INTERACTION OF AFLATOXIN B_1 WITH THE RAT MONOOXYGENASE SYSTEM AND RELATIONSHIPS TO ITS ACUTE TOXIC EFFECT

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SUMMARY

The effect of aflatoxin B_1 (AFB_1) on the liver microsomal and nuclear mixed function oxidase system (MFO) of adult male rats was studied at oral doses of 1 and 3 mg/kg. At first both doses increased the activities and concentrations of all P450 components followed. The maximum values were observed between 24 and 48 h and were dose- and enzyme-dependent. After 72 h the values dropped to 15-55% of the initial values. Inhibition of the MFO system lasted even after 120 h, when a trend to return to the normal values was already noticeable.

We assume that AFB_1 acts as an inductor of the monooxygenase system and the reactive electrophilic intermediate(s) bind irreversibly to or autocatalytically inactivate P450.

KEY WORDS

aflatoxin B_1, monooxygenase system, microsomes

INTRODUCTION

Aflatoxin B_1 (AFB_1) is one of the most potent hepatocarcinogens known and environmental contamination by various aflatoxins is a serious problem in many parts of the world. There is epidemiological evidence that AFB_1 is involved in the induction of human liver cancer /1/.

AFB_1 is not particularly biologically active in its native form and activation is required for interaction with DNA and for induction of biological damage.

AFB_1 is activated in the presence of molecular oxygen and NADPH by the cytochrome P-450-dependent hepatic microsomal mixed function oxidase system. AFB_1-8,9-oxide, the active metabolite of AFB_1, binds avidly to cellular macromolecules (including DNA) where the adduct 8,9-dihydro-2-(N^7 guanyl)-9-hydroxy-AFB_1 is formed. Thereafter, the modified purine in the DNA undergoes transformation to a more stable open-ring formamidopyrimidine adduct 8,9-dihydro-8-(N^5-formyl)-2',5'6'-triamino-4'-oxy-N^5-pyrimidyl-9-hydroxy-aflatoxin B_1. The formation, persistence and accumulation of this adduct is believed to be responsible for the carcinogenic and mutagenic effects of AFB_1.

Hepatic mixed function oxidase converts AFB_1 to 8,9-oxide and hydroxylated metabolites including AFM_1, AFP_1 and AFQ_1. Although these hydroxylated metabolites retain the C_8-C_9 double bond, they do not appear to be good substrates for the cytochrome P-450 which activates AFB_1 to AFB_1-8,9-oxide. They are, however, good substrates for phase II glucuronic acid conjugating enzymes /2/. Hydroxylation of AFB_1 at various sites, leading to the formation of AFM_1, AFP_1 and AFQ_1, deactivates AFB_1. These metabolites are less than 5% as active as AFB_1.

AFB_1 is a substrate for several different isoenzymes of activation induced by treatment of rodents with phenobarbital. It is not certain which specific forms of rat liver cytochrome P-450 are involved in AFB_1 activation, and data in the literature do not point clearly to human homologues.

Abbreviations used: AFB_1 - aflatoxin B_1; AHH - aryl hydrocarbon hydroxylase; P450 - cytochrome P-450; b_5 - cytochrome *b*5; MFO - mixed function oxidases; NADPH c red - nicotinamide adenine dinucleotide phosphate cytochrome *c* reductase.

MFO activity was found not only in liver microsomes, but also in cell nuclei /6/. Their participation in the biotransformation of AFB_1 has been repeatedly demonstrated in the literature /3, 6, 7/. On the other hand, aflatoxin B_1 affects the activity of the MFO system, which decreases after aflatoxin intoxication /8-10/.

We decided to analyse the changes in some MFO activities in hepatic microsomes and nuclei of rats, and to compare these changes with the acute toxic effects of AFB_1 during five days after the administration of two different doses of AFB_1.

