13. We have observed that the potassium content of normal erythrocytes shows a less than 6 percent decrease during such incubations. When glucose is present, there is a loss of approximately 20 percent of the starting amount of adenosine triphosphate (ATP). This loss is not altered by the presence of calcium in the incubation solution. In the absence of glucose, there is no alteration in ATP content or the degree of calcium accumulation (G. A. Plishker and H. J. Gitelman, in preparation).

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Aflatoxicol: Major Aflatoxin B_1 Metabolite in Rat Plasma

Abstract. *Aflatoxicol, a carcinogenic metabolite of the foodborne carcinogen aflatoxin B_1 previously known only as a bioreduction product in vitro, was identified as the major aflatoxin metabolite in the plasma of Sprague-Dawley rats, a susceptible species, that had been dosed orally or intravenously with aflatoxin B_1 labeled with carbon-14. Aflatoxicol, however, was not detected in the plasma of similarly dosed mice and monkeys, which are both resistant to aflatoxin B_1–induced carcinogenesis. The formation of aflatoxicol both in vitro and in vivo may be an indicator of species sensitivity to aflatoxin-induced carcinogenesis and may be useful in the prediction of human susceptibility.*

Aflatoxin B_1 (AFB_1), a mycotoxin produced by certain strains of *Aspergillus*, has been recognized as a significant food contaminant because of its widespread occurrence in such foodstuffs as peanuts and corn (*1*) and its potent hepatocarcinogenicity in rats and other test animals (*2*). The concern over AFB_1 is substantiated by epidemiologic studies which show a correlation between the current incidence of primary liver cancer in certain human populations and the ingestion of AFB_1-contaminated diets (*3*).

Recently, studies of metabolism and mode of action have provided evidence that AFB_1 requires metabolic activation to elicit its carcinogenic effects (*4*), that the ultimate carcinogenic form may be the 8,9-epoxide of AFB_1 (*5, 6*), and that the marked differences in species susceptibility to the carcinogenic effects of AFB_1 can be correlated with species differences in the metabolism of AFB_1 (*7, 8*).

Aflatoxin B_1 can be oxidized in vitro by liver microsomal oxygenases to AFM_1, AFQ_1, AFP_1, and AFB_{2a} (*9*); AFB_1 and AFQ_1 can also be reduced by cytoplasmic reductase to aflatoxicol (AFL) (*10, 11*) (Fig. 1) and $AFLH_1$ (*12*), respectively. Since these metabolites have distinctly different mutagenic activities (*13*), it is reasonable to assume that the carcinogenicity of AFB_1 is influenced by its ultimate partitioning among these competing metabolic pathways if they are operative in vivo. On the basis of metabolism data obtained in vitro, we have found that the susceptibility of a species to the carcinogenic effect of AFB_1 is directly correlated with hepatic transformation of AFB_1 to AFL but inversely correlated with conversion of AFB_1 to AFQ_1 or to aqueous metabolites (*14*). This observed correlation between AFL formation and species susceptibility to aflatoxin-induced carcinogenesis may provide a means for estimating human susceptibility.

Despite these interesting findings, to our knowledge AFL has not previously been isolated in vivo to support a relationship between the biotransformation in vitro and the metabolism and disposition in vivo of AFB_1. So far, of the six metabolites mentioned, only AFM_1 has been consistently isolated from the excreta and tissues of exposed animals (*15*) and humans (*16*). However, in the study reported here, we found that AFL was the major AFB_1 metabolite in the plasma of rats dosed with AFB_1 either intravenously or orally. The identification of AFL as a metabolite of AFB_1 in vivo not only confirms the significance of metabolism studies in vitro but also strengthens the possibility that the AFL-forming activity can be used as a metabolic parameter to estimate species susceptibility to aflatoxin-induced carcinogenesis.

Experiments were conducted with Sprague-Dawley rats weighing 260 to 280 g. The rats were anesthetized with diethyl ether and secured dorsally to a heated surgical platform, and both jugular veins were revealed by blunt dissection for catheterization with a 27-gauge needle cannula. To study the toxicokinetics of AFB_1, 1.0 mg/kg of ring-labeled [^{14}C]AFB_1 (0.6 Ci/mole), dissolved in 0.3 ml of a mixture of physiological saline solution and dimethyl sulfoxide (3:1), was administered intravenously as a rapid infusion into the left jugular vein. Blood samples were then collected every 5 to 10 minutes for 50 minutes from the opposing catheterized jugular vein. In studies where AFB_1 was administered orally by intubation, the dose was tripled and blood samples were taken for 100 minutes at wider intervals.

