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Use of Monkey Liver Microsomes in Production of Aflatoxin Q_1

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Aflatoxin Q_1, a newly identified monkey liver metabolite of aflatoxin B_1, is prepared in milligram quantities in the crystalline form by biotransformation of aflatoxin B_1 using monkey liver microsomal preparations. Parameters needed for quantitation of aflatoxin Q_1 using a spectrodensitometer are determined with the aid of radiolabeled metabolites. An amount as small as 1 ng can be measured with certainty. The availability of aflatoxin Q_1 will make possible studies on its chemical properties, toxicity, and possible role in the carcinogenicity of aflatoxin B_1.

Aflatoxin Q_1 is a major metabolite of aflatoxin B_1 when the latter is biotransformed by the monkey or rat liver preparations (Masri *et al.*, 1973). It has been identified as an isomer of aflatoxin M_1 with the hydroxyl at the carbon β to the carbonyl of the cyclopentenone ring. Its uv, mass, and nmr spectral data as well as structural assignments have been reported (Masri *et al.*, 1974).

In monkey liver microsomal preparations, as much as 52% of aflatoxin B_1 is converted to aflatoxin Q_1; therefore, it is important to determine the chemical properties, toxicity, and biochemical role of this major aflatoxin metabolite. In the present work, the optimum conditions determined in the *in vitro* metabolic studies with the Rhesus monkey livers (Dalezios *et al.*, 1972) were used to produce relatively large quantities of aflatoxin Q_1 for use in analytical, toxicological, and biochemical studies.

EXPERIMENTAL SECTION

Aflatoxins. Radioactive aflatoxin B_1, labeled at the ring carbons, was prepared from cultures of *Aspergillus parasiticus* ATCC 15517 supplemented with [1-^{14}C]acetate according to the method of Hsieh and Mateles (1971) and was identified by cochromatography with the unlabeled standard on thin-layer plates. Radiological purity was monitored by autoradiography of the distribution of radioactivity on thin-layer chromatograms, as described in the following section. Nonradioactive aflatoxin B_1 was purchased from Calbiochem, La Jolla, Calif., and was used after recrystallization from benzene.

Analysis. Thin-layer chromatographic plates 250 μ in thickness were prepared from Adsorbosil-1 silica gel (Applied Science Labs, State College, Pa.). The plates were developed with one of the following solvent systems: chloroform–acetone–water (88:12:1.5, ratios all by volume) (Stubblefield *et al.*, 1969), chloroform–acetone–*n*-hexane (85:15:20) (Teng and Hanzas, 1969), or 3% methanol in chloroform. The fluorescent spots were visualized under long-wave (366 nm) ultraviolet light. Autoradiographs were made using Kodak medical No-Screen X-ray films (Eastman Kodak Co., Rochester, N. Y.). Zones containing radioactivity were scraped into vials containing toluene–Triton X-100 scintillation cocktail (Packard Instrument Co., Downers Grove, Ill.), and the radioactivity was measured using a Packard Tri-Carb Model 2425 liquid scintillation spectrometer.

Ultraviolet absorption spectra were obtained with a Cary 15 recording spectrophotometer. A double-beam spectrodensitometer, Schoeffel Model 3000-2 (Westwood, N. J.), with a mercury–xenon lamp and interference wedge monochromators (400–700 nm) between the tlc plates and the photomultipliers, was used to quantify the aflatoxins. The excitation wavelength was 360 nm, and the interference wedge monochromators were set at 410 nm.

The mass spectrum was obtained with a CEC Model 21-110 mass spectrometer with electron multiplier detection.

Enzyme Preparation. The experimental Rhesus monkeys (male, 3–4 kg) were housed in the National Center for Primate Biology, Davis, Calif. There they were maintained on a Purina chow diet supplemented with fresh fruits.

When needed, the liver (120–160 g fresh weight) was excised and perfused with saline (1.15% KCl), and a 20% (w/v) homogenate was prepared in saline using a Waring Blendor. The homogenate was filtered through a piece of coarse-meshed nylon organdy to remove fibrous debris. The preparation was then centrifuged at 10,000*g* for 30 min in a Sorvall RC-2B centrifuge, after which the supernatant was decanted and centrifuged at 105,000*g* for 1 hr in a Beckman Model L-2 ultracentrifuge equipped with a 50 Ti fixed-angle rotor. The microsomal pellets were resuspended in buffer, and the protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Incubation. The microsomal preparations were incubated with shaking for 1 hr at 37° in ten open 500-ml erlenmeyer flasks. The reaction mixture, final volume 100 ml, contained 500 μmol of phosphate buffer (pH 7.4), 24 μmol of glucose 6-phosphate, 1.1 μmol of $NADP^+$, 100

