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Metabolism of Aflatoxin B_1 and Its Metabolism-Dependent and Independent Binding to Rat Hepatic Microsomes

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SUMMARY

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The NADPH-mediated metabolism of aflatoxin B_1 by rat hepatic microsomes was studied by means of difference spectroscopy. A decrease in absorbance at 360 nm, due to the disappearance of aflatoxin B_1 during its metabolism, was caused by the formation of a metabolite having a spectral peak at 398 nm. From the solubility, spectral, and microsomal binding characteristics, this metabolite appears to be aflatoxin B_{2a} (aflatoxin hemiacetal). Difference spectroscopic studies on microsomes reisolated from incubation mixtures containing aflatoxin B_1 with or without NADPH showed that aflatoxin B_1 was bound to microsomes, giving a complex having a spectral peak at 360 nm; this binding did not require NADPH and the complex could be separated by gel filtration chromatography. A metabolite of aflatoxin B_1, the formation of which was NADPH-dependent, was bound to microsomes, yielding a complex having a spectral peak at 405 nm; this complex was not dissociated by gel chromatography or by treatment with trichloracetic acid or extraction with chloroform and acetone. SKF 525-A and L-cysteine inhibited the formation of this complex, the former by inhibiting the metabolic conversion of aflatoxin B_1 to aflatoxin B_{2a} and the latter by blocking the binding of the metabolite to microsomes. Aflatoxin B_1 and aflatoxin B_{2a} appeared to bind to different sites on the microsomes. A change in pH caused an alteration in the structure of aflatoxin B_{2a} and its extraction with chloroform. pH equilibria for this phenomenon were estimated to be 3.00 and 7.35. Based on these studies, it is proposed that aflatoxin B_{2a}, under alkaline conditions, cleaves to yield a dialdehyde derivative(s) which binds to microsomes, forming Schiff bases with free amino groups. These studies also indicate that, under certain conditions, rat hepatic microsome-mediated conversion of aflatoxin B_1 to a water-soluble metabolite, which appears to be aflatoxin B_{2a}, represents a major metabolic pathway.

INTRODUCTION

Aflatoxin B_1, produced by some strains of *Aspergillus flavus*, is a potent hepatotoxic and hepatocarcinogenic agent in a number of animal species (1, 2). In spite of the extensive literature on aflatoxins since 1960,

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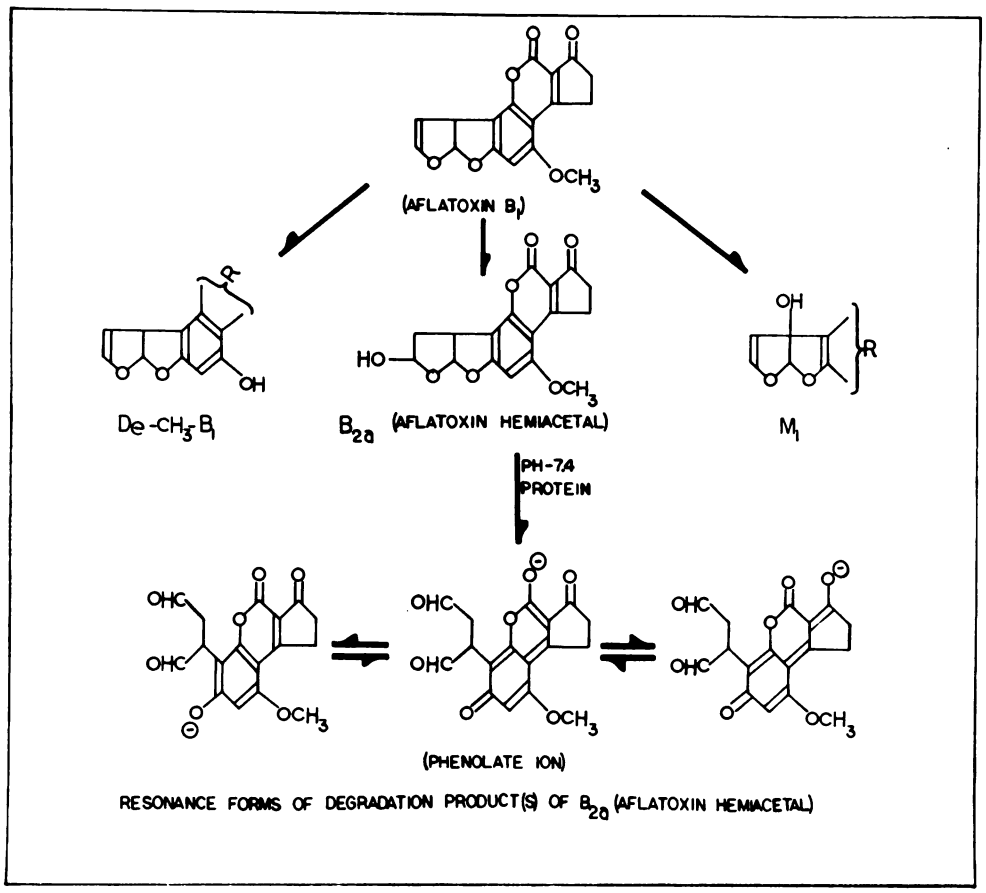

FIG. 1. *Structural formulae of aflatoxin B_1 and aflatoxin B_{2a} (B_{2a}, aflatoxin hemiacetal) and partial structure of NADPH- and hepatic microsome-mediated metabolite, aflatoxin M_1 (M_1).*

The presumed partial structure of demethylated aflatoxin B_1 (De-CH_3-B_1, also called aflatoxin P_1) is also shown, as are cleavage products of aflatoxin B_{2a}. R and R′ denote unchanged portions of the aflatoxin B_1 molecule. This scheme has been adapted after Patterson and Roberts (14) and Pohland *et al.* (18).

when they were first discovered (3), the metabolic fate of even the most potent of all aflatoxins, aflatoxin B_1, is still incompletely understood. Various reports (4–10) regarding the biotransformation of aflatoxin B_1 in different animal species are not in complete agreement. However, it is now generally accepted that both *in vivo* or *in vitro*, in the presence of hepatic microsomes and NADPH, aflatoxin B_1 undergoes metabolic conversion possibly by three different pathways (Fig. 1): (a) 4-hydroxylation to form aflatoxin M_1 (11–13), (b) Hydration of the C_2—C_3 double bond to form aflatoxin B_{2a} (11, 14), and (c) demethylation, resulting in the formation of aflatoxin P_1 (7, 9, 15). The excretion of the hydroxylated derivatives, particularly aflatoxin P_1, is facilitated by subsequent conjugation with glucuronic acid and sulfate (7, 15). Acute toxicity tests showed aflatoxin M_1 to be as toxic as its parent compound, aflatoxin B_1, whereas aflatoxin B_{2a} has been found to be practically innocuous (5, 16–19).

