentage of the total adducted material is found in the peaks eluting at shorter retention times in the liver (49%) than in the kidney (14%). Qualitative differences between organs opposite to those in the rat are noted with peak H. This again may indicate relative levels of O-demethylase or conjugation enzymes in these organs.

A qualitative difference between the two species is present with peak E. This peak is seen in both organs of the rat but is barely detectable or absent in the mouse. If this product is formed by activation of a metabolite of AFB_1 which still has an intact 2,3-vinyl ether bond (e.g., AFM_1), this difference may be indicative of the relative contributions separate metabolic pathways make in these species to the detoxification and activation of AFB_1.

In relation to tissue and species susceptibility, no conclusions can be formulated concerning the role that specific products may play or what biochemical mechanisms may be involved in the toxic responses of these animals to AFB_1. DNA from target and non-target tissues of the rat and mouse produce qualitatively similar patterns of hydrolysis products. Minor differences possibly reflect the relative contributions of pathways by which AFB_1 is metabolized in different organs or species. A good correlation exists between the overall covalent binding of AFB_1 to DNA and tissue susceptibility to its toxic effects. Thus the differential ability to activate AFB_1 and some of its metabolic products (e.g., AFM_1, AFP_1) may

play a role in determining the tissue specificity for toxicity and carcinogenicity in the rat and toxicity in the mouse. Much further work is needed to order to determine the mechanisms by which AFB_1 derivatives, covalently bound to DNA, affect cellular processes and the roles they play in the toxic responses of various organisms to aflatoxin B_1.

ACTIVATION OF AFB_1 BY MOUSE AND RAT LIVER MICROSOMES

The biochemical mechanism(s) responsible for the resistance of the mouse to AFB_1 toxicity is not known. Results of the previous experiment suggest that the Swiss mouse either has a low capacity for producing aflatoxin B_1-2,3-oxide, the activated species, or that this intermediate is efficiently inactivated or removed by cellular detoxification reactions before attack at nucleophilic centers in cellular macromolecules can take place. Microsomal fractions from both rat and mouse liver were used to compare qualitatively and quantitatively the activation of AFB_1 by these two species. [^{3}H] AFB_1 was activated by the microsomal fractions in the presence of calf thymus DNA and an NADPH generating system. The DNA was isolated and the covalently bound ^{3}H activity determined.

MATERIALS AND METHODS

Microsomal fractions were isolated from untreated 100 day old mice (15) and 70 day old rats (3) by the method of Kinoshita et al. (1973) and stored at -70°C. Incubations were carried out in a 500 ml Erlenmeyer flask containing 30-49 ml of solution in a shaking water bath at 37°C. The solution contained the following concentrations of enzymes and substrates: *tris*-HCl (45 mM, pH 7.5), $MgCl_2$ (3 mM), calf thymus DNA (0.8 mg/ml, Sigma Chemical Co.), NADP (0.64 mg/ml, Sigma Chemical Co.), glucose-6-phosphate dehydrogenase (0.8 units/ml, Sigma Chemical Co.), AFB_1 (20 µg/ml, Makor Biochemicals, Jerusalem, Israel), [^{3}H] AFB_1 (1.9 µCi/ml, Moravek Biochemicals) and 0.5 mg/ml

microsomal protein. Incubations were started by the addition of AFB_1. At appropriate time intervals 5.0 ml aliquots were removed and added to volumes of 4 M NaCl and 5 percent SDS to stop the reaction. The final concentrations of NaCl and SDS were 0.1 M and 0.3 percent, respectively.

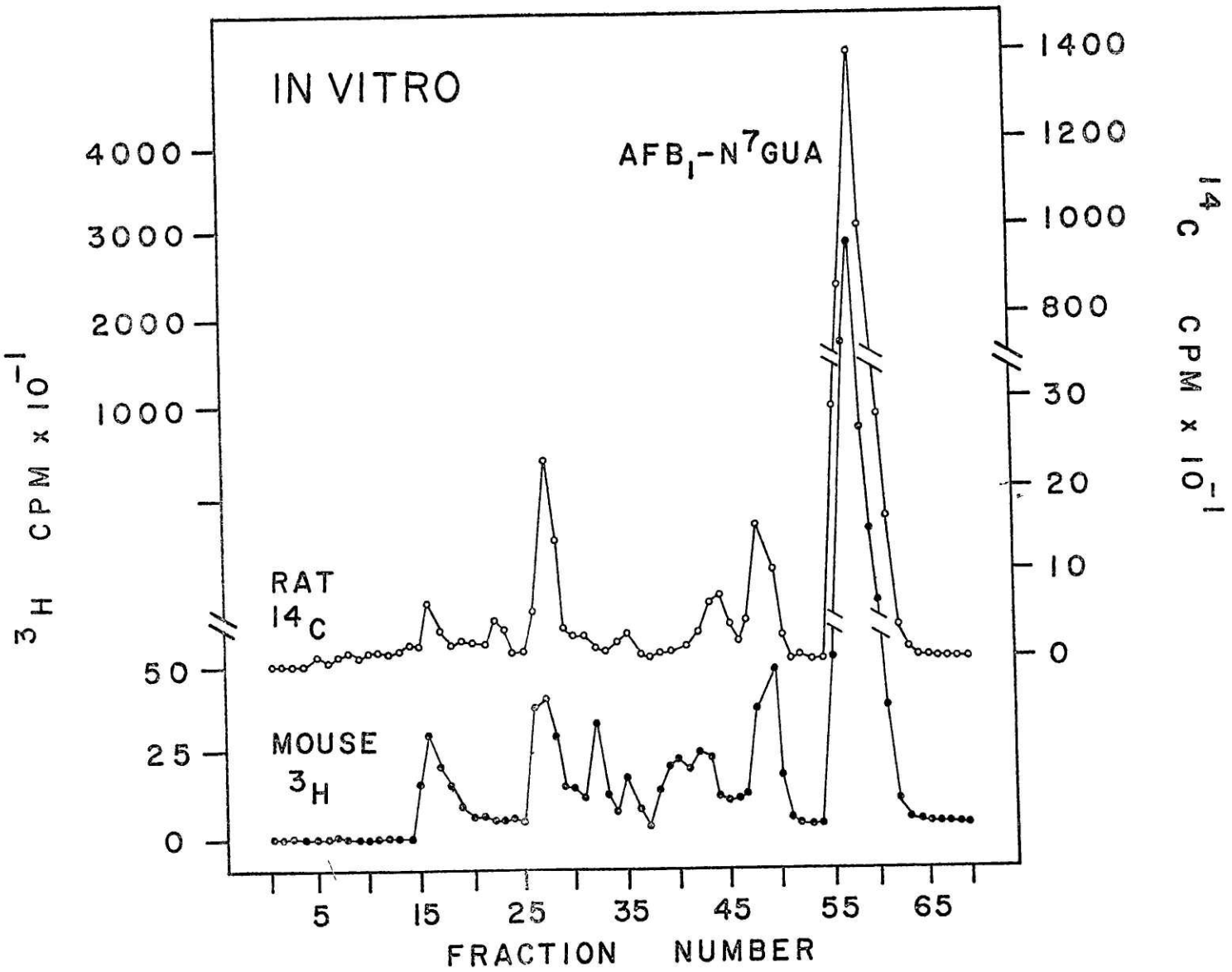
DNA was isolated by extracting the aqueous solution twice with $CHCl_3$:isoamyl alcohol (90:2), separating the aqueous and organic phases via centrifugation at 10,000 rpm after each extraction. The DNA was precipitated from the aqueous solution by the addition of three volumes of cold ethanol and recovered by winding on to glass rods. After being rinsed twice in absolute ethanol, it was dried *in vacuo*.

To determine the amount of [^{3}H] AFB_1 bound per milligram of DNA, the dried DNA was dissolved in 10.0 ml of 0.05 M KAc pH 5.0 and heated at 95°C for 10 min. This treatment resulted in the hydrolysis of most of the bound products from DNA. Aliquots of the solution were then removed for the determination of ^{3}H activity and DNA concentration by the diphenylamine reaction. For chromatographic analysis portions of the dried DNA were hydrolyzed and analyzed as detailed in Appendix I.

RESULTS

The chromatographic patterns of hydrolysis products obtained from calf thymus DNA adducted by AFB_1 which was activated by either rat or mouse liver microsomes are shown in Figure 3-3. AFB_1-N^7-GUA is the principal product obtained from either species, representing 96 percent in the rat and 95 percent in the mouse of the total covalent derivatives. The mouse appears to

Figure 3-3. Comparison of AFB_1 hydrolysis products obtained from calf thymus DNA covalently modified *in vitro* by AFB_1 activated by mouse or rat liver microsomes. 500 µl of material was injected onto a µ Bondapak C_{18} column (Waters Associates) and subsequently eluted using a linear, 25 min, 14 to 18 percent ethanol/H_2O gradient at ambient temperature. Flow rate was 1.0 ml/min. The eluant contained 0.02 M KAc pH 5.0. 30-drop fractions were collected for the determination of ^3H and ^{14}C activity.


IN VITRO
AFB1-N7GUA
RAT
14C
MOUSE
3H
3H CPM x 10-1
14C CPM x 10-1
4000
3000
2000
1000
50
25
0
1400
1200
1000
800
30
20
10
0
5
15
25
35
45
55
65
FRACTION NUMBER

produce a more complicated spectrum of minor products than the rat. Figure 3-4 shows the results of a typical experiment comparing the kinetics of AFB_1 activation by these species. At short periods of incubation mouse microsomes were more active in producing covalently bound products. However, at longer periods, i.e., 15 min, they appeared to be inactivated. Rat microsomes produced a linear increase in the amount of [^{3}H] AFB_1 bound to DNA throughout the experimental period. Additional investigations concerning the mechanism of inactivation of the mouse microsomes are presented in Figure 4-5. In this experiment amounts of substrate (AFB_1), cofactors (glucose-6-phosphate, NADP, and glucose-6-phosphate dehydrogenase) and microsomes equal to those present at the start of the experiment were added to incubation mixtures containing mouse liver microsomes 20 min after the start of the incubation, at which time there was an apparent complete inhibition of activation (c.f., Fig. 3-4). Addition of substrate or cofactors did not produce any significant changes in the levels of covalently bound material, but addition of microsomes produced a rapid increase in bound ^{3}H activity.

DISCUSSION

These results suggest that the microsomal enzymes in the mouse, responsible for activating AFB_1, are inactivated during metabolism of AFB_1 and that this results in their limited capacity, as compared to the rat, for producing covalently bound DNA products. Cautious reasoning must be applied, however,

Figure 3-4. Kinetics of the covalent binding of [^{3}H] AFB_1 activated by mouse or rat liver microsomes to calf thymus DNA *in vitro*.

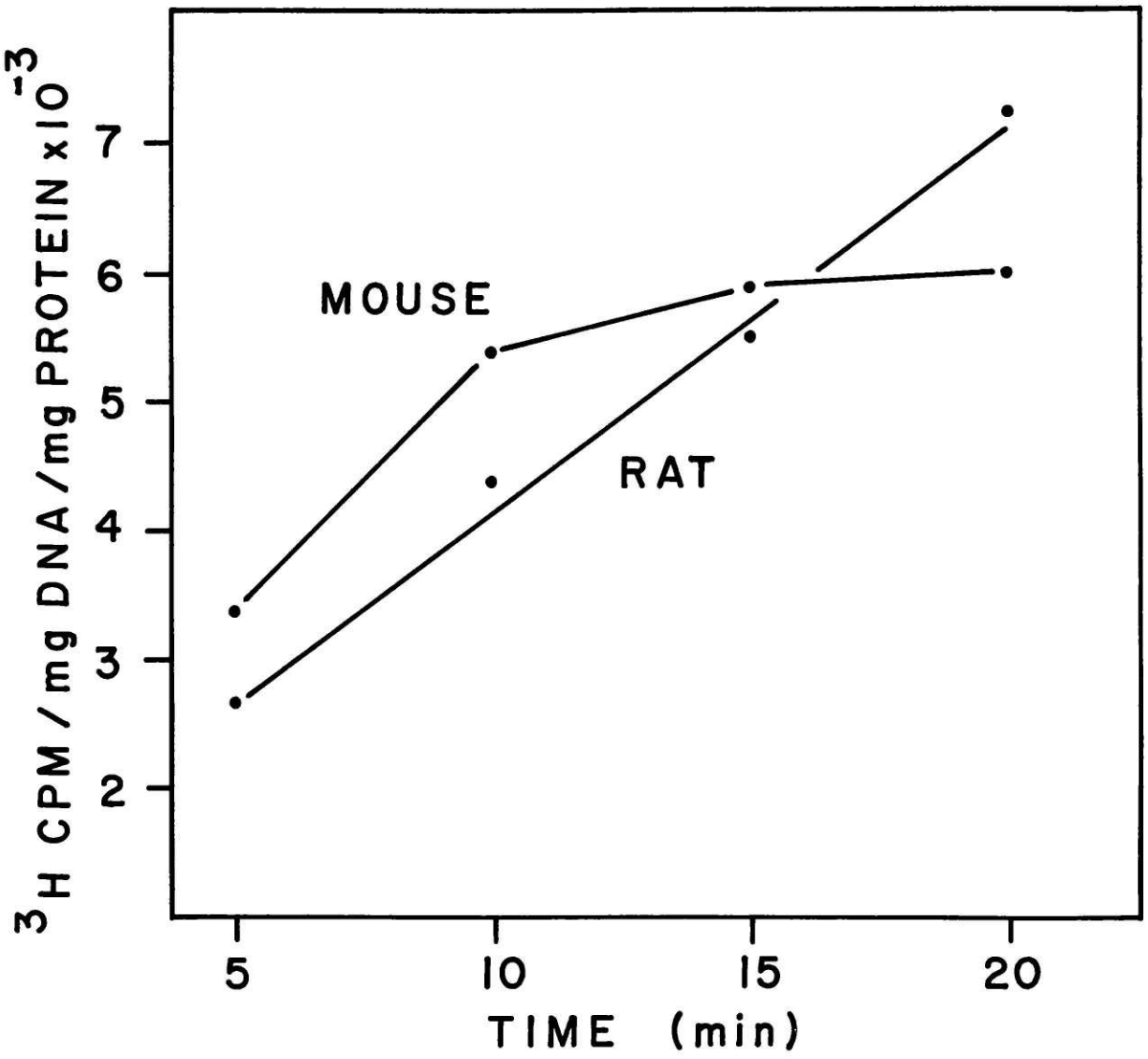
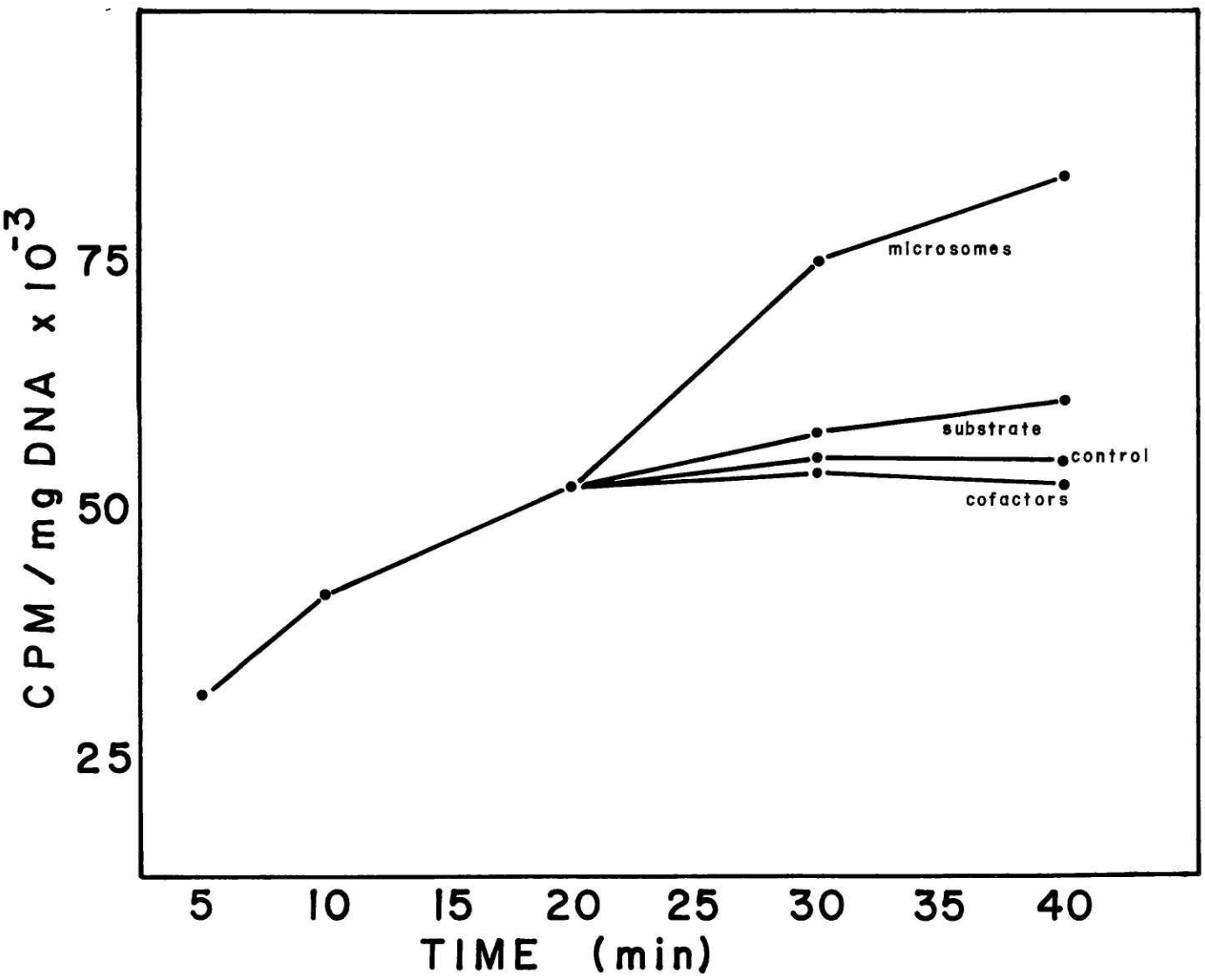

^{3}H CPM / mg DNA / mg PROTEIN x 10^{-3}
7
6
5
4
3
2
MOUSE
RAT
5
10
15
20
TIME (min)

Figure 3-5. Kinetics of binding of [^{3}H] AFB_1 activated by mouse liver microsomes to calf thymus DNA *in vitro* before (5-20 min) and after (20-40 min) the addition of supplementary factors to the *in vitro* system.


CPM / mg DNA x 10^{-3}
75
50
25
5
10
15
20
25
30
35
40
TIME (min)
microsomes
substrate
control
cofactors

when these results are used to explain the resistance of the mouse to the toxic effects of AFB_1.

Isolated microsomal metabolism systems, exposed to large concentrations of substrate and cofactors, do not duplicate the complex metabolic pathways by which aflatoxin B_1 is activated and detoxified *in vivo*. This was illustrated by the comparison of the hydrolysis products of DNA isolated from the livers of rats after exposure of AFB_1 and the products obtained from DNA adducted by a rat liver microsomal activation system (Fig. 2-1). Several of the qualitative and quantitative differences which exist are probably due to the secondary activation of AFB_1 metabolites *in vivo*.

Consideration of enzyme kinetics may provide a plausible explanation for these differences. K_m values of the more polar aflatoxin derivatives such as P_1 and M_1 for the enzyme(s) responsible for epoxidation of the 2,3 region of the molecule would be expected to be higher than that for AFB_1, considering the presumed hydrophobic environment around the active site of the P_{450} mixed function oxidases. Therefore the large concentration of AFB_1 present in the *in vitro* microsomal system would prevent activation of the relatively small amount of aflatoxin derivatives produced. The situation *in vivo* may be presumed quite different. A dose of AFB_1 injected into an animal is distributed into a variety of physical and metabolic compartments. Distribution may limit concentration of the primary substrate at the active site of the 2,3-oxidase and allow products of other processes such as hydroxylation (M_1) or

O-demethylation (P_1) to effectively compete with AFB_1 for activation. These products may also be formed in other organs of the body and be redistributed to the liver where activation occurs.

In addition to metabolic differences, it should be obvious that the physical environment of the mixed function oxidase enzymes in the microsomal preparation may be considerably altered from their native state in the cellular endoplasmic reticulum. This may induce conformational changes exposing sensitive sites and increasing the susceptibility of these enzymes to inactivation.

Despite these two formidable qualifications this phenomenon provides a simple and plausible explanation for observations concerning AFB_1 toxicity in the mouse and deserves further investigation.

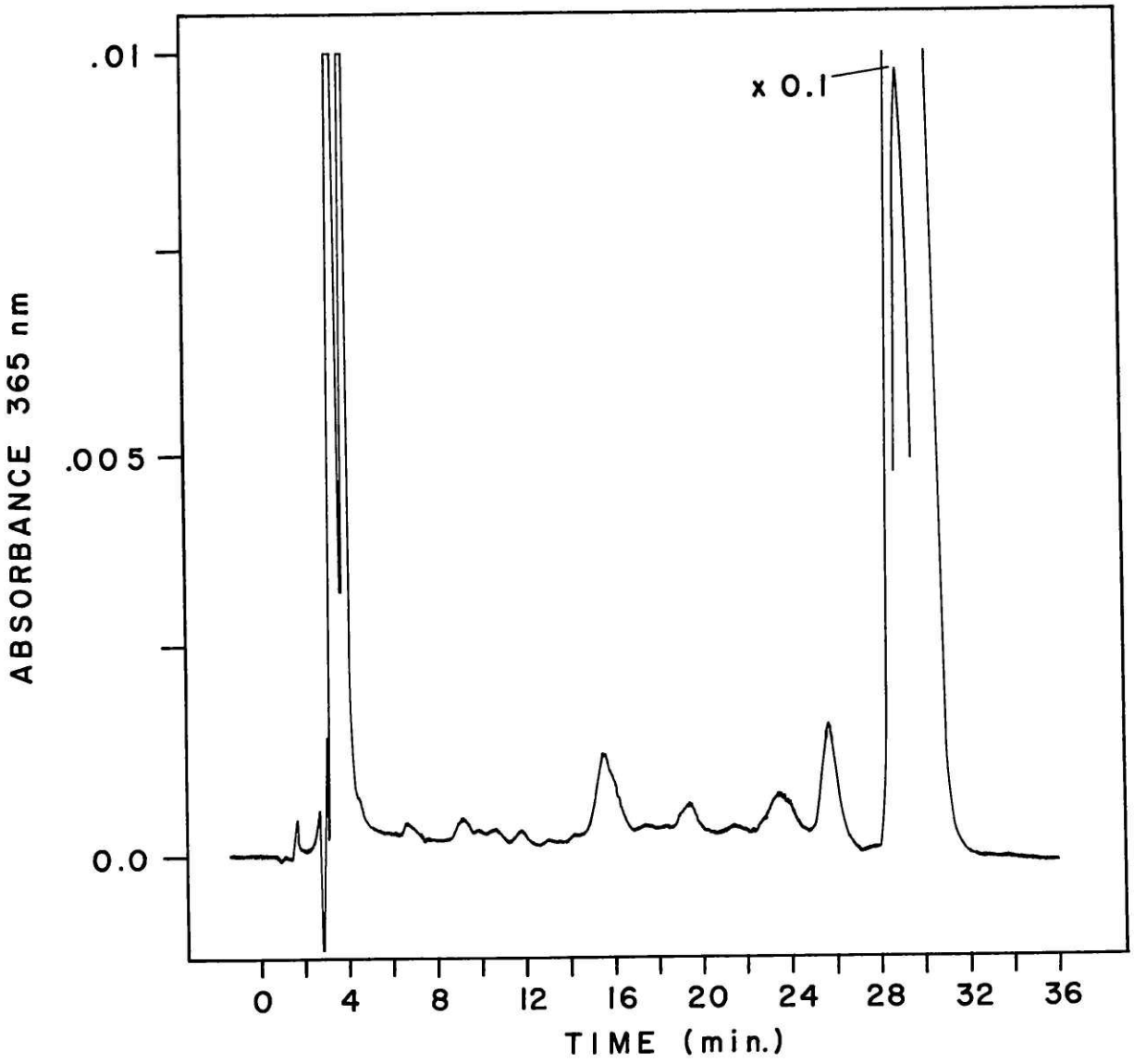
IDENTIFICATION OF THE PRINCIPAL AFB_1-DNA ADDUCT PRODUCED BY MOUSE LIVER MICROSOMES *IN VITRO*

The low level of DNA modification in the mouse liver and kidney after AFB_1 administration prevents direct characterization of the AFB_1 hydrolysis products formed *in vivo*. Although the chromatographic properties of the principal products from rat and mouse liver DNA were the same, other evidence was sought to confirm their identity. A sufficient quantity of AFB_1-DNA derivatives were produced *in vitro* using a mouse liver microsomal activation system.

Isolation of mouse liver microsomes, *in vitro* incubations, and the isolation of DNA were performed as described previously for the kinetic studies with the following modifications: microsomal protein concentration was 4 mg/ml; [^{3}H] AFB_1, specific activity was 10 mCi/mM; incubation volume was 40 ml; and the incubation time was 40 min. The reaction was terminated by addition of appropriate volumes of 4 M NaCl and 5 percent SDS.

The isolated DNA was hydrolyzed and a portion analyzed chromatographically as described in Figure 3-6. The principal product had a retention time of 29.5 min, identical to that of authentic 2,3-dihydro-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N^7-GUA) identified as the principal product produced in DNA by rat liver both *in vitro* and *in vivo*. Four to five µg of this compound was isolated from 20 ml of hydrolysate which contained approximately 15 mg of DNA adducted with AFB_1 by mouse liver microsomes.

Figure 3-6. HPLC reversed-phase separation of AFB_1 acid hydrolysis products from calf thymus DNA, covalently modified *in vitro* by AFB_1 activated by mouse liver microsomes. Separation was accomplished using a μ Bondapak C_{18} column (Waters Associates) eluted with a 25 min, linear gradient of 14 to 18 percent ethanol/H_2O at ambient bemperature. Flow rate was 1.0 ml/min. The eluant contained 0.02 M KAc pH 5.0, and was monitored at both 254 (not shown) and 365 nm.


.01
.005
0.0
ABSORBANCE 365 nm
x 0.1
0
4
8
12
16
20
24
28
32
36
TIME (min.)

The UV-VIS spectrum was recorded in 0.1 N HCl. Absorbance maxima at 238, 263, and 364 nm were seen and remained unchanged after adjustment of the solution to pH 10 with 1.0 N NaOH.

Approximately 1.0 µg of material was dissolved in N,N-dimethylacetamide, reacted with dimethylsulfate for 6 hours at room temperature and then hydrolyzed with 70 % $HClO_4$ at 100°C for 1 hour (Essigmann et al., 1977). Ion-exchange chromatographic analysis of the hydrolysate revealed the presence of one methylated base N-9 methyl guanine, implying that the guanine moiety is substituted by the AFB_1 molecules at the N-7 position.

These results are identical to those obtained with authentic AFB_1-N^7-GUA isolated from rat liver (Croy et al., 1978). They provide further evidence that the principal AFB_1 adducts produced by the mouse and rat are identical.

CHAPTER FOUR

DIFFERENTIAL REPAIR OF AFB_1 LESIONS IN RAT LIVER DNA *IN VIVO*

INTRODUCTION

Functional and structural changes in rat liver nuclei are apparent soon after the administration of aflatoxin B_1. A dose of 1 mg/kg body weight caused rapid inhibition of RNA synthesis and nucleolar segregation. Restoration of transcriptional activity and nucleolar structure was seen 24 to 36 hours later (Pong and Wogan, 1970). Although the transcriptional process may be disrupted at several steps, experiments *in vitro* have shown that covalent modification of the DNA template by AFB_1 is in some way responsible for most of the inhibition of this essential cellular process (Edwards and Wogan, 1970). The recovery of translational activity to pretreatment levels suggests repair of this damage in DNA.

Repair of covalent lesions in DNA involves both their removal and the subsequent restoration of the base sequence in DNA to its original order. The inability of a cell to complete either of these steps may result in its death or a change in the expression and/or information contained in its genetic complement.

The rate at which removal and repair of various lesions may occur may also be important. Mispairing of modified bases may occur during replication or translation resulting in the fixation of damage in daughter cells or the accumulation of altered products. Lesions which are persistent or slowly repaired will have a greater probability of causing damage through these mechanisms.

The previous chapters of this thesis have investigated the covalently bound AFB_1 adducts present in DNA isolated from various organs of animals treated with AFB_1. The present chapter examines the rate of removal of these adducts from the DNA of rat liver. In addition, to access the structural integrity of the DNA at various stages in the repair process, the relative size of single-stranded DNA molecules has been measured during repair by the alkaline sucrose gradient technique.

