

Some High-Performance Liquid-Chromatographic Studies of the Metabolism of Aflatoxins by Rat Liver Microsomal Preparations

By GORDON E. NEAL* and PETER J. COLLEY

Toxicology Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey, U.K.

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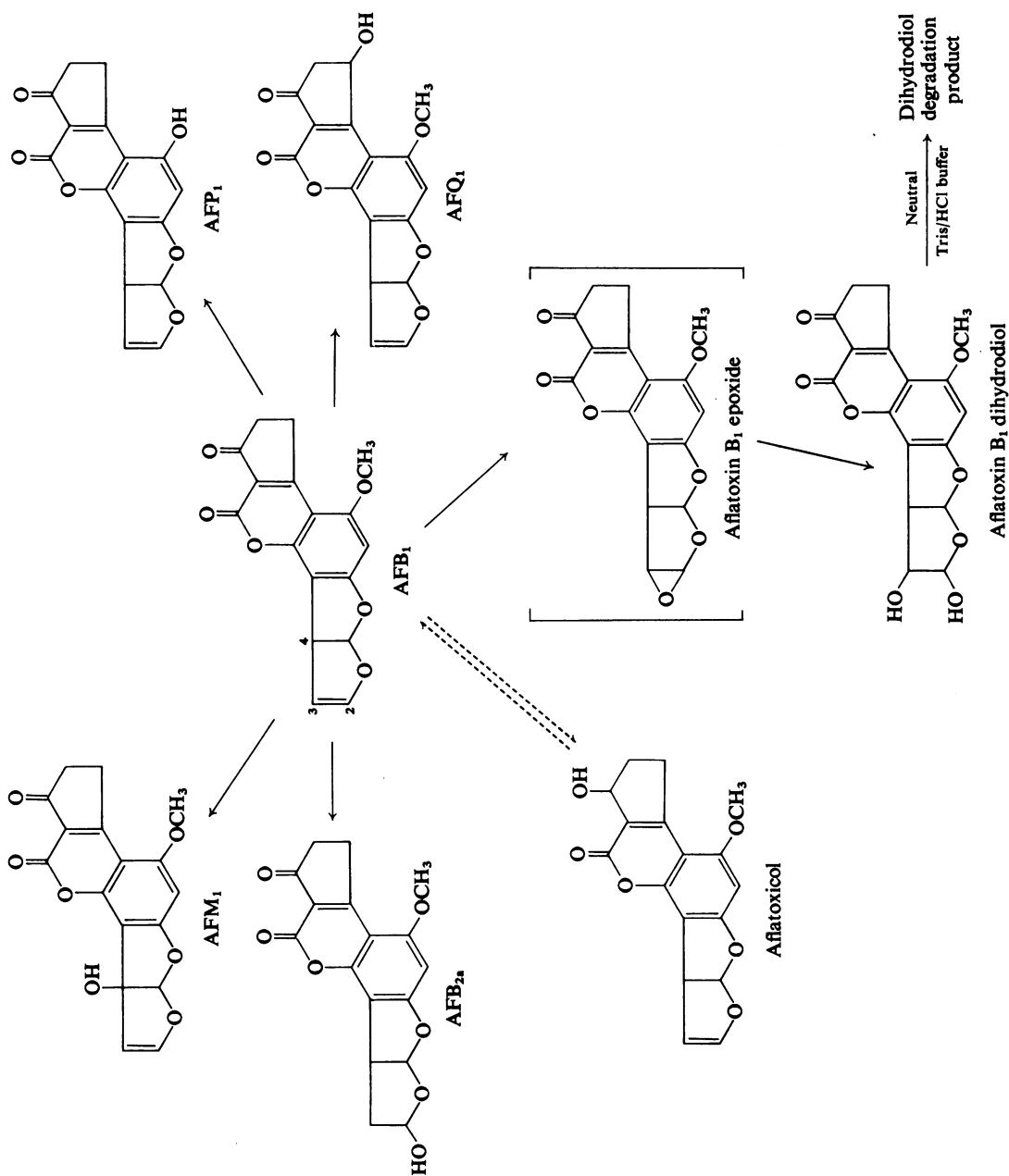
1. The metabolism of aflatoxin B₁ *in vitro* was examined in rat liver microsomal preparations. 2. H.p.l.c. (high-performance liquid-chromatographic) systems were used. A silica column was used to separate non-polar metabolites. A system utilizing a reversed-phase column which separates both polar and non-polar metabolites was also developed. 3. The principal metabolites of aflatoxin B₁ found were aflatoxin M₁, aflatoxin Q₁ and a compound which co-chromatographed with a degradation product of aflatoxin B₁, 2,3-dihydrodiol. 4. The time course of metabolism of aflatoxin B₁ by microsomal preparations isolated from control and phenobarbitone-pretreated rats was examined. The rate and extent of metabolism was greater with microsomal preparations from the latter. The formation of aflatoxin Q₁ was enhanced 4-5-fold by phenobarbitone pretreatment, whereas the production of aflatoxin M₁ was only increased 1-2-fold. The formation of the degradation product of aflatoxin B₁, 2,3-dihydrodiol was increased 4-5-fold by the pretreatment with phenobarbitone. 5. The microsomal metabolism of aflatoxins M₁, P₁ and Q₁ was examined. Aflatoxin M₁ apparently underwent very limited microsomal metabolism to more polar compounds. Aflatoxin P₁ was not metabolized. The situation with aflatoxin Q₁ was complicated in that it was metabolized in the absence of NADPH to an unidentified metabolite. Aflatoxin B₁ appeared as a metabolite of aflatoxin Q₁ only when NADPH was present, and the formation of more polar metabolites was also then observed.

Aflatoxin B₁, the potent hepatotoxin and hepatocarcinogen, is metabolized by rat liver microsomal enzymes to a variety of metabolites, including phenols, hydroxy compounds and probably an epoxide (Scheme 1). The epoxide has not yet been isolated and it is presumed to undergo further conversion either metabolic or non-enzymic to form a dihydrodiol (for review see Campbell & Hayes, 1976). The only major non-microsomal route of metabolism reported is reduction to aflatoxicol, which is mediated by the cytosolic fraction. This range of metabolites reflects the existence in hepatocytes of diverse metabolic pathways. Considering these alternative pathways of metabolism in terms of detoxification or activation of aflatoxin B₁, the biological potencies of the non-polar chloroform-soluble metabolites, except for aflatoxin M₁, all appear to be less than that of the parent compounds and so may be considered detoxication products (Campbell & Hayes, 1976). Aflatoxin M₁ is as acutely toxic to rats as aflatoxin B₁, but has a very much smaller carcinogenic capacity (Wogan & Paglialunga, 1974). The production of 2,3-dihydro-2,3-dihydroxyaflatoxin B₁ (aflatoxin B₁ 2,3-dihydro-