Since metabolites of various carcinogens are known to bind to cellular components (20), it is essential to know the nature of such metabolites in order to obtain a reasonable understanding of the possible consequences arising from such binding. Wogan *et al.* (21) reported that when ring-labeled [^{14}C]aflatoxin B_1 was injected into rats, the highest

amount of radioactivity was incorporated into liver, and eventually most of the radioactivity was associated with the microsomal fraction. These findings and our earlier observations concerning the binding of aflatoxin B_1 metabolite to microsomes prompted us to conduct the investigations reported in this paper (22, 23).

The metabolite binds to microsomes, forming a complex with a difference spectral peak at 400 nm region (22, 23). We now report on the properties of this biotransformation pathway, the possible identification of this metabolite, and its characteristics and possible nature of its binding to microsomes.

MATERIALS AND METHODS

Chemicals. Chromatographically pure aflatoxin B_1 was obtained from Calbiochem. The purity of this compound was established by the migration of a single spot on repeated thin-layer chromatography, using coated silica gel thin-layer plates of 0.25-mm thickness developed with chloroform–methanol (95:5) and chloroform–acetone (80:20). Any batch of aflatoxin which had more than a single spot on thin-layer chromatography was repurified by the same process. Additional verification of the purity of the batch was obtained by examination of the spectrum in methanol. Aflatoxin B_1 was tritiated by New England Nuclear according to Lijinsky *et al.* (24). The purification of [^{3}H]-aflatoxin B_1 was carried out by repeated thin-layer chromatography, essentially according to Hanna and Campbell (25). Within a week before use [^{3}H]aflatoxin B_1 was rechromatographed, diluted with unlabeled aflatoxin B_1 in dimethyl sulfoxide, divided in aliquots of 200 μl, and stored at $-20°$. On the day of the experiment [^{3}H]aflatoxin B_1 in dimethyl sulfoxide was thawed just before use, refrozen in a Dry Ice–acetone bath immediately after being used, and then stored at $-20°$; this procedure was found to prevent degradation, which was otherwise significant during storage at room temperature or at 4°. Ring-labeled [^{14}C]aflatoxin B_1 was prepared essentially according to Adye and Mateles (26); the labeled aflatoxin B_1 was purified from the crude extract by thin-layer chromatography by the method of Hanna and Campbell (25). Aflatoxin B_{2a} and [^{3}H]aflatoxin B_{2a} were prepared from aflatoxin B_1 and [^{3}H]aflatoxin B_1, respectively, by the method of Giegler and Peterson (27), as modified by Garner *et al.* (28). This method involved acid-catalyzed hydration of aflatoxin B_1. Aflatoxin B_{2a} was extracted from the reaction mixture with chloroform, the extracts were pooled, reduced in volume, and purified using 0.25-mm-thick silica gel plates (Merck, Darmstadt), developed with chloroform–methanol (95:5, v/v). Aflatoxin B_{2a}, visible as an intensely fluorescent band near the baseline (R_F 0.15), was eluted with methanol and repurified by repeated thin-layer chromatography. No aflatoxin B_1 was seen under these conditions. Aflatoxin B_1, when cochromatographed with aflatoxin B_{2a}, moved behind the solvent front (R_F 0.84) under these conditions. Glass-redistilled methanol was used in all operations. The purity of aflatoxin B_{2a} was established by thin-layer chromatography (28) and by infrared, ultraviolet, and visible spectroscopy (27, 18, 14).

Aflatoxin B_1, labeled and unlabeled, was dissolved in dimethyl sulfoxide. Spectral grade dimethyl sulfoxide, used for dissolving aflatoxins and obtained from Aldrich Chemical Company, was purified by fractional distillation. Three fractions were collected, and the fraction containing dimethyl sulfoxide free of any oxidation products, as verified by infrared spectroscopy, was used as the solvent. Solutions of aflatoxin B_1 in 200-μl aliquots were stored frozen at $-20°$, checked for the stability of aflatoxin by thin-layer chromatography and spectroscopy and used within 1 week.

SKF 525-A[1] was kindly provided as a gift by Smith Kline & French. The sources of the other chemicals were described previously (22).

Preparation of microsomes. Hepatic microsomes were isolated from untreated or phenobarbital (sodium salt)-treated male Sprague-Dawley rats (190–260 g). Treated rats received phenobarbital intraperitoneally at a dose of 40 mg/kg, given once on day 1 and

[1] The abbreviations used are: SKF 525-A, diethylaminoethyl 2,2-diphenylvalerate HCl; AFB_1, aflatoxin B_1; AFB_{2a}, aflatoxin B_{2a}.

twice daily (8:00 a.m. and 4:30 p.m.) on days 2–4. On day 5 at 1:00 p.m. the animals were killed, and their livers removed and perfused with cold 0.9% NaCl until free of blood; each group consisted of four to six livers, which were pooled and stored frozen at −20° for 72–96 hr. On the day of assay the microsomes were isolated as follows. The livers were thawed at room temperature and were homogenized in 3 volumes (1 g/3 ml) of ice-cold sucrose (0.25 M) containing 1 mM EDTA. The homogenate was centrifuged at 14,000 × *g* for 20 min. The resulting supernatant solution was centrifuged again for 10 min to remove light mitochondria and smaller cell fragments, and the supernatant fraction was centrifuged at 105,000 × *g* for 90 min in a Beckman model L2-65B ultracentrifuge. The microsomal pellet was floated off the glycogen pellet and resuspended in 0.1 M potassium phosphate buffer (pH 7.4) by light homogenization. All operations during the isolation of microsomes were carried out at 0–4°. Protein in the microsomal suspension was assayed by the method of Lowry *et al.* (29). The functional integrity of the microsomes and the inductive effect of phenobarbital treatment of the animals was verified in preliminary experiments by assaying the microsomes for aminopyrine demethylase (30) and cytochrome *c* reductase activities (31) and the cytochrome P-450 and b_5 contents (32). In general phenobarbital was found to induce aminopyrine demethylase and cytochrome *c* reductase activities and to increase the content of cytochrome P-450 about 2-fold.