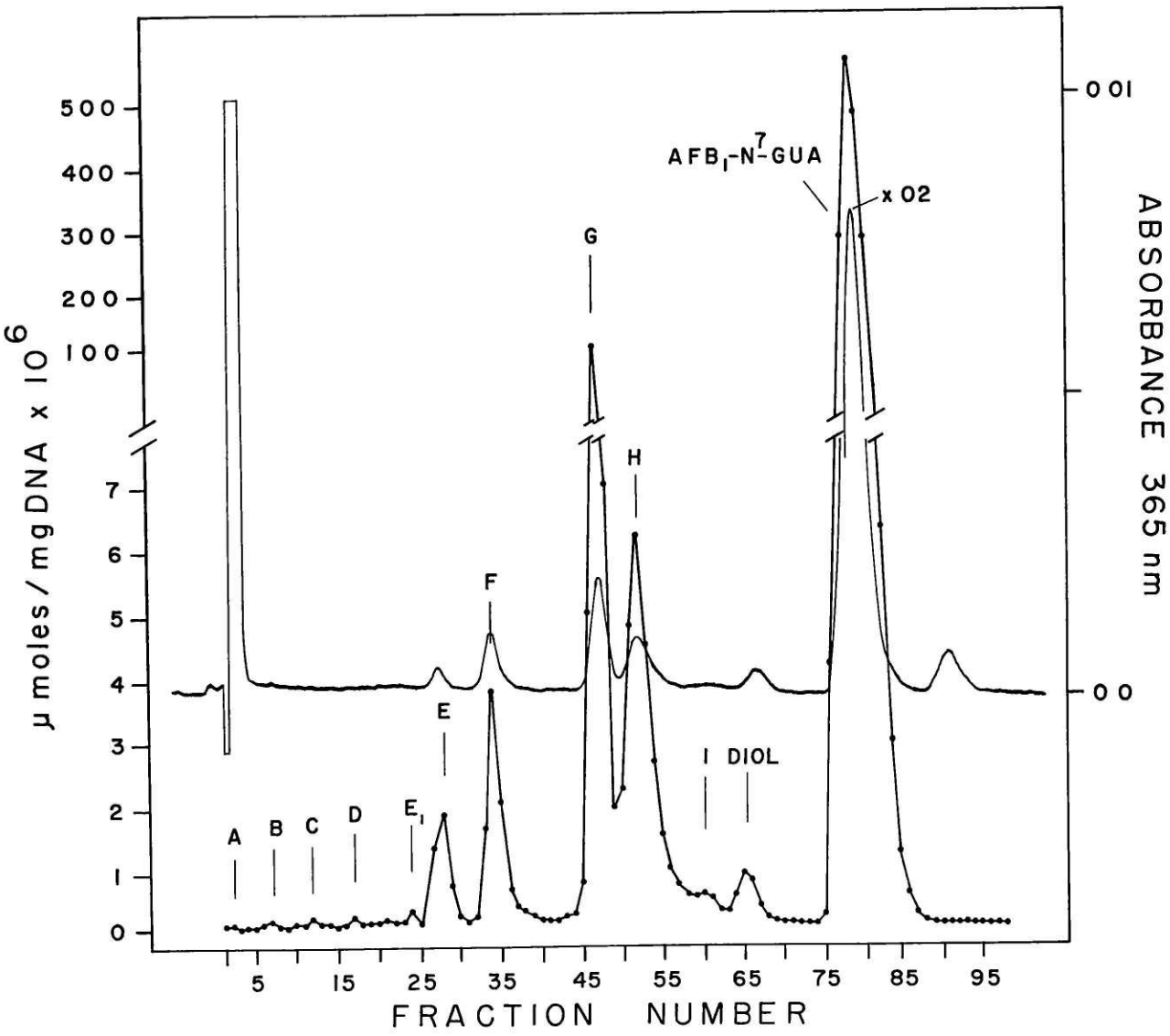
MATERIALS AND METHODS

Male Fischer rats were obtained as weanlings from Charles River Laboratories (North Wilmington, MA), housed in suspended, wire bottom cages, and fed a semi-synthetic agar gel diet (Wogan and Newberne, 1967) *ad libitum*. Six 50 day old animals weighing 130 to 135 grams were injected i.p. with 0.6 mg/kg body weight AFB_1 (Makor Biochemicals) containing [^{3}H] AFB_1 (specific activity 20 Ci/mM, Moravek Biochemicals) in 50 µl DMSO. The specific activity of the injected AFB_1 was 346 mCi/mM. Rats were sacrificed at 2, 4, 12, 24, 48, and 72 hours after AFB_1 administration. Livers were perfused *in situ*, excised, and DNA isolated from a crude preparation of liver cell nuclei. After hydrolysis of the isolated DNA the identity and amount of the AFB_1 derivatives present were determined using preparative and analytical chromatographic methods.

RESULTS

The chromatographic analysis of hydrolized DNA isolated two hours after AFB_1 administration is shown in Figure 4-1.

Figure 4-1. HPLC reversed phase separation of acid hydrolysis products obtained from rat liver DNA, 2 hours after administration of 0.6 mg/kg [^{3}H] AFB_1. Hydrolysis products were injected onto a μ Bondapak C_{18} column (Waters Associates) in a volume of 500 μl. The column was eluted with a linear, 65 min, 12 to 18 percent ethanol/H_2O gradient at ambient temperature. The eluant contained 0.02 M KAc pH 5.0. It was monitored at 254 (not shown) and 365 nm. 30-drop fractions were collected for the determination of ^{3}H activity.


μ moles / mg DNA x 10^6
500
400
300
200
100
7
6
5
4
3
2
1
0
A
B
C
D
E1
E
F
G
H
I
DIOL
AFB1-N7-GUA
x 02
0 01
0 0
ABSORBANCE 365 nm
5
15
25
35
45
55
65
75
85
95
FRACTION NUMBER

The number of picomoles of material in each fraction and the absorbance at 365 nm are plotted. A good correspondence is found between the two with the exception of the peak eluting in fractions 90 to 93. No radioactivity was found in these fractions and the magnitude of the peak (365 nm) in duplicate runs did not have a constant relationship with the other peaks. It is most probably not an AFB_1 derivative.

Quantitative data for each peak at various intervals after dosing is presented in Table 4-1. Numbers in parentheses indicate the percentage of the total amount of adducted material represented by each peak. The unretained peak, A, has not been measured. At short periods after dosing, peak I was not resolved from the comparatively large amounts of H present. However, with the rapid disappearance of H, resolution of quantification of I, which apparently has a longer half-life, was possible. Peaks A, B, C, D, I, and DIOL, collectively, represent less than one percent of the hydrolyzed products, and are detectable in small variable amounts at every time period. The five remaining peaks, E, F, G, H, and AFB_1-N^7-GUA, account for 99 percent of the hydrolyzed products. Figure 4-2 shows that the relative proportions of these peaks change dramatically during the 72 hour period. F and G, the products of the putative hydrolysis of the 7,9-disubstituted imidazole ring of the AFB_1-N^7-GUA adduct, represent a greater percentage of the covalently associated AFB_1 at later time periods.

Figure 4-3 shows the changes in the absolute levels of the five major hydrolysis products measured during the 72 hour

TABLE 4-1

QUANTIFICATION OF ACID HYDROLYSIS PRODUCTS OF[a] AFB_1-MODIFIED DNA AT VARIOUS TIMES AFTER DOSING

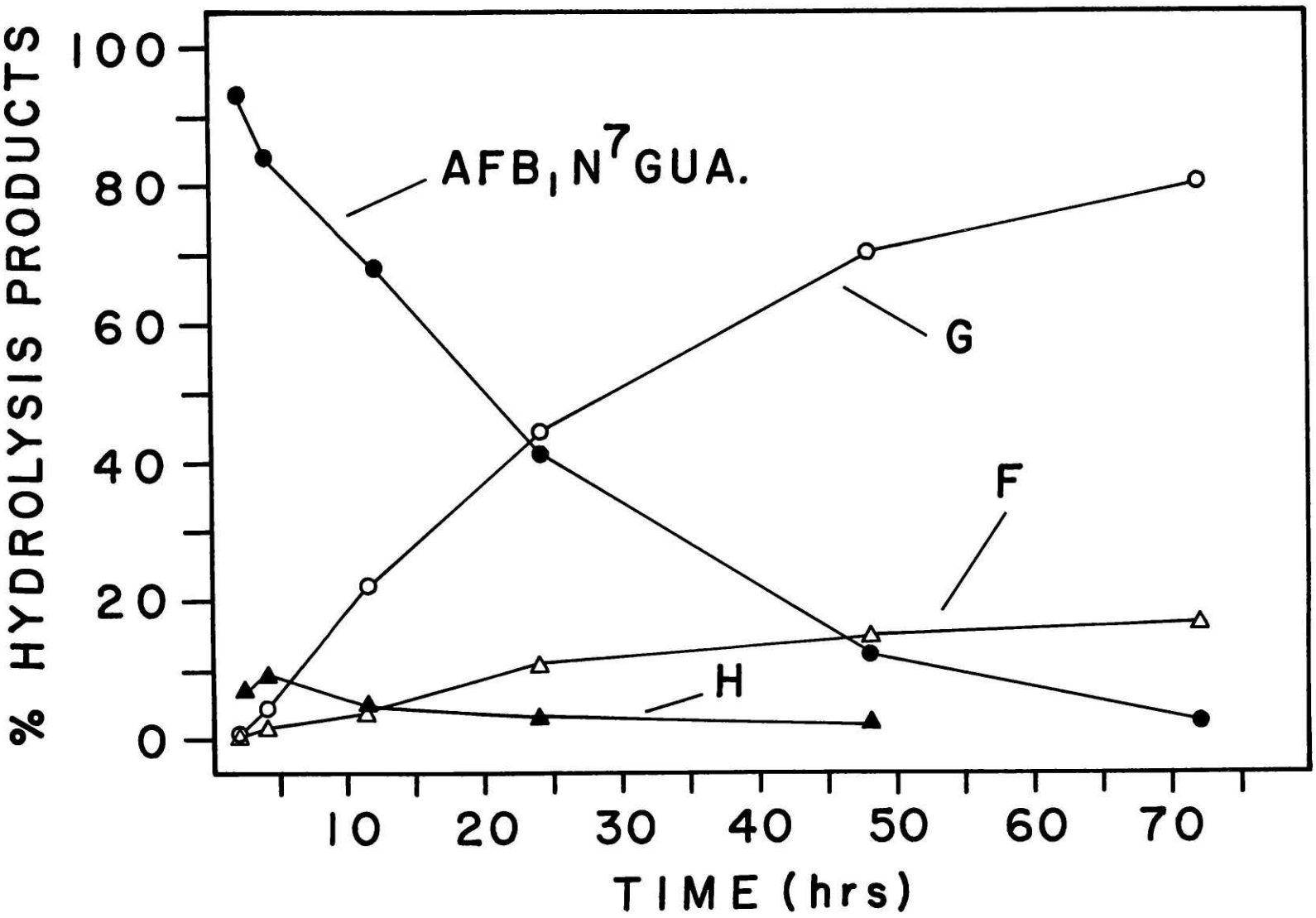
[μmoles/mg DNA x 10^8]

TIME (hrs)	A	B	C	D	E_1	E	F	G	H	I	DIOL	AFB_1-N^7-GUA	TOTAL
2	-	2.4	2.0	2.8	1.7	300[b] (1)	890 (3)	1900 (7)	1600 (6)	--	60	22,000 (82)	26,800
4	-	11	12	5.1	3.6	430 (1)	880 (4)	2500 (10)	2400 (10)	--	29	18,000 (74)	24,300
12	-	4.1	5.4	1.6	1.0	190 (1)	840 (7)	3300 (27)	520 (4)	6.1	13	7,500 (60)	12,400
24	-	14	6.4	3.4	1.6	120 (1)	1000 (11)	4100 (47)	260 (3)	7	10	3,200 (37)	8,700
48	-	4.9	4.9	1.5	0.3	42 (1)	410 (15)	1900 (70)	56 (2)	7	5	260 (10)	2,700
72	-	12	5.4	1.7	0.8	29 (1)	360 (17)	1700 (81)	--	4	3	26 (1)	2,100

[a]Calculations are detailed in Appendix II.

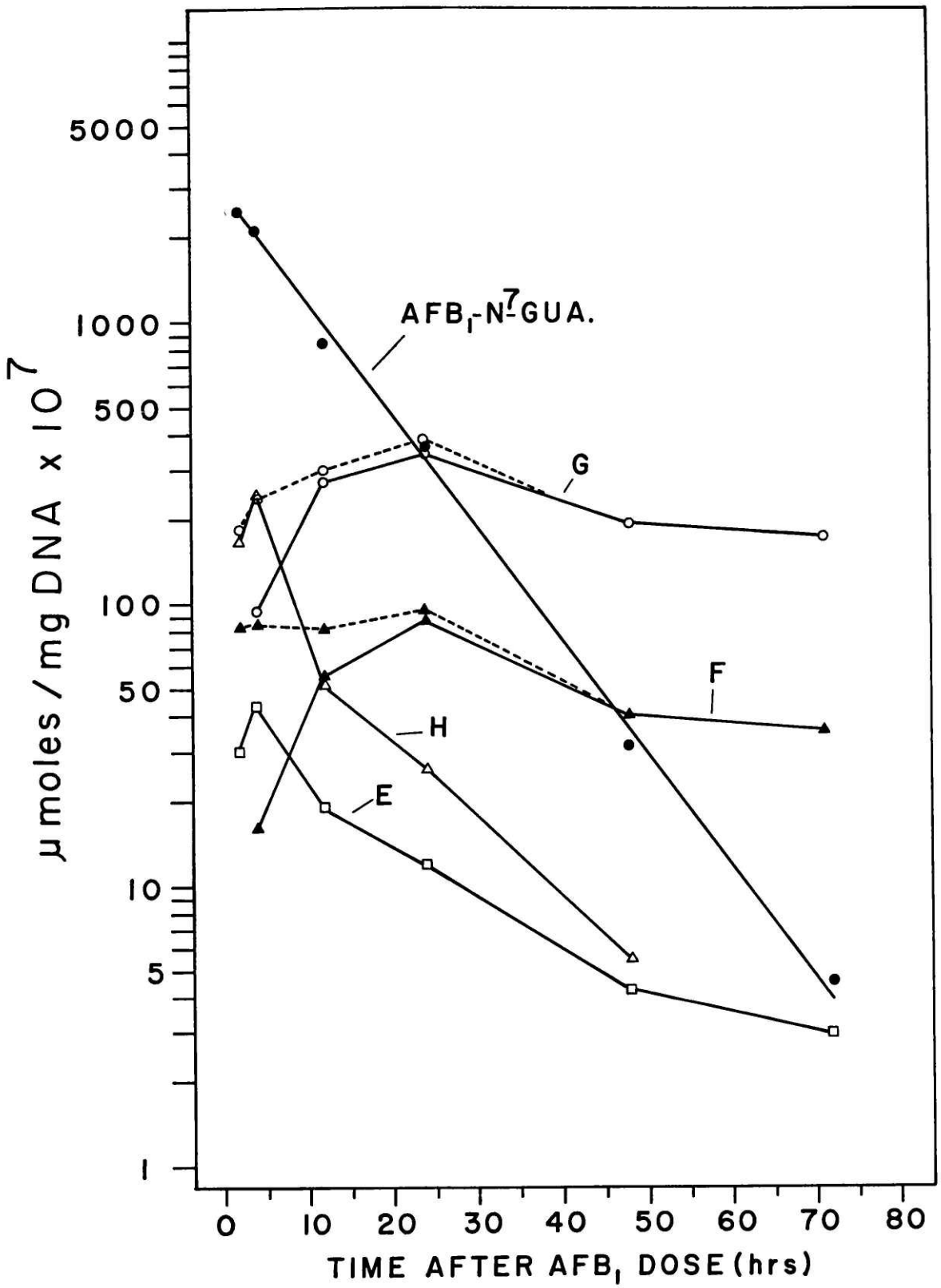
[b]Numbers in parentheses indicate the percentage of the total bound material represented by each peak. Values less than 0.5% have not been indicated.

Figure 4-2. Percent of total AFB_1 acid hydrolysis products represented by the four principal products obtained from rat liver DNA isolated at various times after the administration of 0.6 mg/kg AFB_1, i.p., in 50 µl DMSO to male Fischer rats. The data plotted here are contained in the numbers in parentheses in Table 4-1.


% HYDROLYSIS PRODUCTS
100
80
60
40
20
0
AFB1 N7 GUA.
G
F
H
10
20
30
40
50
60
70
TIME (hrs)

period. The levels of F and G measured at short time periods after AFB_1 administration are overestimates of their *in situ* levels. Preliminary experimental evidence indicates that approximately 5 percent of the AFB_1-N^7-GUA adduct may be converted to F and G during the DNA isolation procedure. At relatively long periods of time (e.g., 24 hours) this artifact will not change the measured amounts of F and G appreciably since as the amount of AFB_1-N^7-GUA decreases the amount of F and G formed from this precursor during the isolation process will be small compared to their *in situ* levels. Because of this artifact the rate constant(s) for the formation of F and G from AFB_1-N^7-GUA cannot be accurately estimated since the absolute levels of these products must be determined. The persistence of these adducts is clearly shown by their relatively constant level throughout the time period. In Figure 4-3 the measured levels of F and G are shown by dotted lines. The solid lines represent the calculated amounts of these derivatives present *in situ* from 4 to 72 hours after dosing. Correction for the amount of F and G formed during isolation at each time point was made by subtracting from the measured values the estimated 5 percent and 3.8 percent (for F and G, respectively) of the AFB_1-N^7-GUA present at each time point which is converted to these products during isolation. A close correspondence between these values is reached at 24 hours, when the amount of the AFB_1-N^7-GUA adduct has diminished to 10 percent of its two hour value. Comparisons of the amounts of peaks F and G present at 24 hours (51 picomoles/mg DNA) with the amount of AFB_1-N^7-GUA initially present

Figure 4-3. Concentrations of the five principal hydrolysis products obtained from [^{3}H] AFB_1-DNA isolated from rat liver at various times after administration of 0.6 mg/kg AFB_1. These data are enumerated in Table 4-1. The dotted lines represent measured values of peaks F and G which were not corrected for the conversion of AFB_1-N^7-GUA to these products during the isolation of DNA (see text).


5000
1000
500
100
50
10
5
1
μ moles / mg DNA x 10^7
AFB1-N7-GUA.
G
F
H
E
0
10
20
30
40
50
60
70
80
TIME AFTER AFB1 DOSE (hrs)

(250 picomoles/mg DNA) indicates that approximately 20 percent of the original N-7 guanine adductwas converted to F and G in 24 hours.

The other three products shown in Figure 4-3 are rapidly removed from DNA with apparent half-lives of 7 hours, 11 hours, and 16 hours for AFB_1-N^7-GUA, H, and E, respectively. Examination of the kinetics of formation of these products up to four hours after dosing indicates that maximum levels are reached at different times. The AFB_1-N^7-GUA adduct attains its highest level at or shortly before two hours, while peaks E and H are not at maximum levels until at least four hours. These observations are consistent with the fact that peak H is an aflatoxin P_1 (AFP_1) derivative requiring two enzymatic steps, demethylation and epoxidation, for formation from AFB_1. These enzymatic steps need not occur in the same organ. Furthermore, activation of AFP_1 immediately after dosing may be limited by competition between it and AFB_1 for the active site of the epoxidase favoring other pathways for its removal. The similarities between the kinetics of formation and removal of peaks H and E further support the hypothesis (see Chapter Two) that E is the product of activation of another AFB_1 metabolite such as the 4-hydroxylated drivative, aflatoxin M_1.

ALKALINE SUCROSE GRADIENT STUDIES

INTRODUCTION

Removal of covalently modified bases from the DNA molecule constitutes the first part of the repair process. Insertion of the correct base at the damaged site and restoration of breaks in the covalent sturcture of the deoxyribose-phosphate backbone are necessary to complete the process. The previous experiment has indicated that approximately 68 and 92 percent of the covalent aflatoxin adducts in DNA are removed 24 and 72 hours, respectively, after AFB_1 exposure. These results do not indicate the structural integrity of the DNA molecule.

Alkaline sucrose gradients have been used by a number of investigators to indicate damage to DNA caused by alkylating agents such as N-methyl-N-nitrosourea (Zubroff and Sarma, 1976).

Lysis of cells or nuclei in alkaline layers on top of these gradients is thought to produce single stranded DNA molecules of large molecular weight. The evidence on the nature of sedimenting DNA in these gradients is incomplete (Parodi et al., 1975), and its molecular weight cannot be accurately determined from s values because of anomalous sedimentation characteristics (Burgi and Hershey, 1963). However, differences in sedimentation rates can be shown between control and carcinogen treated animals (Cleaver, 1975). The slower rate of sedimentation of DNA from animals or cells treated with chemical or physical agents has been interpreted to indicate a reduction in its molecular weight resulting from either alkali-labile sites or single strand breaks produced by repair endonucleases. The

persistence of these differences seen with azaserine was interpreted to indicate the slow repair of damage to DNA caused by this carcinogen (Lilja et al., 1977).

Some types of DNA damage have been shown to cause only minor changes in the sedimentation characteristics of DNA in alkaline sucrose. Changes in the sedimentation rate of DNA complexes caused by exposure to UV light were detectable only at short times after exposure using special techniques (Cleaver, 1975). This is consistent with the hypothesis that single strand breaks are transiently present during the excision repair process and act as sites for strand separation in alkali.

In order to detect the presence of alkali-labile sites and/or linkages in the DNA sugar-phosphate backbone during the removal of lesions *in vivo* from DNA following exposure to AFB_1, the sedimentation characteristics of liver DNA were examined in rats 4 and 24 hours after AFB_1 administration. A control experiment was initially performed using N-methyl-N-nitrosourea.

MATERIALS AND METHODS

TREATMENT OF ANIMALS

Male Fischer rats weighing approximately 145 grams were administered N-methyl-N-nitrosourea (MNU) or aflatoxin B_1 (AFB_1). MNU (Aldrich Chemical Co.), 50 mg/kg body weight was injected i.p. in 0.5 ml of saline solution. The animal was sacrificed 4 hours later. 0.6 mg/kg body weight AFB_1 (Makor Chemicals) was injected i.p. in 50 µl of DMSO. Animals treated with AFB_1 were sacrificed 2 and 24 hours after dosing. The isolation of nuclei and alkaline sucrose gradient studies were performed

using methods essentially the same as those described by Lilja et al. (1978). The exact procedure is described here to enumerate the minor changes.

ISOLATION OF NUCLEI

The livers of treated animals were perfused *in situ* with phosphate buffer containing 0.14 M NaCl, 10 mM EDTA, 8 mM Na_2HPO_4, 15 mM KH_2PO_4, and 3 mM KCl, pH 7.50. All succeeding steps were performed at 2°C. Three grams of liver were weighed and minced using a tissue press, then gently homogenized using a teflon-glass homogenizer in 40 ml of phosphate buffer. The homogenate was strained through 100 µm nylon mesh and centrifuged at 750 x *g* for 10 min. The supernatant was removed by aspiration and the brown pellet suspended in 10 ml phosphate buffer. The nuclei in an aliquot of this suspension were stained with an equal volume of 0.05 percent trypan blue and the concentraion of nuclei in each suspension determined in a 1:20 dilution using a hemocytometer. An appropriate dilution of each suspension was then made to obtain a concentration of approximately 1.5×10^6 nuclei/ml.

ALKALINE SUCROSE GRADIENTS

32 ml, 5-25 percent linear sucrose gradients were formed over a 2 ml cushion of 50 percent sucrose. Sucrose solutions for the gradient and cushion contained 0.3 M NaOH, 0.9 M NaCl. The lysis layer consisted of 1.5 ml of a solution containing 0.3 M NaCl, 0.03 M EDTA, 0.5% sodium dodecylsulfate (SDS), and 0.1 M *tris*, pH 12.5. Precipitation of SDS in the lysis medium

by contact with the high salt gradient was minimized by a 2.0 ml interface between the solutions containing 3 percent sucrose, 0.3 M NaOH.

100 µl of nuclei suspension containing 1.5 x 10^5 nuclei was added to the top of the lysis layer followed by another 0.5 ml of lysis solution. After a lytic period of one hour in the dark, the gradients were centrifuged in a Beckman SW27 rotor at 113,000 x *gmax* (25,000 rpm) for 50 min at 25°C. Following centrifugation the gradients were fractionated from the top (20 fractions/gradient). DNA was precipitated overnight at 2°C after addition of 0.5 ml 0.02% bovine serum albumin, 0.5 ml 1 N HCl, and 0.6 ml 50% trichloroacetic acid (TCA). Precipitated DNA was washed three times with cold 5% TCA, once with 95% ethanol containing 0.1 M KAc, once with absolute ethanol, and dried at 70°C for 30 min.

The relative DNA content of each fraction was estimated fluorometrically (Kissane and Robins, 1958). Precipitates were covered with 0.2 ml of 0.6 M diaminobenzoic acid (DABA, Aldrich Chemical Co.) which had been mixed with Norit (Fisher Scientific Co.), 1 g/50 g DABA, for ten minutes just prior to use, and incubated at 60°C for 30 min. After cooling and addition of 1.8 ml 1 N HCl the fluorescence of each fraction was read using a Turner Fluorometer Model 110. Wrattan filter 47-B was used to provide the 436 nm wavelength for excitation, and filter 2A-15 to obtain 520 nm for reading the fluorescence.

RESULTS

The results of the experiment with MNU are shown in Figure 4-4. DNA from the MNU treated animal did not sediment as far into the gradient as control DNA. These results are similar to those obtained by other investigators (Zubroff and Sarma, 1976), and indicate the possible presence of alkaline labile sites and/or single strand breaks in the DNA of treated animals which have caused a reduction in the molecular weight and consequently of the sedimentation rate of this DNA.

Figure 4-5 shows the results of one experiment in which animals were dosed with 0.6 mg/kg aflatoxin B_1. Each experiment was repeated twice with similar results. No significant differences could be shown between the sedimentation characteristics of control and treated animals at either 4 or 24 hours after dosing.

It is apparent from these results that the damage produced by MNU and AFB_1 is quite different. Similar levels of modification of the N-7 atom of guanine are produced in rat liver DNA by the administered doses of MNU (1 modification/7000 base residues; Swann and Magee, 1968) and AFB_1 (1 modification/12000 base residues, Croy et al., 1978). Since the half-life of the N-7 methyl derivative, 72 hrs (Lawley, 1976), is approximately ten times the half-life of AFB_1-N^7-GUA, changes in sedimentation rate resulting from single strand breaks produced by repair endonucleases or alkaline labile apurinic sites would be expected if modification of the N-7 guanine atom resulting in depurination was responsible for the reduction of the sedimentation

Figure 4-4. Sedimentation patterns in alkaline sucrose gradients of DNA from the livers of rats treated with methyl nitrosourea (MNU) and control. Rats received 50 mg/kg MNU i.p. in 0.15 ml 0.9% NaCl. Control rats received an equal volume of 0.9% NaCl. Rats were sacrificed four hours later.