MATERIALS AND METHODS

Chemicals

AFB_1 was isolated from the mould *Aspergillus flavus* in compliance with the Czechoslovak Patent 08/1278, AO 189 852 by cultivation on dietary biscuits. Its chemical purity was determined by TLC, UV spectrophotometry and NMR. 3,4-Benzo(*a*)pyrene, NADPH and glucose-6-phosphate were obtained from Merck (Darmstadt, Germany), glucose-6-phosphate dehydrogenase from Boehringer (Mannheim, Germany), cytochrome *c* from Serva (Heidelberg, Germany), $NADP^+$ and NADH from Reanal (Budapest, Hungary). Other chemicals were obtained from Lachema (Brno, Czechoslovakia).

Animals

Adult male Wistar rats (180-230 g body weight) obtained from the conventional random bred population of SPOFA (the farm of Konárovice, Czechoslovakia) were allowed free access to standard food pellets (DOS 2b VELAZ; Prague, Czechoslovakia) and water. AFB_1 in a 4% suspension of gum arabic was gavaged into the stomach using a metal tube after 17 hours of fasting.

Preparation of microsomes and nuclei

The effect of AFB_1 on the liver monooxygenase system was studied at various time intervals after p.o. administration of 1 and 3 mg/kg

body wt. After 24, 48, 72 and 120 h the liver samples were rapidly removed and homogenized in a Potter-Elvjehjem homogenizer in four volumes of 0.25 M sucrose pH 7.4. The microsomes were obtained from centrifugation of the postmitochondrial fraction at 100,000 *g* for 60 min at 4°C, washed with an isotonic solution of KCl and resuspended in phosphate buffered 0.25 M sucrose pH 7.4 /11/. The nuclei were isolated from the mitochondrial pellet, which was homogenized in 15 ml of 0.24 M sucrose. This homogenate was layered into a cuvette over 13 ml of 2.3 M sucrose /12/. It was centrifuged in a SW 25.1 rotor on a Spinco Beckman L2 65B for 30 min at 41,000 *g*. The nuclear pellet was washed five times in 1 M sucrose with 1 mM $CaCl_2$ and centrifuged for 10 min at 3,000 *g*. The purity of the nuclear preparation was checked microscopically after staining with 0.1% crystal violet.

Microsomal and nuclear fractions were frozen immediately and stored at -50°C. Proteins were determined according to the method of Lowry *et al.* /13/ using bovine albumin as the standard.

Enzyme determination

The P450 and b_5 concentrations were assayed in microsomes in accordance with Omura and Sato /11/. NADPH c reductase (NADPH c red) activities were determined in microsomes and nuclei spectrophotometrically according to Jeffery *et al.* /14/. Aryl hydrocarbon hydroxylase activity was measured in microsomal or nuclear fractions using the fluorometric procedure described by Cumps *et al.* /15/. Fluorescence of hydroxylated products of benzo(*a*)pyrene was determined using a Ciampolini CCG 3000 spectrofluorometer (excitation 467 nm, emission 522 nm). 0.1% quinine sulphate was used as a standard. The results are expressed as rel. Δ $F.mg^{-1}.min^{-1}$. The results were evaluated statistically using Student's t-test.

RESULTS

The effects of AFB_1 on the liver MFO system in the nuclei and in the microsomes were comparable after both doses administered. In microsomes, after the dose of 3 mg/kg, the concentration of P450 was found slightly increased on day 2; on day 3, in contrast, we observed

a marked decrease (to 24% of the initial concentration) (Fig. 1). The changes of b_5 content were not so remarkable even though they followed a similar trend. Both NADPH c red and NADH c red increased with the maximum on day 2 (139% of the original activity); on day 3, we found a minimum of activity (34 and 56% of the initial activity). The changes in the activity of AHH appear to reflect, in principle, the changes of P450, the inhibition being even more marked and longer-lasting.