Treatment of microsomes in vitro. For difference spectroscopic examinations, unless otherwise stated, microsomes (2.75 mg/ml) were incubated in potassium phosphate buffer (0.07 M, pH 7.4) at 37° in a total volume of 24 ml with various combinations of aflatoxin B_1, [^{3}H]aflatoxin B_1, aflatoxin B_{2a}, [^{3}H]aflatoxin B_{2a}, L-cysteine, glutathione, and SKF 525-A in the presence or absence of an NADPH-generating system consisting of NADP (0.33 mM), DL-isocitrate (8 mM), and Sigma type IV isocitrate dehydrogenase (10 μg of protein per milliliter; specific activity, 5 units/mg of enzyme protein). In addition, incubation mixtures contained $MgCl_2$ (5 mM) and $MnCl_2$ (1 mM). $MnCl_2$ was included to prevent changes in the microsomal turbidity caused by lipid peroxidation (33); this treatment does not affect the metabolism of drugs (34). Before the addition of microsomes, each incubation mixture lacking aflatoxin was incubated for 10 min to ensure the presence of an adequate amount of NADPH (where the NADPH-generating system was included); this was followed by the addition of microsomes and aflatoxin (in 50 μl of dimethyl sulfoxide). Incubations were carried out at 37° for 40 min. After incubation each mixture was immediately cooled in ice and centrifuged at 105,000 × *g* for 2 hr, and the microsomes sedimented as a pellet.[2]

Microsomes were suspended in 0.02 M buffer (pH 7.4) by light homogenization and recentrifuged at 105,000 × *g* for 2 hr. The washed microsomes were collected following centrifugation and suspended in 3 ml of 0.1 M potassium phosphate buffer (pH 7.4), and, where indicated, a portion was passed through a Sephadex G-25 column. Microsomal protein was determined before and after Sephadex gel filtration.

Sephadex gel filtration. Microsomes reisolated from incubation mixtures and subsequently washed were suspended in 0.1 M potassium phosphate buffer (pH 7.4), and a portion of this suspension, equivalent to approximately 25 mg of microsomal protein, was adsorbed onto a Sephadex G-25 gel column (1.5 × 20 cm) provided with an overhead reservoir and a flow control valve (Pharmacia). Microsomes were eluted from the column with 0.02 M potassium phosphate buffer (pH 7.4) at a flow rate of 10–12 drops/min. Fractions 13–20 (1 ml each) of the effluent were pooled; 90% of the microsomal protein initially loaded onto the column was

[2] In some cases microsomes sedimented as a pellet over a very thin opaque film which contained less than 3% of the protein in the whole pellet. Microsomes were carefully scraped off from this underlying film in order to prevent any interference with the spectroscopic examination. This procedure did not in any way alter the results, as whole microsomal pellets were used in studies with labeled aflatoxins. This film is probably related to small amounts of glycogen which must have been carried over.

contained in these fractions. It was also found that about 80% of the [^{14}C]aflatoxin B_1 bound noncovalently to microsomes was removed by Sephadex gel filtration. Microsomes reisolated from different incubations were always chromatographed on the same day under similar conditions. The results of chromatography conducted in this way were highly reproducible.

Difference spectroscopy. Two types of materials were used for difference spectroscopic examination: (a) incubation mixtures containing microsomes from phenobarbital-treated rats and an NADPH-generating system (NADP, DL-isocitrate, and isocitrate dehydrogenase) in the presence and absence of aflatoxin B_1 and (b) reisolated microsomes, i.e., microsomes which were incubated under different experimental conditions, reisolated, washed, and used before and after Sephadex gel filtration. Difference spectra were obtained using Cary models 14 and 15 dual-beam recording spectrophotometers. Split cells (shown in Fig. 3), used for obtaining some difference spectra, were obtained from Pyrocell.

Difference spectra on reisolated microsomes were obtained with the microsomal protein concentration adjusted in the sample cuvette to within ± 8% of that in the reference cuvette, which contained microsomes at a concentration of 0.2 mg/ml; this adjustment of the microsomes in the sample cuvette was necessary and was within the variation noted in the determination of protein concentration. The spectra presented in this paper are representative of two or more experiments in each case. For quantification and confirmation of the spectral data, studies were conducted using [^{3}H]aflatoxin B_1 and [^{3}H]aflatoxin B_{2a}.

Determination of radioactivity. One-milliliter aliquots of the microsomal suspensions obtained before and after Sephadex gel filtration, with protein adjusted to 2 mg/ml, unless otherwise stated, were counted in duplicate or triplicate in 10 ml of toluene-based scintillator by a procedure reported elsewhere (35). A Packard liquid scintillation counter, model 3320, was used.

RESULTS

Metabolism of aflatoxin B_1. A difference spectrum of whole incubation mixtures (Fig. 2) showed a time-dependent decrease in the absorbance at 360 nm, associated with an increase in the absorbance in the 400 nm region. No change occurred in the initial spectrum of aflatoxin B_1 when NADPH[3] or microsomes were omitted, suggesting that the decrease at 360 nm was due to the

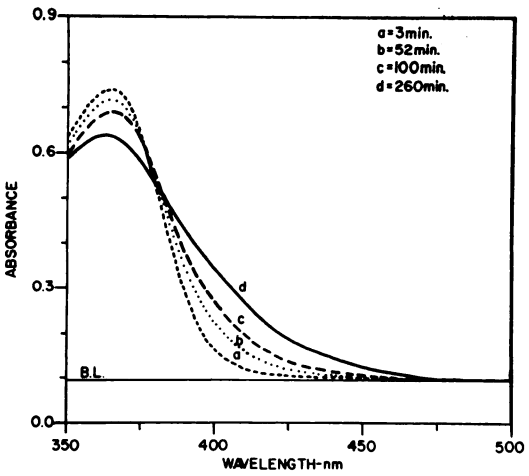

FIG. 2. *Difference spectra between two incubation mixtures differing from each other by presence and absence of aflatoxin B_1*

Cuvettes with 3.5-ml capacity and a 1-cm light path were used. All incubation mixtures were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing $MnCl_2$ (1 mM) and $MgCl_2$ (5 mM). The sample cuvette contained microsomes from phenobarbital-treated rats (0.167 mg/ml), an NADPH-generating system (NADP, 0.33 mM; DL-isocitrate, 8 mM; isocitrate dehydrogenase, 10 μg of protein per milliliter), and aflatoxin B_1 (0.10 mM). The reference cuvette contained all ingredients except aflatoxin B_1. Both cuvettes were incubated for 20 min at 23° in the absence of microsomes and aflatoxin B_1 in order to ensure the presence of an adequate amount of NADPH. Then the reaction, carried out at 23°, was started with the addition of microsomes (equilibrated to 23°) followed by aflatoxin B_1 in dimethyl sulfoxide (10 μl) to the sample cuvette alone, whereas an equivalent amount of microsomes and solvent was added to the reference cuvette. Before the spectra were recorded the contents of each cuvette were mixed by inversion.

[3] Throughout these studies an NADPH-generating system was used as the source of NADPH. The two terms have been used interchangeably.