diol), which has been reported to degrade spontaneously in Tris/HCl buffer, pH 7-8, to polar chloroform-insoluble products (Garner, 1973a), could reflect a detoxifying hydrazase reaction subsequent to the primary activation of aflatoxin B₁ by epoxidation. The formation of the dihydrodiol may therefore indicate the aflatoxin B₁-activating capacity of the microsomal preparation. Most studies of aflatoxin B₁ metabolism to date have involved examinations of the chloroform-soluble non-polar metabolites on t.l.c. or paper chromatograms (Campbell & Hayes, 1976). These studies, however, are time-consuming and difficulties arise because of the instability of some of the metabolites. H.p.l.c. offers many advantages for examination of the metabolism of aflatoxin B₁. Quantitative as well as qualitative data could be obtained in a short period of time. Although several authors have published h.p.l.c. techniques for separating aflatoxins [principally the parent aflatoxins B₁, B₂, G₁ and G₂ (e.g. Pons, 1976; Garner, 1975)], the use of these systems in actual metabolic or diagnostic studies has so far been limited (Hsieh *et al.*, 1976; Unger *et al.*, 1977). Furthermore, all these studies have utilized silica columns to examine non-polar metabolites of aflatoxin B₁, although it is known that a large percentage of the microsomal metabolism of this

Abbreviation used: h.p.l.c., high-performance liquid chromatography.

* To whom requests for reprints should be sent.



Scheme 1. Metabolism of aflatoxin B₁.
 →, Microsomal; - - - - ->, cytosolic. AF, Aflatoxin.

compound results in polar water-soluble compounds (Campbell & Hayes, 1976). In those few studies in which the production of polar metabolites has been compared with metabolism to non-polar metabolites in the same microsomal preparations, the extent of formation of the polar compounds has been assessed by determining the fraction of the total label remaining in the aqueous phase after the extraction of metabolites of labelled aflatoxin B₁ with chloroform; the individual polar components have not been resolved (Roebuck & Wogan, 1977; Faris & Hayes, 1975). In other studies a different technique has been used. After microsomal metabolism of aflatoxin B₁, the non-polar compounds have been separated and estimated by t.l.c. The activation of aflatoxin B₁ by these same microsomal preparations has been assessed in parallel experiments in which the binding of labelled aflatoxin metabolites to DNA has been measured (Gurtoo & Motycka, 1976) without examination of the identities of the binding compounds. In order to assess the relative effects of any particular treatment on the several metabolic pathways present in microsomal preparations for metabolizing aflatoxin B₁, it would be desirable to examine the complete range of polar and non-polar metabolites by one extraction and separation procedure. In the present study we used silica h.p.l.c. columns to examine non-polar metabolites, but we have also compared the results obtained with this technique with those obtained by using a reversed-phase h.p.l.c. system which we have developed. We have used both of these systems to examine the metabolism of aflatoxin B₁ by rat liver microsomal preparations *in vitro*.

Materials and Methods

Animals and subcellular preparations

Adult male Fischer rats, maintained on MRC 41B diet and weighing approx. 250g, were killed by decapitation. Microsomal fractions were prepared essentially by the method of Omura & Sato (1964): 5g amounts of liver, normally obtained from the pooled livers of two animals, were homogenized in 3 vol. of 150mM-KCl and, after a 20min centrifugation at 9000g, microsomal fractions were sedimented from the supernatant by centrifuging at 100000g for 1h. Microsomal pellets were washed by resuspension in 150mM-KCl and resedimented at 100000g for 1h. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Chemicals

Aflatoxin B₁ was obtained from Makor Chemical Co., Jerusalem, Israel and was routinely dissolved in dimethyl sulphoxide (special for spectroscopy grade)

obtained from BDH, Poole, Dorset, U.K. [¹⁴C]-Aflatoxin B₁ was prepared by culturing *Aspergillus parasiticus* N.R.R.L. 3990 (a generous gift from Dr. Lillehoj, Northern Regional Research Laboratory, Peoria, IL, U.S.A.) in a medium containing sodium [U-¹⁴C]acetate (sp. radioactivity 58mCi/mmol), which was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [¹⁴C]Aflatoxin was extracted and purified on t.l.c. essentially by the method of Hsieh & Mateles (1971) as modified by Garner (1973b). The radiochemical purity of the labelled aflatoxin B₁ was checked immediately before use in experiments by scanning t.l.c. plates on a Packard thin-layer radiochromatogram scanner and ensuring the coincidence of the labelling both with the fluorescence observed on viewing the plates under u.v. light and the R_F of an aflatoxin B₁ marker. Glucose 6-phosphate, NADP⁺ and reduced glutathione were obtained from Sigma (London) Chemical Corp., Kingston-upon-Thames, Surrey, U.K. Glucose 6-phosphate dehydrogenase was obtained from Boehringer Corp. (London), Lewes, Sussex, U.K. 1,2-Epoxy-3,3,3-trichloropropane was obtained from Ralph Emmanuel, Wembley, Middx., U.K. Chloroform and dichloromethane, the solvents used for h.p.l.c., were analytical-grade reagents obtained from Fisons, Loughborough, Leics., U.K. The chloroform contained 2% ethanol as a stabilizer. The solvents were not dried before use. All other chemicals and solvents used were A.R. grade obtained from BDH. Preparative and analytical t.l.c. was performed on precoated silica-gel G plates either 2.0mm or 0.25mm thick, obtained from Anachem, Luton, Beds., U.K. The aflatoxin standards used were generous gifts from the following sources: aflatoxin M₁, Dr. P. L. Schuller, Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands; aflatoxin Q₁, Dr. D. P. H. Hsieh, University of California, U.S.A.; aflatoxins P₁ and M₁, Dr. G. N. Wogan, M.I.T., Cambridge, MA, U.S.A. Aflatoxins B₂, G₁ and G₂ were present in a fungal extract obtained from M.R.E., Porton, Wilts., U.K.

After confirmation of the identity of aflatoxins Q₁ and M₁ produced in microsomal incubations *in vitro* by reference to the authentic standards, these compounds were purified by h.p.l.c. and preparative t.l.c. and subsequently used as substrates in the metabolism studies.

Aflatoxicol was prepared by metabolism of aflatoxin B₁ by the 100000g supernatant prepared from homogenates of chicken liver by the method of Patterson & Roberts (1971) and also by the reduction of aflatoxin B₁ with NaBH₄ as described by Garner *et al.* (1972). Aflatoxin B_{2a} was prepared by refluxing aflatoxin B₁ with 0.03M-citric acid for 4h [Ciegler & Peterson (1968) as modified by Garner *et al.* (1972)].

Aflatoxin 2,3-dihydrodiol was prepared from aflatoxin B₁ by reaction of aflatoxin B₁ with *m*-chloroperbenzoic acid in dichloromethane and subsequent hydrolysis with methanolic KOH (Garner, 1973a). As reported by Garner (1973a) this compound spontaneously degraded in the presence of the Tris/HCl buffer, pH 7–8, which was used in the metabolic incubations, forming a water-soluble fluorescent derivative. The identity of all these products was confirmed by comparing their u.v. spectra and t.l.c. behaviour with published data.