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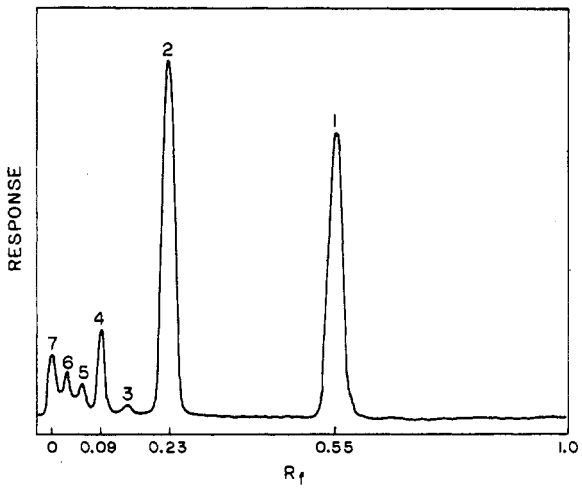

Figure 1. Mobility of aflatoxin B_1 and its monkey liver metabolites on an Adsorbosil-1 silica gel thin-layer plate developed with chloroform–acetone–*n*-hexane (88:15:20), as recorded on a spectrodensitometer: (1) B_1; (2) Q_1; (3) X; (4) M_1; (5) B_{2a}; (6) Y; (7) origin.

units of glucose-6-phosphate dehydrogenase, and 27 μmol of KCl. The substrate, a mixture of [ring-^{14}C]- and nonradioactive aflatoxin B_1, was dissolved in dimethylformamide or methanol immediately before use, and a 2-ml portion containing 3 mg of substrate was added to each reaction mixture. The reaction was initiated by addition of 10 ml of microsomal suspension (~3 mg of protein/ml or 0.2 g of liver/ml). Liver microsomes from male Sprague-Dawley rats (250 g) were also isolated and incubated in the same manner.

Extraction and Purification. The reaction was terminated by adding 60 ml of ice-cold methanol to each flask and immediately placing the flasks into ice. The mixtures were extracted five times with 100-ml aliquots of chloroform using a mechanical shaker (Extracto-Matic, Virtis Co., Gardiner, N. Y.). Each preparation was shaken for 15 min, and the resulting emulsion was transferred to separatory funnels and centrifuged in an I.E.C. centrifuge Model EXD at 800*g* for 10 min to sharpen the phase partition. The chloroform extracts were pooled, reduced in volume under vacuum with a flash evaporator at 37°, and then evaporated to dryness under nitrogen at room temperature. The residue was redissolved in 10 ml of 3% methanol in chloroform.

The number and amounts of the chloroform-soluble metabolites of aflatoxin B_1 were determined by thin-layer chromatography (tlc) followed by autoradiography and liquid scintillation counting. The per cent of aflatoxin B_1 biotransformed to water-soluble metabolites was determined by scintillation counting of samples of the aqueous phase.

Half of the chloroform extract (5 ml, corresponding to five flasks) was concentrated to 1 ml and chromatographed on a column (1.2 cm × 30 cm) containing 10 g of Merck silica gel (0.05–0.20 mm, activated at 105° for 1 hr) packed as a slurry in chloroform. One gram of anhydrous sodium sulfate was layered at both the top and the bottom of the column. The column was irrigated with 200 ml of chloroform to remove the unmetabolized aflatoxin B_1. The more polar metabolites were then eluted stepwise with 150-ml mixtures of 0.5, 1.5, 3.0, and 5.0% of methanol in chloroform. The flow rate was adjusted to 30 ml/hr, and 10-ml fractions were collected. The fractions containing aflatoxin Q_1 (fractions numbered 27–39 as monitored by tlc) were combined and Q_1 was repeatedly crystallized from benzene.

Table I. Relative Fluorescence Intensity of Aflatoxins on Plates Coated with Adsorbosil-1

Aflatoxin	Radioact., dpm[a]	Amount, nmol	Area, cm^2	Rel response	R_f[b]
B_1	252 ± 18	0.320	3.81 ± 0.37	1.00	0.55
Q_1	414 ± 32	0.525	4.54 ± 0.61	0.73	0.27
M_1	171 ± 16	0.217	2.60 ± 0.13	1.01	0.13

[a] Specific activity of [^{14}C]aflatoxin B_1 used: 788 dpm/nmol. [b] Thin-layer plates developed with chloroform–acetone–*n*-hexane (85:15:20).