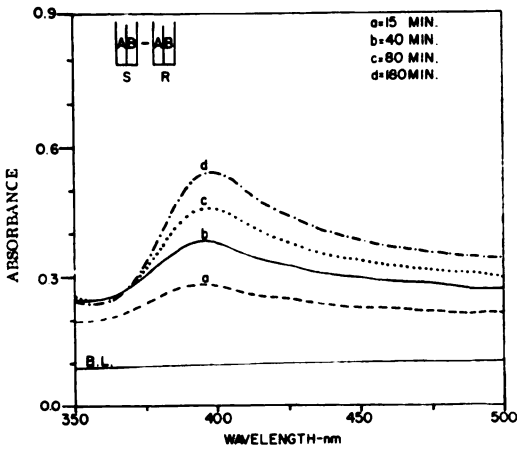

FIG. 3. *Difference spectra between an incubation mixture metabolizing aflatoxin B_1 and one not metabolizing aflatoxin B_1*

Split cells were used; the light path length of each compartment was 0.45 cm. All solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing $MnCl_2$ (1 mM). Both sample and reference cuvettes contained mixtures of identical composition: compartment A, microsomes from phenobarbital-treated rats (0.34 mg) and aflatoxin B_1 (0.14 mM) in buffer in a total volume of 1.20 ml; compartment B, an NADPH-generating system (NADP, 0.40 mM; DL-isocitrate, 4.20 mM; isocitrate dehydrogenase, 17 μg of protein) in buffer in a total volume of 1.20 ml. Both cuvettes were incubated at 37° for 10 min to ensure the presence of an adequate amount of NADPH before the start of the reaction in the sample cuvette (S), which was done by mixing the contents of compartments A and B by inversion; no mixing was done in the reference cuvette (R). Spectra were recorded at the indicated time points, and at each time point sample cuvette contents were mixed by inversion just before the spectrum was recorded.

metabolism-related disappearance of aflatoxin B_1, and the increase at 400 nm, to the formation of metabolite(s). From the decrease in absorbance at 360 nm and using an $\epsilon_{M^{-1}}^{cm^{-1}}$ of 21,800 at 360 nm for aflatoxin B_1 (36), it can be calculated that 5.14 nmoles/ml of aflatoxin B_1 (14% of the total) were metabolized.

Difference spectra were also obtained by using an identical pair of split cells (Fig. 3). A difference spectral peak with a maximum at 398 nm appeared within 15 min, and its magnitude increased with time. This peak was not seen when either NADPH, aflatoxin B_1, or microsomes were omitted from the incubation. The compound responsible for these spectral changes appears to be aflatoxin B_{2a}, as suggested by spectroscopic examination of aflatoxin B_{2a} and ethyl acetate extracts of various incubation mixtures obtained by a procedure to be described later. Furthermore, the presence of aflatoxin B_{2a} in these incubation mixtures was detected by thin-layer chromatography (5, 10, 14).

Additional evidence in support of the metabolism of aflatoxin B_1 was derived from visual examination of the incubation mixtures. During studies on the metabolism of aflatoxin B_1 the appearance of a peak at 398 nm in the difference spectrum coincided with a yellow coloration in the incubation mixture. This yellow color did not develop in the absence of NADPH or the presence of SKF 525-A. On the other hand, incubation of aflatoxin B_{2a} with microsomes also led to the development of a yellow color.

Binding of aflatoxin B_1 metabolite and aflatoxin B_{2a} to hepatic microsomes. The results reported in this and subsequent sections, unless otherwise stated, were obtained not with incubation mixtures but with microsomes previously incubated with various assay ingredients and then reisolated, washed, and in some cases subjected to Sephadex gel filtration.

When microsomes are treated with aflatoxin B_1 in the absence of NADPH, aflatoxin B_1 per se binds to microsomes, forming a complex with a difference spectral peak at 360 nm. However, in the presence of NADPH, both aflatoxin B_1 and its metabolite bind to microsomes, forming a complex with two spectral peaks, one at 360 nm and the other at 405 nm (Fig. 4). These results are in agreement with an earlier report (22). The spectral peak at 405 nm was also observed with an aflatoxin B_{2a}–microsome complex prepared by treating microsomes with aflatoxin B_{2a} in the presence and absence of NADPH. The binding of aflatoxin B_1 to microsomes is a reversible phenomenon, as this complex is separable by Sephadex gel filtration, in contrast to the complex formed between aflatoxin B_1 metabolite or aflatoxin B_{2a} and microsomes, which resists such treatment.

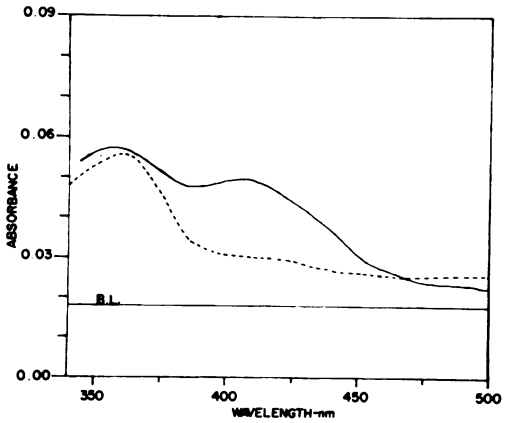

FIG. 4. *Difference spectra of liver microsomes, showing binding of aflatoxin B_1 and its metabolite*

Microsomes were incubated with aflatoxin B_1 (0.10 mM) in the presence and absence of an NADPH-generating system as described in the text. Control incubations contained everything except aflatoxin B_1. At the termination of the incubation microsomes were reisolated by centrifugation, washed, suspended in buffer, adjusted in protein concentration to 0.2 mg/ml as described in the text, and used for recording the spectrum. The reference cuvette in each case contained microsomes treated with NADPH or solvent alone; no significant difference was observed between microsomes treated with solvent alone and solvent plus NADPH. - - -, sample cuvette, microsomes treated with aflatoxin B_1; ——, sample cuvette, microsomes treated with aflatoxin B_1 and NADPH. B.L., baseline obtained with sample cuvette (same as in reference).

Radiochemical studies (Table 1), using [^{3}H]aflatoxin B_1, also show that a metabolite of aflatoxin B_1 binds to microsomes; the formation of the [^{3}H]aflatoxin B_1 metabolite–microsome complex is inhibited by SKF 525-A (70–73%), which is an inhibitor of microsomal mixed-function oxygenase (37), by L-cysteine (75%), and by glutathione (58%). Spectral studies showing similar effects of these three inhibitors have been reported previously (23).

The results of studies on the binding of [^{3}H]aflatoxin B_{2a} to microsomes (Table 1) show that [^{3}H]aflatoxin B_{2a} binds to microsomes independently of NADPH; this binding is partially blocked by L-cysteine (40%) but not by SKF 525-A. Furthermore, the extent of binding is dependent upon the concentration of [^{3}H]aflatoxin B_{2a} in the incubation mixture. In additional studies it was found that at least 80% of the radioactivity remained associated with microsomes following the precipitation of the [^{3}H]aflatoxin B_{2a}–microsome complex with 8% trichloracetic acid.