Microsomal metabolism

Incubations, with washed microsomal fractions prepared as described above, were carried out in a shaking water bath at 37°C under 100% oxygen. Routinely the incubations were carried out in 15 ml thick-walled test tubes set at an angle in the shaking bath to aid shaking the incubation medium, which contained the following: 50 μmol of KCl, 150 μmol of Tris/HCl, pH 7.4, 12.5 μmol of MgCl₂, 0.4 ml of microsomal suspension (normally containing approx. 4 mg of protein in 150 mM-KCl), 0.5 unit of glucose 6-phosphate dehydrogenase, 10 μmol of glucose 6-phosphate, 0.75 μmol of NADP⁺ and 20 μl of dimethyl sulphoxide alone or containing 40 μg (128 nmol) of aflatoxin B₁ in a total volume of 1.89 ml. Tubes were preincubated at 37°C in the bath for 5 min before the addition of aflatoxin B₁ or other aflatoxin substrates. In those experiments in which the metabolism of aflatoxin B₁ metabolites was examined, the incubations were scaled down to a total of 0.94 ml. All the other constituents of the incubations were decreased proportionally, except for aflatoxin B₁ or other aflatoxin substrates, which were added at the level of 10 μg per incubation flask.

Upon completion of the incubation period, in those experiments in which only the non-polar chloroform-soluble metabolites were to be examined, 1 ml of ice-cold chloroform was added to stop the incubation and the contents were thoroughly mixed by using a Vortex mixer. Tubes were stored in ice for 30 min with additional Vortex-mixing at 10 min intervals. The tubes were then centrifuged at 2000 rev./min in an MSE 4L centrifuge for 15 min at 4°C to separate the aqueous and organic phases. The precipitated protein was located at the interface. The chloroform layer was carefully removed and the aqueous phase together with the precipitated protein was re-extracted twice with further 1 ml portions of ice-cold chloroform. The combined chloroform extracts were blown to dryness at room temperature in a stream of oxygen-free nitrogen. The dry residues were redissolved in 200–500 μl of chloroform, and samples used for h.p.l.c. and t.l.c. analysis.

In experiments in which non-polar and polar metabolites were examined, metabolism was stopped

by adding 1 ml of ice-cold methanol and the contents of the tube were thoroughly mixed by using a Vortex mixer. Internal standards could be added at this stage. Tubes were stored in ice for 30 min with remixing, in the Vortex mixer, at 10 min intervals. The precipitated protein was removed by centrifuging in an MSE Mistral 4L centrifuge at 2000 rev./min (1000g) for 15 min, at 4°C. The supernatant was removed and the pellet washed twice with further 1 ml portions of ice-cold methanol, followed by centrifugation [2000 rev./min for 15 min (1000g)]. The combined supernatants, in 15 ml thick-walled tubes, were reduced in volume at 37°C in a stream of oxygen-free nitrogen, until the volume indicated that most of the alcohol had been removed. The remaining solution was then freeze-dried overnight in the same tubes in a type SB3 freeze-drier obtained from Chem Lab Instruments, Hornchurch, Essex, U.K., fitted with a manifold. Ice-cold methanol (1 ml) was added to the freeze-dried residues and the contents were thoroughly mixed on a Vortex mixer. The tubes were centrifuged at 2000 rev./min (1000g) in an MSE Mistral 4 L centrifuge set at –10°C for 1 h. The supernatants were carefully removed and transferred to small glass vials fitted with plastic caps. The residues were washed twice with further 0.5 ml portions of ice-cold methanol, thoroughly mixed on the Vortex mixer and centrifuged at 2000 rev./min (1000g) at –10°C for 30 min. The combined supernatants were evaporated to dryness in a stream of oxygen-free nitrogen, the vials being held above a 37°C water bath. The residues were dissolved in 200 μl of methanol and samples used for h.p.l.c. or t.l.c. analysis. The salts carried over from the original incubation media were largely left behind after this methanol-extraction procedure.

H.p.l.c. analysis

The apparatus used was a Du Pont 830 liquid chromatograph fitted with an 838 gradient elution accessory, an 837 absorption detector and a Rheodyne 7120 syringe loading sample injector. The apparatus was connected to a Kontron series 1100 chart recorder fitted with an event marker. In those experiments utilizing [¹⁴C]aflatoxin B₁, the h.p.l.c. effluent was fractionated by connection to a Gilson Microcol TDC8 fraction collector [Gilson Medical Electronics (France) Ltd.]. Chloroform extracts were chromatographed on a Du Pont Zorbax-SIL column (25 cm × 2.1 mm internal diameter). An isocratic elution system was used, with chloroform/dichloromethane (1:1, v/v, with or without 1% propan-2-ol) as solvent (Pons, 1976). The apparatus was operated at a constant column pressure of 13.78 MPa, which gave a flow rate of 0.45 ml/min.

The following technique was developed for examining polar and non-polar metabolites together.

The samples, dissolved in methanol, were chromatographed on a Du Pont Zorbax-ODS column (25 cm × 2.1 mm internal diameter). Elution was by a linear gradient of 10–60% methanol in water over 40 min. Phosphoric acid at a final concentration of 0.01% was added to both primary and secondary solvents. This avoided changes in the pH during elution and resulted in a better resolution of aflatoxins M_1 and Q_1 . The apparatus was operated at a column pressure of 20.67 MPa, giving an initial flow rate of 0.48 ml/min, which increased to 0.55 ml/min during the course of elution. The column chamber was thermostatically controlled at 37°C. The effluent was monitored at 365 or 400 nm. When ^{14}C -labelled samples were used, fractions were collected at 1 min intervals and rinsed into counting vials with two portions of 5 ml of Insta-Gel scintillation solution (Packard Instrument Ltd., Caversham, Berks., U.K.). Radioactivity counting was carried out in a Philips liquid-scintillation analyser, fitted with automatic quench correction utilizing an external standard. The results of the experiments with [^{14}C]aflatoxin indicated overall recoveries of 70–80% by the extraction and h.p.l.c. procedures detailed above. During the course of this study, however, it became apparent that, in certain h.p.l.c. experiments, quantification could be improved by the use of internal standards. We have found aflatoxin G_1 to be a suitable internal standard. This is because, firstly, it is similar chemically to aflatoxin B_1 and its immediate metabolites, and so is likely to yield an accurate assessment of the degree of extraction of these compounds; secondly, experiments have shown that it is not formed from aflatoxin B_1 in the incubation systems we have used; thirdly, it chromatographs in a part of the elution profile, in both of the h.p.l.c. systems we have used, not occupied by any other metabolite of aflatoxin B_1 ; and lastly, its u.v.-absorption spectrum allows it conveniently to be monitored at the same wavelength as the aflatoxin metabolites.