After the dose of 1 mg/kg (Fig. 2) the induction of P450 was more manifest (140%) than after the larger dose. Other changes in the concentration of P450 and b_5 and activity of NADPH c red were less pronounced. AHH was inhibited during all intervals under study. The concentration of microsomal protein increased to 120% of the original concentration in the first two days, whereas on day 3 a decrease to 46% of the original concentration was recorded. On day 5 the concentration approached the original value (93%).

The concentration of P450 in nuclei was assayed only in the control rats, and 0.0371 ± 0.0143 μmol/mg of nuclear protein was found, which accounted approximately for 5.4% of the microsomal concentration. In the course of administration of AFB_1 the concentrations of P450 and b_5 in the nuclei were not examined. After the dose of 3 mg/kg body weight AFB_1 (Fig. 3) the activities of nuclear NADPH c red were increased on day 1 (118 and 140%), and between days 2 and 3 they decreased to 17-28%. On day 5 they were decreased only slightly. Similar changes were found after the dose of 1 mg/kg AFB_1 (Fig. 4), when on day 1 the activity of NADPH c red was increased to 145% and that of AHH to 113% of the original activity, whereas at longer intervals a decrease in the activities was found, again with the maximum on day 3 (60 and 42% of the original activities).

DISCUSSION

We found that inhibition of the MFO system in rat liver microsomes and nuclei was dose dependent and began 48 h after AFB_1 administration with a statistically significant maximum at 72h. The inhibition of the MFO system was, however, expected. Our results are in concordance with the findings of, among others, Mgbodile *et*