In combined radiochemical and spectral studies, using microsomes reisolated from incubations devoid of NADPH but containing [^{3}H]aflatoxin B_1 (0.11 and 0.18 mM) and aflatoxin B_{2a} (0.027 and 0.040 mM), it was found that the microsomal binding of aflatoxin B_{2a} does not interfere with either the microsomal binding of aflatoxin B_1 or its dissociation from the complex by Sephadex gel filtration, which was calculated to remove over 75% of the aflatoxin B_1 bound noncovalently to microsomes.

Effect of pH on spectral structure of aflatoxin B_{2a}. The spectral structure of aflatoxin B_{2a} is modulated by pH in such a way that the 360 nm peak at pH 1.0, 2.2, and 4.4 gradually shifts to 398 nm with increasing pH (Fig. 5A). The peak at 398 nm at pH 8.0 does not shift with increasing pH but instead shows hyperchromicity as the pH is raised to 9.2 and 11.3. When each of these mixtures was extracted with 1.25 volumes of chloroform and the spectrum of the aqueous phase was obtained (Fig. 5B), the 360 nm peak, seen at low pH values, was substantially reduced, possibly because of the extraction of aflatoxin B_{2a} into chloroform, whereas the 398 nm peak, seen at higher pH values, remained associated with the aqueous phase. These results suggest that at lower pH values the chemical nature of aflatoxin B_{2a} renders it less soluble in the aqueous phase but that, as the pH is increased, its structure is altered in such a way that its extractability into the aqueous phase is increased substantially in contrast to that in chloroform.

The results shown in Fig. 5A further suggest that at least three chemical species occur during the pH-induced transition of the 360 nm peak. To examine this further, three parameters—absorbance at 360 and 398 nm and at the isosbestic points—were

TABLE 1

Effects of inhibitors on formation of [^{3}H-aflatoxin B_1 metabolite–microsome complex and on binding of [^{3}H]aflatoxin B_{2a} to microsomes

Experimental details for the incubation of microsomes are described in the text and specifically in the legend to Fig. 4, except that [^{3}H]AFB_1 (0.10 or 0.11 mM) was used instead of AFB_1. In the first set of experiments microsomes in phosphate buffer and in the presence of an NADPH-generating system were incubated with (a) [^{3}H]AFB_1 alone (b) [^{3}H]AFB_1 plus SKF 525-A (0.8 mM), (c) [^{3}H]AFB_1 plus L-cysteine (10 mM), or (d) [^{3}H]AFB_1 plus glutathione (10 mM). Corresponding controls for each incubation mixture were prepared under identical experimental conditions in the absence of an NADPH-generating system. At the termination of the incubation, microsomes were reisolated, washed, and adjusted in protein concentration to 2 mg/ml, and 1-ml aliquots were counted in triplicate for radioactivity by the procedure described in the text. The amount of microsome-bound metabolite shown here was calculated from the difference in the radioactivity bound to microsomes reisolated from the test incubation mixture minus microsomes reisolated from control incubations, which contained everything except the NADPH-generating system, which is necessary for the generation of the microsome-mediated metabolite of AFB_1. The specific activity of [^{3}H]AFB_1 was 0.27 or 0.44 mCi/mmole. In the second set of experiments the binding of [^{3}H]AFB_{2a} to microsomes was studied. All incubations were performed in the absence of NADPH in a total volume of 12 ml of 0.1 M potassium phosphate buffer containing $MnCl_2$ and $MgCl_2$. Other experimental details were similar to those described above. Microsomes, reisolated from the incubation mixture and subsequently washed, were adjusted to a protein concentration of 5 mg/ml; 1-ml aliquots were counted in triplicate, and the counts were averaged. Three incubation mixtures were processed in each experiment: (a) [^{3}H]AFB_{2a} (0.01 and 0.02 mM) plus microsomes (3 mg of protein per milliliter), (b) [^{3}H]AFB_{2a} plus microsomes and SKF 525-A (0.70 mM), and (c) [^{3}H]AFB_{2a} plus microsomes and L-cysteine (12 mM). The specific activity of [^{3}H]AFB_{2a} was 0.184 mCi/mmole.

Binding of [^{3}H]AFB_1 metabolite to microsome

Incubation conditions prior to reisolation of microsomes	Metabolite bound	[^{3}H]AFB_1 metabolite bound to microsomes
	nmoles/2 mg protein	*% control*
(Test[a]) − (control[b])	5.1, 6.3, 6.8[c]	100
(Test + SKF 525-A) − (control + SKF 525-A)	1.4, 1.5[d]	30, 27[d]
(Test + L-cysteine) − (control + L-cysteine)	1.7	25
(Test + glutathione) − (control + glutathione)	2.9	42

Binding of [^{3}H]aflatoxin B_{2a} to microsomes

Incubation conditions prior to reisolation of microsomes	[^{3}H]AFB_{2a} bound		[^{3}H]AFB_{2a} bound to microsomes[e]
	0.01 mM AFB_{2a}	0.02 mM AFB_{2a}	
	nmoles/5 mg protein		*% control*
[^{3}H]AFB_{2a} + microsomes	7.2	12.2	100
[^{3}H]AFB_{2a} + microsomes + SKF 525-A	8.1	12.7	108
[^{3}H]AFB_{2a} + microsomes + L-cysteine	4.4	7.3	60

[a] The test incubation mixture contained microsomes, NADPH, and [^{3}H]AFB_1.

[b] The control incubation mixture contained everything in the test incubation except NADPH.

[c] Results of three separate experiments.

[d] Results of two separate experiments.

[e] Average.