Non-polar metabolites were chromatographed on silica-gel G t.l.c. plates, with chloroform/acetone (9:1, v/v) as the developing solvent. The polar metabolites, immobile in the above solvent, were developed in chloroform/methanol (4:1, v/v). When samples containing both polar and non-polar metabolites were examined by t.l.c., the above two solvent systems were used sequentially on the same plates.

Results

Separations of non-polar chloroform-soluble aflatoxin standards and metabolites by t.l.c. and h.p.l.c. on a silica column

Identities of metabolites. Aflatoxin standards were separated on a Zorbax-Sil silica column. Chloro-

form/dichloromethane (1:1, v/v) was used for the separation given in Fig. 1(a), which is similar to that described by Pons (1976) and Garner (1975) except that we did not use solvents saturated with water. This improved the reproducibility of the separations. Difficulties with water-saturated solvents have been reported by Hsieh *et al.* (1976). Our system separated the parent aflatoxins in the order B_1 , G_1 , B_2 and G_2 instead of B_1 , B_2 , G_1 and G_2 in the system used by Pons (1976) and Garner (1975). Linear detector responses for recovery of aflatoxin B_1 were obtained for both peak area and peak height.

To identify the major non-polar metabolites, microsomal incubations were carried out for 30 min as described in the Materials and Methods section. Samples were extracted with chloroform and chromatographed in t.l.c. and silica h.p.l.c. systems. The results of the t.l.c. separations are given in Table 1. Fluorescence was normally detected only at the locations corresponding to the R_F values of aflatoxins B_1 , M_1 and Q_1 . In only a few experiments was a trace of fluorescence detected at the R_F of aflatoxin P_1 . The presence of aflatoxin B_{2a} and aflatoxicol was not detected. To elute aflatoxin M_1 and Q_1 standards from the Zorbax-Sil h.p.l.c. column it was necessary to add 1% propan-2-ol to the eluting solvent. The resulting separation (Fig. 1b) shows that the resolution of aflatoxins B_1 , G_1 , B_2 and G_2 was largely lost in achieving a reasonably short retention time for aflatoxin M_1 .

When a chloroform extract of a microsomal incubation of aflatoxin B_1 , incubated either in the presence or absence of NADPH, was injected on to the Zorbax-Sil column and subsequently eluted with the chloroform/dichloromethane/propanol solvent, the results indicated in Fig. 2 were obtained. These results confirmed the t.l.c. findings that in the presence of NADPH aflatoxins M_1 and Q_1 were the principal non-polar metabolites produced by microsomal metabolism of aflatoxin B_1 . No conversion into aflatoxins M_1 and Q_1 occurred in the absence of NADPH. The identities of the compounds were further checked by adding small quantities of authentic aflatoxin M_1 or Q_1 to the extracts before injection and confirming increases in heights and areas of the appropriate detector peaks.

Time course of aflatoxin B_1 metabolism. Aflatoxin B_1 -metabolizing incubations were carried out with microsomal fractions prepared from either control rats or animals pretreated with phenobarbitone in drinking water (0.1%, w/v, for 2 weeks). The time course of the metabolism was examined with the h.p.l.c. silica column system, and the results are given in Fig. 3(a). Under the conditions used, metabolism of aflatoxin B_1 virtually ceased within 30 min of incubation with microsomal fractions from either control or phenobarbitone-pretreated rats. The

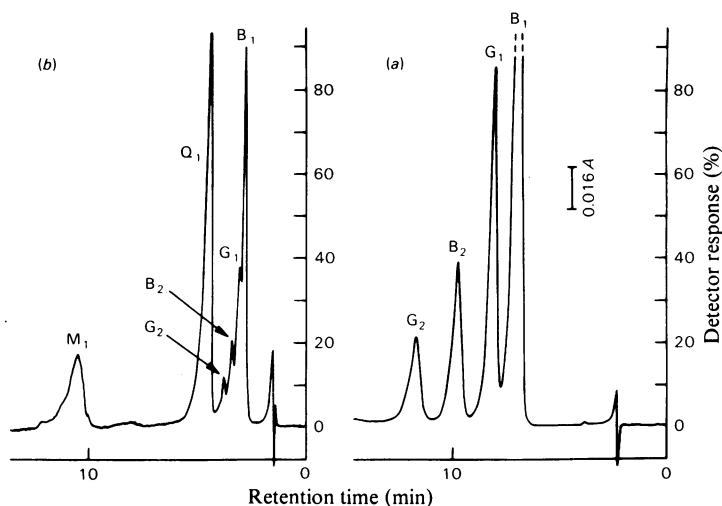


Fig. 1. Separation of aflatoxin standards on a silica h.p.l.c. column

(a) Separation of a mixture of parent aflatoxin standards. A fungal aflatoxin mixture (1.6 μg ; 55% aflatoxin B₁, 25% aflatoxin G₁, 13% aflatoxin B₂ and 7% aflatoxin G₂) was injected on to Zorbax-SIL silica column (25 cm \times 2.1 mm internal diameter). Isocratic elution was with methanol/dichloromethane (1:1, v/v) at constant column pressure (13.78 MPa) and ambient temperature. Flow rate was 0.45 ml/min. Effluent was monitored at 365 nm. Detector sensitivity is indicated by the vertical bar. (b) Separation of parent aflatoxin standards and aflatoxins Q₁ and M₁. Aflatoxin mixture was as in (a), but with addition of approx. 1 μg of aflatoxin Q₁ and 0.25 μg of aflatoxin M₁. H.p.l.c. conditions were as in (a) except that the eluting solvent contained 1% propan-2-ol.

Table 1. T.l.c. examination of metabolites of aflatoxin B₁

T.l.c. was carried out on silica-gel G plates without fluorescent indicator. Compounds were located by fluorescence when viewed under long-wavelength u.v. light. Solvent 1:chloroform/acetone (9:1, v/v); solvent 2:chloroform/methanol (4:1, v/v). The presence of metabolite was detected in chloroform or methanol extracts of microsomal incubations as described in the Materials and Methods section.

Compound	R_F		Fluorescence colour	Present in	
	Solvent 1	Solvent 2		Chloroform extract	Methanol extract
Aflatoxin B ₁	0.57		Blue	+	+
Q ₁	0.41		Grey-green	+	+
M ₁	0.31		Blue	+	+
P ₁	0.21		Pale-fawn	±	±
B _{2a}	0.17		Blue	-	-
Aflatoxicol	0.65		Blue	-	-
Aflatoxin B ₁ 2,3-dihydrodiol		0.69	Blue	-	-
Aflatoxin B ₁ 2,3-dihydrodiol (in neutral Tris buffer)		0.33	Blue	-	+

results in Fig. 3(a) show that only 50% of the aflatoxin B₁ was metabolized in the presence of control microsomal preparations, whereas more than 90% was metabolized by preparations from phenobarbitone-pretreated rats. The microsomal protein content of the two incubations was similar (approx. 4 mg of protein/incubation). The effect of phenobarbitone pretreatment on the formation of the

chloroform-soluble aflatoxin B₁ metabolites detected is shown in Figs. 3(b) and 3(c).