Approximately 95 percent of the total blood radioactivity was found in the plasma, and the total plasma radioactivity was partitioned into the protein, chloroform-extractable, and residual aqueous fractions. Time-concentration profiles of various radioactive species in the plasma of rats administered [^{14}C]AFB_1 are shown in Fig. 2. In the rats dosed intravenously, the major identifiable constituents of the chloroform-extractable fraction were residual AFB_1, AFM_1, and a metabolite subsequently identified as AFL. Aflatoxin M_1, which is invariably the major identifiable urinary metabolite, was present only in trace amounts, accounting for less than 0.2 percent of the total plasma radioactivity. Aflatoxicol was found to be the principal metabolite, representing 15 to 25 percent of the total plasma radioactivity and consistently accounting for more than 25 percent of the chloroform-extractable radioactivity.

The major plasma metabolite was identified as AFL by thin-layer chromatography (TLC) and ultraviolet and mass spectroscopic analyses, using AFL standards prepared by biotransformation in vitro (*10, 11*) and by chemical reduction of AFB_1 (*17*). Analytical evidence for the bioreduction of AFB_1 to its cyclopentanol structure, AFL, is summarized in Table 1. The identification of AFL was further confirmed by its relatively potent mutagenic activity to *Salmonella typhimurium* strain TA98 upon metabolic activation (*13*).

It was observed that oral administration of [^{14}C]AFB_1 resulted in a much lower total plasma radioactivity, which peaked at 1 hour (Fig. 2). The concentrations of AFL and AFM_1 relative to that

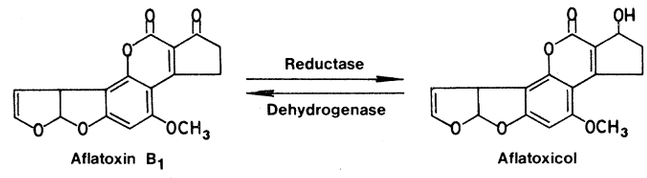

Fig. 1. Interconversion of aflatoxin B_1 and aflatoxicol. Aflatoxicol is formed by reduction of the cyclopentanone function of aflatoxin B_1.

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of the residual AFB_1 were much higher than those determined after intravenous administration, but AFM_1 levels remained below 0.1 percent of the administered dose. The most obvious difference in the time-concentration profiles representing the two routes of administration is the marked increase in the proportion of water-soluble products in the orally dosed rats. The values obtained after oral administration probably reflect greater bioavailability of AFB_1 to the liver and hence higher levels of metabolites. The results also indicate active hepatic extraction of AFB_1; this is additional evidence for the organ specificity of AFB_1, which causes mainly hepatic lesions.

The biotransformation of AFB_1 to water-soluble products and plasma protein–bound species probably indicates an active multiple detoxification system. Formation of AFL as the principal metabolite in a susceptible species such as the rat is consistent with the positive correlation between AFL-forming activity in vitro and species susceptibility to AFB-induced carcinogenesis, as mentioned earlier. Theoretically, AFL could be associated with toxicity since its intact vinyl ether double bond can undergo direct bioactivation by forming a reactive 8,9-epoxide. In addition, AFL is the most potent mutagen among the known AFB_1 metabolites (*13*). It has been suggested that the reversibility of the transformation of AFB_1 to AFL in target hepatocytes can serve to prolong cellular exposure to the carcinogen and hence enhance the carcinogenic effect (*11, 18*). The ratio of AFB_1 reductase activity to AFL dehydrogenase activity in vitro (Fig. 1) was recently observed to be higher in species that are extremely sensitive to acute aflatoxicosis; however, no correlations were apparent between these activities and species susceptibility to aflatoxin-induced carcinogenesis (*19*). The actual role of AFL in the etiology of AFB_1 tumorigenesis remains to be further elucidated.

In our similar studies with rhesus monkeys (*20*) and mice (*21*), which are both relatively resistant to AFB_1-induced carcinogenesis (*2, 22*), AFL was not detected in the plasma of [^{14}C]AFB_1-treated animals, but the plasma levels of water-soluble metabolites were considerably higher in the rhesus monkey than in the rat. Since the total AFB_1 metabolic activity of the resistant species is much greater than that of the rat (*8, 14*), the low AFL concentration in monkey and mouse plasma is probably attributable to the relatively high microsomal oxygenase activity in these resistant species, which depletes the available intracellular AFB_1 for reduction to AFL. Thus, the formation of water-soluble products and oxidative metabolites such as AFQ_1 and AFP_1 could be indicative of species resistance to aflatoxin-induced carcinogenesis. The findings reported here have strengthened our conviction that it is possible to predict animal and human susceptibilities to aflatoxin-induced carcinogenesis by using metabolic parameters.