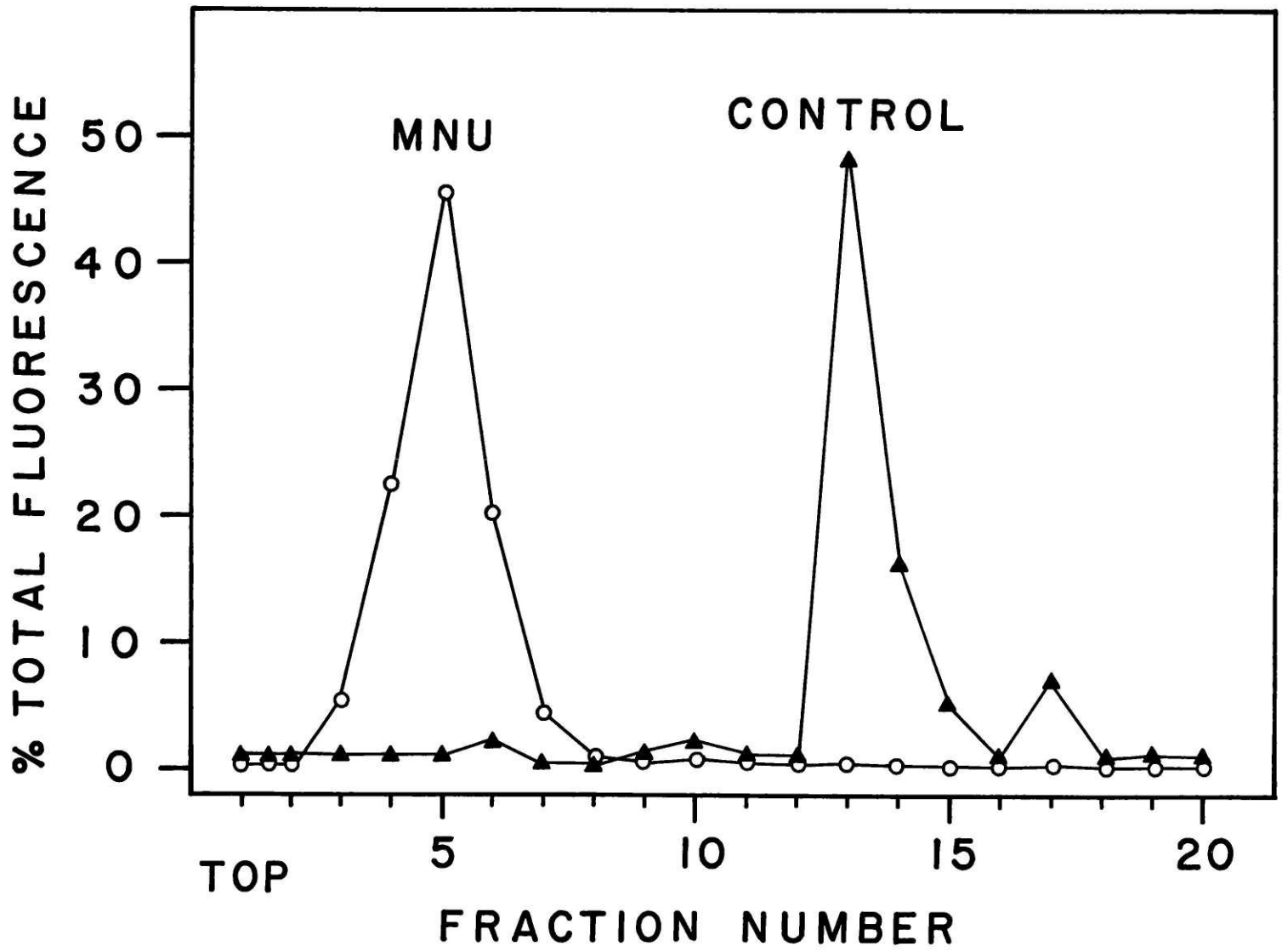
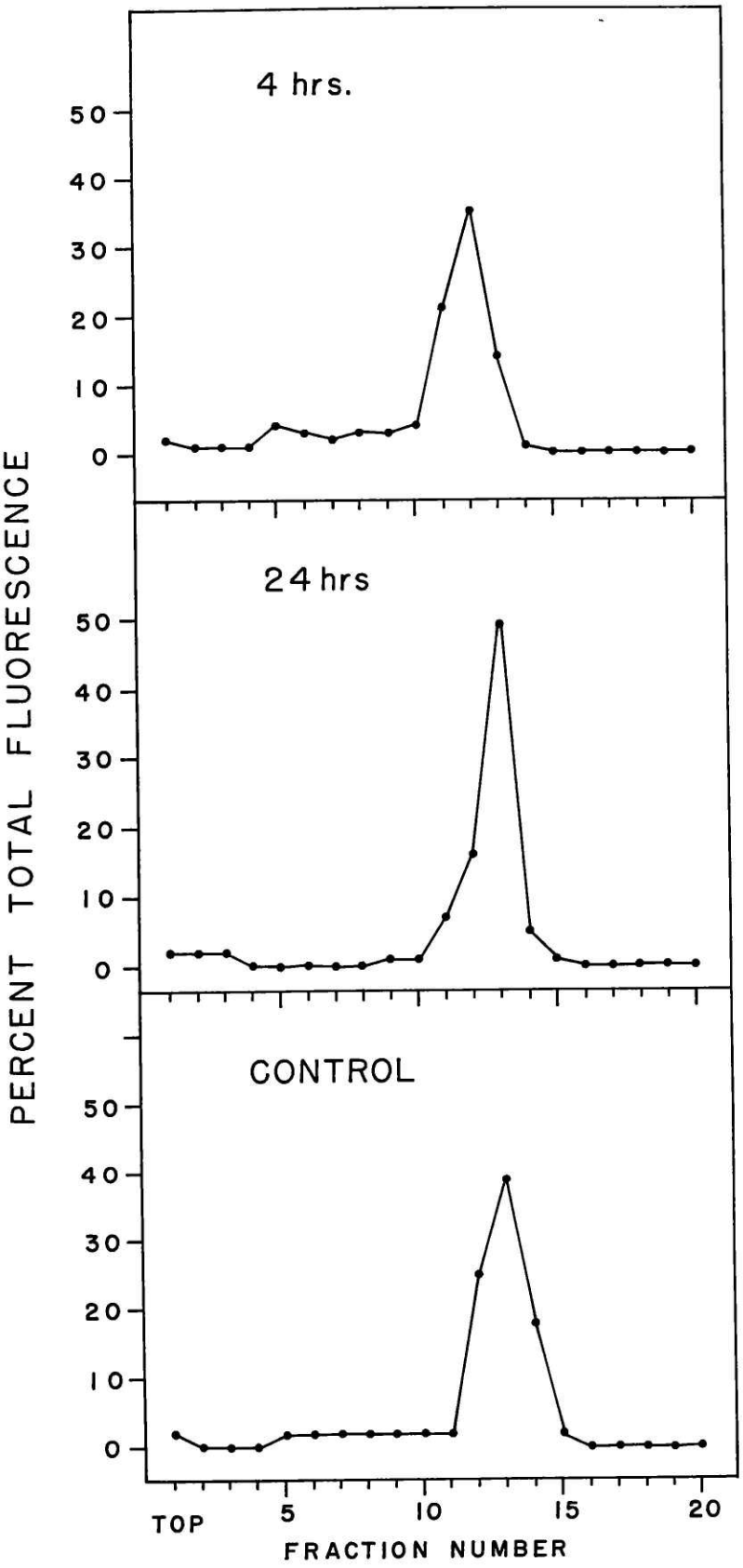

MNU
CONTROL
% TOTAL FLUORESCENCE
50
40
30
20
10
0
TOP
5
10
15
20
FRACTION NUMBER

Figure 4-5. Effect of administration of AFB_1 on the sedimentation patterns of liver DNA in alkaline sucrose gradients. Male Fischer rats were administered 0.6 mg/kg AFB_1 i.p. in 50 µl DMSO. Control rats received 50 µl DMSO. Rats were sacrificed 4 hours and 24 hours later. The curves represent the results of two experiments for which identical results were obtained.


4 hrs.
24 hrs
CONTROL
PERCENT TOTAL FLUORESCENCE
0
10
20
30
40
50
TOP
5
10
15
20
FRACTION NUMBER

rate by MNU. It is more likely that this phenomenon is caused by the formation of alkaline-labile methylphosphotriesters, which are known to be persistent products in DNA (Bannon and Verley, 1972).

These results imply that the spontaneous or enzymatic removal of N-7 guanine lesions produced in DNA by aflatoxin B_1 does not result in the formation of a large number of single strand breaks or depurinated alkaline-labile sites. If the mechanism of removal of the covalent lesions involves the generation of these potential points of strand separation in alkali, they are probably present transiently during the repair process and the methods used here were not sensitive enough for their detection.

Similar results and conclusions have been reached in investigations of the repair of AFB_1 damage to DNA in WI38 human fibroblasts (Sarasin et al., 1977). No alkaline labile lesions could be detected in the DNA of these cells using alkaline sucrose gradients after their exposure to AFB_1. DNA damage was demonstrated in rat liver following exposure *in vivo* to AFB_1 using the alkaline elution assay (Petzold et al., 1978). It was reported that treatment with AFB_1 increased the elution of single stranded DNA from polyvinyl chloride filters by alkaline media. This technique may be more sensitive than alkaline sucrose gradients in detecting alkaline-labile damage.

DISCUSSION

Two types of lesions are present in liver DNA following exposure of the animal to AFB_1. Primary lesions are formed by the activation of AFB_1 or some of its metabolic products to the 2,3-epoxide which attacks the nucleophilic N-7 guanine moiety. Secondary lesions are formed by the hydrolysis of the positively charged imidazole ring of this adduct, producing the putative aflatoxin-formamido-pyrimidine derivative, covalently bonded to the deoxyribose-phosphate backbone of the DNA molecule. These secondary lesions are chemically more stable in DNA because of the elimination of the positive charge on the nitrogen atom which formed the glycosidic bond in the 7,9-disubstituted guanine.

The primary lesions which have been identified, AFB_1-N^7-GUA, H (AFP_1-N^7-GUA), and possibly E, are readily removed from the DNA molecule. Spontaneous depurination, enzymatic excision, or a combination of these processes may be responsible for their removal. Recent studies have recognized a number of enzymes collectively known as N-glycosidases which excise damaged bases from DNA by the hydrolysis of their glycosidic bond, without perturbing the DNA sugar-phosphate backbone (Lindahl, 1976; Brent, 1977; Laval, 1977; Riazuddin and Lindahl, 1978). The resulting apurinic or apyrimidinic sites may be repaired either by an excision repair pathway involving the sequential action of an endonuclease, exonuclease, DNA polymerase, and finally a ligase or possibly by the recently discovered purine insertase. This enzyme activity has been isolated from human fibroblasts

and found to directly insert purine basis into apurinic sites in a DNA molecule (Deutsch and Lin, 1979). The results of sedimentation studies of damaged DNA in alkaline sucrose gradients suggest that if spontaneous depurination or removal by N-glycosidases occurs, the resulting apurinic sites are rapidly repaired, since a large number of these sites were not detected.

The persistence of the secondary, ring-opened formamido derivative may be partially attributed to the chemical stability of the glycosyl fond formed between the C^1-1 hemiacetal of the sugar and the secondary amine of the AFB_1-formamido moiety. Its stability *in vivo* indicates that repair enzymes are inefficient in its removal. This implies that despite the presence of the large AFB_1 molecule and the hydrolytic change in the purine base, little distortion occurs in the helical structure of the DNA molecule which might be recognized by an excision repair endonuclease. Studies with molecular models support this hypothesis. The most likely orientation of the AFB_1 molecule in both the 7,9-disubstituted guanine and ring-opened formamido derivatives is parallel to the major groove of the double helix resulting in little molecular perturbation in the surrounding bases.

CHAPTER FIVE

AFB_1 HYDROLYSIS PRODUCTS FROM DNA ISOLATED FROM RAT LIVER DURING EXPOSURE OF RATS TO CARCINOGENIC DOSES OF AFB_1

INTRODUCTION

Induction of hepatocellular carcinoma by AFB_1 in the rat is strongly dependent upon the dosage regimen to which the rats are subjected. Single administration of a toxic dose has been found ineffective, while continuous administration at low levels in the diet or small multiple doses given i.p. or i.g. have produced a dose-dependent incidence of tumor induction of up to 100 percent in the male Fischer rat (see Wogan, 1973).

The previous studies have investigated the identity of the AFB_1 hydrolysis products isolated from the DNA of animals after the administration of a single dose of AFB_1, and described the disappearance of these lesions as a function of time. The present study concerns the identity and amounts of these hydrolysis products during the administration of multiple doses of AFB_1 to male Fischer rats on a schedule which has been shown to induce a 100 percent incidence of hepatocellular carcinoma.

EXPERIMENTAL DESIGN

Administration of 40, 25 μg doses of AFB_1 over an eight week period (5 consecutive doses each week) produces 100 percent incidence of liver carcinoma in the male Fischer rat (Wogan, 1973). Rats studied in this experiment were subjected to the first two weeks of this protocol. During this 14 day period 25 μg of AFB_1 labeled with ^3H was administered i.p. in 25 μl of DMSO to rats at 2:00 PM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11. Two rats were sacrificed each day and the DNA of their livers isolated, hydrolyzed, and analyzed chromatographically.

With the exception of day 0, animals were not administered AFB_1 on the day of sacrifice.

MATERIALS AND METHODS

Thirty 45 day old rats, weighing approximately 100 grams, were obtained from Charles River Laboratories (North Wilmington, MA). These were individually housed in suspended, wire-bottom cages, and fed a semi-synthetic agar gel diet (Wogan and Newberne, 1967) *ad libitum*. Following a 7 day period to allow adjustment to the new diet and environment, the animals were injected i.p. with 25 µg AFB_1 (Makor Biochemicals) containing 60.7 µCi [^{3}H] AFB_1 (20 Ci/mM, Moravek Biochemicals) dissolved in 25 µl DMSO, on the regimen previously described. Two rats were sacrificed per day and DNA isolated from their livers. The DNA was then hydrolyzed and the [^{3}H] AFB_1 hydrolysis products analyzed by the use of preparative and analytical chromatographic methods.

RESULTS

No overt signs of toxicity were apparent during the experimental period, either in the behavior of the animals or the gross appearance of their livers at the time of sacrifice. The animals weighed approximately 125 grams at the beginning of the experiment and increased to 145-150 grams at the end of the two week period.

The amount of DNA recovered from the rat livers varied from 2.46 mg to 7.55 mg (Table 5-1). The lowest recoveries and most variation between duplicate samples occurred with animals

TABLE 5-1

AMOUNT OF DNA RECOVERED FROM RAT LIVER FOLLOWING MULTIPLE DOSES OF AFB_1

DAY NUMBER	AMOUNT OF DNA RECOVERED (mg) RAT I	RAT II
0	7.40	7.55
1	3.34	4.60
2	2.46	4.68
3	5.61	2.90
4	3.16	3.04
5	6.10	5.10
6	5.03	5.34
7	6.46	5.92
8	7.11	7.10
9	5.33	5.86
10	6.80	6.00
11	5.99	6.94
12	4.98	6.32
13	6.00	7.20
14	5.75	6.40

sacrificed during the first five day period. The highest recovery was obtained from animals sacrificed two hours after a single 25 μg AFB_1 dose on day 0. The reasons for this variation are not known. Possible explanations include a low recovery of nuclei because of increased fragility caused by AFB_1 or an increased association of proteins or conformational changes. However, despite the variations in recovery between some duplicate samples (e.g., days 2 and 3), the levels of AFB_1 derivatives in these hydrolysates did not have any greater apparent variations than duplicates with closely matched recoveries (e.g., day 4).

Figure 5-1 shows the chromatographic profile of AFB_1-DNA hydrolysis products in rat liver two hours after the injection of the initial 25 μg dose of AFB_1 (day 0). The products identified in this figure are qualitatively identical to those isolated from the liver DNA of rats which were treated with 1.0 and 0.6 mg/kg body weight (Chapters Three and Four), with the exception of peak F_1. The identity of this peak is discussed in Chapter Two. It is believed to be an isomeric 2-hydroxyaflatoxin B_1 derivative. The reasons for its presence in this experiment and absence in previous ones are not known. Quantitative data on the levels of each peak are presented in Table 5-2. The average values range from 1.9×10^{-5} modifications/base (50,000 bases/modification) for AFB_1-N^7-GUA to 4.0×10^{-9} modifications/base (300,000,000 bases/modification) for peak D which represents less than 0.1 percent of the covalently bound material. After the two hour period, 7.3×10^{-5} μmoles

Figure 5-1. Reversed-phase HPLC pattern of [^{3}H] AFB_1 hydrolysis products from rat liver DNA two hours after administration of 25 µg AFB_1 containing 60.7 µCi ^{3}H activity. 400 µl of material was injected onto a µ Bondapak C_{18} column (Waters Associates), which was eluted with a linear, 65 min, 14 to 18 percent ethanol/H_2O gradient at ambient temperature. The eluant contained 0.02 M KAc pH 5.0, and was monitored at 254 (not shown) and 365 nm. 30-drop fractions were collected for the determination of ^{3}H activity.

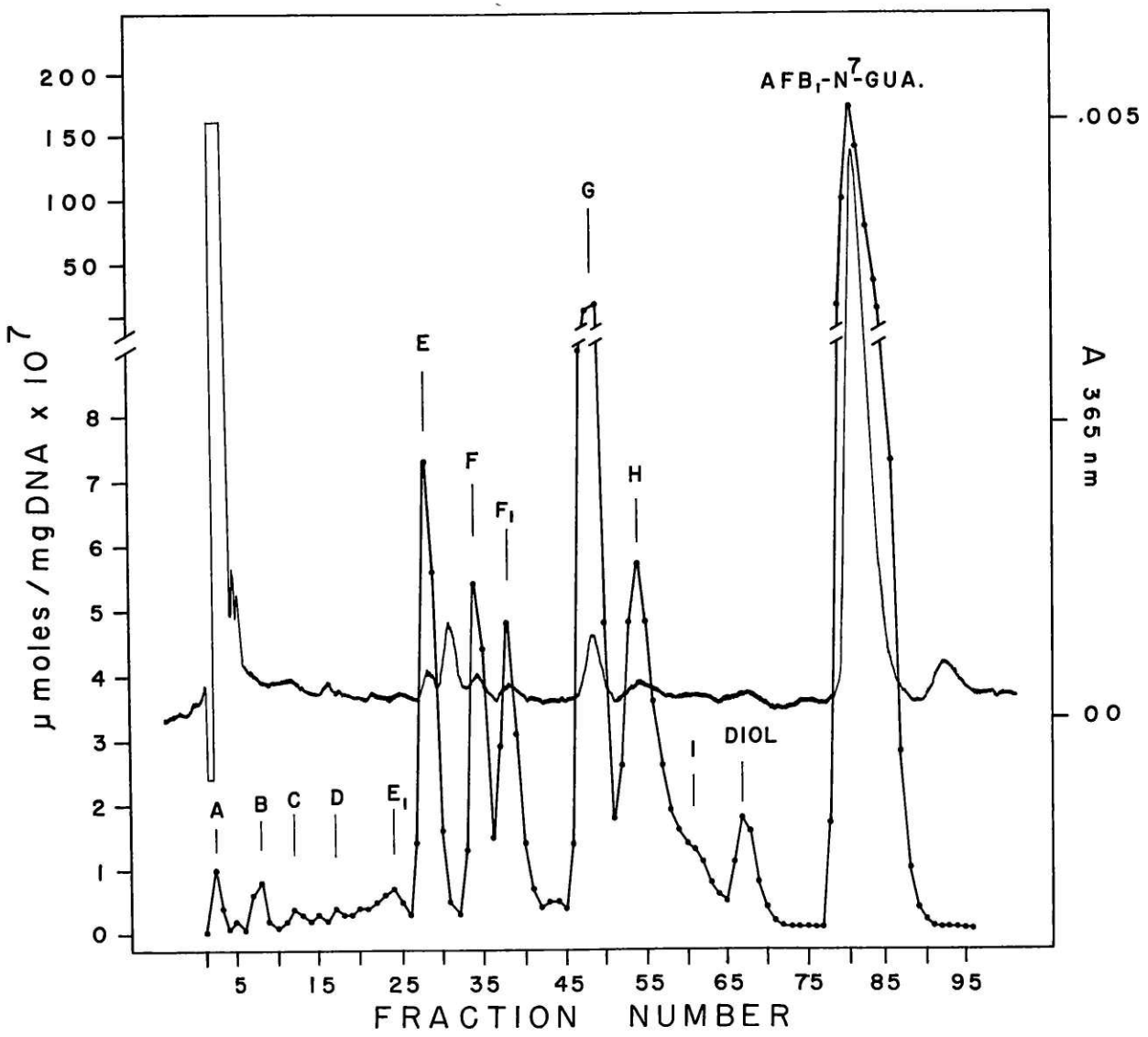

AFB1-N7-GUA.
.005
00
A 365 nm
μ moles / mg DNA x 10^7
200
150
100
50
8
7
6
5
4
3
2
1
0
A
B
C
D
E1
E
F
F1
G
H
I
DIOL
5
15
25
35
45
55
65
75
85
95
FRACTION NUMBER

TABLE 5-2

AMOUNTS OF HYDROLYSIS PRODUCTS PRESENT IN RAT LIVER DNA[a] TWO HOURS AFTER A 25 μg DOSE OF AFB_1

(Modifications/Base x 10^8)

PEAK DESIGNATION	LIVER 1	LIVER 2	AVERAGE
A	--	--	--
B	3.0	4.5	3.8 (0.2)[b]
C	0.7	1.3	1.0 (--)
D	0.3	0.5	0.4 (--)
E_1	4.9	3.6	4.3 (0.2)
E	51.0	53.0	52.0 (2.2)
F	49.0	43.0	46.0 (2.0)
F_1	39.0	43.0	41.0 (1.8)
G	170.0	160.0	165.0 (7.1)
H	93.0	86.0	90.0 (3.8)
I	26.0	24.0	25.0 (1.1)
DIOL	10.0	12.0	11.0 (0.5)
AFB_1-N^7-GUA	1900.0	1900.0	1900.0 (81.2)
TOTAL	2347	2331	2340

[a]Calculations are detailed in Appendix II.

[b]Numbers in parentheses indicate the percentage of total represented by each peak. Values less than 0.1 not shown.

of AFB_1 were covalently bound per milligram of DNA. Assuming 12 mg of DNA per liver, a total of 8.8×10^{-4} μmoles AFB_1 were covalently associated with liver DNA two hours after dosing. This is approximately one percent of the administered dose of 8.0×10^{-2} μmoles AFB_1.

The kinetics of removal of this material during the first 24 hour period were similar to those described in the previous chapter for a 0.6 mg/kg body weight dose. The amounts of AFB_1 derivatives present after 24 hours (day 1) are shown in Table 5-3. (The duplicate for this day was lost because of a chromatographic error.) Large reductions are seen in the amounts of E, F, H, and AFB_1-N^7-GUA. Peaks F and G show little apparent change. However, the amounts of these products measured after the two hour period are most probably overestimates of their true *in situ* levels because of the conversion of a small percentage of AFB_1-N^7-GUA to these derivatives during the DNA isolation procedure. The remaining peaks, I, diol, E, D, C, and B, show slight reductions and are present at levels 10 to 100 times lower than the AFB_1-N^7-GUA derivative. The low levels of these compounds make their measurement difficult, resulting in wide variations between duplicate samples.

After 24 hours, 88 percent of the covalently bound AFB_1 has been removed from DNA. The remaining 12 percent is found primarily in three peaks, F, G, and AFB_1-N^7-GUA, containing 11, 51, and 34 percent, respectively, of this residual material. The remaining four percent is distributed among seven other peaks representing one percent or less each. During the two

TABLE 5-3

AMOUNTS OF HYDROLYSIS PRODUCTS PRESENT IN RAT LIVER[a]
24 HOURS AFTER A 25 µg DOSE OF AFB_1
(Modifications/Base x 10^8)

PEAK DESIGNATION	LIVER 1[b]
A	--
B	0.9 (0.3)[c]
C	1.3 (0.5)
D	0.5 (0.2)
E_1	0.2 (--)
E	1.9 (0.7)
F	31.0 (11.3)
F_1	-- (--)
G	140.0 (50.9)
H	-- (--)
I	2.7 (1.0)
DIOL	3.2 (1.2)
AFB_1-N^7-GUA	93.0 (33.9)
TOTAL	275

[a]Calculations are detailed in Appendix II.

[b]The duplicate for this day was lost.

[c]Numbers in parentheses indicate the percentage of total represented by each peak. Values less than 0.1 are not shown.

week period the three major peaks account for greater than 90 percent of the AFB_1 derivatives hydrolyzed from DNA.

Figure 5-2 shows the levels of hydrolysis products of G, F, and AFB_1-N^7-GUA obtained from rat liver DNA during the two week period. Peak E is included in this figure to be representative of the relative levels of the minor AFB_1 hydrolysis products in relation to the three major products. Peak G is present at the highest concentration in DNA and attains a relatively constant level of 1.8×10^{-5} μmoles/mg DNA at the end of the first five day dosing period. Its level does not significantly change with cessation of dosing on days 5 and 6 or during the second dosing period, days 7 through 11. A similar behavior is seen with peak F at a lower level of modification.

The amount of AFB_1-N^7-GUA remaining 24 hours after the AFB_1 administration shows a gradual decline during the first five day period. On day 6, 48 hours after cessation of dosing, a precipitous decline in the residual level is seen, which is further apparent on day 7, 72 hours after the last AFB_1 dose. Resumption of treatment on day 7 for a second five day period results in an increase on day 8 to approximately ten times the level seen on day 7. This level remains constant during this period until day 13, when dosing is again stopped and a second decline in the residual amount of AFB_1-N^7-GUA is apparent. The level of peak E and other minor products B, C, D, E, I, and diol remain relatively constant throughout the entire period.

Figure 5-3 shows the changes in the relative percentage of total covalently bound material represented by the three

Figure 5-2. Levels of various AFB_1 acid hydrolysis products from the liver DNA of rats during administration of multiple doses of AFB_1. 25 µg AFB_1 was injected i.p. in 25 µl DMSO to male Fischer rats on days 0 (not shown), 1, 2, 3, 4, 7, 8, 9, 10, and 11. Two rats were sacrificed each day on days 1 through 14 and the DNA isolated from their livers analyzed separately. Rats did not receive AFB_1 on the day of sacrifice.

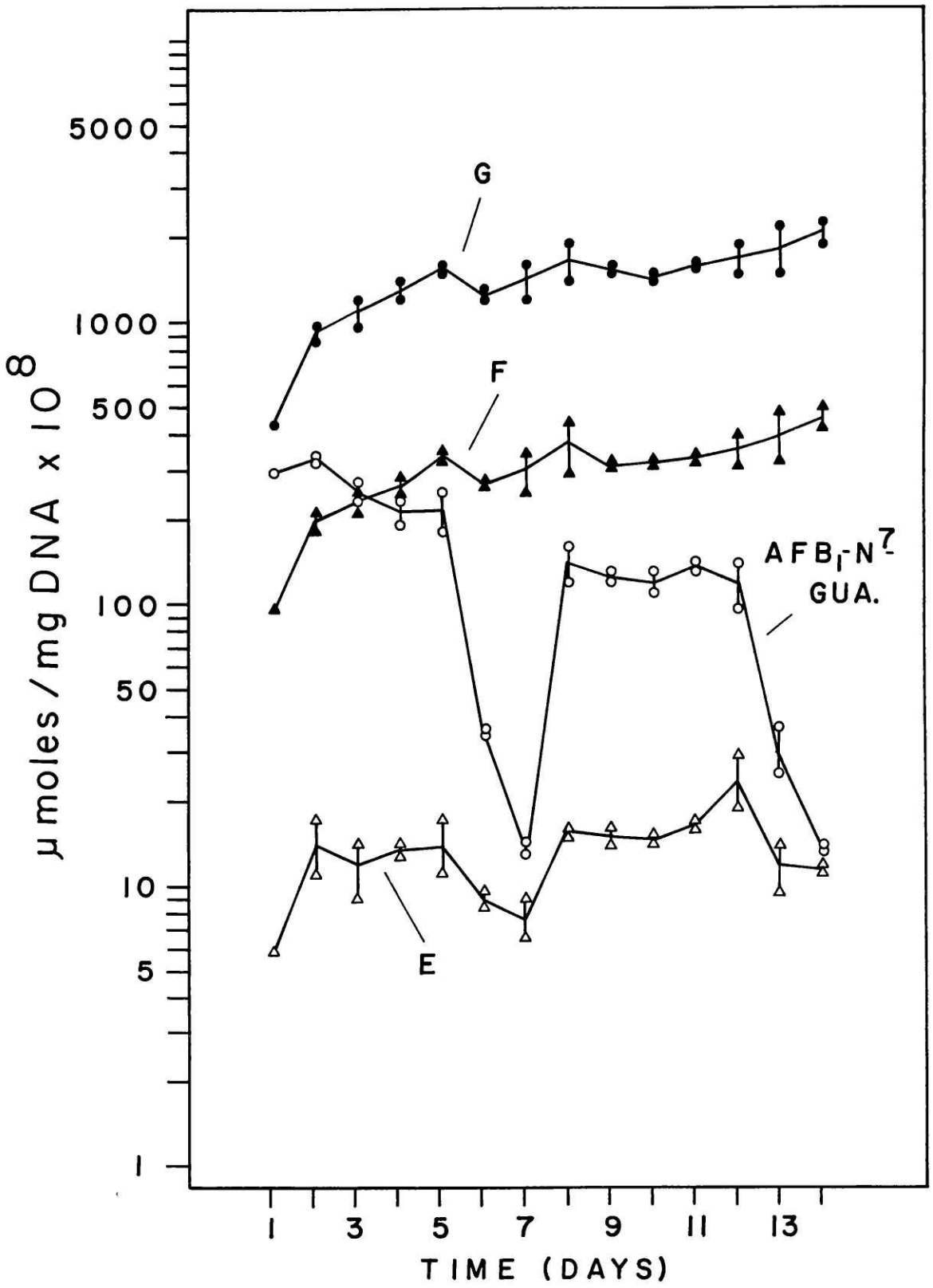
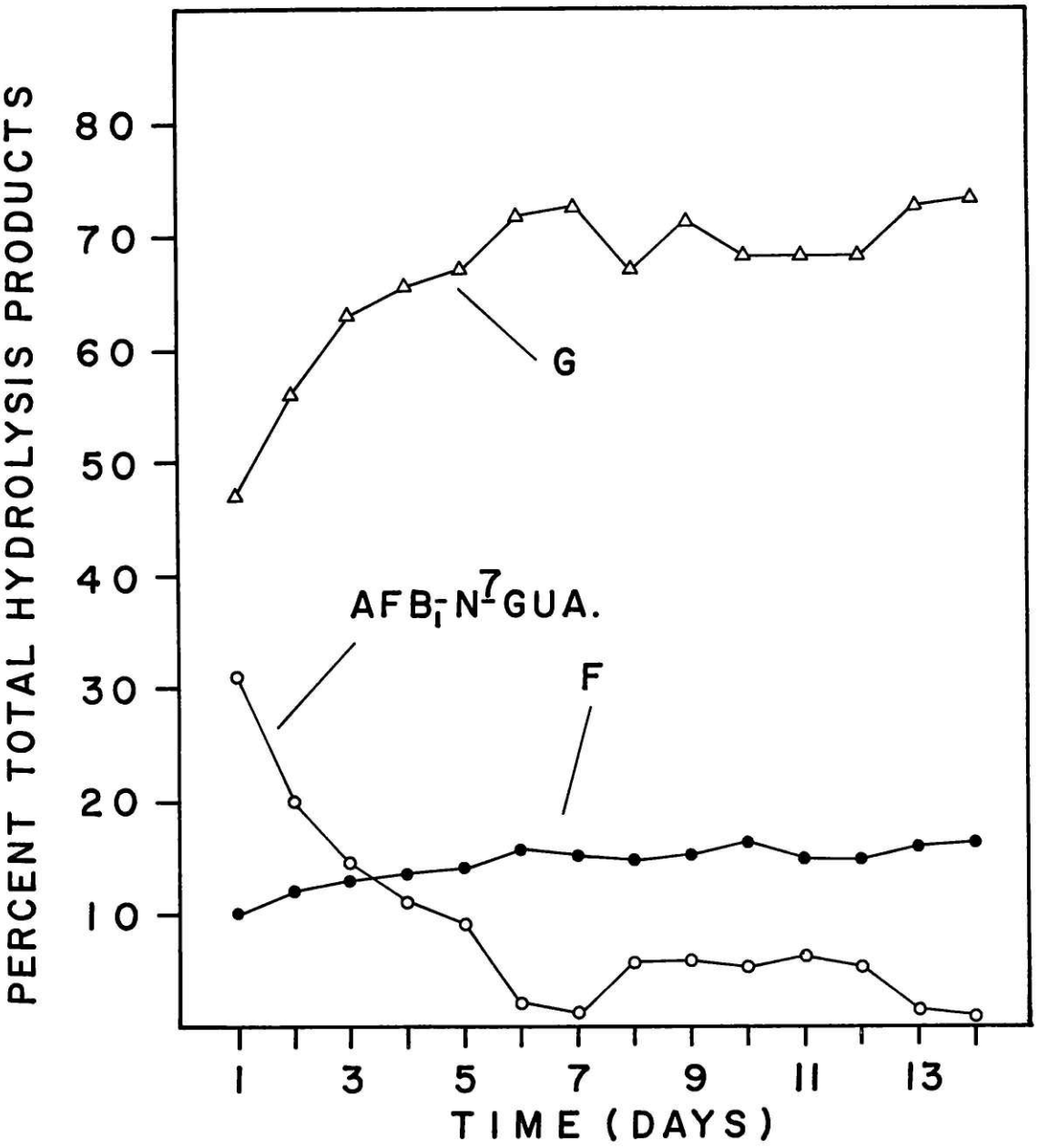

5000
1000
500
100
50
10
5
1
μ moles / mg DNA x 10^8
G
F
E
AFB1-N7 GUA.
1
3
5
7
9
11
13
TIME (DAYS)

Figure 5-3. Percentage of total AFB_1 acid hydrolysis products represented by the three major AFB_1 derivatives during administration of multiple doses of AFB_1 to male Fischer rats. Animals recevied 25 µg AFB_1 administered i.p. in 25 µl DMSO on days 0 (not shown), 1, 2, 3, 4, 7, 8, 9, 10, and 11.