Separations of both polar and non-polar aflatoxin standards and metabolites by t.l.c. and h.p.l.c. on a reversed-phase column

Identities of metabolites. Microsomal metabolism of aflatoxin B₁ was carried out *in vitro*, and total

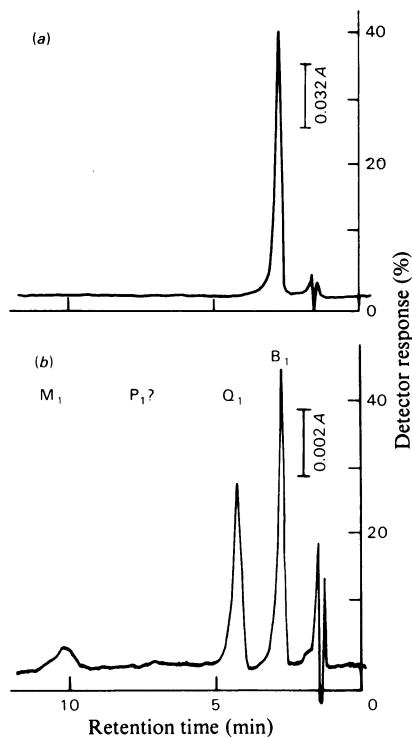


Fig. 2. Separations of chloroform-soluble metabolites of aflatoxin B₁ on a silica h.p.l.c. column

Samples injected on to h.p.l.c. columns represented equal fractions of the original incubation medium. H.p.l.c. conditions were as in Fig. 1(b). Microsomal preparations were obtained from the liver of rats pretreated with phenobarbitone. (a) Incubated in the absence of NADPH; (b) incubated in the presence of NADPH. Note that as shown by the vertical bars there are different detector sensitivities.

metabolites were extracted with methanol as described in the Materials and Methods section. These extracts were chromatographed on t.l.c. plates and the results are given in Table 1. When compared with the chloroform extracts, additional fluorescent products were observed, which were immobile in chloroform/acetone but mobile in the chloroform/methanol solvent revealing, a major fluorescing material with *R_F* similar to that of the degradation product obtained when aflatoxin B₁ 2,3-dihydrodiol was exposed to neutral Tris/HCl buffer. As expected from its behaviour in neutral Tris/HCl buffer, the presence of undegraded aflatoxin B₁ 2,3-dihydrodiol was not detected.

The reversed-phase h.p.l.c. system utilizing a Zorbax-ODS column was developed to permit column chromatography of the range of aflatoxin metabolites observed by t.l.c. to be present in the

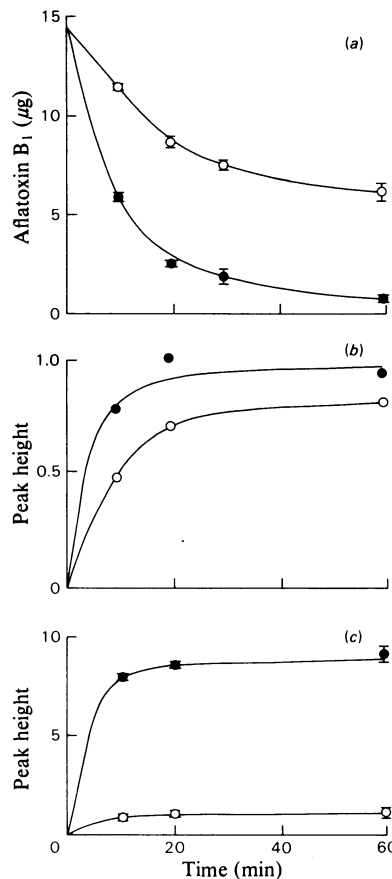


Fig. 3. Time course of microsomal metabolism of aflatoxin B₁ with chloroform extracts and a silica h.p.l.c. column. H.p.l.c. conditions were as in Fig. 1(b). Microsomal preparations used were obtained from control (○) or phenobarbitone-pretreated rats (●). Variations of duplicate determinations of aflatoxins B₁ and Q₁ are indicated by bars. (a) Metabolism of aflatoxin B₁; (b) production of aflatoxin M₁; (c) production of aflatoxin Q₁.

methanol extracts of microsomal incubations of aflatoxin B₁. Figs. 4(a) and 4(b) show the elution profiles obtained when methanol extracts of microsomal incubations of aflatoxin B₁, in the presence or absence of NADPH, were chromatographed on a Zorbax-ODS column.

The identity of the compounds present in the peaks was confirmed by coincidence on t.l.c. plates and also by additions of the individual authentic compounds to the metabolite extracts before h.p.l.c. The degradation product of aflatoxin B₁ 2,3-dihydrodiol was present in the extracts. The formation of these compounds by microsomal metabolism of aflatoxin

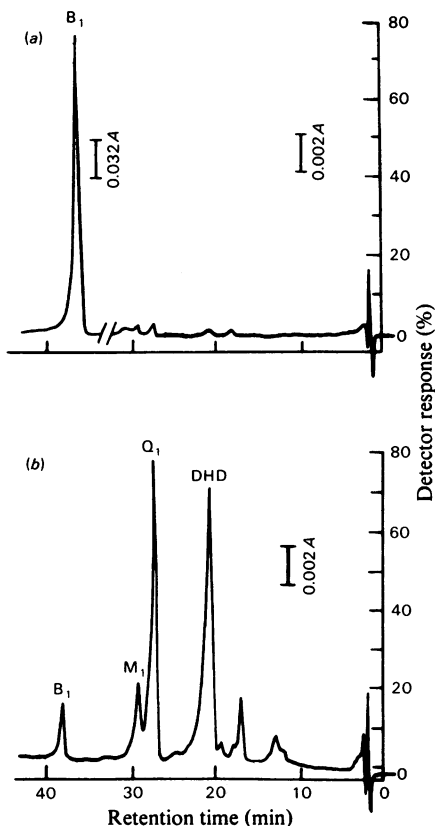


Fig. 4. Separation of methanol-soluble metabolites of aflatoxin B_1 on a reversed-phase h.p.l.c. column

Samples representing equal fractions of the original incubation medium were injected on to a Zorbax-ODS column (25cm \times 2.1mm internal diameter). Elution was carried out by a linear gradient of 10–60% methanol in water. Eluting solvents contained 0.01% phosphoric acid. A constant column pressure of 20.67MPa was maintained, with a flow rate of 0.45ml/min initially and 0.55ml/min finally. Microsomal preparations were from livers of rats pretreated with phenobarbitone. Note that as shown by the vertical bars there are different detector sensitivities. Incubations were without (a) or with (b) NADPH. DHD, 2,3-dihydrodiol.