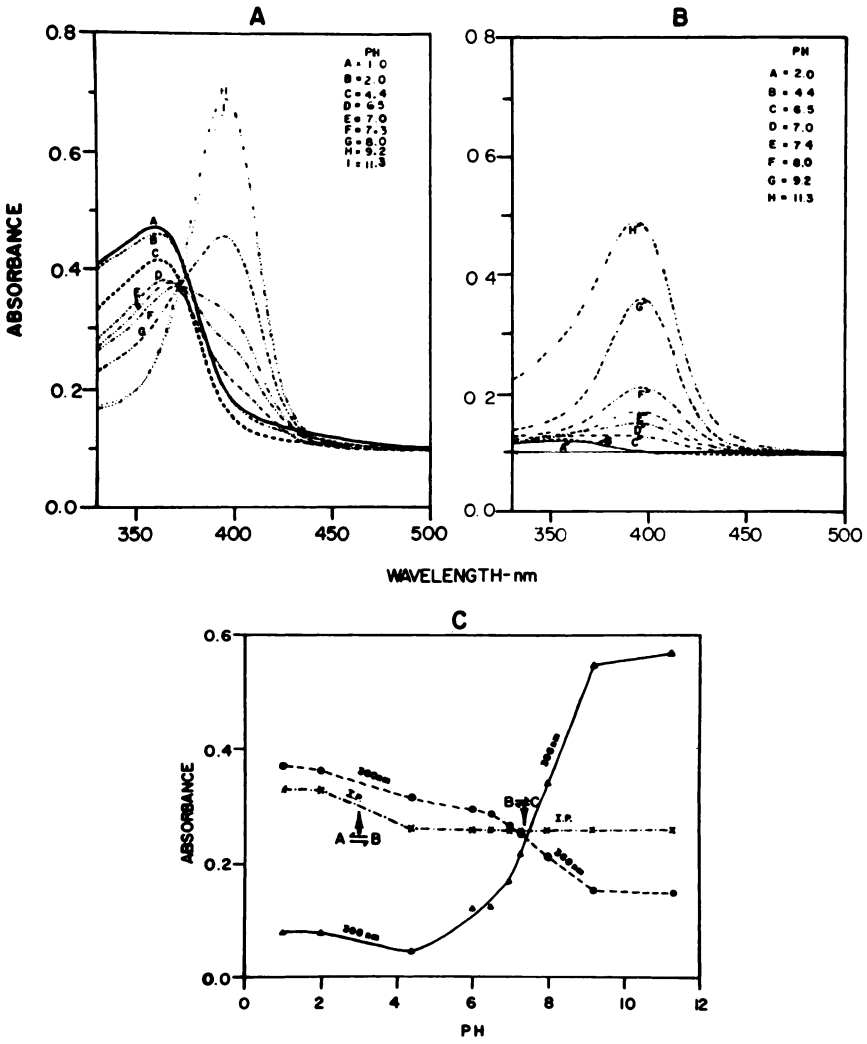

FIG. 5. *Effect of pH on spectrum of aflatoxin* B_{2a}

Aflatoxin B_{2a}, prepared and purified as described in the text, was dissolved in methanol, 200-μl aliquots were mixed with 3.80-ml portions of potassium phosphate buffers of varying pH, and spectra were recorded (A). A solution having a pH of 1.0 was prepared by mixing a 200-μl methanolic solution of aflatoxin B_{2a} in water, the pH of which had been adjusted with 0.1 N HCl; 0.01 N NaOH was used to obtain the solution with pH 11.3. The reference cuvette in each case contained the appropriate methanol-buffer mixture, which was devoid of aflatoxin B_{2a}. After the spectra had been recorded each solution (4 ml) was extracted by shaking (5 min) and centrifuging (5 min) with 5 ml of chloroform, and the aqueous phase was removed and used for recording the spectra (B). The baseline was adjusted with an instrument balance. Plots of the absorbance at isosbestic points (I.P.) and at 398 and 360 nm of aflatoxin B_{2a} vs. pH are also shown (C).

plotted as a function of pH (Fig. 5C). The drop in the isosbestic point curve at low pH values and the lack of any further alteration after this initial drop suggest that aflatoxin B_{2a} is first converted to an intermediate (probably a monoaldehyde in Fig. 1), the equilibrium pH for this conversion being in the midpoint of the drop (pH 3.0). This intermediate (*B*) seems to be subsequently converted to the final product (*C*) (possibly

the dialdehyde derivative in Fig. 1), and the equilibrium pH of this transition lies somewhere in the triangle described by the three curves. A very close estimate of this pH value, obtained from the pointed of intersection of the 360 and 398 nm curves, is pH 7.35.

The pH-dependent alteration of the aflatoxin B_{2a} spectrum shown in Fig. 5A was found to be reversible. The reversal of the spectral peak shift from 398 nm to 360 nm by acidification of an alkaline solution of aflatoxin B_{2a} was accompanied by a decreased ability of the aqueous phase, upon extraction with chloroform, to retain the aflatoxin B_{2a} chemical species responsible for the appearance of a spectral peak at 398 nm.

Effect of inhibitors on formation and microsomal binding of water-soluble metabolite from [^3H]aflatoxin B_1. If the metabolite in question has same solubility, spectral, and microsomal binding characteristics as aflatoxin B_{2a}, it should be possible to study the effect of inhibitors of the microsomal mixed-function oxygenase on the formation and microsomal binding of the metabolite derived from [^{3}H]aflatoxin B_1. This could be done by making the incubation mixture alkaline, extracting it with chloroform to remove parent [^{3}H]aflatoxin B_1 and its chloroform-soluble metabolites, and then counting the microsomal precipitate and an aliquot of the aqueous phase. The results (Table 2) clearly show that aflatoxin B_1 is converted by microsomal mixed-function oxygenase into a polar metabolite which binds tenaciously to microsomes.

The total amount of polar metabolite, whether free in the aqueous phase or bound to microsomes, was estimated (Table 2) to account for more than 88% of the metabolized aflatoxin B_1. In additional studies, the aqueous phase after extraction with chloroform was acidified (pH 2.0) and extracted with ethyl acetate. The latter extract was then evaporated to dryness, and the residue was dissolved in water and used for recording the spectrum between 350 and 500 nm. This spectrum and the spectrum of aflatoxin B_{2a} were similar in respect to absorption in the 360 and 400 nm regions, pH dependence (see Fig. 5A), and reversibility of the pH-induced spectral alteration.

DISCUSSION

The results of these studies indicate that (a) in the presence of rat liver microsomes and NADPH, aflatoxin B_1 is converted into a metabolite which is similar to aflatoxin B_{2a} and, under certain conditions, this conversion constitutes a major pathway; (b) aflatoxin B_1 per se binds loosely to microsomes, whereas its metabolite binds much more firmly; and (c) the binding of aflatoxin B_{2a} is mediated via its cleavage products.

When the metabolism of aflatoxin B_1 by microsomes was studied *in vitro* by means of difference spectroscopy, a temporal relationship was evident between the decrease in absorbance at 360 nm, caused by the disappearance of aflatoxin B_1, and an increase in absorbance in the 400 nm region. The change at 400 nm was related to the microsomal mixed-function oxygenase-mediated metabolism of aflatoxin B_1. The wavelength of maximum difference, related to the formation of the metabolite, was located at 398 nm, suggesting a similarity between the metabolite in question and aflatoxin B_{2a} or a product related to it, since pure aflatoxin B_{2a} at pH 7.4 (in solutions containing microsomes) and above was found to have a peak at 398 nm. Furthermore, the aflatoxin B_1 metabolite and aflatoxin B_{2a} were both found to have similar spectral, thin-layer chromatographic and solubility characteristics. Additional evidence for this metabolic pathway was also provided by the observation that spectral characteristics of the aflatoxin B_1 metabolite–microsome complex and the aflatoxin B_{2a}–microsome complex were identical. These data strongly suggest that aflatoxin B_{2a}, or a very closely related species, is formed from aflatoxin B_1 by rat liver microsomes, contrary to some reports (5, 14) that aflatoxin B_{2a} was scarcely formed by rat liver microsomes.