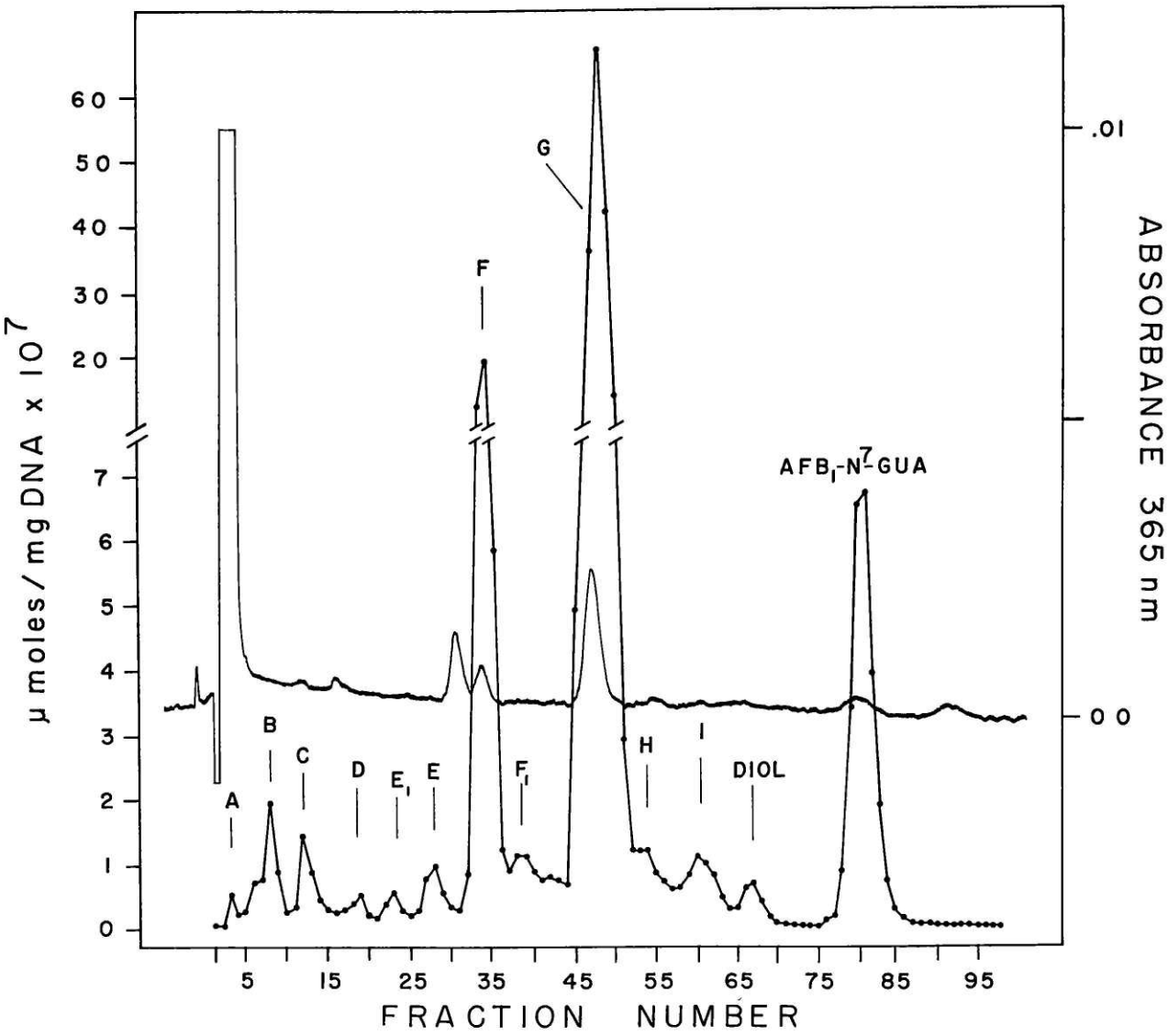

PERCENT TOTAL HYDROLYSIS PRODUCTS
80
70
60
50
40
30
20
10
G
AFB1-N7GUA.
F
1
3
5
7
9
11
13
TIME (DAYS)

principal hydrolysis products during the two week period. These changes reflect the accumulation of G and F and the decrease in the amount of the AFB_1-N^7-GUA derivative with time.

The chromatographic patterns of hydrolyzed AFB_1-DNA derivatives isolated from the liver DNA of a rat on day 5, 24 hours after the fifth consecutive 25 μg dose of AFB_1, is shown in Figure 5-4. Comparison with Figure 5-1 reveals that the products are qualitatively identical to those observed two hours after a single 25 μg dose. However, quantitative changes in their distribution are apparent. The quantitative changes in each peak during the 15 day experimental period are enumerated in Table 5-4 and 5-5, which list the values of duplicate analyses. The concentration of these products in DNA is expressed in terms of the number of molecules of adduct present per nucleic acid base residue. The unretained peak, A, was not measured. Peaks F_1 and H could not be measured accurately because they were present in small amounts and poorly resolved as shoulders on the much larger adjacent peaks F and G (see Fig. 5-4). The data in Table 5-4 and 5-5 for individual peaks are plotted in Figures 5-5 through 5-10.

Figures 5-5 and 5-6 show the changes in the level of AFB_1-N^7-GUA and the accumulation of its putative imidazole ring hydrolyzed derivatives, F and G, over the two week period. Interanimal variations are most apparent for peak F. F is in some way related to G (Chapter Two) and the magnitude of variation between duplicate samples in these two peaks is similar. Figure 5-6 also shows the total level of modification of rat

Figure 5-4. Reversed-phase HPLC pattern of [^{3}H] AFB_1 hydrolysis products from DNA isolated from the liver of a rat 24 hours after administration of five daily doses of 25 µg [^{3}H] AFB_1 containing 60.7 µCi ^{3}H each. 450 µl of material was injected onto a µ Bondapak C_{18} column (Waters Associates) which was eluted with a linear, 65 min., 14 to 18 percent ethanol gradient at ambient temperature. The eluant contained 0.02 M KAc pH 5.0, and was monitored at 254 nm (not shown) and 365 nm. 30-drop fractions were collected for the determination of ^{3}H activity.


μ moles / mg DNA x 10^7
60
50
40
30
20
7
6
5
4
3
2
1
0
A
B
C
D
E1
E
F
F1
G
H
I
DIOL
AFB1-N7-GUA
5
15
25
35
45
55
65
75
85
95
FRACTION NUMBER
.01
1
0 0
ABSORBANCE 365 nm

liver DNA by all the AFB_1 products measured. Figures 5-7 through 5-10 show the levels of the seven remaining peaks. As noted earlier, large variations are seen in the duplicate determinations of these minor hydrolysis products because of the low concentrations at which they are present. These data are presented only to indicate the relative level at which they occur with respect to the major products.

TABLE 5-4

CONCENTRATIONS OF AFB_1 ACID HYDROLYSIS PRODUCTS IN THE LIVER DNA[a] OF RATS EXPOSED TO MULTIPLE DOSES OF AFB_1

(Molecules of AFB_1 Derivative/Nucleic Acid Base Residue x 10^8)

DAY NUMBER	A	B	C	D	E_1	E	F	F_1	G	H	I	DIOL	AFB_1-N^7-GUA	TOTAL
1[b]	--	0.9	1.3	0.5	0.2	1.9	31	--	140	--	2.7	3.2	93	274
2[b]	--	1.1	2.4	1.1	0.8	3.7	59	--	280	--	5.6	4.1	110	464
3[b]	--	5.6	2.8	1.2	1.9	2.9	80	--	400	--	5.1	4.5	86	596
4[b]	--	2.6	5.3	1.5	0.9	4.1	93	--	450	--	8.6	6.2	75	644
5	--	10	4.9	1.9	2.2	3.6	100	--	490	--	6.6	4.1	59	689
6	--	8.6	2.4	1.8	1.9	2.8	86	--	390	--	5.3	2.7	12	514
7[b]	--	11	9.3	1.8	1.2	2.2	80	--	390	--	5.3	2.3	4.3	509
8[b]	--	16	8.0	2.2	2.4	4.8	140	--	620	--	7.0	7.5	51	871
9[b]	--	1.9	2.4	1.1	0.9	4.5	100	--	510	--	6.6	5.9	41	686
10[b]	--	2.1	6.2	1.8	0.9	4.5	100	--	490	--	7.5	8.6	43	654
11[b]	--	9.3	7.4	2.3	1.9	5.3	110	--	510	--	5.1	4.9	45	700
12	--	16	11	1.9	2.0	6.2	100	--	490	--	4.5	4.0	47	670
13	--	15	9.3	2.3	2.5	3.0	100	--	490	--	4.3	4.1	8.0	650
14	--	7.8	13	3.2	1.8	4.7	140	--	620	--	6.6	9.3	4.1	825

[a] Calculations are detailed in Appendix II.

[b] Rats received 25 µg [^{3}H] AFB_1 on these days.

TABLE 5-5

CONCENTRATIONS OF AFB_1 ACID HYDROLYSIS PRODUCTS IN THE LIVER DNA[a] OF RATS EXPOSED TO MULTIPLE DOSES OF AFB_1

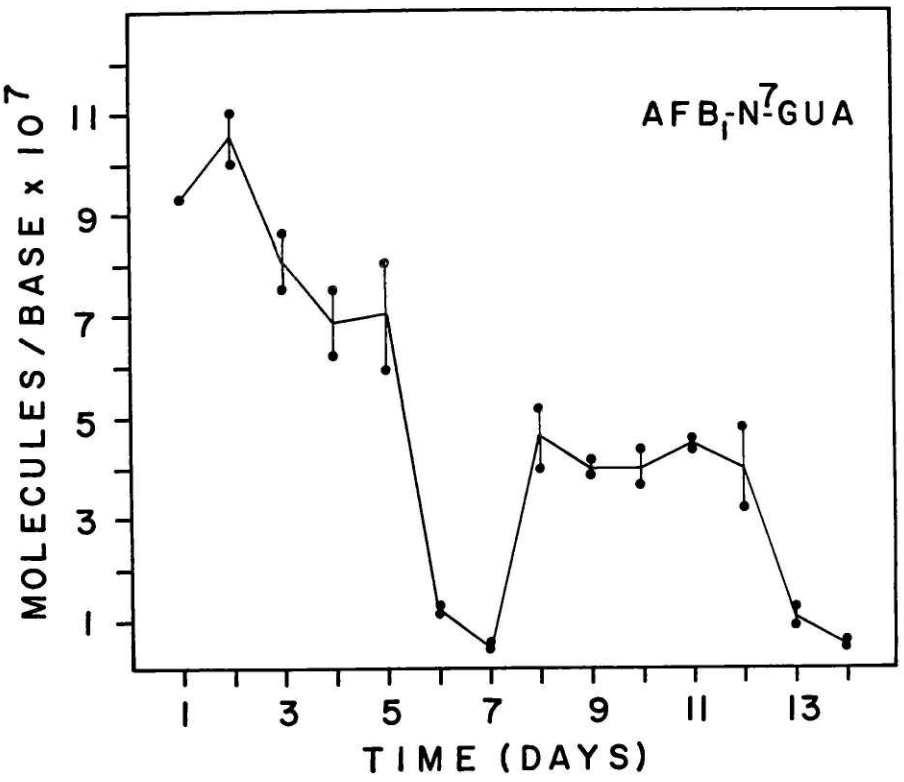
(Molecules of AFB_1 Derivative/Nucleic Acid Base Residue x 10^8)

DAY NUMBER	A	B	C	D	E_1	E	F	F_1	G	H	I	DIOL	AFB_1-N^7-GUA	TOTAL
1[b]	--	--	--	--	--	--	--	--	--	--	--	--	--	---
2[b]	--	1.1	1.9	0.9	0.7	3.9	70	--	320	--	4.3	5.6	100	506
3[b]	--	2.2	3.2	1.2	1.4	4.5	65	--	310	--	4.3	2.6	75	476
4[b]	--	8.0	5.3	1.6	1.6	4.5	80	--	390	--	4.0	2.9	62	550
5	--	11.0	7.0	1.9	2.1	5.3	110	--	530	--	6.6	4.0	80	752
6	--	13.0	4.9	1.6	1.8	3.2	86	--	430	--	4.7	2.4	11	556
7[b]	--	5.9	6.2	1.9	1.3	2.9	110	--	510	--	8.0	5.1	4.5	653
8[b]	--	22	7.5	1.9	2.9	5.1	93	--	460	--	3.0	3.2	39	642
9[b]	--	1.7	4.1	1.9	1.0	5.3	100	--	490	--	7.5	5.9	38	652
10[b]	--	10	5.9	1.9	1.8	4.9	110	--	460	--	4.9	5.1	36	653
11[b]	--	13	6.2	2.0	1.9	5.6	110	--	530	--	4.7	4.9	43	722
12	--	12	12	4.5	2.8	9.3	130	--	620	--	6.6	8.0	31	834
13	--	4.6	10	2.7	1.5	4.7	150	--	700	--	10	12	12	934
14	--	10	10	2.9	2.2	4.1	160	--	750	--	8.6	10	4.5	958

[a] Calculations are detailed in Appendix II.

[b] Rats received 25 µg [^{3}H] AFB_1 on these days.

Figure 5-5. Daily levels of acid hydrolysis products AFB_1-N^7-GUA and peak G found in rat liver DNA during the administration of multiple doses of AFB_1 to male Fischer rats. 25 µg of AFB_1 was administered i.p. on days 0 (not shown), 1, 2, 3, 4, 7, 8, 9, 10, and 11. Animals did not receive AFB_1 on the day of sacrifice. Values plotted for each day were obtained from determinations made on separate animals and are contained in Tables 5-4 and 5-5.


AFB1-N7-GUA
MOLECULES / BASE x 10^7
1
3
5
7
9
11
1
3
5
7
9
11
13
TIME (DAYS)

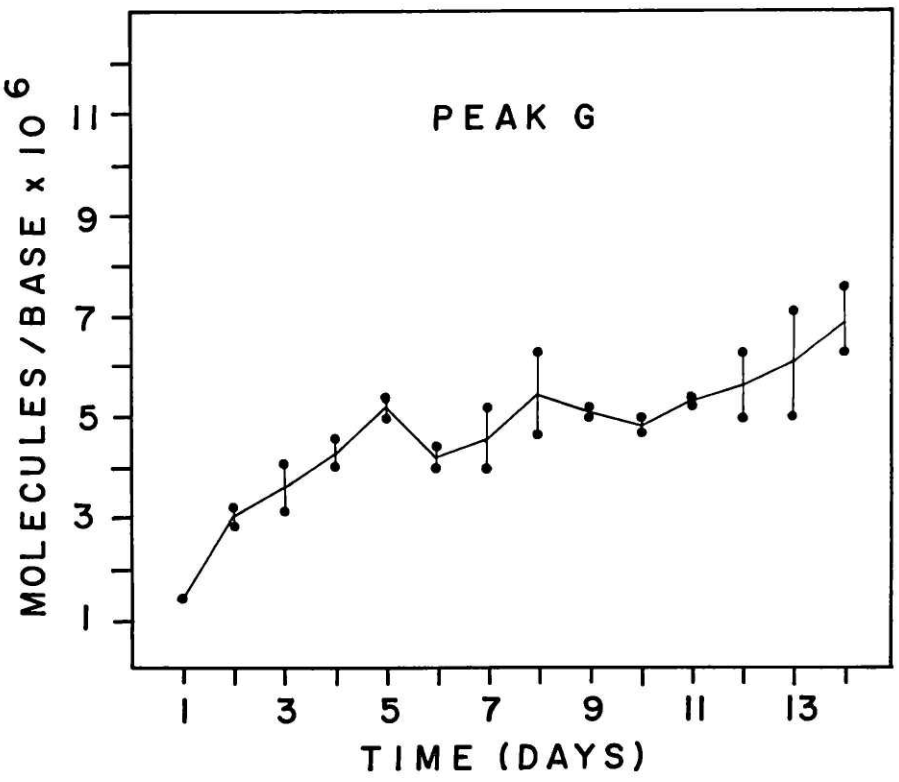
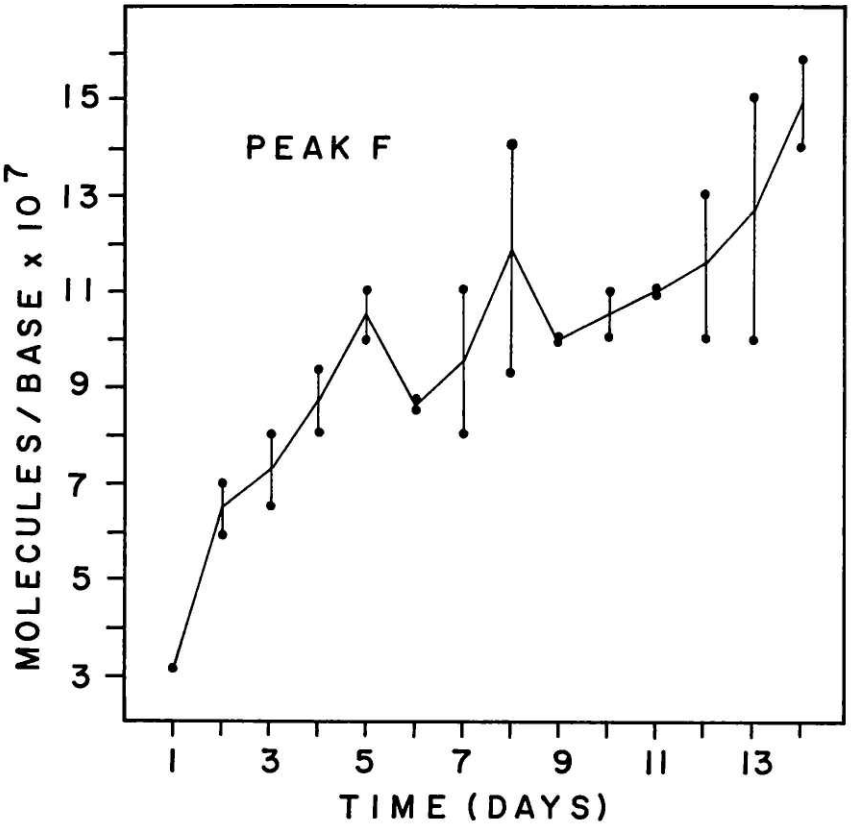

PEAK G
MOLECULES / BASE x 10^6
1
3
5
7
9
11
1
3
5
7
9
11
13
TIME (DAYS)

Figure 5-6. Daily levels of peak F and the total amount of modification represented by all hydrolysis products found in rat liver DNA during the administration of multiple doses of AFB_1 to male Fischer rats. 25 μg of AFB_1 was administered i.p. on days 0 (not shown), 1, 2, 3, 4, 7, 8, 9, 10, and 11. Animals did not receive AFB_1 on the day of sacrifice. Values plotted for each day were obtained from determinations made on separate animals and are contained in Tables 5-4 and 5-5.


PEAK F
MOLECULES / BASE x 10^7
15
13
11
9
7
5
3
1
3
5
7
9
11
13
TIME (DAYS)

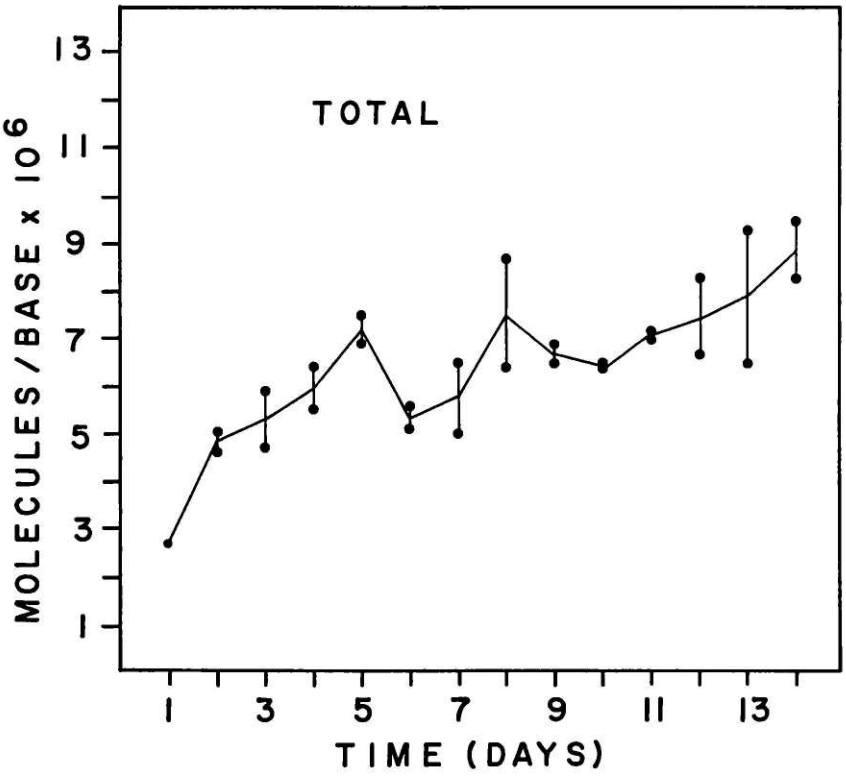
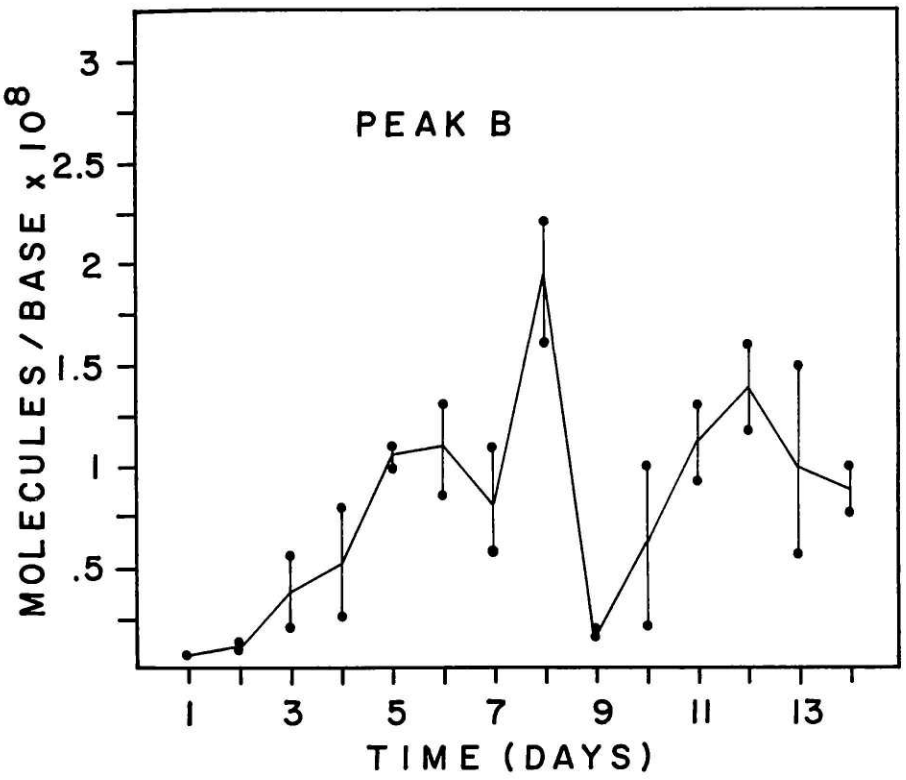

TOTAL
MOLECULES / BASE x 10^6
13
11
9
7
5
3
1
3
5
7
9
11
13
TIME (DAYS)

Figure 5-7. Daily levels of acid hydrolysis products peak B and peak C found in rat liver DNA during the administration of multiple doses of AFB_1 to male Fischer rats. 25 µg of AFB_1 was administered i.p. on days 0 (not shown), 1, 2, 3, 4, 7, 8, 9, 10, and 11. Animals did not receive AFB_1 on the day of sacrifice. Values plotted for each day were obtained from determinations made on separate animals and are contained in Tables 5-4 and 5-5.


PEAK B
MOLECULES / BASE x 10^8
3
2.5
2
1.5
1
.5
1
3
5
7
9
11
13
TIME (DAYS)

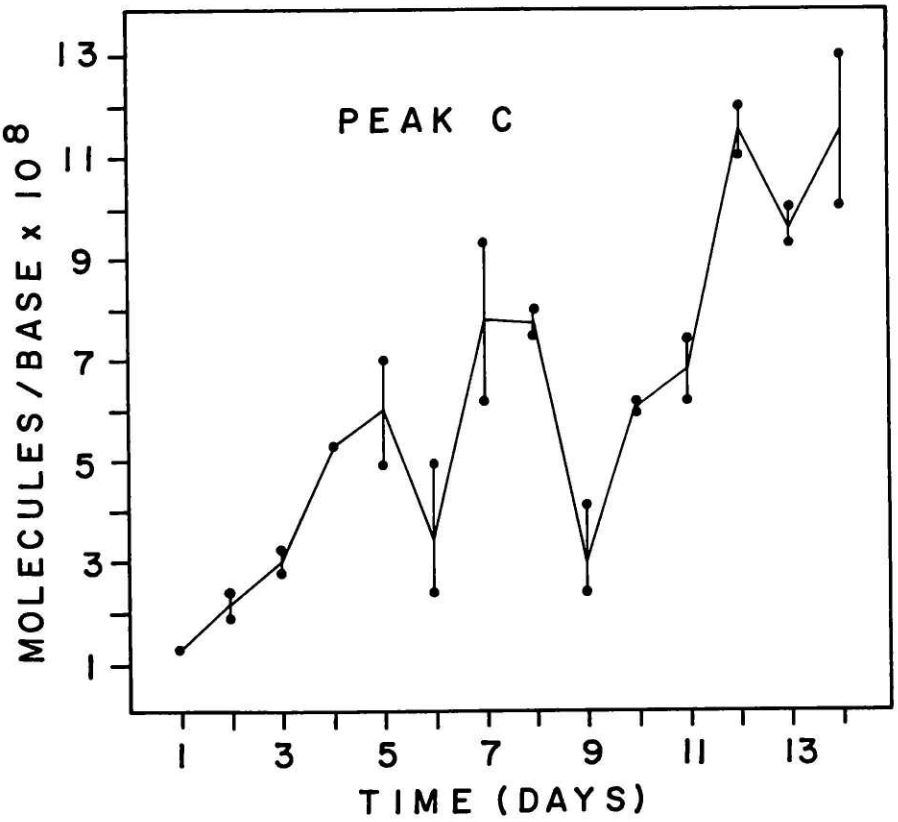
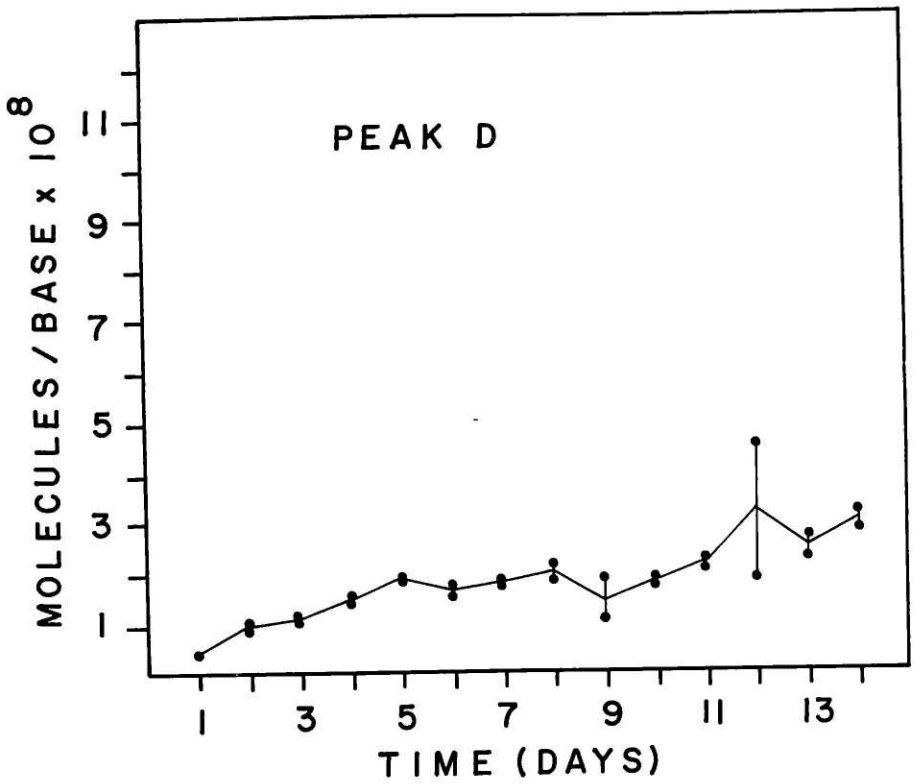

PEAK C
MOLECULES / BASE x 10^8
13
11
9
7
5
3
1
1
3
5
7
9
11
13
TIME (DAYS)

Figure 5-8. Daily levels of acid hydrolysis products peak D and peak E_1 found in rat liver DNA during the administration of multiple doses of AFB_1 to male Fischer rats. 25 µg of AFB_1 was administered i.p. on days 0 (not shown), 1, 2, 3, 4, 7, 8, 9, 10, and 11. Animals did not receive AFB_1 on the day of sacrifice. Values plotted for each day were obtained from determinations made on separate animals and are contained in Tables 5-4 and 5-5.