B_1 was confirmed in experiments in which ^{14}C -labelled aflatoxin B_1 was used as substrate (Figs. 5a and 5b). The recoveries of ^{14}C at various stages during these experiments is indicated in Table 2. Further experiments were carried out to compare the results obtained when the patterns of metabolites formed by microsomal preparations from control or phenobarbitone-pretreated animals were examined by the silica and the reversed-phase column systems. The results obtained with the Zorbax-ODS column

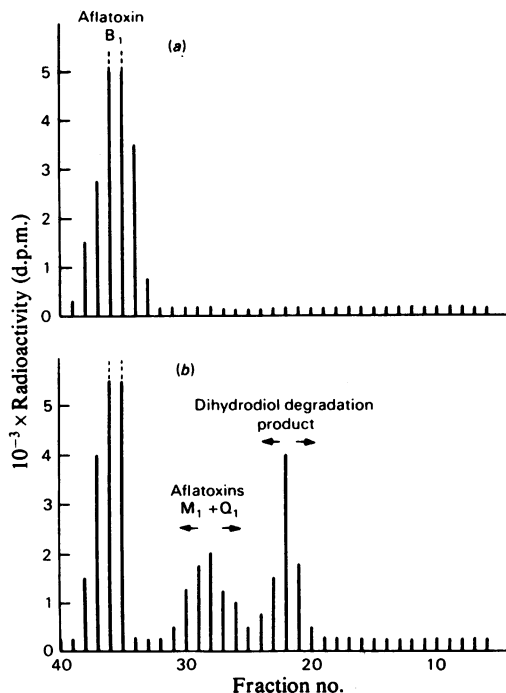


Fig. 5. Separation of methanol-soluble ^{14}C -labelled metabolites on a reversed-phase h.p.l.c. column after microsomal metabolism of [^{14}C]aflatoxin B_1

H.p.l.c. conditions were as in Fig. 4. Fractions were collected at 1min intervals. Microsomal preparations were from rats pretreated with phenobarbitone, and were incubated with (b) or without (a) NADPH.

are summarized in Table 3. Comparing the results given in Fig. 3, obtained by using the silica column, with those in Table 3, obtained by using the reversed-phase column, shows that both techniques indicated that a 4–5-fold increase in production of aflatoxin Q_1 and a 1–2-fold increase in aflatoxin M_1 production resulted from phenobarbitone pretreatment of the animals. In addition the Zorbax-ODS technique also indicated that a 4–5-fold increase in aflatoxin B_1 , 2,3-dihydrodiol production resulted from the pretreatment of the animals with phenobarbitone.

Metabolism of aflatoxins M_1 , Q_1 and P_1 . In view of the possible significance of the production of more polar metabolites in terms of the activation of aflatoxin B_1 (Campbell & Hayes, 1976) and the differing toxicities of the various aflatoxins, we have examined the possible metabolism of aflatoxins M_1 , Q_1 and P_1 to more polar compounds by microsomal incubations with the Zorbax-ODS systems *in vitro*. Because only small amounts of these materials were available, the standard incubation volume was decreased by 50%. The results of these incubation

Table 2. Recoveries of ^{14}C during extraction of microsomal metabolites of [^{14}C]aflatoxin B_1 . For details of incubation and extraction procedures see the Materials and Methods section. Results are means of duplicate assays from at least duplicate incubations and are expressed as nmol of aflatoxin $\text{B}_1 \pm$ the percentage range of the results.

Fraction	[^{14}C]Aflatoxin recovered		
	Control		Phenobarbitone-induced
	-NADPH	+NADPH	+NADPH
Initial methanol precipitate*	0.44 \pm 3	2.26 \pm 8	3.88 \pm 6
Methanol-insoluble freeze-dried material†	0.21 \pm 12	0.18 \pm 22	1.23 \pm 4
Methanol-soluble freeze-dried material‡	29.3 \pm 1	25.38 \pm 1	24.12 \pm 5
Total accounted for	30	28	29
Percentage of initial [^{14}C]aflatoxin added to incubation flasks	88	81	85

* Mainly microsomal proteins

† Mainly methanol-insoluble salts derived from the incubation medium + small amount of protein.

‡ Final extract as applied to Zorbax-ODS column.

Table 3. Effect of pretreatment with phenobarbitone *in vivo* on the disappearance of aflatoxin B_1 and the production of aflatoxins M_1 , Q_1 and 2,3-dihydrodiol by liver microsomal preparations *in vitro*

Aflatoxins were extracted in methanol and separated on a Zorbax-ODS column as detailed in legend to Fig. 4. Incubations were carried out as detailed in the Materials and Methods section. Values under 'Contents' are amounts of the aflatoxin B_1 and its metabolites found to be present after incubation with microsomal preparations from phenobarbitone-pretreated rats, expressed as % of the amount of the same compounds present after incubations with microsomes isolated from control animals. Means of duplicate incubations are shown. Variation was <10% of the means.

Aflatoxin	Contents
B_1	45
M_1	155
Q_1	445
B_1 2,3-dihydrodiol	430

metabolite was found, but, in addition, a metabolite having the same retention time as aflatoxin B_1 appeared as well as small amounts of more polar metabolites. The identity of the former metabolite as aflatoxin B_1 was confirmed by co-chromatography with authentic aflatoxin B_1 .

Lack of effect of adding reduced glutathione or 1,2-epoxy-3,3,3-trichloropropane to incubations. It appeared possible that a strongly electrophilic epoxide and its subsequent hydration by epoxide hydase activity could be involved in the production of the dihydrodiol. Therefore in some experiments the nucleophilic compounds reduced glutathione (1mM) and the epoxide hydase inhibitor 1,2-epoxy-3,3,3-trichloropropane (0.5mM) were added to incubations with the standard incubation procedures and aflatoxin B_1 . Microsomal preparations from phenobarbitone-pretreated rats were used because of their greater metabolizing capacity. No differences in either the pattern or the amounts of metabolites resulted from adding these compounds to the incubation medium.

Discussion

Our data on the principal non-polar chloroform-soluble metabolites of aflatoxin B_1 produced by rat liver microsomal preparations obtained with both silica and reversed-phase h.p.l.c. columns in the present study, and their relative abundances, are generally in agreement with reports of other workers. In agreement with Dahms & Gurtoo (1976) and Roebuck & Wogan (1977), who used t.l.c. methods, we found approximately twice as much aflatoxin Q_1 as aflatoxin M_1 to be formed in the incubations of microsomal fractions isolated from control rats. In

studies are given in Figs. 6(a), 6(b) and 6(c). Aflatoxin P_1 was not metabolized in the microsomal incubation systems either in the presence or absence of NADPH. Aflatoxin M_1 underwent very limited further metabolism in the presence of NADPH to produce more polar compounds which in view of their retention times on the Zorbax-ODS system qualitatively appeared similar to the metabolic products of aflatoxin B_1 . The metabolism of aflatoxin Q_1 was complicated. In the presence of microsomal fraction but absence of NADPH, there was a considerable conversion into a compound which was eluted later than aflatoxin Q_1 from the Zorbax-ODS column. When NADPH was present, less of the unidentified