Since AFL is the only AFB_1 metabolite formed by a cytoplasmic reductase system that is correlated with susceptibility (*14, 23*), and it is the most carcinogenic (*24*) and mutagenic (*13*) metabolite of AFB_1, its formation in the rat but not in the rhesus monkey and mouse may indeed indicate that AFL does contribute to the carcinogenic activity of AFB_1. The

Table 1. Analytical evidence that AFL is the principal plasma metabolite of AFB_1. Aflatoxicol was produced and isolated in vivo for positive identification by a procedure similar to that described for the intravenous studies (see text). A 5.0 mg/kg dose of nonradioactive AFB_1 was used, and the total blood volume of the rat was collected by catheterization 5 minutes after AFB_1 administration. Purification of AFL for identification was accomplished by TLC, using diethyl ether as the developing solvent. The developing solvents used in the TLC identification of AFL were: solvent 1, benzene and ethanol (40 : 6); solvent 2, ethyl acetate and chloroform (3 : 1); solvent 3, diethyl ether; and solvent 4, chloroform, acetone, and isopropanol (85 : 15 : 2.5). Abbreviations: λ_{max}, wavelength; A_{330}, A_{260}, and A_{253}, absorbance at 330, 260, and 253 nm, respectively; *m/e*, mass-to-charge ratio; and +, mutagenicity of plasma metabolite and AFL relative to AFB_1 was 12 percent.

Measurement	Plasma metabolite	Aflatoxicol
Thin-layer chromatography		
Solvent 1 (R_F)	0.57	0.57
Solvent 2 (R_F)	0.73	0.73
Solvent 3 (R_F)	0.59	0.59
Solvent 4 (R_F)	0.73	0.73
Ultraviolet spectroscopy		
λ_{max} (nm)	325, 260, 253	325, 260, 253
A_{330}/A_{260}	1.12	1.18
A_{253}/A_{260}	0.72	0.75
Mass spectroscopy [*m/e* (%)]	314 (100), 296 (47), 268 (22)	314 (100), 296 (46), 268 (24)
Mutagenicity to *S. typhimurium*	+	+

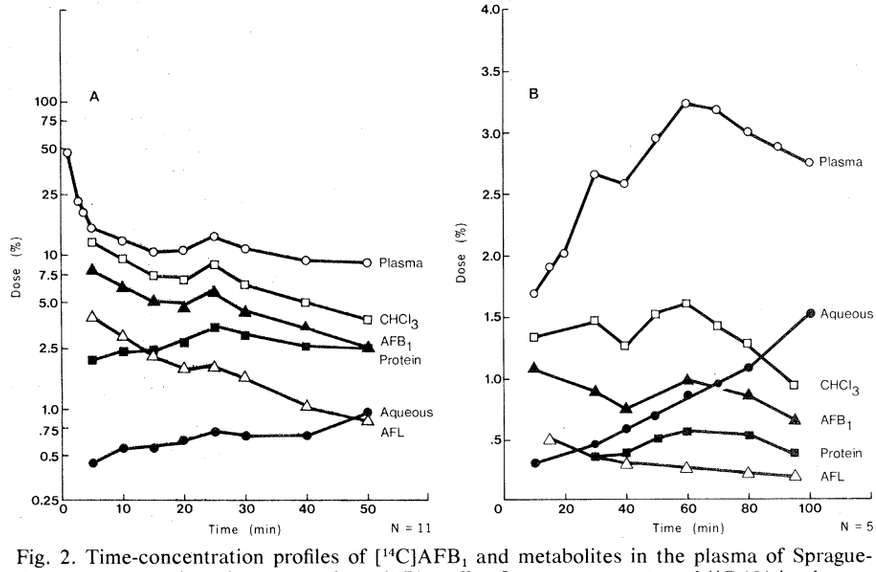

Fig. 2. Time-concentration profiles of [^{14}C]AFB_1 and metabolites in the plasma of Sprague-Dawley rats dosed (A) intravenously and (B) orally. Curves represent total ^{14}C (○) in plasma, (□) extractable from plasma with $CHCl_3$, (■) plasma protein–bound, (●) residual aqueous, (▲) AFB_1 in $CHCl_3$, and (△) AFL in $CHCl_3$. Ring-labeled [^{14}C]AFB_1 was prepared from cultures of *Aspergillus parasiticus* (ATCC 15517) supplemented with [1-^{14}C]acetate (*25*). The composite profiles were determined from plasma samples (0.1 to 0.2 ml) obtained from blood taken with a 27-gauge needle catheter and a heparinized syringe. The total plasma volume in the rats was assumed to be 40.4 ml/kg. In determining the distribution of radioactive aflatoxin in plasma, the plasma proteins were precipitated with methanol, and the remaining plasma was extracted with chloroform. Chloroform-extractable activity representing AFB_1 and metabolites was separated on silica gel thin-layer chromatograms developed first in diethyl ether and then in a mixture of chloroform, acetone, and isopropanol (85 : 15 : 2.5). Radioactivity in these fractions was determined by liquid scintillation counting, and the points plotted are mean values for (A) four and (B) five rats. Profiles are not shown for AFM_1 because concentrations were very low. The data in (A) are plotted on a semilogarithmic scale to express first-order kinetics.

identification of AFL as the major plasma metabolite of AFB_1 in the rat indicates that AFL-forming activity in vitro may be useful as a metabolic parameter for the estimation of animal and human susceptibilities to the carcinogenic effects of AFB_1. Similar studies with other species with different susceptibilities are warranted.