PEAK D
MOLECULES / BASE x 10^8
1
3
5
7
9
11
1
3
5
7
9
11
13
TIME (DAYS)

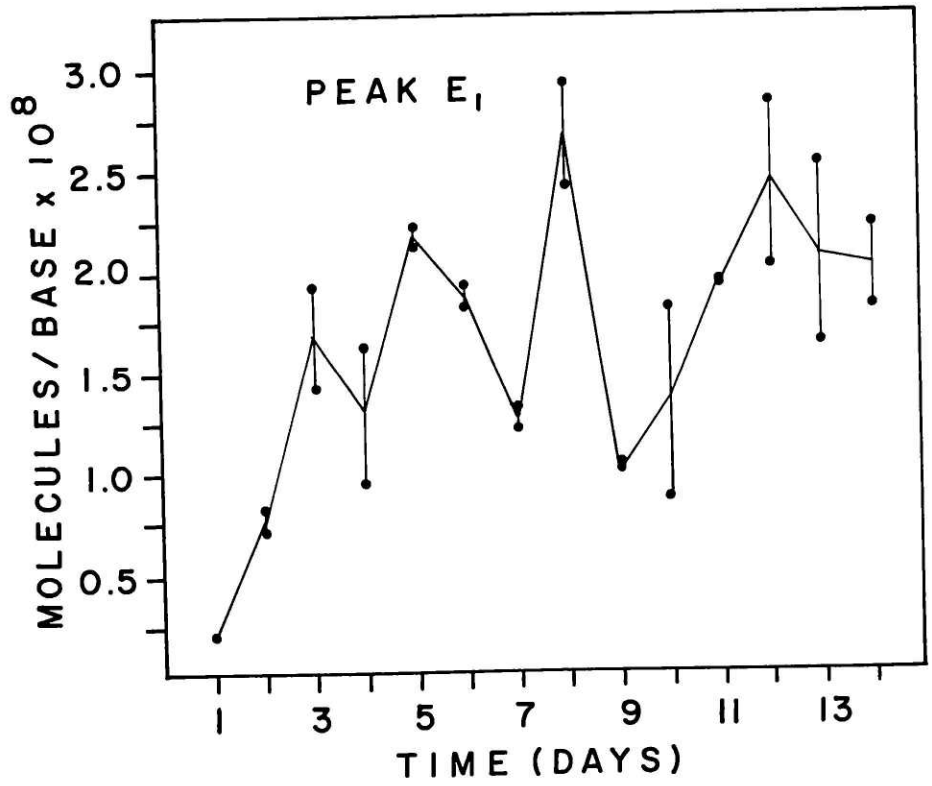
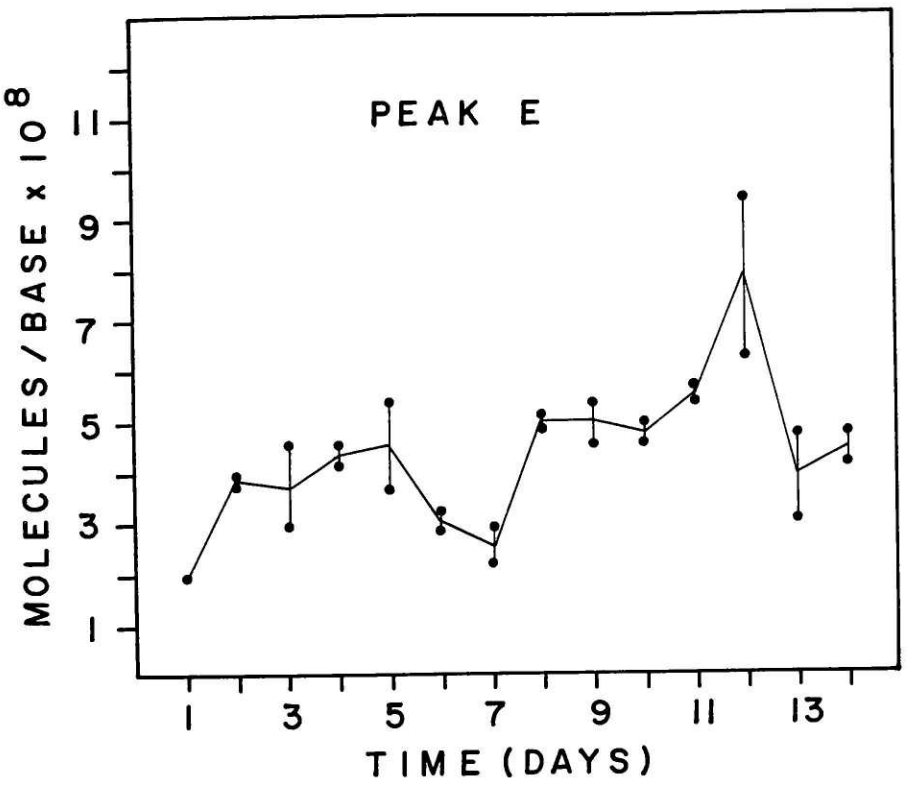

PEAK E_1
MOLECULES / BASE x 10^8
0.5
1.0
1.5
2.0
2.5
3.0
1
3
5
7
9
11
13
TIME (DAYS)

Figure 5-9. Daily levels of acid hydrolysis products peak E and peak I found in rat liver DNA during the administration of multiple doses of AFB_1 to male Fischer rats. 25 µg of AFB_1 was administered i.p. on days 0 (not shown), 1, 2, 3, 4, 7, 8, 9, 10, and 11. Animals did not receive AFB_1 on the day of sacrifice. Values plotted for each day were obtained from determinations made on separate animals and are contained in Tables 5-4 and 5-5.


PEAK E
MOLECULES / BASE x 10^8
1
3
5
7
9
11
13
TIME (DAYS)

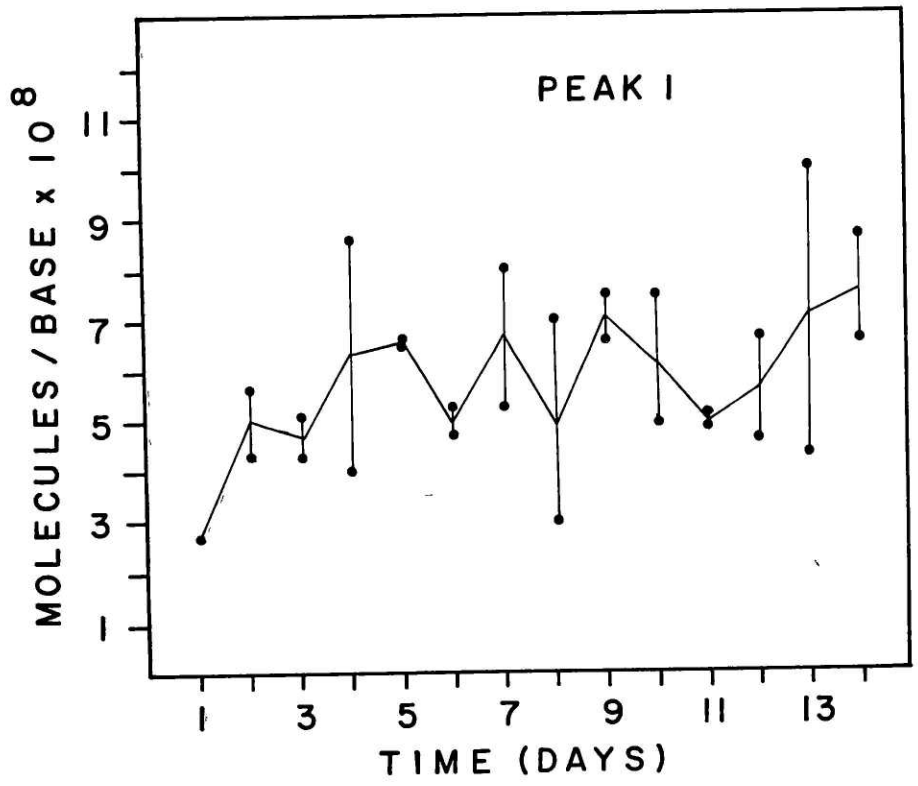
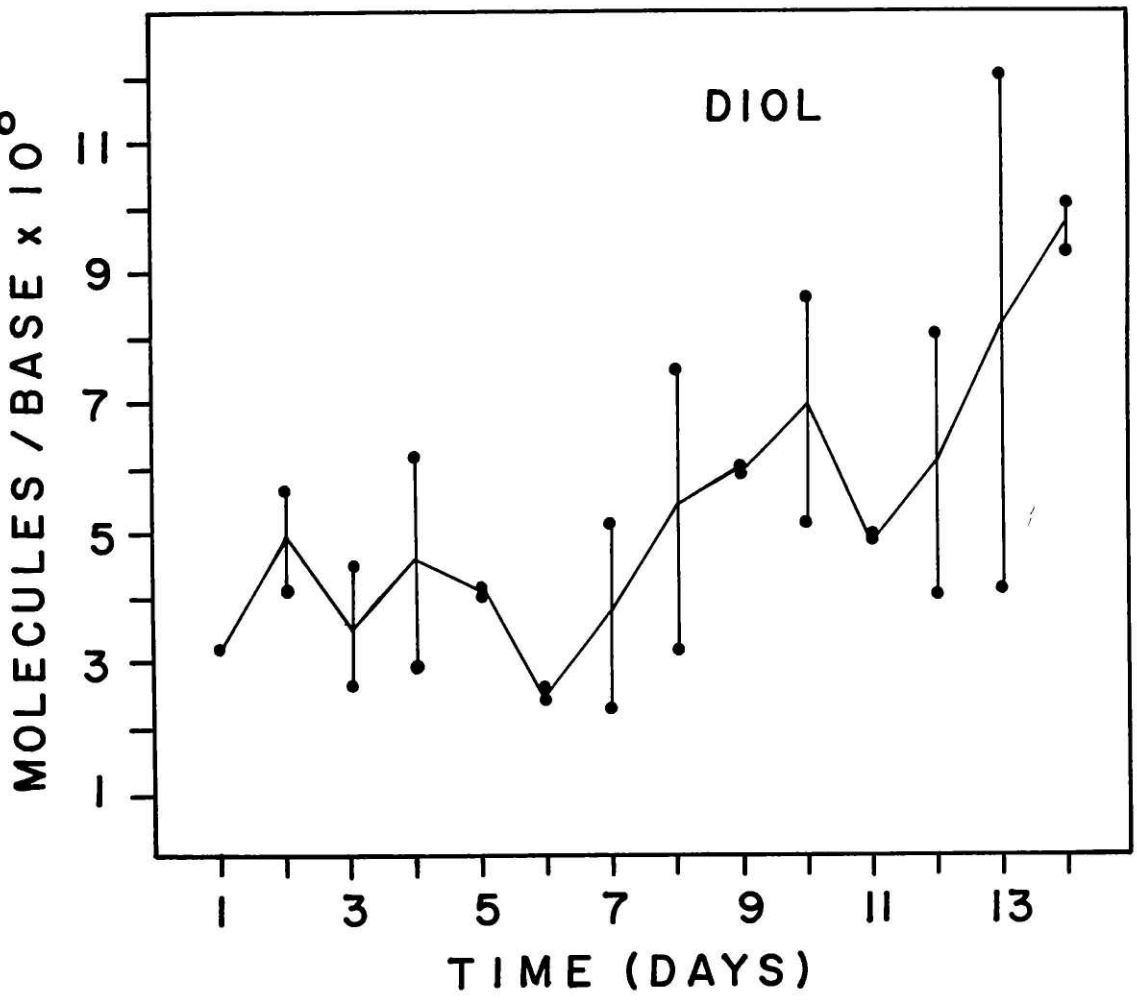

PEAK I
MOLECULES / BASE x 10^8
1
3
5
7
9
11
13
TIME (DAYS)

Figure 5-10. Daily levels of 2,3-dihydro-2,3-dihydroxy AFB_1 obtained from the acid hydrolysis of DNA isolated from the livers of rats during the administration of multiple doses of AFB_1 to male Fischer rats. 25 μg of AFB_1 was administered i.p. on days 0 (not shown), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11. Animals did not receive AFB_1 on the day of sacrifice. Values plotted for each day were obtained from determinations made on separate animals and are contained in Tables 5-4 and 5-5.


DIOL
MOLECULES / BASE x 10^8
1
3
5
7
9
11
1
3
5
7
9
11
13
TIME (DAYS)

DISCUSSION

Covalent modification of liver DNA by AFB_1 during the two week experimental period produced both persistent and repaired lesions. Primary modifications which were formed by the attack of an activated AFB_1 derivative on the N-7 atom of guanine were readily removed either spontaneously or enzymatically from the DNA molecule. Secondary modifications formed by the hydrolysis of the positively-charged imidazole ring in the 7,9-disubstituted guanine molecule have long biological half-lives resulting in their accumulation in DNA during multiple doses.

Two hours after a 25 μg dose of AFB_1 to a rat, 8.8×10^{-4} μmoles of AFB_1 were covalently associated with the liver DNA. This represents a level of modification of one adduct per 43,000 nucleic acid base residues. Twenty-four hours later, 88 percent of this material had been removed. The remaining 12 percent, representing a modification level of one adduct per 365,000 base residues, was distributed primarily between peaks F (11.3%), G (51%), and AFB_1-N^7-GUA (34%). In terms of potential genetic damage to a rat liver cell, the total number of covalent AFB_1 modifications present in a cellular genome may be calculated assuming a DNA content of 7 pg per cell and an average molecular weight of 326 for a mononucleotide unit in DNA. Thus, if a rat liver genome contains approximately 3×10^{10} base residues, 6.9×10^5 and 8.2×10^4 AFB_1 lesions are estimated to be present at 2 and 24 hours, respectively. These calculations assume a uniform adduction of DNA in all cells of the

liver. This is most probably not true since some groups of parenchymal cells (i.e., the periportal areas) are known to be more sensitive than others to the acute toxic effects of AFB_1. Some liver cells may be subject to more damage than is estimated here and others less.

Figures 5-5 and 5-6 show that most of the increases in the levels of the persistent derivatives F and G occur within the first five day period. Relatively constant levels of these products are maintained over the remaining nine days. A possible explanation for this pattern is derived from the examination of the kinetics of the precursor AFB_1-N^7-GUA in Figure 5-5. During the first five day period a decrease in the amount of this product remaining after 24 hours is seen. In the subsequent five day period of AFB_1 administration, the residual level is constant at a lower value than at any time during the first dosing period. Two processes could provide an explanation for these observations: the increased rate of removal of AFB_1-N^7-GUA from DNA, or a decreased level of modifications of DNA by successive doses of AFB_1. Either of these mechanisms would reduce the residual level of AFB_1-N^7-GUA and the amounts of F and G produced in DNA by a given dose of AFB_1. Increased repair is considered the least probable explanation since the half-life of AFB_1-N^7-GUA calculated for the 24 hour period between days 5 and 6 is greater than its half-life during the first 24 hour period on day 0 (5.5 and 9.0 hours, respectively). The induction of other metabolic pathways which would limit AFB_1 activation or inactivate the 2,3-epoxide provides a more

likely explanation since these mechanisms would decrease the initial level of DNA modification. Induction of P_{450} mixed function oxidase by AFB_1 has been reported (Schabort and Steyn, 1969). This phenomenon is well documented for other xenobiotics and has been described for rats fed N-acetylaminofluorene, another hepatocarcinogen (Sporn and Dingman, 1966).

Continuation of this dosing schedule for an additional six weeks would have produced a 100 percent incidence of hepatocellular carcinoma in these animals. The results of this experiment indicate that during the first week of AFB_1 administration changes in the metabolism of AFB_1 probably occur in the liver and reduce the amount of covalent DNA modification produced by successive doses. Otherwise, the persistent products F and G would have accumulated in liver DNA at the same rate in the second week as they did in the first, and the residual levels of AFB_1-N^7-GUA 24 hours after dosing would have been equal or have shown a slight increase during the two dosing periods. The roles of changes in metabolic pathways, the persistence of some lesions, and the repair of other lesions in the carcinogenic process cannot be determined from these experiments.

Equally plausible hypothesis may be formulated for either of these types of lesions in terms of DNA damage during repair or fixation of damage in daughter strands during replication which may lead to mutation and cancer. Certainly, the number of persistent lesions in the cellular genome after the two week period, estimated to be 2.7×10^5, would provide adequate opportunity for mutagenic events to occur. Studies using human

lymphoblasts (unpublished) and *Salmonella typhimurium* (Stark et al., 1979) report a mutagenic efficiency of one mutation per 200 AFB_1 lesions and one mutation per 37 AFB_1 lesions, respectively, in these biological models. No information is yet available on the mutagenic efficiency of AFB_1 in non-dividing somatic cells.

Metabolic changes which produce differences in the sensitivities of various cell populations to the toxic effects of AFB_1 may be equally important in terms of the appearance of hepatocellular carcinoma in the rat liver following this eight week dosing protocol. Nodules of hyperplastic parenchymal cells which can eventually progress to neoplasia are characteristic of the first perneoplastic changes seen during chronic AFB_1 administration (Newberne and Wogan, 1968). Daughter cells from which these nodules originate may come from populations of cells which have become resistant to the toxic effect of AFB_1 after initial covalent damage to their DNA has induced a neoplastic potential.

The covalent binding of AFB_1 to cellular macromolecules may thus play different roles at various stages of the carcinogenic process, initially inducing heritable changes in a cellular genome and subsequently establishing a selective advantage for initiated cells through toxic effects (Scherer and Emmelot, 1976). Because of the possibility of the dual role covalent products may have at stages of initiation and promotion, elucidation of the functional significance of a particular lesion to the subsequent appearance of neoplasia will be difficult using *in vivo* models.

CHAPTER SIX

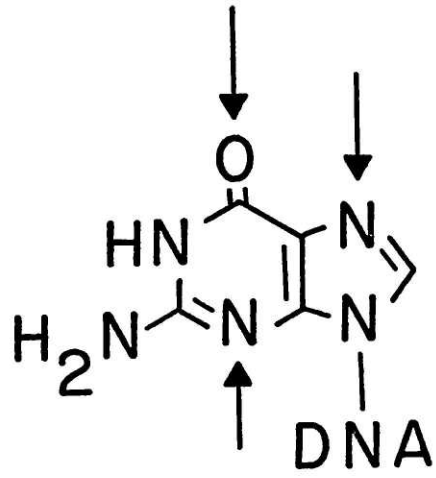
GENERAL DISCUSSION

GENERAL DISCUSSION

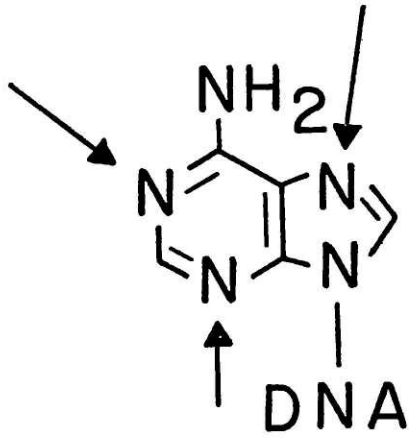
It has become generally accepted that the induction of neoplasia by chemical carcinogens requires the interaction of their activated, electrophilic derivatives with nucleophilic sites in cellular macromolecules such as DNA, RNA, and protein (Miller, 1978). These activated electrophiles may be formed spontaneously as with nitrosamides and nitrosamines, or they may be products of metabolic oxidation such as the epoxide derivatives of various polycyclic hydrocarbons. The interaction of these agents with DNA has been most extensively studied in recent years because of the essential role that temporary alterations or permanent changes in this molecule are thought to play in the initiation of the carcinogenic process. Basic insights into carcinogenesis may be gained by the identification of covalent DNA interactions and elucidation of their functional significance. Although the latter task remains a formidable experimental challenge, considerable progress has been made in describing the covalent interactions of several carcinogens with DNA *in vitro* and *in vivo*.

Virtually all nucleophilic atoms in the purine and pyrimidine bases of the DNA molecule have been shown to be substituted by various carcinogens (Singer, 1975). Figure 1 shows the nucleophilic positions on DNA bases at which alkylated derivatives have been identified. Phosphotriester derivatives have been identified as products of several alkylating reagents (Lawley and Shaw, 1973; Lawley et al., 1973). The positions of covalent modification in DNA seem to be determined by both

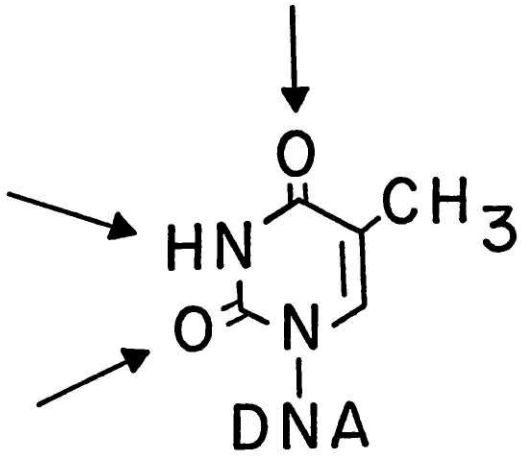
Figure 6-1. Nucleophilic positions on DNA bases at which alkylated derivatives have been identified (data from Singer, 1975, 1976).



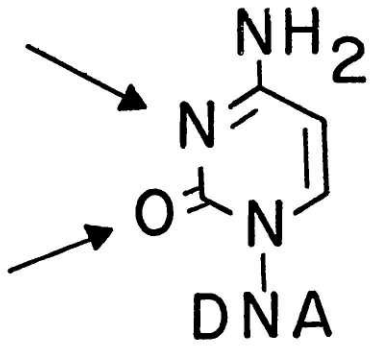
GUANINE



ADENINE



THYMIDINE



CYTIDINE

the stereochemistry and mechanism of the reaction involved (for review see Brown, 1975). Nucleophilic atoms, such as the N-1 position of purine bases, which are positioned in the interior of the DNA molecule and involved in interstrand hydrogen bonding, are relatively immune from attack; this may be true especially for large molecules such as the polycyclic hydrocarbons. Atoms which are oriented on the exterior of the helical structure, such as the N-7 position of guanine which is situated in the major groove, are very susceptible to attack by most electrophilic agents. In addition to these simple stereochemical considerations, the relative nucleophilicites of the various atoms in DNA and the electrophilic reactivity of the activated carcinogen will be determinants in the proportions of products formed (Brown, 1975). For example, the methylating agents dimethylsulfate and methylmethanesulfonate yield a low proportion of O-alkylated products in DNA, while N-methyl-N-nitrosourea and N-methyl-N^1-nitro-N-nitrosoguanidine give relatively high proportions of alkylated products. These differences have been attributed to the greater S_N2 character of the reaction mechanisms of the former agents (Lawley, 1976).

Extensive investigations have been carried out concerning the identification and fate of the covalent products formed in DNA by carcinogenic alkylating agents (for review see Singer, 1975). Initial attempts to ascertain the significance of DNA alkylation in terms of carcinogenesis examined the organotropism of various agents such as N-methyl-N-nitrosourea (MNU). No relationship was found between the total amount of covalent

DNA modification in an organ and its susceptibility to tumor formation. MNU induces neural (Kleihues and Margison, 1974) and renal neoplasms (Nicoll et al., 1975) in rats; however, the highest levels of DNA modification were found in the liver after a single dose (Swann and Magee, 1968). Additional investigations studying the rate of excision of alkylated bases from the DNA molecules of target and non-target tissues revealed a correlation between the persistence of O-alkyl derivatives of DNA bases and susceptibility to neoplasia (Frei and Lawley, 1975). Specifically, in the intact animal the persistence of O-6-alkylguanine was found in target tissues (Kleihues and Bucheler, 1977; Goth and Rajewsky, 1975; Margison and Kleihues, 1975). Because the O-6 atom of guanine is involved in the base pairing interactions with cytosine, modification of this position may cause mispairing during DNA replication, producing mutations in daughter strands. Evidence for mispairing of O-6-methylguanine during transcription *in vitro* indicates that this lesion can be considered promutagenic (Loveless, 1969). The carcinogenic potency of several alkylating agents has shown a positive correlation with the extent of their reaction with oxygen atoms in DNA bases (Lawley, 1974). Two important corollaries concerning the functional significance of covalent DNA interactions have been derived from these investigations: that all types of DNA damage are not equivalent and that the persistence of minor amounts of damage to the cellular genome may be more important than the initial amount of damage in producing long-term effects such as cancer.

In contrast to alkylating agents, less is known concerning the interactions of larger, activated molecules such as aromatic amines or polycyclic aromatic hydrocarbons with DNA. Table 6-1 lists the nucleophilic positions in DNA and RNA at which covalent adducts formed by electrophilic derivatives of larger carcinogenic molecules have been identified. Stereochemical considerations are expected to play an increased role in the reactions of these molecules with nucleophilic sites in DNA. In addition, hydrophobic and charge-transfer interactions between the aromatic and aliphatic portions of these molecules may orient the electrophilic centers of the attacking species with specific nucleophilic atoms in the DNA molecule directing and promoting specific interactions (Slifkin, 1973).

The following discussion provides a summary of what is known concerning the nature of the covalent interactions and their functional significance in two classes of macrocyclic compounds which have been extensively studied because of the early identification of some of them as carcinogens. Two compounds representative of each of these groups, benzo(a)pyrene [B(a)P] and 2-acetylaminofluorene (AAF), have received particular attention recently because of the identification and availability of their ultimately reactive forms.

AROMATIC HYDROCARBONS

Benzo(a)pyrene is the most extensively studied carcinogenic, polycyclic aromatic hydrocarbon. Recent evidence indicates a two-step metabolic activation process is responsible for generation of the principal ultimately reactive form which binds to

TABLE 6-1

NUCLEOPHILIC SITES IN PURINE BASES[a] AT WHICH COVALENT DERIVATIVES OF MACROCYCLIC CARCINOGENIC COMPOUNDS HAVE BEEN IDENTIFIED

CARCINOGEN	GUA	ADE	REFERENCES
2-acetylamino-fluorene (AAF)	C-8		Lin et al., 1975
	N-2		Westra et al., 1976
Benzo(a)pyrene		N-6	Meehan et al., 1977
	N-2		Weinstein et al., 1976
	N-7		Osborne et al., 1978
β-Napthylamine	O-6		Kadlubar et al., 1979
Sterigmatocystin	N-7		Essigmann et al., 1979
Aflatoxin B_1	N-7		Essigmann et al., 1977
			Lin et al., 1977
N-methyl-4-amino-azobenzene (DAB)	C-8		Lin et al., 1975

[a]No pyrimidine base adducts have been identified.