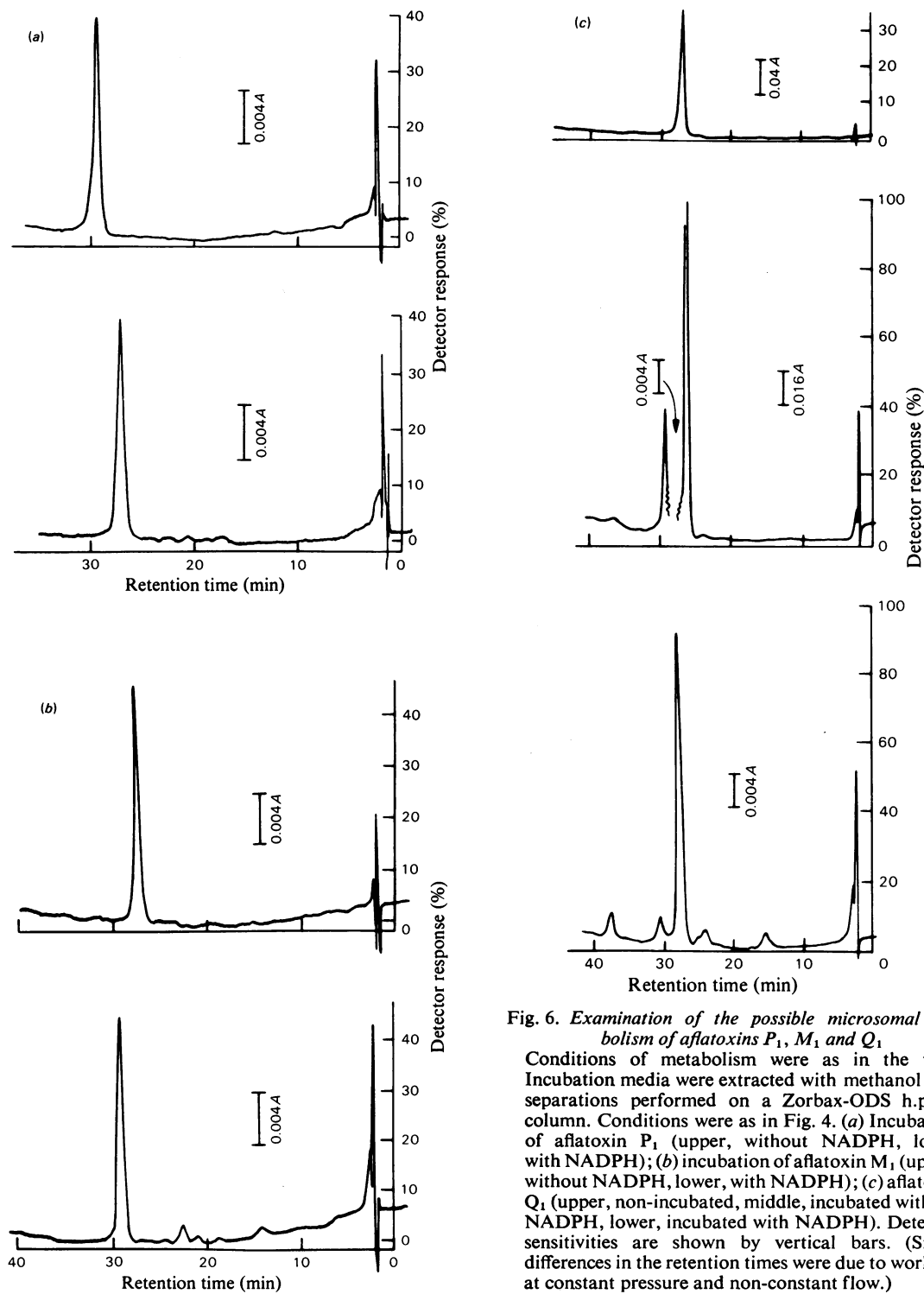


Fig. 6. Examination of the possible microsomal metabolism of aflatoxins P₁, M₁ and Q₁

Conditions of metabolism were as in the text. Incubation media were extracted with methanol and separations performed on a Zorbax-ODS h.p.l.c. column. Conditions were as in Fig. 4. (a) Incubation of aflatoxin P₁ (upper, without NADPH, lower, with NADPH); (b) incubation of aflatoxin M₁ (upper, without NADPH, lower, with NADPH); (c) aflatoxin Q₁ (upper, non-incubated, middle, incubated without NADPH, lower, incubated with NADPH). Detector sensitivities are shown by vertical bars. (Small differences in the retention times were due to working at constant pressure and non-constant flow.)

our experiments, however, pretreatment with phenobarbitone resulted not only in a greatly increased rate and extent of overall metabolism of aflatoxin B₁, but also in a disproportionate increase in aflatoxin Q₁ compared with aflatoxin M₁. This result is in contrast with that of Dahms & Gurtoo (1976), who found in their experiments that the relative proportions of aflatoxins Q₁ and M₁ in the medium after microsomal incubations *in vitro* did not alter as a result of pretreating the animals with phenobarbitone. The finding in our experiments that the production of aflatoxin Q₁ by microsomal fractions increased by 4–5-fold as a result of pretreating the rats with phenobarbitone, whereas the production of aflatoxin M₁ was increased by 1–2-fold, indicates that the different metabolic pathways leading to formation of these two metabolites, although both presumably mediated by cytochrome P-450 since they are both stimulated by phenobarbitone pretreatment, are not so tightly integrated that stimulation of the metabolic formation of one compound automatically results in a proportionate stimulation in the production of the other. Multiple forms of cytochrome P-450 with different substrate specificities have been reported (Lu & Levin, 1974). It was also found that pretreatment with phenobarbitone increased the formation of the compound formed by the spontaneous degradation of aflatoxin B₁ 2,3-dihydrodiol in neutral Tris/HCl buffer to approximately the same extent as the increase in production of aflatoxin Q₁ but not aflatoxin M₁. In agreement with Roebuck & Wogan (1977) and Dahms & Gurtoo (1976), we found aflatoxin P₁ production to be at or below the minimum level of detection. Aflatoxin B_{2a} and aflatoxicol were not found in the medium after microsomal incubations. Schabort & Steyn (1969) made the original claim that the hemiacetal, aflatoxin B_{2a}, could be produced by microsomal metabolism of aflatoxin B₁. Subsequently it has appeared that, although aflatoxin B_{2a} may be a major metabolite of aflatoxin B₁ in microsomal fractions isolated from a wide variety of species, it binds strongly to microsomal proteins and so is often not detectable in extracts of free metabolites. In agreement with this, Dahms & Gurtoo (1976) also failed to detect aflatoxin B_{2a} in their experiments with microsomal fractions isolated from control and phenobarbitone-stimulated rats and mice. Gurtoo (1973) has shown the probable presence of bound aflatoxin B_{2a} on rat liver microsomal preparations by means of the absorption spectrum of protein-bound aflatoxin B_{2a}. In our experiments with [¹⁴C]aflatoxin B₁, 5% of the added label became bound to microsomal protein when control microsomal fractions were used and 15% with microsomal fractions obtained from phenobarbitone-pretreated rats. These values might therefore give estimates of the amounts of aflatoxin B_{2a} formed in these incubations. However, the possibility

that the protein binding could have occurred via formation of the aflatoxin B₁ 2,3-epoxide must also be considered.