In systems *in vitro* utilizing hepatic subfractions, aflatoxin B_{2a} is known to be a major metabolite of aflatoxin B_1, accounting for 85–100% of the metabolized aflatoxin B_1; however, under similar conditions only traces of aflatoxin M_1 are detected (5, 11, 14). Furthermore, it has been recently reported (38) that aflatoxins M_1 and P_1 together account for less than 3% of the aflatoxin B_1 metabolised *in vitro* by human

TABLE 2

Effect of inhibitors on formation and microsomal binding of water-soluble metabolite of aflatoxin B_1

Incubation mixtures in potassium phosphate buffer (0.07 M, pH 7.4) contained an NADPH-generating system (unless otherwise indicated), $MgCl_2$, and $MnCl_2$. The concentrations of the assay components are described in the text. After incubation the mixture was rendered alkaline by adding 1 ml of 0.5 M K_2HPO_4 (pH 9.3); immediately afterward the mixture was extracted with 20 ml of chloroform (in order to remove unmetabolized aflatoxin B_1 and its chloroform-soluble metabolites) by shaking for 10 min and centrifugation at 2000 rpm for 30 min. The aqueous phase, free of precipitate, was collected, and an aliquot was counted in duplicate. In another set of identical experiments the microsomal precipitate obtained after chloroform extraction was carefully collected and once again extracted with 20 ml of chloroform. The precipitate after the second extraction was collected and then extracted with 10 ml of acetone. Acetone was aspirated, and the pellet was dried, digested with 0.5 ml of 1 N NaOH, and counted in 18 ml of scintillation mixture. The details for counting and calculating the radioactivity are described in the text. It is presumed that organic solvent extraction removed lipids from the microsomes. The concentrations of inhibitors are given below. All incubation mixtures, in the absence of the inhibitor, microsomes, and [^{3}H]aflatoxin B_1, were first incubated for 10 min at 37°; then microsomes and the inhibitor (where indicated) were added, in that order, and reaction was started with the addition of [^{3}H]-aflatoxin B_1 in 20 µl of dimethyl sulfoxide. In experiments using 0.12 mM AFB_1 the total volume of each incubation mixture was 1.6 ml; microsomal protein; 0.63 mg/ml; period of incubation, 40 min at 37°; specific activity of [^{3}H]AFB_1, 0.127 mCi/mmole. The corresponding figures for experiments in which 0.18 mM AFB_1 was used were 2 ml, 2.5 mg/ml, 1 hr, and 0.120 mCi/mmole. Microsomes used in these experiments were isolated from phenobarbital-treated rats as described in the text.

The complete incubation mixture contained microsomes, an NADPH-generating system, and [^{3}H]-AFB_1 in phosphate buffer.

Incubation conditions	AFB_1 metabolite in aqueous phase[a]		AFB_1 metabolite bound to microsomal precipitate[d] at 0.18 mM AFB_1
	0.12 mM AFB_1[b]	0.18 mM AFB_1[c]	
	% control		*% control*
Complete	100	100	100
+NaCN (1 mM)	108	110[e]	91[e]
Boiled microsomes	2	0	7
−NADPH	5	6	15
+SKF 525-A (2 mM)	21	28[g]	33[g]
+CO[h]	28	30	5

[a] These values were calculated after subtracting the radioactivity in the blank (incubation mixture containing everything except microsomes), which was 800 dpm/ml for 0.12 mM and 1300 dpm/ml for 0.18 mM AFB_1.

[b] These values represent an average of two experiments for each incubation. Radioactivity (disintegrations per minute per milliliter of aqueous phase) from the complete incubation (after subtracting the blank) in the two experiments was 4356 and 4662.

[c] Radioactivity (disintegrations per minute per milliliter of the aqueous phase) from the complete incubation (after subtracting the blank) was 6995.

[d] Radioactivity (disintegrations per minute) in the microsomal precipitate from the complete incubation was 7890. This is the average of two experiments, with individual values of 8005 and 7775. Two experiments were carried out for each incubation.

[e] The concentration of NaCN was 1.5 mM.

[f] Microsomes were boiled for 3 min, allowed to cool, and then used.

[g] The concentration of SKF 525-A was 1.5 mM.

[h] The reaction was carried out in rubber-stoppered 25-ml Erlenmeyer flasks, and CO (Matheson) was bubbled through the incubation mixture for a total of about 3 min, 2.5 min before the addition of microsomes and 40 sec after the addition of microsomes. The reaction was started with [^{3}H]aflatoxin B_1.

liver. Other investigators, using thin-layer chromatography, have reported the formation *in vitro* of aflatoxin B_{2a} from aflatoxin B_1, but the extent of this biotransformation was not studied (11). Also, phenobarbital treatment of rats has been shown to enhance the microsome-mediated metabolism of aflatoxin B_1 *in vitro* (11, 12).

In view of these observations, it was of interest to determine whether the conversion of aflatoxin B_1 to the metabolite in question, particularly by microsomes from phenobarbital-treated rats, is a major pathway in the rat. Using the decrease and increase in absorbance at 360 and 400 nm, respectively (Fig. 2), and the extinction coefficients of 21,800 for aflatoxin B_1 at 360 nm (36) and of 34,276 for aflatoxin B_{2a} at 400 nm [calculated from the data of Patterson and Roberts (14) for pH 7.4], it was calculated that under our experimental conditions most of the metabolized aflatoxin B_1 was converted to the metabolite responsible for the increase in absorption in the 400 nm region. However, using [^{3}H]aflatoxin B_1, the water-soluble metabolite, which appears to be similar to aflatoxin B_{2a}, was estimated to account for at least 88% of the metabolized aflatoxin B_1. These findings suggest that, at least under the conditions *in vitro* of our experiments, the metabolite in question, presumably aflatoxin B_{2a}, appears to be a major metabolite of aflatoxin B_1. If the effect of phenobarbital treatment of rats on aflatoxin B_1 metabolism *in vitro* is a reflection of its effects *in vivo* one might explain how such treatment of rats affords protection against aflatoxin B_1-induced toxicity and carcinogenicity (39–41), since aflatoxin B_{2a} is relatively nontoxic (5, 16–19).