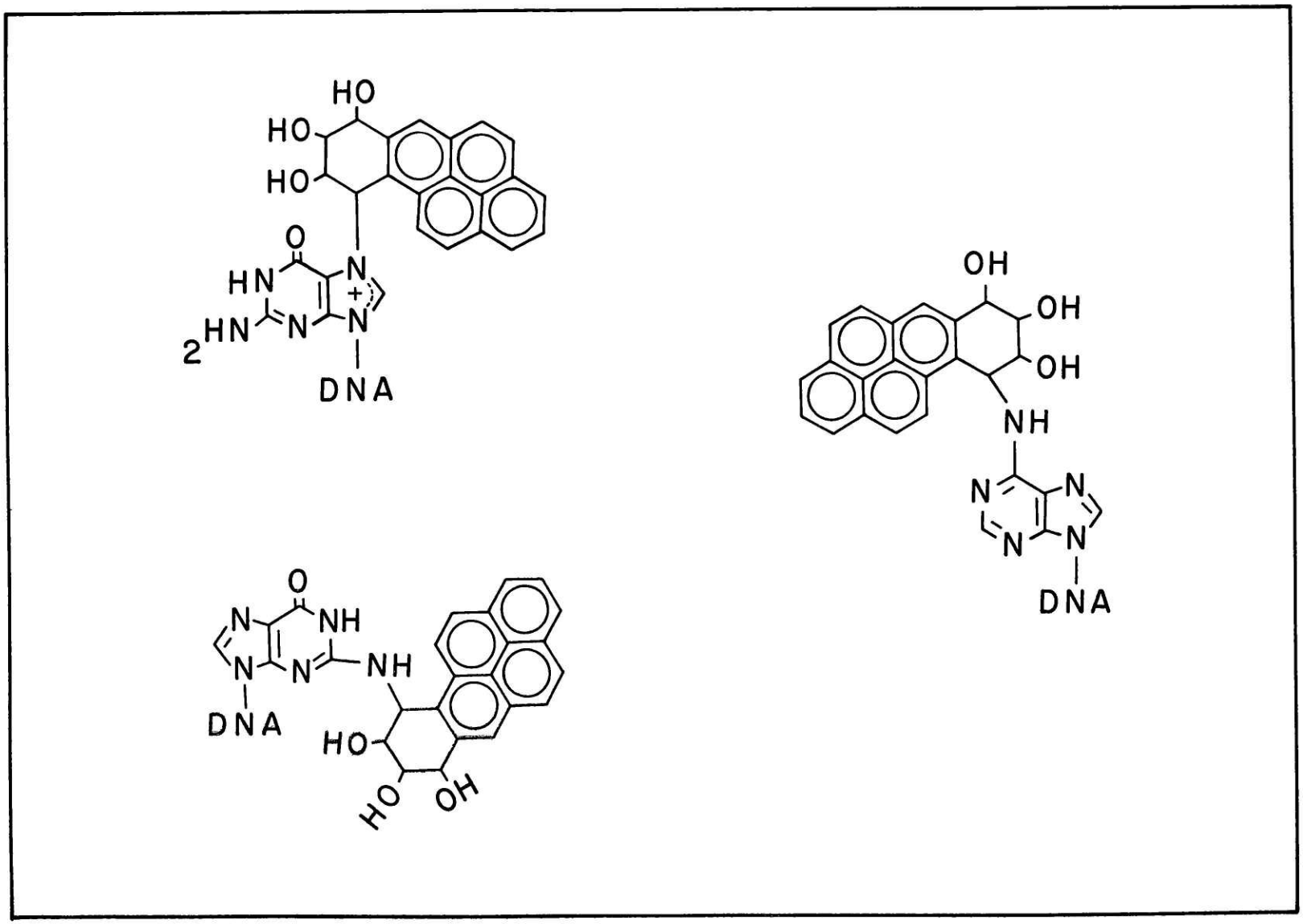
DNA *in vivo*. The first step involves the production of a 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene. This is further metabolized to an epoxide, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (Sims et al., 1974; Thakker et al., 1976, 1977; Weinstein et al., 1977). The major reaction product of this diolepoxide with polyguanylic acid or nucleic acids involves a covalent bond formed between the 2-amino group of guanine residues and the C-10 of the diolepoxide (Koreena et al., 1976; Jeffrey et al., 1976). Adducts formed between the C-10 of the diolepoxide, the N-7 atom of guanine (Osborne et al., 1978), and the exocyclic amino group of adenine (Meehan et al., 1977) have also been identified. These derivatives are shown in Figure 6-2. The principal N-2 guanine adduct has been identified chromatographically in nucleic acids isolated from bronchial explants that were incubated with [^{3}H] benzo(a)pyrene (Weinstein et al., 1976) and mouse skin, a target organ, following toxic application (Moore et al., 1977).

Diolepoxide derivatives of benz(a)anthracene (Wood et al., 1977), 7-methylbenz(a)anthracene (Tierney et al., 1977), and 7,12-dimethylbenz(a)anthracene (Moschel et al., 1977) have also been implicated as the ultimately reactive forms of these molecules which bind to DNA. However, specific products of these interactions have not been identified.

AROMATIC AMINES

The aromatic amine N-methyl-4-aminoazobenzene is activated through N-hydroxylation and sulfonation to a reactive sulfate

Figure 6-2. Structures of identified DNA adducts of benzo(a)-pyrene [B(a)P].

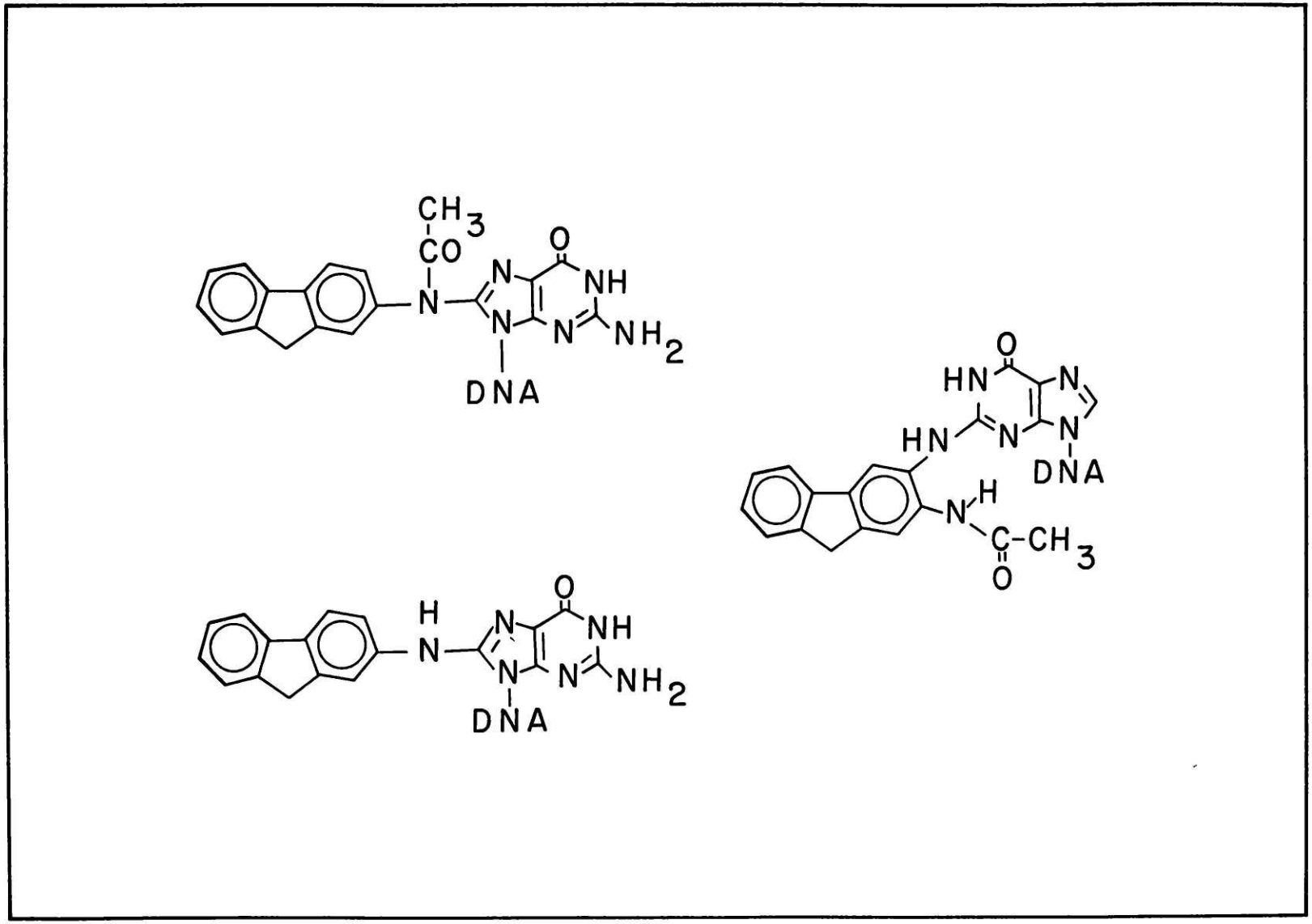

HO
HO
HO
O
HN
N
+
2 HN
N
N
DNA
OH
OH
OH
NH
N
N
N
N
DNA
O
N
NH
N
N
NH
DNA
HO
HO
OH

(Kadlubar et al., 1976a,b) ester. The principal nucleic acid adduct formed by the reaction of this electrophile involves the C-8 of guanine and the nitrogen atom of N-methyl-4-aminoazobenzene (Lin et al., 1975).

A more complex situation, both in activation and formation of covalent derivatives, was found during investigations of 2-acetylaminofluorene (AAF). Following N-hydroxylation (Cramer et al., 1960), electrophilic species may be formed by sulfation (King and Philips, 1968; DeBaum et al., 1970), O-glucuronide formation (Miller et al., 1968), peroxide-catalyzed oxidation followed by dismutation (Bartsch and Hecker, 1971), or enzymatic transfer of the acetyl group from the nitrogen to the oxygen atom of the hydroxylamine-forming N-acetoxy-2-aminofluorene (Bartsch et al., 1972).

Both acetylated and non-acetylated adducts have been isolated from the livers of rats treated with N-hydroxy-2-acetylaminofluorene (Kreik, 1974; Westra et al., 1977). Two DNA adducts have been identified *in vitro* and *in vivo*; both result from substitution of the guanine moiety. The principal adduct is formed by substitution at the C-8 of guanine by the nitrogen atom of the AAF molecule (Lin et al., 1975). A minor product is formed by a covalent bond between the N-2 of guanine and the C-3 atom of the aminofluorene molecule (Westra et al., 1976). The structures of these products are shown in Figure 6-3.

Figure 6-3. Structures of identified DNA adducts of N-acetyl-2-aminofluorene (AAF).


CH3
CO
N
O
NH
N
N
N
NH2
DNA
O
HN
N
HN
N
N
DNA
H
N
C-CH3
O
H
N
O
NH
N
N
N
NH2
DNA

STRUCTURAL AND FUNCTIONAL CONSEQUENCES OF DNA MODIFICATION BY B(a)P and AAF

A single 30 mg/kg body weight dose of N-hydroxy AAF to a rat rapidly inhibited liver nuclear RNA synthesis (Grunberger et al., 1973). Analysis of nucleoplasmic and nucleolar RNA polymerase activities indicated that both enzyme inactivation and template modification were responsible for this reversible phenomenon (Yu and Grunberger, 1976). Studies *in vitro* have been facilitated by the availability of N-acetoxy-N-2-acetylaminofluorene (N-acetoxy AAF). This derivative does not require metabolic activation and reacts directly with DNA *in vitro*. Inhibition of template function was observed when native, duck reticulocyte DNA was modified by N-acetoxy AAF *in vitro*. Despite marked impairment of template function, however, modification did not grossly alter the ability of the DNA to interact with chromosomal proteins to form apparently normal nucleosome sturctures (Yamasaki et al., 1977a).

Physical studies on the binding of N-acetoxy AAF to calf thymus DNA showed that guanine bases modified at the C-8 position were shifted outside the double helix while the fixed aminofluorene molecule was inserted. Circular dichroism and melting curve analysis indicated that after binding of the fluorene residues in native DNA, the guanine moiety rotates around the C'_1-N-9 glycosidic bond, inserting the fluorene ring between the two neighboring bases of the G-C pair (Fuchs and Daune, 1972). This results in a general destabilization of the helical DNA structure in the vicinity of modification. Destabilization

has been detected by an increased reactivity and consequently denaturability with formaldehyde (Fuchs and Duane, 1974), and sensitivity of the modified DNA to the single strand specific endonuclease S_1 from *Aspergillus oryzae* (Fuchs, 1975). This enzyme was found to release 5 to 35 base pairs from DNA for every N-2 aminofluorene residue released (Yamasaki, 1977b). It is hypothesized that these conformational changes may cause mispairing as well as deletion of single or multiple bases during DNA replication. This so-called base displacement model (Grunberger and Weinstein, 1976) is consistent with the base-pair substitution, frameshift, and deletion mutations which have been observed with two AAF derivatives (Maher et al., 1968; McCann et al., 1975).

Investigations concerning the formation and removal of AAF adducts from liver DNA *in vivo* found that the two guanine adducts disappeared from DNA at different rates. Following a 15 mg/kg body weight dose of N-hydroxy AAF to a rat, maximum levels of covalent modification were found at 16 to 18 hours. The C-8 guanine adduct represented 80 percent of the covalently bound products and disappeared rapidly from DNA with a half-life of approximately seven days. The n-2 guanine derivative, accounting for the additional 20 percent of adducted material, was persistent and remained associated with the liver DNA up to eight weeks (Kreik, 1972). Preferential loss of the C-8 adduct also occurred when AAF-modified DNA was treated with S_1 nuclease (Yamasaki, 1977b). These investigations indicate that modification of the N-2 position of guanine by aminofluorene

residues produces only minor structural aberrations in the DNA molecule. It is hypothesized that the fluorene residue may simply occupy the minor groove of the DNA helix.

Modification of the N-2 position of guanine by the benzo(a)-pyrene molecule was found to produce dose dependent conformational changes in the DNA helix. Calf thymus DNA which was modified from 1.5 to 2.2 percent by reaction with 7α,8β-dihydroxy,9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene showed a slight decrease in the T_m of denaturation and a slight increase in susceptibility to nuclease S_1 at the lower level of modification. Kinetics of reaction with formaldehyde indicated that 1 to 7 base plates in the DNA molecule were destabilized in the local region of modification (Pulkrabek et al., 1977). Conformational studies of a modified dinucleoside monophosphate, G_pU, indicated that the benzo(a)pyrene molecule was probably inserted coplanar with the uridine moiety by rotation of the modified guanine about its glycosidic bond (Frenkel et al., 1978). Evidence suggesting this conformation in double stranded helical molecules is provided by studies which examined the unwinding of supercoiled SV40 DNA by the covalent binding of benzo(a)-pyrene derivatives (Drinkwater et al., 1978).

Functional changes in template activity of benzo(a)pyrene-modified DNA were identified by measuring the transcriptional activity of *E. coli* DNA-dependent RNA polymerase using modified calf thymus DNA (Leffler et al., 1977). Increasing levels of adduction produced progressive inhibition of transcription. Analysis of the RNA transcripts produced under conditions

allowing continuous reinitiation or only a single initiation suggested that modified sites blocked the movement of polymerase molecules along the template and prevented recycling.

These studies do not provide us with any details on the specific roles that these modifications have in producing either the acute toxic or carcinogenic responses in animals exposed to these agents. This can hardly be expected, however, when many of the details of the biological processes which they affect, such as nucleic acid metabolism and cellular differentiation, remain unknown. Until these processes are fully dissected or *in vitro* models are available to study the effects of specific interactions, comparative studies between chemicals of similar structure or animals with markedly different responses may identify important interactions and biochemical effects. These studies may also help elucidate fundamental aspects of these biological processes.

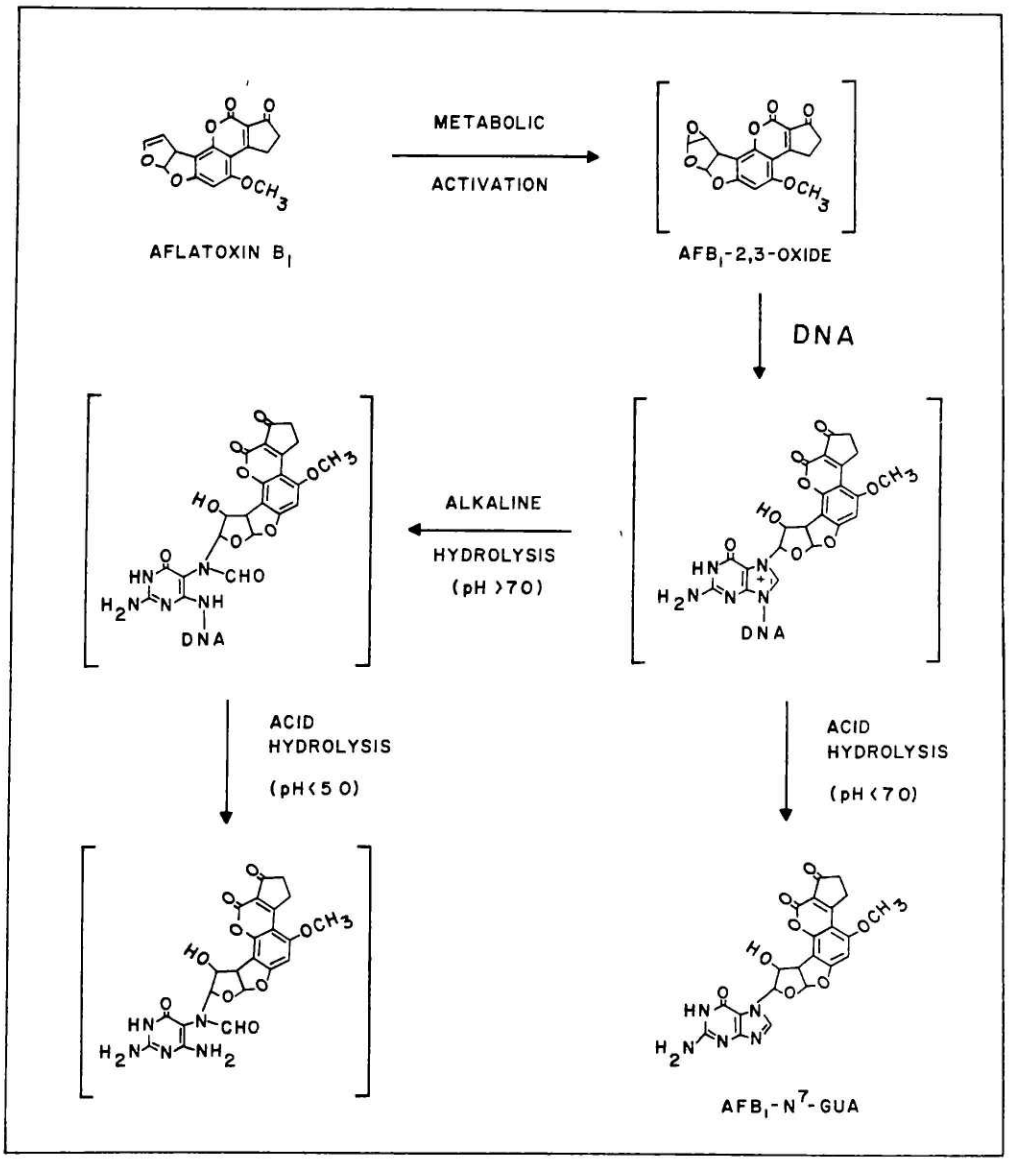
The studies detailed in this thesis were undertaken to identify the nature and amount of covalent derivatives formed by AFB_1 in DNA *in vivo* and to investigate their repair. This information sould enable more detailed studies on the possible roles these lesions have in acute toxicity, carcinogenesis, and mutagenesis in various organisms.

Structural studies have provided insight into the mechanisms of aflatoxin activation, identified target sites in DNA, and disclosed the chemical transformations of covalent AFB_1-DNA derivatives. The stereochemistry of the principal adduct formed *in vitro*, AFB_1-N^7-GUA, indicated that it had been

produced by the attack of the N-7 atom of the guanine moiety in DNA on the 2,3-epoxide of AFB_1 (Essigmann, 1977). Evidence was also found for the activation of metabolic derivatives of AFB_1 — specifically, the O-demethylated aflatoxin P_1 and possibly the 4-hydroxylated derivative aflatoxin M_1. Epoxidation of the 2,3-vinyl ether bonds of these derivatives also leads to substitution at the N-7 atom of guanine.

The 7-substituted guanine moiety in DNA was found to undergo reactions typical of 7,9-disubstituted purines. The positive charge on the imidazole ring facilitated two reactions (Figure 6-4). The acid catalyzed hydrolysis of the glycosidic C'-1-N-9 bond, resulting in release of the AFB_1-N^7-GUA moiety from the DNA molecule with concomitant formation of an apurinic site; and the base catalyzed hydrolysis of the imidazole ring, forming an AFB_1-formamido-pyrimidine derivative, attached to the DNA backbone through a glycosidic bond from the sugar C'1 to a secondary amino nitrogen. This seond hydrolytic transformation results in the increased stability of the damage caused by AFB_1 at this site. Analogous formamido derivatives of the N-7 guanine adducts formed by AFP_1 and possible AFM_1 have not been identified because of their low concentration in DNA; however, their existence is likely. Thus, *in vivo* activation of AFB_1 and its metabolic derivatives results in the subsequent formation in DNA of a spectrum of unstable and stable products. All of these which have been identified so far involve initial substitution at the N-7 atom of guanine.

Figure 6-4. Metabolic activation of AFB_1 and the reactions of the N-7 substituted guanine adduct in DNA.


METABOLIC
ACTIVATION
AFLATOXIN B1
AFB1-2,3-OXIDE
DNA
ALKALINE
HYDROLYSIS
(pH >7.0)
ACID
HYDROLYSIS
(pH <5.0)
ACID
HYDROLYSIS
(pH <7.0)
AFB1-N7-GUA

Both stereochemical and mechanistic factors are probably involved in determining the apparent specificity of this reaction. The N-7 atom of guanine is positioned in the large groove of the DNA double helix, readily available for reaction. In addition, this atom is the most nucleophilic in the DNA molecule — i.e., it has the highest electron density (Brown, 1974). It has been suggested that the reaction mechanism for covalent bond formation between the N-7 guanine and C-2 of AFB_1-2,3-oxide is of S_N2 character (Lin et al., 1977). This may also play a role in the specificity of the reaction.

In relation to the toxic effects of AFB_1, comparison of the covalent AFB_1-DNA products formed in a susceptible (Fischer rat) and resistant (Swiss mouse) species did not reveal any qualitative differences which would allow speculation as to the significance of any specific modification. However, a good correlation was found between the total covalent binding levels in the livers and kidneys of these species and the organotropic and species specific effects of AFB_1. After administration of an LD_{50} dose to each species, the highest level of AFB_1 binding to DNA was found in the rat liver, which is most sensitive to its biological effects. Rat kidney DNA was modified at 1/10 this level. In the mouse, kidney DNA was modified to the greatest extent, which correlates well with the opposite organotropism of AFB_1 in this species. *In vitro* experiments with microsomal fractions from rat and mouse liver studied the kinetics of activation of AFB_1 in these species. The results indicated that the rat has a greater capacity to activate AFB_1

and produce covalently bound DNA products. Microsomes isolated from mouse liver initially had a greater activity than those of the rat. However, mouse microsomes were apparently inactivated after a short period of time, while rat microsomes produced a linear increase in the amount of AFB_1 bound to DNA during the entire experimental period. The resistance of the mouse liver therefore probably determined its limited capacity to activate AFB_1 rather than the efficient removal or circumvention of damage from DNA or other critical targets.

In the rat, however, survival following an acute dose of AFB_1 is dependent upon removal or circumvention of the damage produced by covalent modification of critical cellular targets. Chapter Four enumerates the removal of various covalent products from DNA following a single sublethal dose of AFB_1. These investigations disclosed the differential removal of the N-7 guanine and formamido-pyrimidine derivatives of AFB_1 from DNA. The principal covalent product, AFB_1-N^7-GUA, was rapidly removed from DNA with an apparent biological half-life of 7.5 hours. The AFB_1-formamido-pyrimidine derivative persisted in DNA with little decrease in its maximum concentration attained at 24 and 72 hours after the initial exposure to AFB_1.

The rapid removal of the primary lesion AFB_1-N^7-GUA was shown not to produce a large number of alkali-labile sites in the DNA molecule, indicating that its removal did not lead to persistent formation of apurinic sites or single strand breaks in the sugar-phosphate backbone of DNA. This lesion may be removed from DNA by spontaneous chemical or enzymatic hydrolysis

of its glycosidic bond. In the latter process, the resulting apurinic site could be repaird by the sequential action of an endonuclease, exonuclease, polymerase and ligase enzymes, or possibly by the direct insertion of a guanine base by a purine insertase. Alternatively, an excision repair mechanism involving the recognition of distortions in the DNA double helix by an endonuclease which excises the lesion and a number of surrounding nucleotides may be responsible. The resulting gap would then be repaired by the sequential action of a polymerase and ligase. Insufficient information is available with which to evaluate the importance of any of these mechanisms.

Nuclear RNA synthesis is inhibited maximally 15 minutes after a dose of 1 mg AFB_1/kg body weight. Comparison of the time course of inhibition of RNA synthesis with that of the removal of AFB_1 lesions from DNA reveals a good correlation between the removal of the AFB_1-N^7-GUA adduct and the recovery of RNA synthesis to pretreatment levels. The dose-response relationships between the dose of AFB_1 and inhibition of RNA polymerase activity (Pong and Wogan, 1970) and the formation of AFB_1-N^7-GUA (Croy et al., 1978) indicate no inhibition of polymerase activity occurred with a dose of 0.05 mg AFB_1/kg body weight to a male Fischer rat. This dose would produce approximately one AFB_1 modification per 100,000 DNA base residues. The time source of inhibition of RNA polymerase activity following a 1 mg AFB_1/kg body weight dose shows maximal inhibition of approximately 70 percent is maintained from 15 minutes until after 12 hours post dosing. Recovery of activity to

pretreatment levels was seen at 36 hours. The data in Chapter Four indicate that 12 hours after a 0.6 mg/kg dose, one AFB_1 lesion is present per 33,000 DNA bases, corresponding to approximately 70 percent inhibition. At 24 hours the level of modification has decreased to one AFB_1 lesion per 96,000 DNA bases, the level at which no inhibition of RNA polymerase activity can be detected. Twenty-four hours after dosing, the quantitative distribution of AFB_1 modifications has changed. AFB_1-N^7-GUA represents 37 percent of the adducted material and has decreased to approximately 14 percent of its maximal level at two hours. The persistent imidazole ring opened products attain their maximal levels in DNA at approximately 24 hours and represent 58 percent of the adducted AFB_1 at this time. In terms of the functional significance of these changes, the time course of inhibition of RNA synthesis is correlated better with the total levels present in DNA than with the formation of these minor products.

The possible significance of these persistent minor products during multiple or continuous exposure of animals to AFB_1 is suggested by the experiments detailed in Chapter Five. Multiple doses of AFB_1 given to rats during a two week period resulted in the accumulation of these products, F and G, the putative AFB_1-formamido-pyrimidine derivatives. Most of the accumulation took place during the first week of dosing, after which a relatively constant level of these products was present. The residual level of the precursor of F and G 24 hours after each dose decreased during the first week but remained constant

during the second. It is speculated that enzyme induction was responsible for this decreased binding, since phenobarbital administration was shown to have the same effect (Garner, 1975) and an identical phenomenon has been noted in rats fed AAF (Dingman and Sporn, 1966).

During the two week period, ten 25 μg doses of AFB_1 were administered to rats. The animals exhibited no overt signs of toxicity and no gross pathologic damage could be seen in their livers at the time of sacrifice. The levels of modification of the liver DNA ranged from one AFB_1 lesion per 43,000 base residues two hours after the initial dose to one AFB_1 lesion per 365,000 base residues 24 hours later. At the end of the first seven day period, the level of persistent products had risen from one lesion per 585,000 bases to one per 172,000 bases. Following a second five day exposure, the level of these products increased to approximately one lesion per 112,000 bases. Assuming a DNA content of 7 pg per rat liver cell (3×10^{10} nucleotides/cell) this represents 270,000 modified bases per cellular genome. This calculation assumes a uniform level of modification of nuclei in all cell populations of the liver, which is most certainly not true. This figure therefore represents the minimum damage to a liver parenchymal cell genome produced by the accumulation of persistent products. The total amount of potential damage produced by lesions which are readily removed from DNA following each dose, e.g., AFB_1-N^7-GUA, will be much greater.

These patterns of adduction are not revealing with respect to the mechanisms responsible for the carcinogenicity of AFB_1. It is evident, however, that since AFB_1 has been shown to be a potent mutagen, ample opportunity exists for the induction of somatic mutations in the liver cell population. The structural sequence of a 1000 base-pair gene would contain approximately 1.8×10^{-2} persistent lesions at the end of the 14 day dosing period. Since there are approximately 3×10^{9} parenchymal cells in a rat liver, 5×10^{7} copies of this sequence in the liver will contain a lesion. If these lesions are promutagenic, their efficiency in producing mutations would not have to be large to produce a substantial number of mutants.

Elucidation of the roles which these lesions have in the production of somatic mutations and the relationship of somatic mutation to cancer must await the development of appropriate *in vitro* models and a more in-depth understanding of basic biological processes such as mutation and differentiation.

SUMMARY

Aflatoxin B_1 was found to produce two general types of lesions in DNA, both of which occurred at guanine moieties. Primary lesions resulted from the activation of AFB_1 or one of its metabolically produced derivatives to the 2,3-epoxide which subsequently formed covalent derivatives at the N-7 atom of guanine. Secondary lesions resulted from the hydrolysis of the positively charged imidazole ring of this 7,9-disubstituted guanine in DNA, forming a putative AFB_1-formamido-pyrimidine derivative.

Covalent binding of AFB_1 to DNA in a susceptible and resistant species correlated with its toxic and organotropic effects. In the rat, which is highly susceptible to the toxic effects of AFB_1 (LD_{50}, 1 mg/kg), the liver showed the highest level of DNA modification, while the kidney had the highest level of DNA modification in the mouse (LD_{50}, 12 mg/kg). The qualitative pattern of AFB_1-DNA adducts was not revealing with respect to the mechanisms responsible for tissue susceptibility in either species.

Differential rates of removal of various AFB_1 lesions were seen in rat liver DNA following a single acute dose of AFB_1. Primary N-7 guanine lesions were readily removed from DNA with apparent biological half-lives of 7 to 11 hours. Approximately 20 percent of the primary lesions initially formed are converted to secondary ones during the first 24 hour period. Secondary AFB_1-formamido-pyrimidine products have long half-lives in DNA. Little change in the level of these persistent products was

seen 72 hours after initial exposure. The removal of lesions either spontaneously or enzymatically did not result in an amount of damage to the covalent sugar-phosphate backbone of DNA which could be detected using alkaline sucrose gradients. No conclusions as to the mechanisms of repair could be inferred.

During multiple exposure of rats to AFB_1, which was shown previously to induce a 100 percent incidence of hepatocellular carcinoma, accumulation of secondary lesions and changes in AFB_1 metabolism were seen. Increases in AFB_1-formamido-pyrimidine derivatives were seen subsequent to successive doses of AFB_1. However, the increments decreased with the level of AFB_1-N^7-GUA remaining 24 hours after each dose during the first week. These observations were interpreted in terms of decreased activation or increased detoxification of AFB_1 as a result of the induction of metabolizing enzymes. In relation to carcinogens, no conclusions could be formulated as to the significance of these interactions in inducing neoplasia in the rat liver.