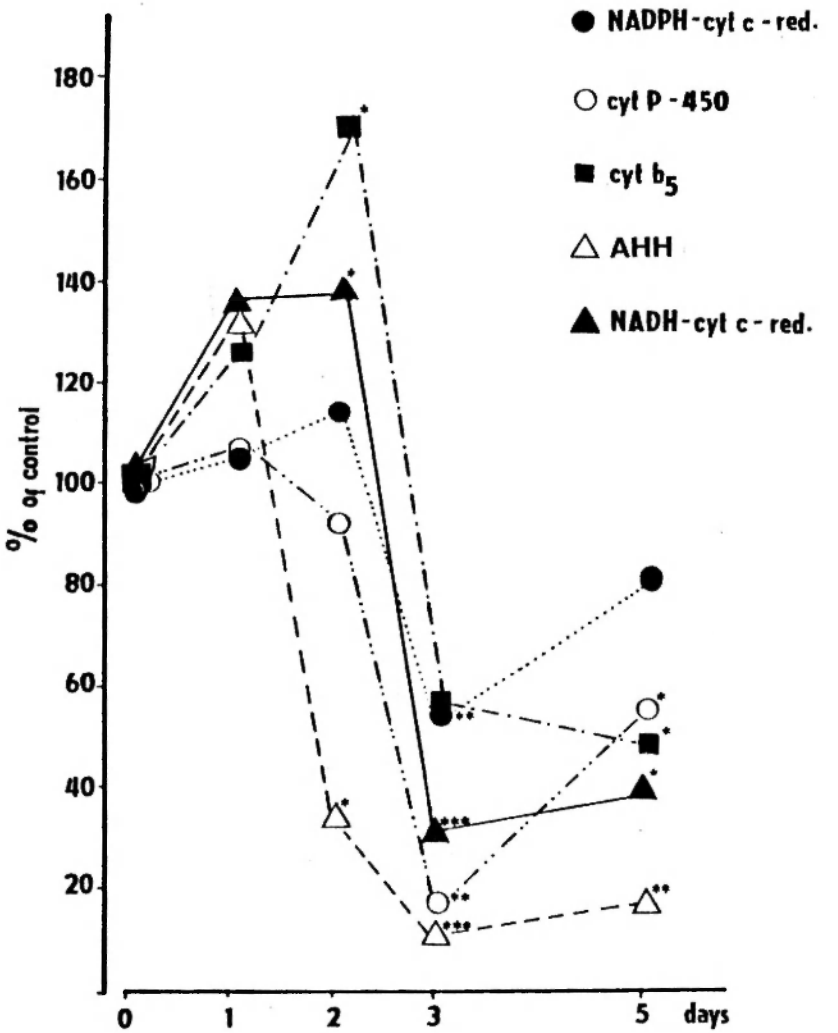

Fig. 1: Time course of the changes in activities of microsomal NADH and NADPH c red and AHH, and concentrations of P450 and b_5 after peroral intoxication with AFB_1 at a dose of 3 mg/kg in male rats. Values are means for 3-4 rats per group. Standard errors (< 20% of the mean) were omitted. The activities of the control group were 1181.1 and 673.2 nmol cyt c/mg/min for NADPH and NADH c red respectively. The concentration of P450 and b_5 was 0.509 and 0.393 nmol/mg of microsomal protein, respectively. Significantly different from control as determined by Student's t-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

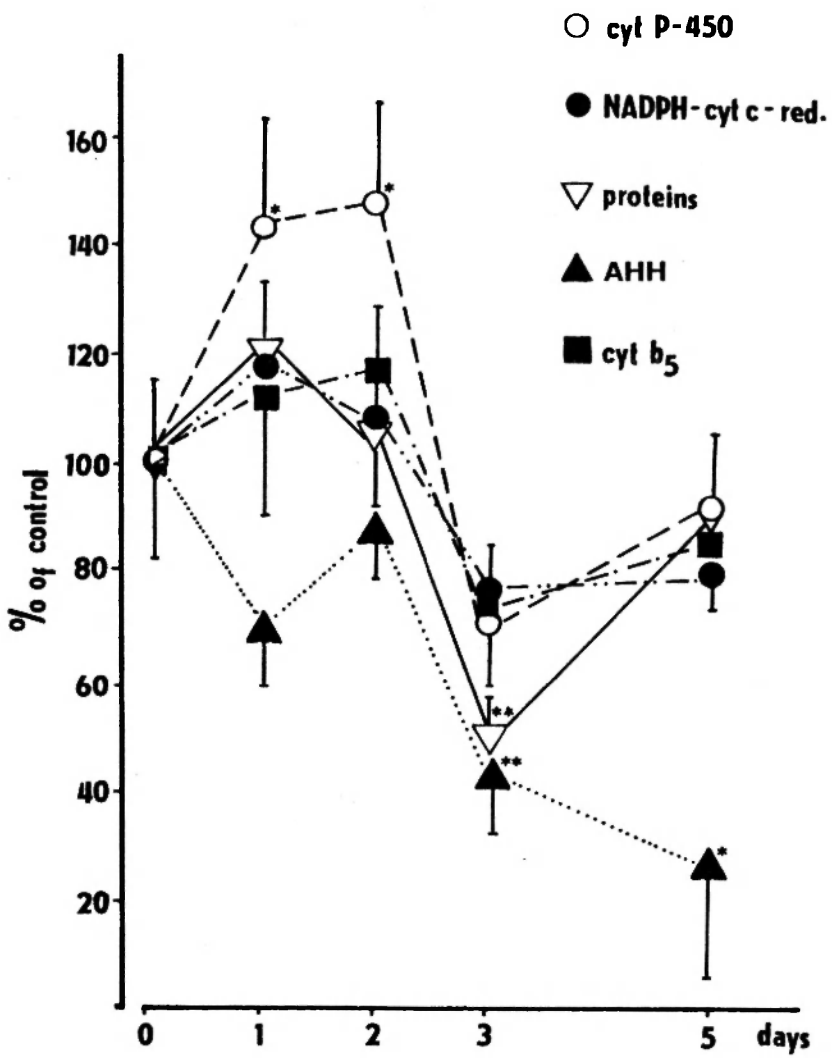

Fig. 2: Changes in the activities of microsomal NADPH c red, AHH, P450 and b_5 and microsomal protein after the p.o. administration of 1 mg/kg AFB_1 to rat males during the 5 days after intoxication. Values are means for 4 animals. Standard errors (< 20%) were omitted. The activity of NADPH c red in control animals was 132.1 nmol/min/mg of microsomal protein. AHH activity was measured as relative fluorescence with regard to fluorescence of 0.1% quinine sulphate and was 12.9 ΔF_{522}. $min^{-1}.mg^{-1}$. Significantly different from control: *$P < 0.05$; **$P < 0.01$.