The formation of aflatoxin B₁ 2,3-dihydrodiol could be dependent upon primary activation of aflatoxin B₁ via epoxidation on C-2 and C-3. Although the epoxide has not been detected, owing presumably to its highly unstable nature, Swenson *et al.* (1975) have synthesized aflatoxin B₁ 2,3-dichloride and have found it to have the strong electrophilic nature and direct carcinogenic potential predicted for the aflatoxin B₁ epoxide. The amount of the dihydrodiol or its degradation product could give a quantitative estimate as to the extent of the activation in the absence of the free epoxide. The results obtained in the present study with microsomal fractions from phenobarbitone-pretreated animals indicate that epoxide formation might have increased as a result of this treatment. This is in agreement with the greater potency of microsomal preparations obtained from phenobarbitone-pretreated animals *in vitro*, assessed by the lethality of aflatoxin B₁ towards micro-organisms (Garner *et al.*, 1972), and also by the inhibition of RNA synthesis in hepatic nuclei *in vitro* (Neal & Godoy, 1976). However, the results obtained when 1,2-epoxy-3,3,3-trichloropropane or reduced glutathione were added to the incubations, appear to be in conflict with the proposal that the primary activation of aflatoxin B₁ depends on the formation of a strongly electrophilic epoxide, which is subsequently converted into a dihydrodiol via epoxide hydrase. Using benzopyrene in microsomal metabolizing systems, Freundenthal *et al.* (1975) found that the addition of 0.05 mM 1,2-epoxy-3,3,3-trichloropropane to the incubation system resulted in almost complete inhibition of the production of benzopyrene dihydrodiols and more polar metabolites, presumably by inhibiting the activity of epoxide hydrase, and a striking increase in the production of less polar metabolites. A similar result might have been expected in the present study when 1,2-epoxy-3,3,3-trichloropropane at the same concentration as that used by Freundenthal *et al.* (1975) (0.05 mM) was added to the aflatoxin B₁-metabolizing microsomal system. However, if aflatoxin B₁ is first activated to form a 2,3-epoxide and then is further converted to form the 2,3-dihydrodiol, our results would indicate that this latter step is either non-enzymic or else takes place in a system not susceptible to inhibition by 1,2-epoxy-3,3,3-trichloropropane. Furthermore the nucleophile reduced glutathione did not, as might have been expected, decrease the production of the 2,3-dihydrodiol by reacting with an electrophilic metabolite of aflatoxin B₁ (Guengerich, 1977). Such unexpected behaviour in aflatoxin B₁-metabolizing systems *in vitro* has been previously reported by Garner & Wright (1973), who found that in the presence of a range of nucleo-

philic compounds at the concentration used in this present study (1mM) there was no decrease in the lethality of the microsomal formed aflatoxin B₁ metabolite *in vitro*. We have found similar results on examining the formation *in vitro* of aflatoxin B₁ metabolites that are capable of inhibiting nucleic acid synthesis in isolated nuclei (G. E. Neal & D. J. Judah, unpublished results). It would appear therefore that the mechanism of the activation of aflatoxin B₁ by microsomal systems needs further attention and the reverse-phase h.p.l.c. system described in this paper could facilitate such studies.

In view of the possible importance of metabolic activation of aflatoxin B₁ to its biological potency the possible further metabolism of the aflatoxin B₁ metabolites aflatoxins P₁, M₁ or Q₁ would be of interest. Aflatoxins P₁ and Q₁ have low biological potencies, whereas aflatoxin M₁ is as acutely toxic in many species as aflatoxin B₁, but has a much diminished carcinogenic potential (Wogan & Paglialunga, 1974).

Aflatoxin M₁

If the toxicities of the aflatoxins are mediated via microsomal activation followed by nuclear binding, and the extent of the microsomal activation is indicated by the amount of polar metabolites formed, it is difficult to see from the present results how the acute toxicities of aflatoxins B₁ and M₁ could be similar. Aflatoxin M₁ underwent minimal metabolism and formed only very small amounts of a compound which was eluted at approximately the same retention time as the degradation product of aflatoxin B₁ 2,3-dihydrodiol. At present little is known about the nuclear binding of metabolites of aflatoxin B₁. It has been shown (Neal & Godoy, 1976) that aflatoxin M₁ requires activation by microsomal preparations in order to inhibit nuclear RNA synthesis in isolated nuclei. The possibility still exists, however, that in both aflatoxin M₁ and B₁ the nuclear binding is significant only in terms of the carcinogenic response and that binding to various subcellular organelles, for example, rough endoplasmic reticulum and smooth endoplasmic reticulum (Williams *et al.*, 1973), which does not depend on metabolism, could be responsible for the acutely toxic reaction.

Aflatoxin P₁

Aflatoxin P₁ was not metabolized by our microsomal systems. The absence of this metabolite from the incubations therefore cannot be explained in terms of its rapid further metabolism. This result is somewhat puzzling, since Wogan *et al.* (1967) have reported, on the evidence of relative rates of evolution of ¹⁴CO₂ after the administration *in vivo* of [methoxy-

¹⁴C]- or [*ring*-¹⁴C]-aflatoxin B₁ to rats, that demethylation (presumably forming aflatoxin P₁) was a major metabolic pathway in rats *in vivo*. In contrast with rat liver microsomal preparations, aflatoxin P₁ has been reported to be a major metabolite when aflatoxin B₁ is metabolized by mouse microsomal preparations (Dahms & Gurtoo, 1976). The reason for this discrepancy between the results obtained *in vivo* and *in vitro* in rats requires further study.

Aflatoxin Q₁

Aflatoxin Q₁ was stable in the incubation medium in the absence of microsomal preparation. In the presence of microsomal fraction and absence of NADPH, however, there was metabolism of aflatoxin Q₁. It is of interest that, in the presence of NADPH, metabolism of aflatoxin Q₁ to aflatoxin B₁ and also to more polar metabolites with similar retention times to those seen in aflatoxin B₁ incubations was observed. It has been suggested that the limited mutagenicity of aflatoxin Q₁ (a major metabolite in incubations *in vitro* with human and monkey microsomal preparations; Roebuck & Wogan, 1977) may reflect a requirement for its prior conversion into aflatoxin B₁ (Wong & Hsieh, 1976). The results of the present study would be in agreement with the feasibility of this reaction. It is noteworthy that the limited production of polar metabolites observed when aflatoxin M₁ was incubated was not accompanied by the appearance of aflatoxin B₁.

In conclusion, the reversed-phase h.p.l.c. system described in this paper has much to recommend it as a means of evaluating the spectrum of aflatoxin metabolites, polar and non-polar, present in the same extract.

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