PROPOSALS FOR FUTURE RESEARCH

The information which has been obtained by this work provides an initial description of the interactions of AFB_1 with DNA *in vivo* in the rat and mouse. Future studies will hopefully look at those interactions in more detail and gain insight into the biochemical mechanisms by which these AFB_1 interactions produce their biological effects. Some areas of additional investigation may include:

A. Structural Elucidation of the Unidentified Minor AFB_1-DNA Hydrolysis Products

Characterization of the principal adduct, AFB_1-N^7-GUA, enabled the development of appropriate analytical methods for quantifying *in vivo* AFB_1-DNA hydrolysis products and has given insight into the structure of related products such as the formation of persistent, imidazole ring hydrolyzed derivatives. Discovery of the AFP_1 adduct has indicated that pathways of AFB_1 metabolism previously thought to be inocuous can produce covalently bound products. Structural elucidation of other minor derivatives will provide further insights into the processes which activate and detoxify AFB_1 and those that repair the damage caused by covalent lesions in DNA *in vivo*. It is also necessary to have a complete description of the covalent products which are present in DNA *in vivo* in order to construct and evaluate appropriate *in vitro* models.

B. Comparative Studies on Rates of Formation and Removal of AFB_1 Lesions in DNA or Target and Non-Target Tissues

The organ specificity of AFB_1 carcinogenesis in the rat may be exploited to gain insight as to which, if any, DNA lesion caused by AFB_1 is a causative factor in carcinogenesis. These investigations have the potential to provide additional experimental evidence to test empirical hypotheses which have been put forth to explain the organotropism seen with the simple alkylating agents. The organ specificity and carcinogenic potency exhibited by these carcinogens is better correlated with the formation of persistent O-alkylated DNA bases than the overall initial amount of covalent damage produced. The persistence of these promutagenic lesions is believed to increase the probability of a mutagenic event and thus play a causative role in the initiation of neoplasia. By analogy with these studies the organotropism of AFB_1 carcinogenesis should be correlated with the formation and persistence of the AFB_1-formamido-pyrimidine products in the target organ if these hypotheses can be applied as general principles of chemical carcinogenesis.

C. Mechanisms of Repair of AFB_1 Lesions in DNA

The reasons for the differential rates of removal of AFB_1 lesions from DNA *in vivo* are not known. Spontaneous chemical or enzyme catalyzed hydrolysis of the glycosyl bond of the aflatoxin-N^7-GUA adducts is equally plausible. Investigations of the spontaneous rate percent release of AFB_1-N^7-GUA from DNA *in vitro* and comparison of this with the *in vivo* rate of

excision may provide insight into these processes. Investigations concerning the enzymology of the repair process may further the understanding of DNA metabolism and the types of structural aberrations in the helical DNA molecule caused by these lesions.

D. Mechanisms of Activation and Detoxification Relating to Organ and Species Sensitivity

Comparative investigations of the modification of DNA by AFB_1 in the mouse and rat have revealed differences between both species and organs in the activation of AFB_1. *In vitro* studies have provided preliminary evidence which suggests that the inactivation of microsomal enzyme(s) is responsible for the resistance of the mouse liver to the acute toxic and possibly also the carcinogenic effects of AFB_1. Predictions concerning the susceptibility of other species to the biological effects of AFB_1 and other toxins may be possible with knowledge of the properties of the enzymes which activate AFB_1 and the mechanisms by which they may be inactivated. This information may also be used in the design of appropriate agents to block AFB_1 activation in susceptible species.

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APPENDICES

APPENDIX I

METHODS OF ISOLATION, HYDROLYSIS, AND CHROMATOGRAPHIC ANALYSIS OF DNA

ISOLATION OF DNA

The production of artifacts of two general types must be considered when isolating DNA containing possible covalently bound derivatives for purposes of their identification or quantification: the loss of adducted material or the alteration of its chemical structure. From studies with DNA modified by AFB_1 *in vitro*, two reactions were identified which are potential sources of misleading results. The positively charged imidazole ring of the 7,9-disubstituted guanine-aflatoxin adduct labilizes both the glycosyl bond to acid catalyzed hydrolysis and the imidazole ring to alkaline-catalyzed hydrolysis, resulting in loss of adducted material from DNA or a change in adduct structure. It is therefore necessary to isolate this material rapidly in neutral of slightly acidic conditions. Methods such as isopycnic centrifugation in CsCl or long enzymatic digestions are not ideal.

DNA was isolated from a crude preparation of cell nuclei so that it would not have to be separated from a large amount of cytoplasmic RNA in subsequent steps. Nuclei were prepared by a method modified from that of Hymer and Kuff (1964). Organs were perfused *in situ* with approximately 30 ml of ice cold 0.25 M sucrose, 2 mM $CaCl_2$, 0.01 M *tris* pH 6.9. All subsequent procedures were performed at 2°C. After removal of connective tissue with a tissue press, the organs were gently homogenized, using a motor-driven teflon-glass homogenizer at low speed in 50 ml of buffer. The homogenate was filtered sequentially through 250 μm and 100 μm nylon mesh. Additional buffer

containing 25 percent Triton X-100 was slowly added to obtain a final concentration of 5 percent Triton X-100 in a volume which was nine times the original wet weight of the organ. The homogenate was then centrifuged at 1,000 x *g* for 10 min. The supernatant was removed by aspiration and the crude white nuclear pellet resuspended in 5 to 10 ml of buffer. Intact nuclei with little or no cytoplasmic contamination were seen using phase contact microscopy. Nuclei were used immediately for DNA isolation or frozen at -70°C. These techniques recovered 98 to 100 percent of the nuclei present in the rat liver, measured by the quantitative analysis of DNA in the homogenate and suspended nuclei by the diphenylamine reaction.

Nucleic acids (DNA and RNA) were isolated from this preparation of nuclei by a method similar to that of Marmur (1961). Nuclei were suspended in 0.05 M *tris* pH 6.9 buffer at a concentration of 0.2 to 0.3 mg/ml of DNA. After warming the solution to room temperature, appropriate volumes of 5 percent sodium dodecyl sulfate and 4 m NaCl in 0.05 M *tris* pH 6.9 were added, resulting in concentrations of 1% and 1 M, respectively. An equal volume of $CHCl_3$/isoamyl alcohol, 24:1 (v/v) was added and the two phases shaken vigorously for 15 min at room temperature. The aqueous and organic phases of the white emulsion were separated by centrifugation at 7,000 x *g* for 10 min. The aqueous phase was isolated and re-extracted with $CHCl_3$/isoamyl alcohol using the same procedure. After cooling on ice, the nucleic acids were precipitated from the aqueous phase by the addition of three volumes of ice cold ethanol. The precipitated

DNA was recovered by winding it on to glass rods, washed twice with ethanol, and dried *in vacuo*. This procedure routinely recovered 50 to 60 percent of the DNA present in the initial homogenate.

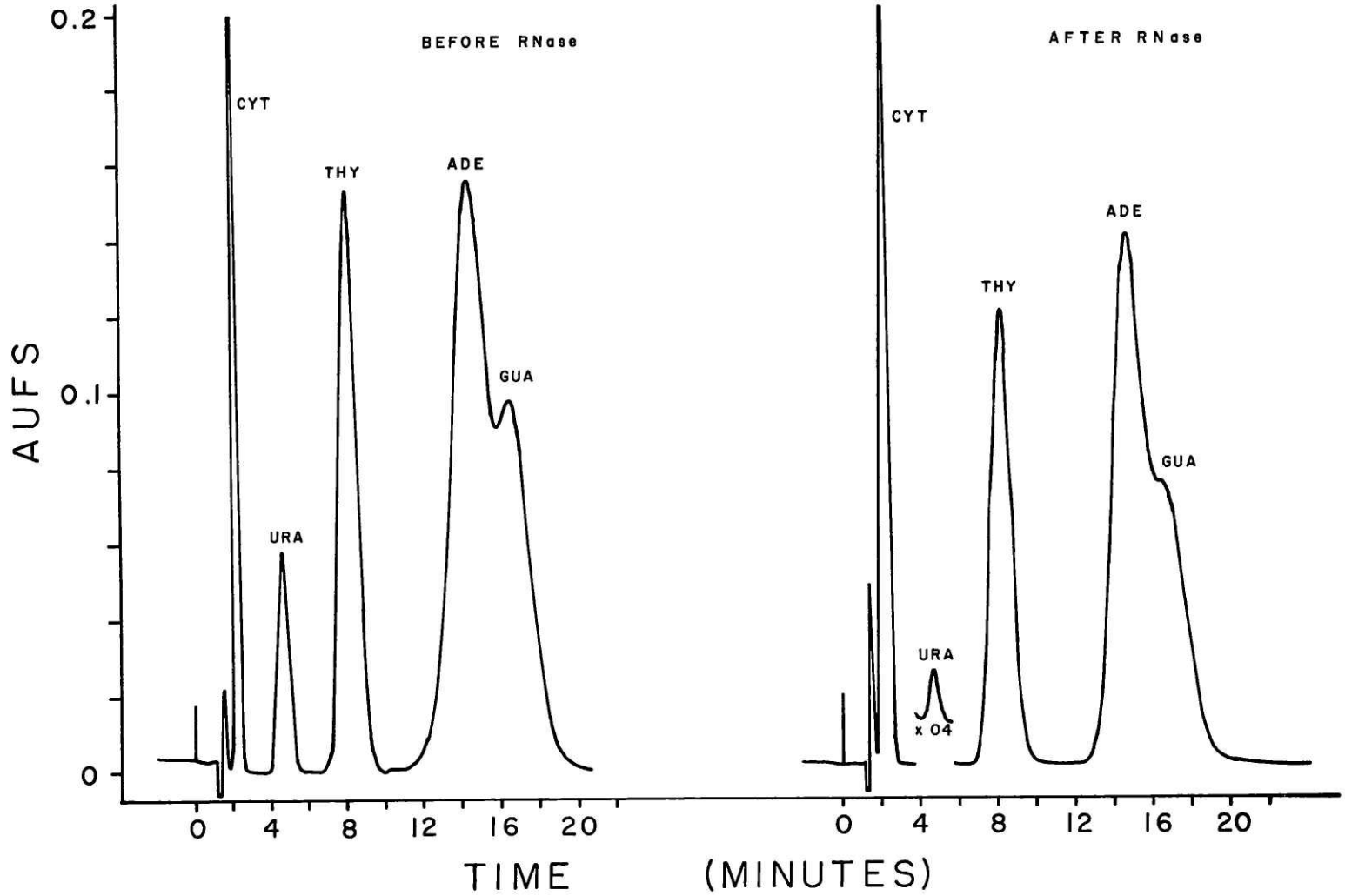
SEPARATION OF DNA FROM RNA

The dried nucleic acids contained 20 percent RNA by weight as determined by the orcinol reaction. Approximately 95 percent of this was removed by enzymatic digestion, followed by reprecipitation of the DNA. In order to avoid loss or change of material adducted to DNA, enzymatic digestions were performed with high concentrations of RNase for short periods of time, limiting the exposure of the AFB_1 derivatives to aqueous conditions.

The isolated nucleic acids (5-7 mg) were dissolved in 10 ml 0.05 M *tris* pH 7.00 on ice. The solution was adjusted to 0.1 M NaCl by addition of 0.25 ml, 4 M NaCl. 2.0 mg of RNase A, specific activity 4321 units/mg (Worthington Biochemicals), was added in 1.0 ml of 0.05 M *tris* pH 7.0, 0.1 M NaCl, and the solution incubated at 37°C for ten min. After cooling on ice, the NaCl concentration was adjusted to 0.9 M by addition of 4 M NaCl in 0.05 M *tris* pH 7.00. The DNA was precipitated with three volumes of cold ethanol and recovered by winding on to glass rods. It was then rinsed twice in ethanol and dried *in vacuo*.

The effectiveness of this procedure in removing RNA was determined by analyzing aliquots of material for uracil content

Figure I-1. Nucleic acid bases present in a $HClO_4$ hydrolysate of isolated rat liver nucleic acids before and after treatment with RNase A. A portion of neutralized hydrolysate was injected on to a 0.3 x 20 cm Aminex A-25 column which was eluted at ambient temperature, 0.5 ml/min with 0.1 M ammonium formate pH 4.72. The eluant was monitored at 254 nm.


0.2
0.1
0
AUFS
BEFORE RNase
AFTER RNase
CYT
URA
THY
ADE
GUA
x 04
0
4
8
12
16
20
TIME (MINUTES)

before and after RNase treatment. A small portion of the nucleic acids (approximately 100 µg) was hydrolyzed with 0.1 ml of 70 percent $HClO_4$ for 1 hr at 100°C. The hydrolysate was neutralized with 0.46 ml of 3.0 NKOH, then adjusted to a pH of approximately 4.0 with two drops of 88 percent HCO_2H. The $KClO_4$ precipitate was removed by centrifugation and a portion (20-30 µl) analyzed by ion-exclusion HPLC using a 0.3 x 20 cm Aminex A-25 column eluted with 0.1 M ammonium formate pH 4.72 0.5 ml/min at ambient temperature. Figure I-1 shows the results of a typical analysis. Based on the uracil/thymine ratio, 94 percent of the RNA had been removed from the sample.

HYDROLYSIS OF DNA

Conditions which have been found effective in removing the AFB_1-N^7-GUA adduct from DNA include hydrolysis with HCO_2H (Essigmann et al., 1977) and heating the adducted DNA dissolved in 0.05 M KAc pH 5.0 (Croy et al., 1978). The positive charge on the imidazole ring of this 7,9-disubstituted purine derivative weakens the glycosyl bond connecting the guanine and deoxyribose molecules. As a result, this bond is readily hydrolyzed in "mild" acidic conditions releasing the AFB_1-N^7-GUA molecule from the DNA backbone. These techniques were used in qualitative and quantitative studies of the AFB_1-N^7-GUA derivative in DNA modified *in vitro* and *in vivo* respectively. The HCO_2H procedure was not used for quantitative studies *in vivo* because of difficulties in removal of the HCO_2H from hydrolysis products to permit quantitative chromatographic analysis.

Additional studies on the hydrolysis of minor products from DNA revealed the necessity for modification of the KAc procedure, as this method was found to be ineffective for the hydrolysis of the putative imidazole ring-opened formamido derivatives, peaks F and G. These derivatives are more stable to acid hydrolysis since they are connected to the sugar-phosphate DNA backbone through a glycosyl bond between the C'-1 hemiacetal carbon of the sugar moiety to the secondary amino nitrogen of the pyrimidine-formamido derivative.

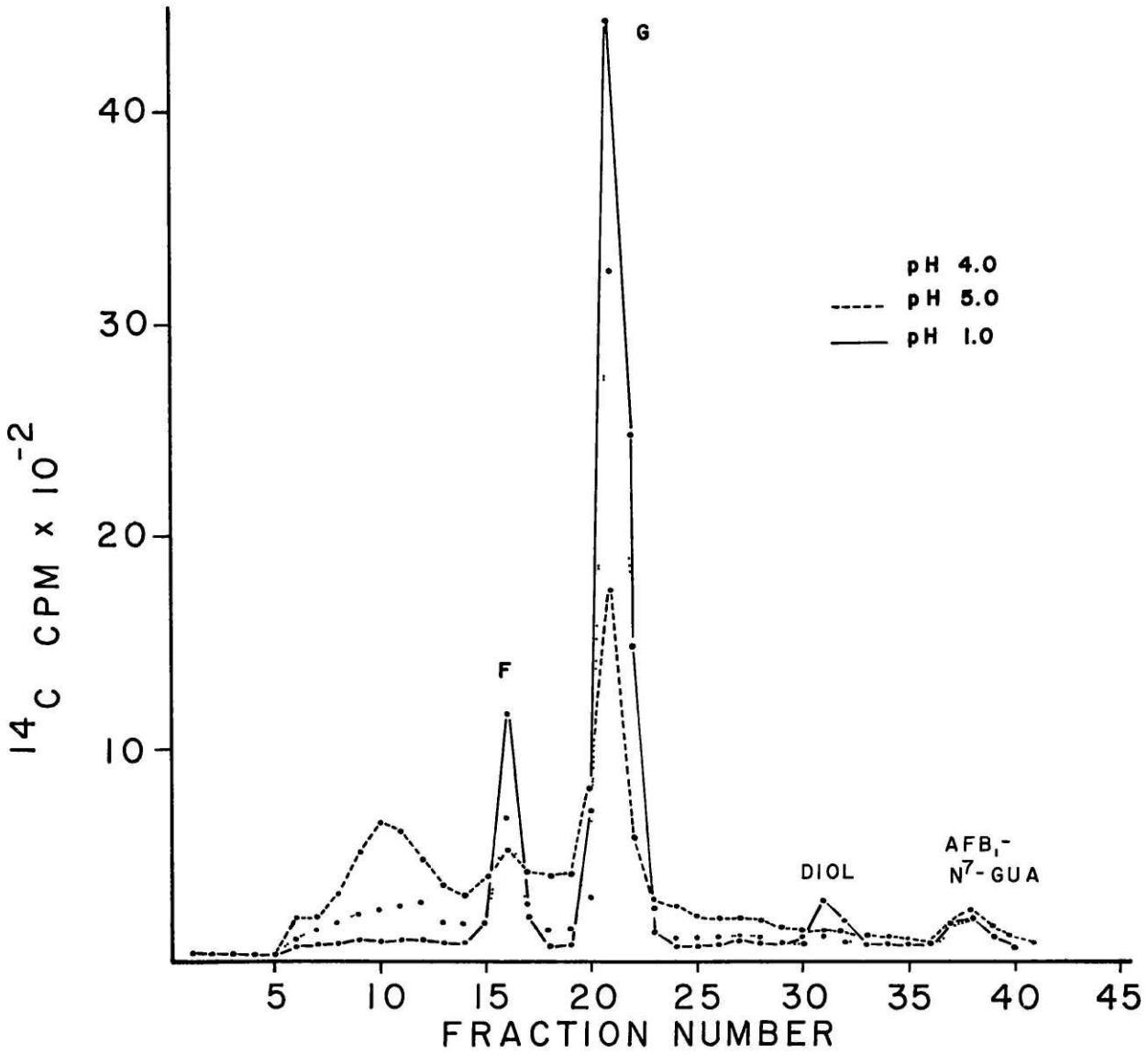
Studies on the hydrolysis of F and G were performed using [^{14}C] AFB_1 covalently bound to calf thymus DNA. 100 µg of AFB_1 (Makor Biochemicals) containing 3 µCi of ^{14}C-labeled AFB_1 (specific activity, 120 mCi/mM, Moravek Biochemicals) was activated by a microsomal activation system containing: *tris*-HCl (pH 7.5, 45 mM), $MgCl_2$ (3 mM), glucose-6-phosphate (5 mM), NADP (0.8 mM, Sigma Chemical Co.), glucose-6-phosphate dehydrogenase (0.5 units/ml, Sigma Chemical Co.), and 30 mg of microsomal protein isolated from untreated rats by the method of Kinoshita et al. (1973), in the presence of 10 mg of calf thymus DNA. Total incubation volume was 15 ml. After incubation at 37°C for 45 min, the reaction was stopped by addition of 3.7 ml 4 M NaCl and 0.9 ml 5% SDS. This solution was extracted twice with equal volumes of $CHCl_3$:isoamyl alcohol (24:1) by vigorous shaking for 10 min followed by separation of the aqueous and organic phases via centrifugation at 7,000 x *g* for 10 min. Following the second extraction, DNA was precipitated from the clear aqueous phase by winding on to a glass rod. The

precipitate was then washed 3 times with absolute ethanol and dried *in vacuo*.

The adducted DNA was exposed to alkaline conditions to convert the AFB_1-N^7-GUA derivative to compounds F and G. The adducted DNA was dissolved in deionized H_2O at an approximate concentration of 0.5 mg/ml and 1.0 N NaOH added to attain a final concentration of 0.1 N (pH 10). After standing at room temperature for 15 min, the solution was neutralized by the slow addition of a volume of 1.0 N HCl equal to that of the 1.0 N NaOH previously added. 1.0 ml aliquots of this solution were then frozen and lyophilized. The fibrous white residues were used for hydrolysis studies.

In these studies the [^{14}C] AFB_1-labeled DNA was dissolved in KAc buffers at pH 4 or 5 or deionized H_2O, which was then adjusted to 0.1 N HCl with 1.0 N HCl. These solutions were heated at 90°C for 10 min and cooled on ice. The products of hydrolysis were then analyzed directly from the KAc solutions and following neutralization of the 0.1 N HCl solution by the addition of 1.0 N NaOH. The analyses are shown in Figure I-2. Identical amounts of hydrolysate were analyzed in each experiment. The material eluting as a broad peak after hydrolysis at pH 4 and 5 indicates the incomplete release of this material and formation of partially hydrolyzed products of unknown structure which are difficult to quantify. This is contrasted with products observed after hydrolysis at pH 1.0 (0.1 N HCl) which consist primarily of peaks F and G.

Figure I-2. Chromatographic patterns of [^{14}C] labeled hydrolysis products from [^{14}C] AFB_1 adducted DNA which was treated with 0.1 N NaOH, neutralized, and subsequently hydrolyzed at the indicated pHs. Identical amounts of hydrolyzed DNA were analyzed in each experiment. Chromatographic separation was performed on a μ Bondapak C_{18} column (Waters Associates) eluted at ambient temperature with 18 percent ethanol/water containing 0.02 M KAc pH 5.0. Flow rate was 1 ml/min.


G
F
DIOL
AFB1-
N7-GUA
pH 4.0
pH 5.0
pH 1.0
14C CPM x 10-2
40
30
20
10
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

Based on these results, the following method was used for the hydrolysis of AFB_1 derivatives from isolated DNA: DNA was dissolved in ice-cold deionized H_2) to a concentration of 0.5 to 1.0 mg/ml. 1.0 N HCl was slowly added to the solution to attain a final concentration of 0.1 N (pH 1.0). A flocculant white precipitate formed which disappeared during subsequent heating of the solution at 95°C for ten min. It was then cooled on ice and 0.5 M KAc pH 5.0 was added to a concentration of 50 mM. A volume of 1.0 N NaOH equal to that of 1.0 N HCl previously added was slowly pipetted into the solution with frequent stirring to adjust the pH to 5.0. In order to facilitate subsequent chromatographic steps, the partially depurinated and denatured DNA molecules were hydrolyzed enzymatically with Nuclease P_1 (Yamasa Shoyu Co., Ltd., Choshi, Japan), a rapid and efficient single-strand specific nuclease. The hydrolysate was incubated for two hours at 40°C in the presence of 10 µg/ml of this enzyme. An aliquot of the hydrolyzed DNA solution was then removed for determination of DNA concentration by the diphenylamine reaction and the remainder analyzed by preparative and analytical liquid chromatography.

The completed method for hydrolysis of AFB_1 adducted DNA involved the following procedures:

1. Dissolve adducted DNA in deionized H_2O on ice at a concentration of 0.5 to 1.0 mg/ml.

2. Adjust colution to 0.1 N HCl with 1.0 N HCl.

3. Heat solution 95°C for 10 min.

4. Cool on ice and add 0.5 M KAc pH 5.0 to a concentration of 50 mM.

5. Slowly add a volume of 1.0 N NaOH equal to that of 1 N HCl in Step 2.

6. Add nuclease P_1 (10 µg/ml). Incubate 2 hrs at 40°C.

7. Removed 0.2 to 0.3 ml aliquot for determination of DNA concentration.

8. Analyze portion chromatographically.

CHROMATOGRAPHIC ANALYSIS

A fundamental problem in analysis and identification of the hydrolyzed products of covalent interactions with nucleophilic centers of the DNA molecule *in vivo* is the separation of the large quantity of unmodified bases and nucleotides from the relatively small amounts of modified derivatives. These products are typically present at a relative concentration of $1/10^4$ to $1/10^7$ of the level of unmodified bases. The initial separation of these two classes of hydrolyzed derivatives was effected by the use of preparative, reversed-phase liquid chromatography. Preparative chromatographic techniques provide poor resolution of individual components with similar retention characteristics; however, they are useful in separating classes of compounds with differing physical properties. The differences in polarity or lipophilicity between AFB_1-modified bases and unmodified hydrolysis products (bases and nucleotides) are

effectively utilized in their separation by the reversed-phase chromatographic technique.

Initial studies were done using a 0.8 x 60 cm column packed with C_{18}-Corasil B (Waters Associates). A 10 to 20 ml volume of hydrolysate was adjusted to 10 percent methanol by addition of 1 to 2 ml of 100 percent methanol. The entire volume was then loaded directly on to the column through the pumping head of a positive displacement pump (Milton Roy Co., Model 196-31) at 0.8 ml/min. After the sample was loaded, the column was eluted with approximately 120 ml of 10 percent methanol. Polar, unmodified nucleic acid bases and nucleotides were removed from the column during this procedure while the relatively less polar AFB_1-containing compounds were retained because of their greater affinity for the hydrophobic C_{18} stationary phase. These retained components were subsequently eluted by increasing the methanol concentration to 80 percent using a linear, 20 min gradient. These procedures and results have been previously described (Essigmann et al., 1977; Croy et al., 1978). These techniques enabled the separation of the AFB_1-N^7-GUA derivative from some minor AFB_1-containing components during the gradient phase, but did not separate individual minor adducts.

Preparative separation of AFB_1 modified and unmodified compounds was considerably simplified by the use of small, disposable, C_{18} reversed-phase columns (Sep Pak, Waters Associates). The hydrolyzed sample was prepared as previously described and loaded on to the Sep Pak column using a syringe.

Elution of these columns successively with 10 and 85 percent methanol effected the same separation as that previously accomplished using the 0.8 x 60 cm column packed with C_{18} Corasil-B. Unretained components were eluted with 15 ml of 10 percent methanol, then less polar AFB_1 derivatives were eluted with 10 ml of 85 percent Methanol. The AFB_1 compounds which were eluted from the Sep Pak were identical both qualitatively and quantitatively with those eluting from the larger preparative column during the gradient phase. However, with this technique a much smaller volume of eluant was obtained. Less than one percent of the ^{14}C activity in a hydrolysate of [^{14}C] AFB_1-modified DNA isolated from rat liver was not retained by the Sep Pak column when eluted with 10 percent methanol, indicating the efficient retention of AFB_1 components. The 85 percent methanol eluant (10 ml) was collected in a 25 ml pear-shaped flask and the volume reduced to approximately 300 µl using a rotary evaporator at 50°C under reduced pressure. The reduced volume of eluant was drawn into a 1.0 ml high-pressure syringe and 2 to 3 ml of 50 percent methanol added to rinse the flask. This volume was reduced by the same procedure to 100-200 µl and collected with the syringe. This procedure was found to quantitatively recover AFB_1 material from the pear-shaped flask.

Analysis of individual AFB_1 derivatives was accomplished using a µ C_{18} reversed-phase column (Waters Associates). The entire 0.5 to 0.5 ml contained in the syringe was injected on to the column, which was subsequently eluted with a linear ethanol/water gradient consisting of 14 to 18 percent ethanol

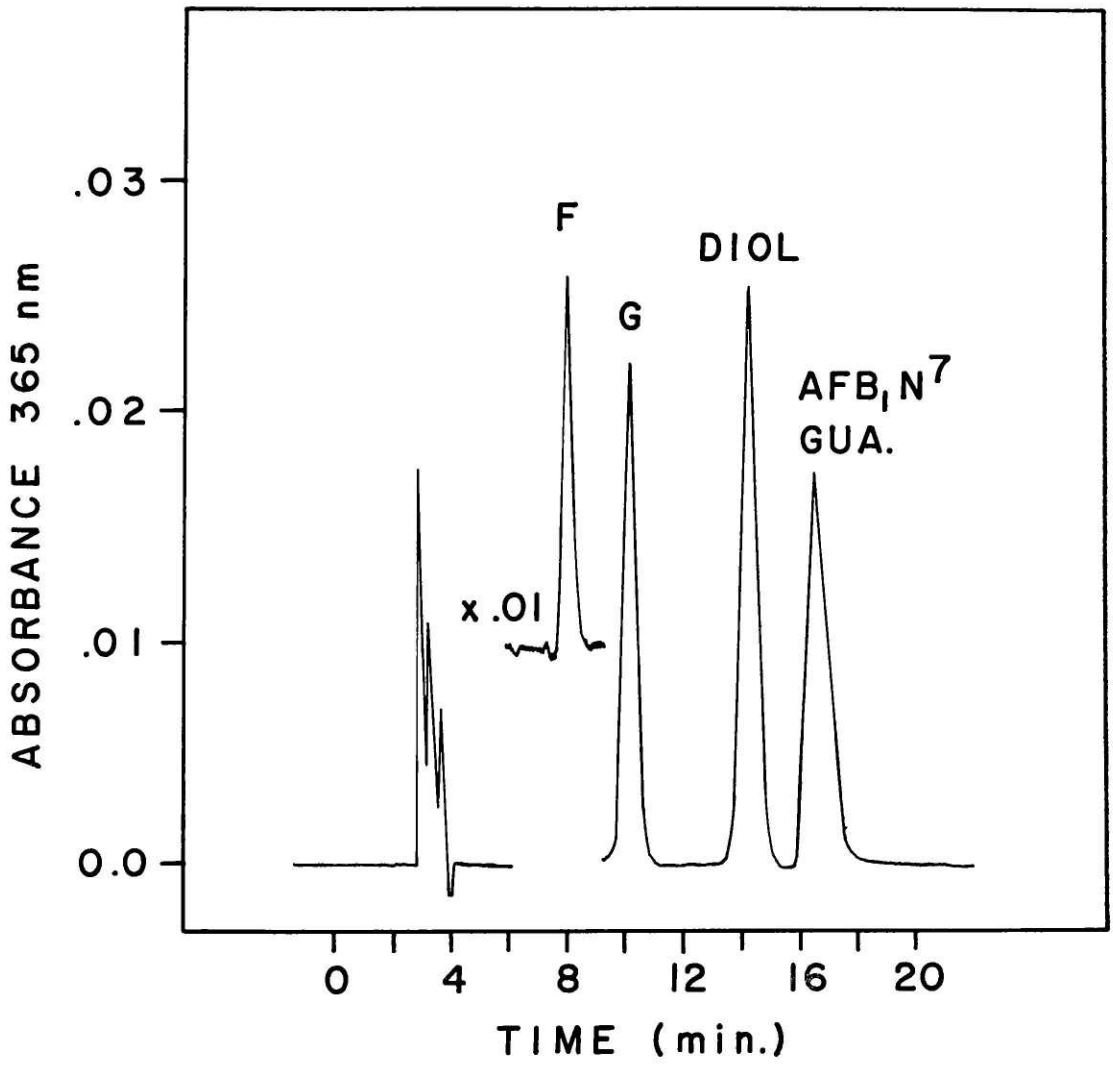
in 25 min or 12 to 18 percent ethanol in 65 min at 1.0 ml/min, ambient temperature. The reversed-phase technique again separated components in accordance with their relative partition coefficients between the polar mobile phase and hydrophobic C_{18} stationary phase. Polar compounds were eluted at short retention times followed by ones with more lipophilic character.