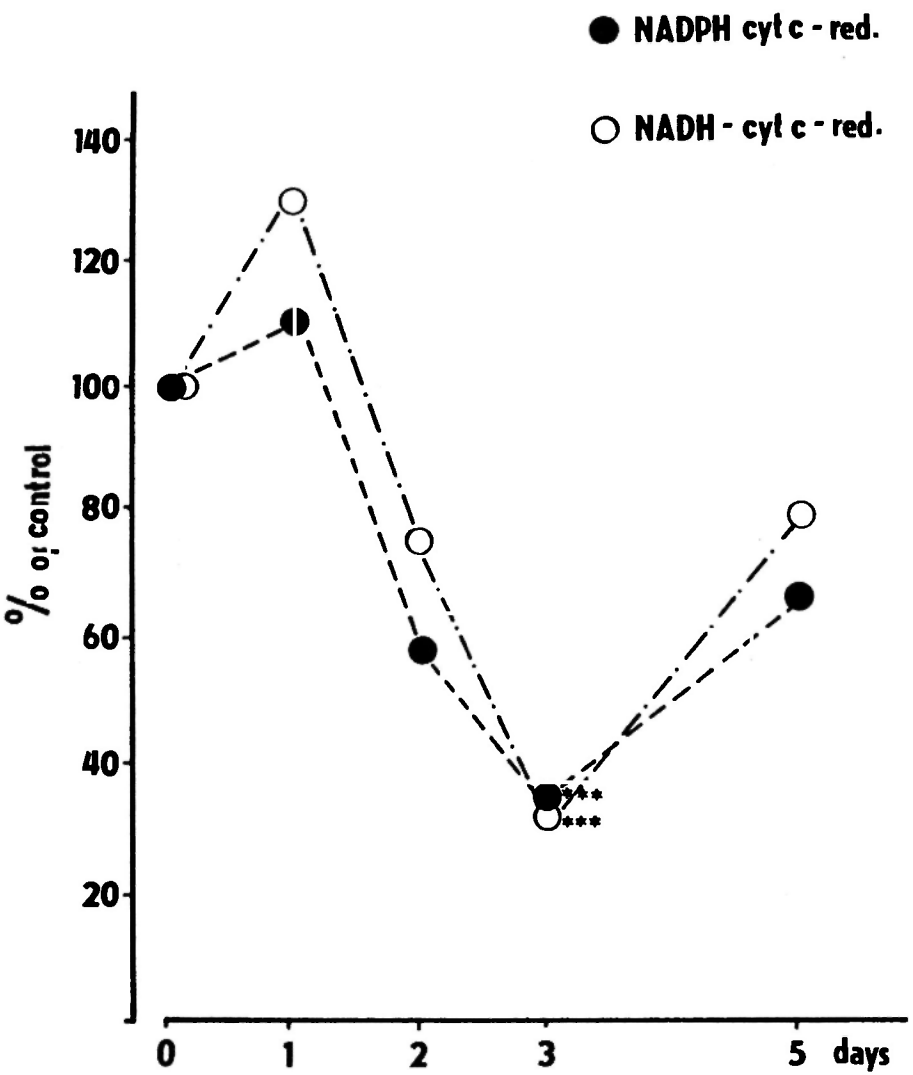

Fig. 3: Time course of the changes in the activities of rat nuclear NADPH and NADH c red after the p.o. administration of 3 mg/kg AFB_1. Points are means for 2-3 rats. Standard errors were omitted (< 30% of the mean). The mean activity in the control animals was 12.5 and 63.3 nmol/min/mg of nuclear protein for NADPH and NADH c red, respectively. Significantly different from control as determined by Student's t-test: ***$P < 0.001$.