A metabolite of aflatoxin B_1 also binds to microsomes, forming a complex with a spectral peak at 405 nm. Aflatoxin B_{2a}, when incubated with microsomes in the presence and absence of NADPH, also binds to microsomes, yielding a complex with identical spectral properties. The formation of either complex, i.e., the complex of aflatoxin B_1 metabolite or of aflatoxin B_{2a} with microsomes, is decreased in the presence of L-cysteine, and neither complex is dissociated by Sephadex gel filtration. SKF 525-A, a potent inhibitor of the microsomal mixed-mixed-function oxygenase, did not interfere with the binding of aflatoxin B_{2a} per se but did decrease the aflatoxin B_1 metabolism-dependent formation of the complex, implying that the inhibitor produced its effect by blocking the metabolism of aflatoxin B_1. On the other hand, L-cysteine had an opposite effect: it did not seem to interfere with the metabolism of aflatoxin B_1 but did substantially reduce (40%) the binding of aflatoxin B_{2a} to microsomes. Comparison of the characteristics of the aflatoxin B_1 metabolite–microsome complex with those of the aflatoxin B_{2a}–microsome complex strongly suggests that either aflatoxin B_{2a} per se or a chemical species related to it is responsible for the 405 nm spectral peak. Although no conclusive evidence is available regarding the nature of this metabolite, a plausible hypothesis is given below.

Pohland *et al.* (18) postulated that under alkaline conditions aflatoxin B_{2a} cleaves to yield a dialdehyde. Also, Patterson and Roberts (14) reported that aflatoxin B_{2a} when incubated with bovine serum albumin adsorbs onto the protein; these investigators also noted a shift in the absorbance of aflatoxin B_{2a} from 400 nm to 450 nm in the presence of such proteins. Furthermore, they suggested that aflatoxin B_{2a} "degrades" before being adsorbed onto the proteins and, in the light of the postulation by Pohland *et al.* (18), implicated the dialdehyde derivative (Fig. 1) in this adsorption phenomenon.

We did not notice any shift in the spectrum of aflatoxin B_{2a} (from 400 nm to 450 nm) in the presence of microsomes, but our data do suggest that aflatoxin B_{2a} undergoes pH-dependent cleavage, which is accompanied by a shift in λ_{max} from 360 nm to 398 nm; furthermore, this pH-induced cleavage was found to be reversible, as indicated by a reversal in the λ_{max} from 398 nm to 360 nm on acidification of the alkaline solution of aflatoxin B_{2a}. However, in the presence of microsomal proteins, aflatoxin B_{2a} binds tightly to microsomes, possibly through the formation of Schiff bases between the aldehyde groups of cleaved aflatoxin B_{2a} and the free amino groups of proteins (42). Such binding seems to be irreversible, as the aflatoxin B_{2a}–microsome complex could not be dissociated by Sephadex gel filtration. Furthermore, radioactivity essentially could not be released from the microsomal proteins by trichloracetic acid precipitation of the [^{3}H]aflatoxin B_{2a}–microsome complex or by solvent extraction (Table 2). The pH-dependent alterations in the solubility characteristics of aflatoxin B_{2a}, found in this study, further support the hypothesis regarding

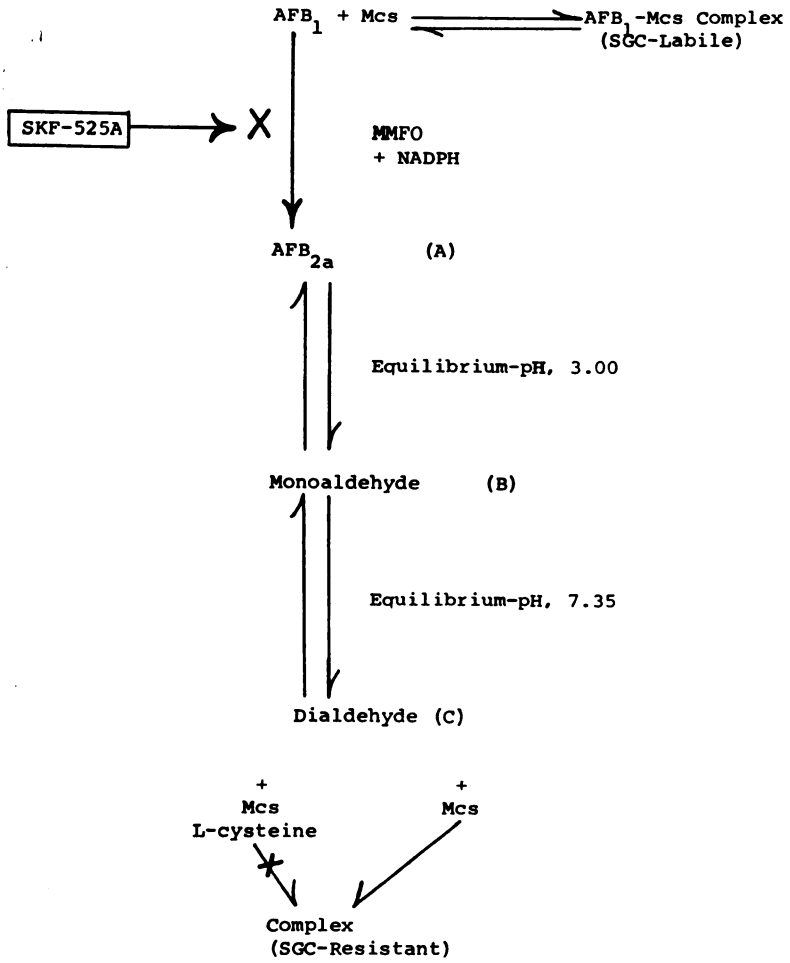

FIG. 6. *Schematic representation of possible mechanisms involved in binding of aflatoxin B_1 and its metabolite (aflatoxin B_{2a}) to rat hepatic microsomes (Mcs)*

X = inhibition; AF = aflatoxin; MMFO = microsomal mixed-function oxygenase; SGC = Sephadex gel chromatography.

pH-induced cleavage of aflatoxin B_{2a} to dialdehyde ionic forms, which would be expected to be more soluble in the aqueous phase than the parent compound. Once aflatoxin B_{2a} is cleaved to the dialdehyde, cysteine could prevent its binding to microsomes by (a) forming a thiazolidine type of addition product, similar to that seen in the structure of penicillin (43, 44), and/or (b) undergoing a Schiff base reaction with the aldehyde groups of aflatoxin B_{2a} cleavage products. Our data further indicate that such a cleavage is probably not spontaneous but involves conversion through an intermediate, which is probably represented by the cleavage product of aflatoxin B_{2a} in which only the first furan ring is cleaved, forming a monoaldehyde derivative. These suggestions, as derived from our data, are schematically summarized in Fig. 6.

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