For determination of radioactivity the eluant was fractionated into 30-drop fractions and collected directly into scintillation vials. 12 ml of scintillation cocktail was added (ACS, Amersham or Aquasol, New England Nuclear) and the activity of each fraction determined using a Beckman LS-8000 liquid scintillation spectrometer.

RECOVERY OF STANDARDS DURING THE HYDROLYSIS AND CHROMATOGRAPHY PROCEDURES

Several compounds were available as standards so that the recovery of the principal products of hydrolysis from AFB_1 adducted DNA could be studied. AFB_1-N^7-GUA and the putative imidazole ring-opened derivatives F and G were isolated from DNA which had been adducted by AFB_1 *in vitro* using a microsomal metabolism system. 2,3-dihydro-2,3-dihydroxy AFB_1 (AFB_1-DIOL) was prepared synthetically. Known amounts of these compounds were added to solutions of unmodified calf thymus DNA which were then hydrolyzed, analyzed chromatographically, and the recovery of these compounds determined by peak height using isocratic conditions of 18 percent ethanol (Figure I-3). AFB_1-N^7-GUA was recovered quantitatively. A decrease in peak G was accompanied by an increase in peak F of approximately the same

Figure I-3. Isocratic HPLC reversed-phase separation of a mixture of the principal hydrolysis products obtained from *in vitro* AFB_1-adducted DNA. Separation was performed using a μ Bondapak C_{18} column (Waters Associates) eluted at ambient temperature with 18 percent ethanol containing 0.02 M KAc pH 5.0 at a flow rate of 1 ml/min. The eluant was monitored at 365 nm.


ABSORBANCE 365 nm
.03
.02
.01
0.0
F
G
DIOL
AFB1N7
GUA.
x .01
0
4
8
12
16
20
TIME (min.)

amount, illustrating the product-precursor relationship which exists between these compounds. Recovery of only 35 percent was determined for the AFB_1-DIOL derivative. Additional studies with this product determined that its recovery could be improved by lowering the pH of the eluants during the preparative and analytical chromatography steps. Recovery studies of all components were then repeated with the following modifications: the aqueous portion of the 10 and 85 percent methanol eluants used in the preparative separation contained 0.05 M KAc pH 4.50 and those used in the analytical separations contained 0.02 M KAc pH 5.00. Recoveries are shown in Table I-1. All the potential principal hydrolysis products available as standards were recovered quantitatively.

The completed method for chromatography analysis of a hydrolysate involves the following procedures:

PREPARATIVE CHROMATOGRAPHY

1. Adjust hydrolysate to 10% methanol (MeOH) by addition of an appropriate volume of 100% MeOH.

2. Rinse Sep Pak with 10 ml 100% MeOH followed by 10 ml of 10% MeOH containing 0.05 M KAc pH 4.50.

3. Elute hydrolysate through Sep Pak and rinse with 15 ml 10% MeOH; 0.05 M KAc pH 4.50.

4. Rinse column with 10 ml 80% MeOH in 0.05 M KAc pH 4.50 and collect eluant directly into 25 ml pear-shaped flask.

5. Reduce volume of eluant to 0.3-0.4 ml with rotary evaporator at 50°C under reduced pressure.

6. Removed reduced volume with 1.0 ml high pressure syringe and add 2 ml 50% MeOH, 0.05 M KAc pH 4.5.

7. Reduce volume to 0.1-0.2 ml and combine with material in syringe.

ANALYTICAL CHROMATOGRAPHY

1. Inject entire contents of syringe on to μ Bondapak C_{18} column.

2. Elute column with ethanol-water gradient containing 0.02 M KAc pH 5.0, 1 ml/min, ambient temperature.

3. Collect eluant in 30-drop fractions directly into scintillation vials.

4. Add 12 ml scintillation cocktail and determine activity in each vial by liquid scintillation spectrometry.

TABLE I-1

RECOVERY OF THE PRINCIPAL AFB_1-DNA HYDROLYSIS PRODUCTS AFTER HYDROLYSIS AND CHROMATOGRAPHIC SEPARATION

PEAK IDENTITY	AMOUNT ADDED[a] TO SAMPLE	AMOUNT RECOVERED	PERCENT RECOVERY
F	8.1	13.7±0.5[b]	169
G	54.8	48.0±2.1	88
DIOL	63.3	63.4±2.7	100
AFB_1-N^7-GUA	37.1	37.2±1.7	100

[a]Absorbance units of material.

[b]Mean and standard deviation of four analyses.

APPENDIX II

DETERMINATION OF ^{3}H LOSS

AFB_1 can be labeled with ^{14}C to a specific activity of 100 to 150 mCi/mM by supplying ^{14}C labeled precursors to an AFB_1 producing strain of *Aspergillus flavus*. This specific activity is too low and the cost of this material too high to permit its routine use in studies such as those reported here. ^{3}H labeled AFB_1 is relatively inexpensive and can be synthesized at a specific activity up to 40 Ci/mM. However, some of the ^{3}H is unstable and can be lost from the molecule under acidic or alkaline conditions. It has been reported (Swenson, 1974) that most of the ^{3}H label is attached to the α carbon of the cyclopentanone ring. In previous investigations using [^{3}H] AFB_1, the loss of ^{3}H during the experiment was measured using a double-label technique (Croy, 1977). Both [^{3}H] and [^{14}C] AFB_1 were administered to a rat and adducts isolated from the liver DNA. Comparison of the $^{3}H/^{14}C$ ratio of the injected material with that present in the hydrolyzed AFB_1 adduct revealed that approximately three percent of the ^{3}H label was lost. Further investigation revealed that this small loss occurred primarily during the acid hydrolysis step of the procedure, which was accomplished by heating the adducted DNA in 0.05 M KAc buffer pH 5.0.

This hydrolysis procedure was subsequently modified (see Appendix I). In order to effect complete hydrolysis of minor AFB_1 adducts, adducted DNA was heated at 95°C in 0.1 N HCl (pH 1.0). Investigations revealed that these conditions caused greater loss of ^{3}H. Three 140 g male Fischer rats were

injected i.p. with a dose of 1 mg/kg (140 µg) of AFB_1 containing 41 µCi [^3H] AFB_1 and 4.3 µCi [^{14}C] AFB_1 (Moravek Biochemicals) in 50 µl DMSO. The rats were sacrificed two hours later and DNA isolated from their livers for analysis of covalently bound AFB_1 products. The adducted DNA was hydrolyzed chemically and enzymatically using 0.1 N HCl and nuclease P_1 as described in Appendix I. The products of hydrolysis were separated chromatographically (Appendix I) and the $^3H/^{14}C$ ratio determined for the AFB_1-N^7-GUA derivative. The $^3H/^{14}C$ ratio of the injected dose was 9.50 and that determined for the AFB_1 derivative isolated from the DNA of three rat livers was 7.62, 7.63, and 7.81 [7.68 ± 0.11 (AVE ± S.D.)]. The decrease in this ratio indicates a loss of 19.2 ± 1.1 percent of the ^3H label had occurred. All determinations of the absolute amounts of hydrolysis products isolated from DNA using ^3H labeled AFB_1 have been corrected for the loss of ^3H by dividing the amount of ^3H activity recovered by 0.80.

DETERMINATION OF DNA CONCENTRATION IN A HYDROLYSATE

The amount of DNA analyzed for the quantification of AFB_1 derivatives was determined by the diphenylamine procedure of Burton (1956) as modified by Giles and Myers (1965). A 0.4 ml aliquot of a DNA hydrolysate was removed following enzymatic digestion. Duplicate determinations were made on 100 or 50 µl of solution. In a typical analysis 50 µl of hydrolysate was added to 2.0 ml of 1 N perchloric acid and the solution heated for ten min at 70°C. After cooling to room temperature, 2.0 ml of 4 percent diphenylamine (Matheson, Coleman, Bell) in glacial

acetic acid was added, followed by 0.1 ml of a 1.6 mg/ml acetaldehyde solution. After standing overnight at room temperature, the $O.D._{595} - O.D._{700}$ was determined.

A standard curve was constructed for each determination. The standard DNA solution was made by dissolving calf thymus DNA (Sigma Chemical Co., Type IV) in deionized H_2O to an approximate concentration of 1 mg/ml. The exact concentration of this solution was then determined by measuring the $O.D._{254}$ of a 1:50 dilution, assuming the relationship of 0.24 A_{254} units equalling a DNA concentration of 10 µg/ml. 1.0 ml aliquots of the DNA solution were frozen and used as needed. A typical standard curve is shown in Figure II-1.

DETERMINATION OF RADIOCHEMICAL PURITY AND ISOLATION OF $-O^{14}CH_3$ LABELED AFB_1

The determination of radiochemical purity of radiolabeled AFB_1 was accomplished by chromatographic analysis. Figure II-2 shows the separation of a standard mixture of aflatoxins by reversed-phase HPLC using a µ Bondapak C_{18} column (Waters Associates) eluted with 1.0 ml/min of 28 percent ethanol/H_2O at ambient temperature. In a typical analysis 0.5-1.0 µCi of labeled material was added to 1 µg of unlabeled AFB_1 and injected on to the column. 30-drop fractions of eluant were collected from 0 to 20 min and the activity of each fraction determined by liquid scintillation spectrometry. Analysis of the ^3H and ^{14}C labeled AFB_1 revealed 100 percent of the activity coeluting with standard AFB_1.

Figure II-1. Standard curve for the determination of DNA by the diphenylamine reaction.

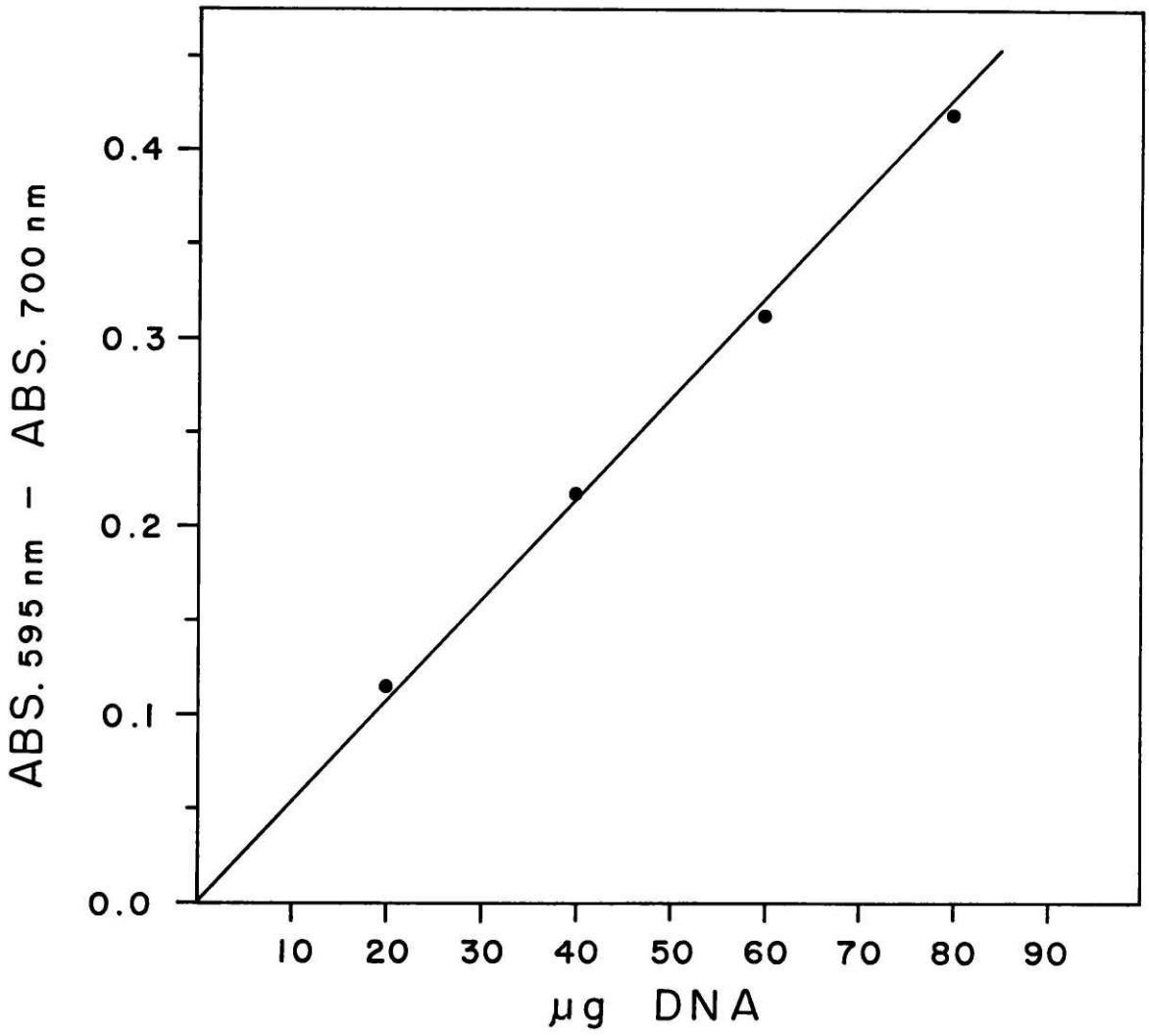
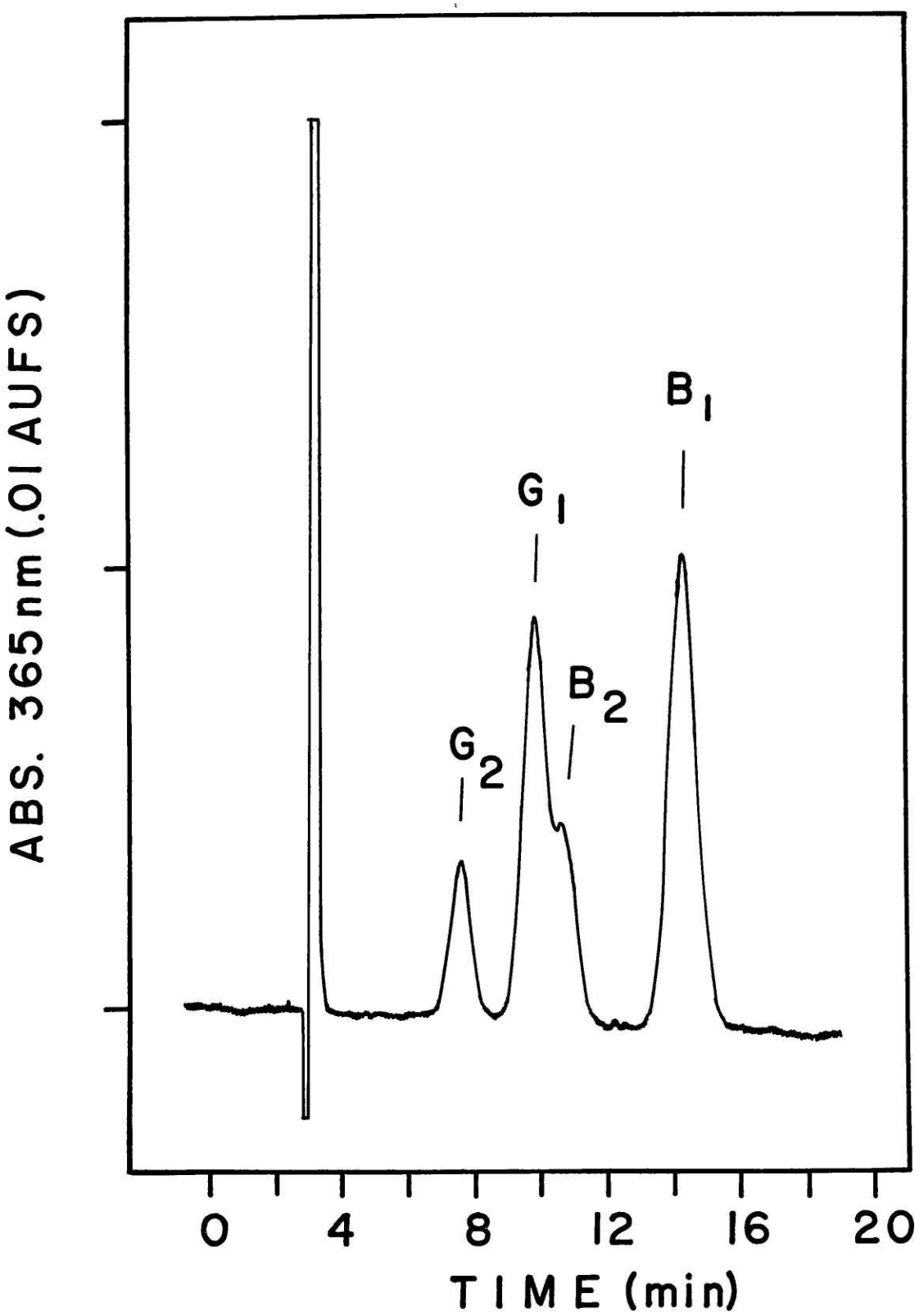

ABS. 595 nm - ABS. 700 nm
0.0
0.1
0.2
0.3
0.4
10
20
30
40
50
60
70
80
90
µg DNA

Figure II-2. Reversed-phase HPLC separation of aflatoxins B_1, B_2, G_1, and G_2. 10 µl of a standard solution of aflatoxins was injected on to a µ Bondapak C_{18} column (Waters Associates) eluted with 28 percent ethanol, 1 ml/min at ambient temperature.


ABS. 365nm (.01 AUFS)
G2
G1
B2
B1
0
4
8
12
16
20
TIME (min)

Identical chromatographic conditions were used to isolate the [^{14}C] methoxy-labeled AFB_1 for experiments to determine the presence of any aflatoxin P_1 derivatives covalently bound to DNA (Chapter Two). Previously synthesized $-O^{14}CH_3$ AFB_1 was dissolved in 200 µl of ethanol (this solution was brown) and injected on to the reversed-phase column in 40 µl aliquots. The AFB_1 peaks collected from each run were pooled resulting in a total volume of approximately 20 ml. This solution was reduced to dryness at 50°C under reduced pressure using a rotary evaporation and the white residue dissolved in 10 ml of absolute ethanol. 320 µg of labeled AFB_1 was recovered with a specific activity of 10.2 mCi/mM.

DETERMINATION OF AFLATOXIN CONCENTRATION AND THE SPECIFIC ACTIVITY OF RADIOLABELED AFB_1

AFB_1 solutions were made up in redistilled ethanol. The approximate amount of AFB_1 desired was weighed out and dissolved in ethanol. The exact concentration of this solution was then determined by measuring the A_{362} of an appropriate dilution, assuming an extinction coefficient of 22,000 for AFB_1 at this wavelength.

For the preparation of ^{14}C or ^{3}H labeled AFB_1 of a predetermined specific activity, the appropriate amount of ^{14}C or ^{3}H AFB_1 of high specific activity (typically 130 mCi/mM for ^{14}C and 20-40 Ci/mm for ^{3}H labeled molecules) was added to a solution of unlabeled AFB_1, typically 0.5-0.8 mg/ml. After dilution the exact concentration of AFB_1 in the solution was determined from its A_{362nm} and the ^{3}H or ^{14}C activity of an

aliquot of the same dilution determined by liquid scintillation spectrometry. After correcting for counting efficiency, the number of mCi per unit volume of solution was calculated (1 mCi - 2.2×10^9 dpm) and divided by the number of mmoles of AFB_1 in the same volume to obtain the specific activity of the AFB_1 in solution.

EXAMPLE

To prepare a solution of [^{3}H] AFB_1 of approximately 100 mCi/mM specific activity, 12.5 mg of AFB_1 was dissolved in redistilled ethanol and 5.0 ml of a 1 mCi/ml solution of [^{3}H] AFB_1, specific activity 20 Ci/mM, was added. The volume of the solution was increased to 25.0 ml with ethanol. 50 µl aliquots of this solution were diluted to 10.0 ml in volumetric flasks. The A_{326} of the solution was found to be 0.197 and 50 µl aliquots contained 36,000 cpm of ^{3}H activity.

AFB_1 concentration:

$$C = \frac{A_{362}}{\varepsilon b}$$

where: C — concentration of AFB_1 in moles/liter

ε — extinction coefficient (22,000)

b — cell path length (1 cm)

$$C = \frac{0.197}{22{,}000\ (1)} = 8.95 \times 10^{-6} \text{ mmole/ml}$$

^{3}H activity:

$$\text{dpm} = \frac{\text{cpm}}{\text{*counting efficiency}} \qquad \text{*C.E.} = 0.379$$

$$\frac{36{,}000/50\ \mu\text{l}}{0.379} = 9.5 \times 10^4 \text{ dpm}/50\ \mu\text{l}$$

OR

$$\frac{1.9 \times 10^6 \text{ dpm/ml}}{2.22 \times 10^6 \text{ dpm/ Ci}} = 0.86 \text{ } \mu\text{Ci/ml}$$

Specific activity:

$$\frac{^3\text{H activity}}{AFB_1 \text{ concentration}} = \frac{8.6 \times 10^{-4} \text{ mCi/ml}}{8.95 \times 10^{-6} \text{ mmoles/ml}}$$

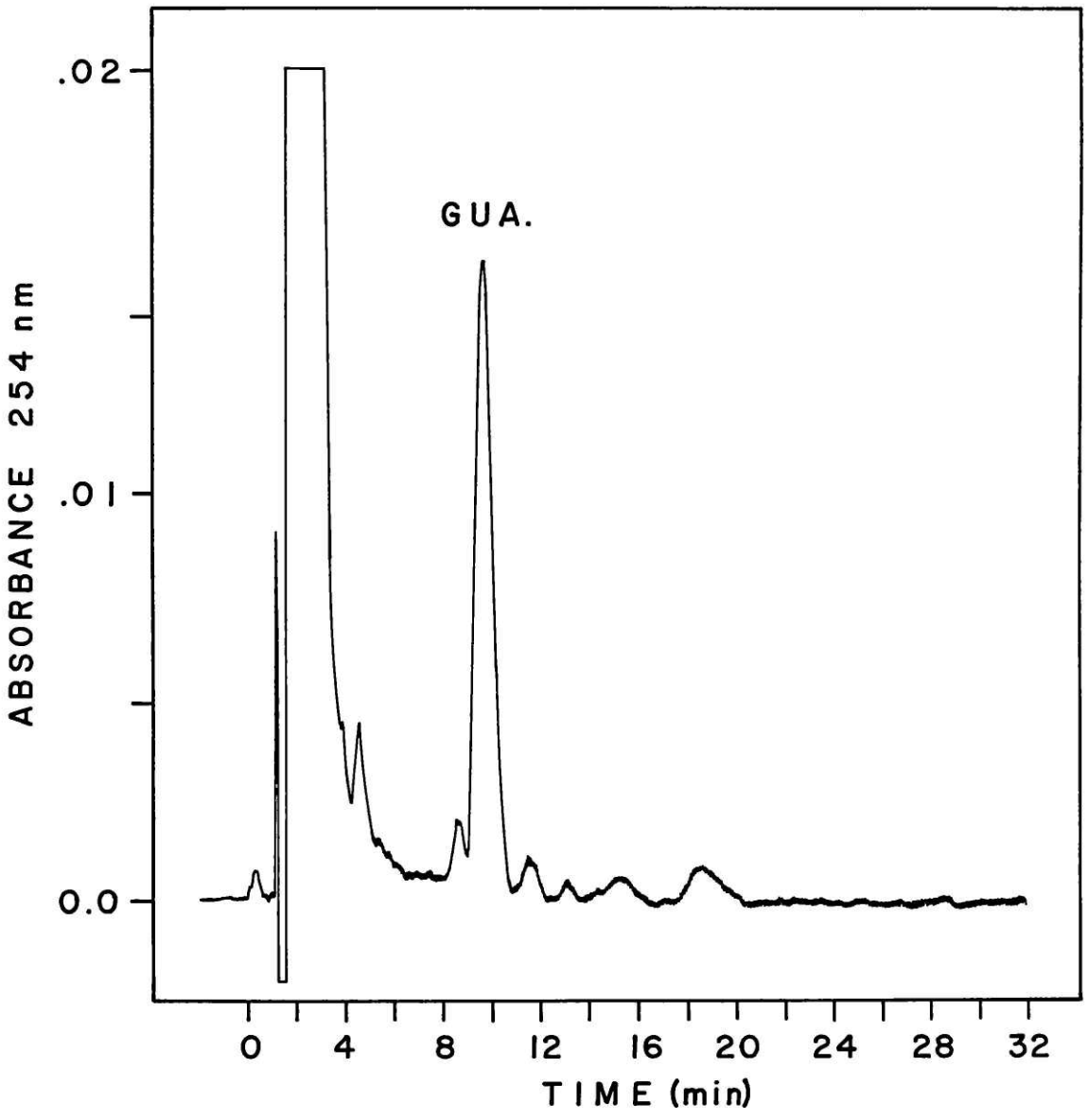
$$= 96.1 \text{ mCi/mM}$$

IDENTIFICATION OF PURINE BASES IN POSSIBLE AFB_1 ADDUCTS BY $HClO_4$ HYDROLYSIS

0.2 to 0.8 µg of the AFB_1 derivative was hydrolyzed by heating with 50 µl of 70% $HClO_4$ at 100°C for 1 hr in a tightly capped 5 ml vial. The vial was then cooled on ice and the solution neutralized by the slow addition of 235 µl of 3 N KOH. One drop of 88% HCO_2H was added to adjust the pH to approximately 4 and the $KClO_4$ precipitate separated from the clear aqueous phase by centrifugation at 2,000 rpm. An aliquot of the aqueous solution was then analyzed by cation exchange HPLC. 40 to 60 µl of solution was applied to a 0.2 x 25 cm column packed with Durrum DC4A resin, eluted at 50°C, 0.3 ml/min with 0.1 M ammonium formate pH 5.08. Using these conditions, the retention time of guanine and adenine in this system was 9.5 and 28.5 minutes, respectively.

The results of this analysis performed on peak H (the AFP_1 derivative) are shown in Figure II-3. A prominent peak is seen at the retention time of guanine, indicating the possible presence of a guanine moiety in the isolated AFB_1 derivative.

Figure II-3. Ion-exchange HPLC analysis of an $HClO_4$ hydrolysate of peak H, the aflatoxin P_1 derivative. 40 µl of neutralized hydrolysate was injected on to a 0.2 x 25 cm column packed with Durrum DC4A resin which was eluted at 50°C, 0.3 ml/min with 0.1 M ammonium formate pH 5.08. The eluant was monitored at 254 nm.


.02
.01
0.0
ABSORBANCE 254 nm
GUA.
0
4
8
12
16
20
24
28
32
TIME (min)

CALCULATIONS

The concentration of a hydrolysis product in DNA expressed as the number of modifications per nucleic acid base residue was calculated using the equation:

$$\text{modifications/base} = \frac{\text{AMW}(\text{CPM/mg DNA})}{(^3H_{exc})(^3H_{eff})(\text{sp. act. } AFB_1)}$$

where: AMW — average molecular weight of a nucleotide residue in DNA (326)

CPM/mg DNA — ^3H counts per minute in the chromatographic peak background per mg of DNA analyzed

$^3H_{exc}$ — correction factor for the amount of ^3H loss due to exchange (0.8)

$^3H_{eff}$ — counting efficiency for the ^3H isotope (0.375)

sp. act. AFB_1 — specific activity of ^3H AFB_1 administered to the animal (dpm/ M AFB_1).

When the data are expressed in terms of μM of product/mg DNA, the equation becomes:

$$\mu M \text{ of product/mg DNA} = \frac{\text{CPM/mg DNA}}{(^3H_{exc})(^3H_{eff})(\text{sp. act. } AFB_1)} .$$

BIOGRAPHICAL SKETCH

Robert Croy was born in Morristown, New Jersey ın 1948, and received his secondary eduction at Morristown Preparatory School in New Jersey. Re received a B.S. degree in Chemistry from Clarkson College of Technology, Potsdam, New York in 1970 and spent two years in a graduate program in Biology at the State University of New York at Potsdam. He was rescued from this program by termination of funds and forced to seek employment which he found at the National Cancer Institute in Bethesda, Maryland. After three years of employment as a technician he was encouraged to continue his educational experience with the financial support of his wife Sue.

He enrolled in the Massachusetts Institute of Technology in September 1975 and is currently a candidate for the Ph.D. degree in Toxicology with Professor Gerald N. Wogan serving as his advisor.