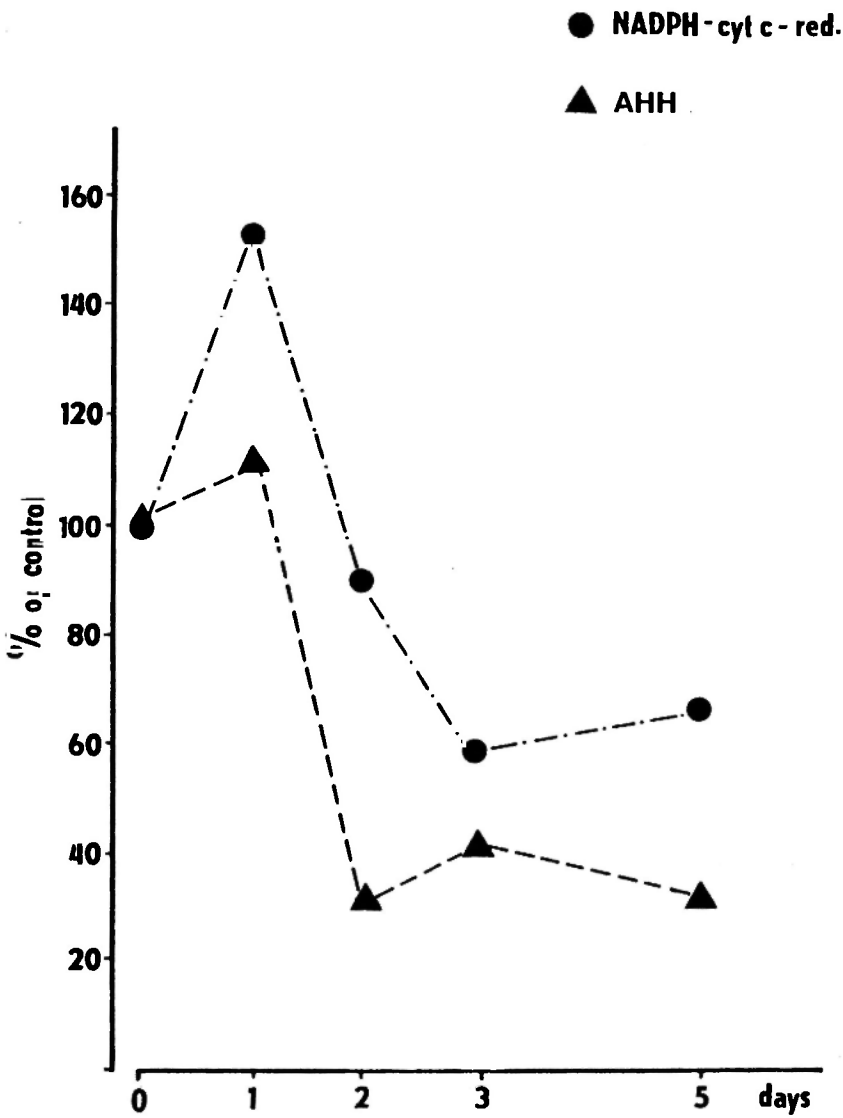

Fig. 4: Changes in the activities of rat nuclear NADPH c red and AHH after the p.o. administration of 1 mg/kg AFB_1. Points represent means for 2-3 rats. Standard errors were omitted (< 30% of the mean). The mean activity in the control animals was 18.8 nmol/min/mg of nuclear protein for NADPH c red and the relative activity of AHH was 2.7 Δ $F.min^{-1}.mg^{-1}$.