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Hv(1), a Variable-Region Genetic Marker of Human Immunoglobulin Heavy Chains

Abstract. *A new antigenic determinant was discovered with a hemagglutination-inhibition assay system. Designated Hv(1), it is located in the variable region of human immunoglobulin heavy chains of the G, M, and A classes. Pedigree and population analyses suggest that it has an autosomal dominant mode of inheritance. This represents the first description of an allotypic determinant in the variable region of human immunoglobulins.*

The inherited polymorphisms present in antigenic determinants on immunoglobulin molecules have aided the study of the genetic control of antibody synthesis. Two of the more successful approaches for examining the diversity and heritability of immunoglobulin genes are primary structure and serologic analyses. The former approach has categorized variable-region genes by their location on heavy chains (V_H) and light chains (V_κ and V_λ); delineated specific subgroups of these three gene families; and localized hypervariable regions, which account for the specificity of the antigen-combining site (*1*). Primary structure analysis is limited by its need for large amounts of homogeneous immunoglobulin. The serologic approach, which does not require isolated immunoglobulin, has been useful in defining constant-region allotypes (that is, inherited antigenic determinants of proteins that are present in some individuals but not in others) in both animal and human immunoglobulin (*2*); variable-region subgroups of human light (*3*) and heavy (*4*) chains; and variable-region allotypes in rabbit immunoglobulins (*5*). Allotypes in the variable regions of human immunoglobulin have not been defined by either of these methods. We now describe the use of an indirect hemagglutination inhibition system to define a human immunoglobulin variable-region genetic marker, and to characterize its mode of inheritance.

Rabbit antiserum to the immunoglobulin M (IgM) protein McE was rendered specific by solid phase immunoadsorption on a column of Sepharose 4B coupled with five different monoclonal IgM proteins (*6*). The serum was further adsorbed with washed, type O, Rh+ erythrocytes. Complement in the serum was inactivated by heating at 56°C for 30 minutes. Monoclonal immunoglobulins of the G, A, and D classes (IgG, IgA, and IgD) were isolated from patients with multiple myeloma, and IgM was isolated from patients with Waldenstrom's macroglobulinemia (*7*). Heavy and light chains were prepared by reducing purified immunoglobulins with 0.01*M* dithiothreitol in 0.1*M* tris-Hcl at 23°C for 2 hours; the component chains were separated on Sephadex G-100 (*8*). The Fab and Fc fragments of IgG (Fabγ and Fcγ) were prepared and isolated (*9*). The Fab and Fc fragments of IgM [Fabμ and (Fc)5μ] were prepared (*10*), separated on Sephadex G-200, and adsorbed on Sepharose 4B coupled with monospecific antibodies to human κ chains.

Hemagglutination inhibition (*11*) was performed with type O, Rh+ erythrocytes, coupled by chromium chloride with intact immunoglobulins or their Fab fragments. Optimal coating was achieved with 1 to 5 mg protein per milliliter and 0.025 to 0.1 percent chromium chloride. In a typical coating experiment, 0.05 ml intact immunoglobulin or Fab solution was added to an equal volume of packed erythrocytes. The mixture was shaken for 1 minute, 0.05 ml of chromium chloride solution was added, and the mixture was shaken for an additional 5 minutes. Coated cells were washed at least three times with a 30-volume excess of 0.15*M* NaCl, and they were suspended to 0.2 percent in 0.15*M* NaCl containing 0.1 percent gelatin.

The absorbed antiserum had an agglutinating titer ranging from 1:128 to 1:512 for red blood cells coated with McE IgM(κ) or its Fabμ fragment, as well as for cells coated with an IgG1(κ) myeloma protein (FER); however, it did not agglutinate cells coated with six other monoclonal immunoglobulins. In order to determine the distribution of antigenic determinants recognized by the antiserum, we tested a panel of 97 isolated human monoclonal immunoglobulins. Approximately 25 percent of the IgG, 16 percent of the IgA, and 29 percent of the IgM inhibited agglutination (Table 1), indicating that the antigenic determinant was not restricted to any particular immunoglobulin class. This was consistent with our previous finding that the same variable-region amino acid sequence was able to associate with constant regions of different heavy-chain classes (*12*). The number of IgD proteins examined was not large enough to give any significant information. Sequence studies from the amino-terminal ends of four inhibiting heavy chains having glutamic acid as

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