al. /8/ and Phillips *et al.* /10/, who described a decrease to 66% in the content of P450 and to 40% in the activity of benzphetamine-N-demethylase. Our results increased our understanding by recording a substantially longer time course of MFO changes. The inhibitory effect of activated AFB_1 metabolite(s) is probably the consequence of the catalytic inactivation of the MFO system /18/. The inhibitory effect of AFB_1 metabolites is, however, more complex with higher doses. AFB_1 does not affect only the MFO system, but also other cells functions, e.g., the respiratory system /19, 20/, synthesis of DNA, RNA and proteins /21-24/, etc. Accordingly, AFB_1 *in vivo* acts simultaneously on different systems. The degree of injury to these systems should be related to the initial concentration of AFB_1, total P450 and to the degree of inhibition or induction of specific isoenzymes of P450.

However, in the initial intervals after AFB_1 administration we found increased activities of MFO. Simultaneously, a similar course of change in concentration of microsomal protein has been noted (Fig. 2). Therefore we postulate that during the first two days after AFB_1 administration there is an induction of microsomal enzymes. The induction is relatively weak (in comparison with halogenated polycyclic aromatic hydrocarbons or flavones) /17/. On the basis of our experiment, we cannot tell which of the P450 species were induced. AHH is dependent on P450 I that is responsible for the formation of AFM_1 and AFQ_1 /25/. The inductors of this type of P450 (e.g., β-naphthoflavone) are capable of inducing specific detoxifying pathways and of decreasing the incidence of hepatic tumorigenesis in rats treated with AFB_1 /29/. However, induction of the entire P450 does not appear to correlate fully with AHH activity (Fig. 2). P450 I is induced by inductors with a planar structure (e.g., 3-methylcholanthrene). The molecule of AFB_1 is not planar and, therefore, AFB_1 should rather induce other P450 isoenzymes (that may, perhaps, include P450 I); in this case, we are dealing with a mixed induction. Inductors of this category do not appear to be so potent. This fact would support our findings.

On the other hand, many compounds which induce P450s also induce Phase II conjugating enzymes. Their activities were not followed in our study. The degree of inducibility of P450s is often higher than the inducibility of Phase II conjugating enzymes and this may create an imbalance between the rate at which the reactive metabo-

lites are inactivated and removed by conjugation. The amount of reactive intermediates formed is totally dependent on the net effect of those pathways competing for activation and deactivation. The rates of these processes may differ among animal species /17/. The activity of hepatic glutathione-S-transferase, rather than the activation of AFB_1 to 8,9-epoxide, is a determining factor in the susceptibility of different animal species to the carcinogenic effects of AFB_1 /25, 26/. In this respect, there is an interesting finding by Grossman *et al.* /16/, that the first dose of AFB_1 modified the sensitivity of the rat to a second dose, dependent on the time interval between the doses. The effect of the two doses (2 x half LD_{50}) is cumulative or potentiated at administration up to 48 h. At a 96 h interval the effect of the first dose was not manifested. Grossman *et al.* demonstrated as well that repeated administration of increasing doses of AFB_1 at an interval of 5 days increased the cumulative lethal dose 12.3 times /16/. From these findings and our results it may be concluded that, in rats, AFB_1 metabolism appears to be the decisive factor in its toxicity.

The activity of biotransforming enzymes (MFO and Phase II enzymes) plays a key role in the degree of AFB_1 toxicity. This fact appears to be related to species differences in biotransforming enzyme induction or inhibition by other substances (environmental pollutants, drugs, etc.). AFB_1 itself appears to stimulate its own metabolism which plays an important part in its toxicity and carcinogenicity, particularly when considering repeated doses. Analysing the effect of low repeated doses of AFB_1 may be useful, especially for areas of the world where AFB_1 contamination of food is endemic.

ACKNOWLEDGEMENTS

The author wishes to thank M. Černá for her excellent technical assistance.

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