Table II. Transformation of Aflatoxin B_1 to Aflatoxins Q_1 and M_1

Runs	Transformation, %[a] Q_1	M_1	W[e]	Residual B_1, %[a]
Monkey microsomes				
1st run[b]	32.4	7.6	20.3	10.6
2nd run[c]	32.2	2.0	21.2	14.2
Rat microsomes				
One run[d]	2.4	2.9	2.0	90.3

[a] Per cent of initial radioactivity of the substrate. Each value is the mean of four samples. Approximately 30% of the radioactivity in the extract of monkey liver reaction mixtures was retained in the origin on the developed tlc plates. [b] 37 mg of protein/flask, one flask. [c] 27 mg of protein/flask, nine flasks. [d] 27 mg of protein, 0.8 mg of substrate/flask, two flasks. [e] Non-chloroform-extractable fraction (aqueous phase).

RESULTS AND DISCUSSION

In three runs with a total of 30 flasks, 90 mg of [^{14}C]aflatoxin B_1 was used. From this about 15 mg of crystallized aflatoxin Q_1 was obtained. The metabolite has mp 295° dec, a principal *m/e* peak at 328, and λ_{max} (in methanol) 336, 267, and 223 nm (Masri *et al.*, 1974). On chromatoplates, aflatoxin Q_1 had an intense green fluorescence under uv and occupied an intermediate position between aflatoxin B_1 and M_1. Its mobility (R_f values) and the relative amount among other metabolites separated by tlc are shown in Figure 1. The monkey liver microsomes catalyzed the transformation of aflatoxin B_1 into five fluorescent metabolites, each more polar than the substrate. The known metabolites other than aflatoxin Q_1 were identified on the basis of their identical chromatographic behavior with authentic standards.

Analysis of these metabolites using the Schoeffel spectrodensitometer was supplemented by radioanalysis. The relative responses are shown in Table I. The fluorescent intensity of aflatoxin Q_1 on a tlc plate under the fluorodensitometric determination at 360 nm appears to be about 70% that of aflatoxin B_1 or M_1; therefore, an amount as little as 1 ng can be detected with considerable certainty. Use of the Schoeffel spectrodensitometer for quantitation of aflatoxins and their metabolites has been described by several investigators (Schabort and Steyn, 1969; Rodricks *et al.*, 1970; Friedman and Yin, 1973; Stoloff, 1973). A limit of detectability as low as 0.1 ng has been established for aflatoxin B_1. The importance of aflatoxin Q_1 in the *in vivo* metabolism is uncertain. In an earlier *in vivo* study, aflatoxin P_1 was the principal metabolite present in the urine of Rhesus monkeys administered aflatoxin B_1 by intraperitoneal injection (Dalezios *et al.*, 1971). In a recent study on the excretion and metabolism of orally administered aflatoxin B_1 by Rhesus monkeys, aflatoxin M_1 rather than aflatoxin P_1 or Q_1 was identified as the principal urinary metabolite (Dalezios *et al.*, 1973).

The per cent of aflatoxin B_1 transformed to aflatoxins Q_1 and M_1, measured as radioactivity, by monkey and rat liver microsomal enzyme systems is shown in Table II.

The aflatoxin Q_1 produced in the rat liver system was identified by co-chromatography with the major aflatoxin metabolite of the monkey liver system using various tlc systems. Approximately one-third of the incubated aflatoxin B_1 was metabolized to aflatoxin Q_1 by the monkey liver microsomes. Another one-third of radioactivity was retained in the origin region of the developed tlc plates. None of the five fluorescent metabolites were formed when heat deactivated microsomes were used in control experiments. When 10-ml volumes of the reaction mixture were incubated in 50-ml baffled conical flasks in the *in vitro* metabolic studies (Dalezios *et al.*, 1972), as much as 52% of aflatoxin B_1 was converted to aflatoxin Q_1. The relatively low percentage conversion observed in the preparatory runs was perhaps due to the insufficient oxygen supply in the 100-ml volumes. The significant conversion of aflatoxin B_1 to Q_1 was also observed in similar experiments using microsomal preparations from livers of two other species of monkeys *Macaca irus* and *Saimiri sciureus*. The extent of conversion ranged from 16 to 41% of the added aflatoxin B_1 at a substrate level of 0.2–1 mg of aflatoxin B_1 per gram equivalent of liver.

Compared to monkey liver preparations, rat liver microsomes possessed much lower catalytic activity for conversion of aflatoxin B_1 to Q_1. Only 2–3% of aflatoxin B_1 was biotransformed to Q_1. Unfortunately this low activity makes readily available rat liver microsomes an unsuitable enzyme source for the production of Q_1. Production of aflatoxin Q_1 so far has not been reported but the green fluorescing metabolite of aflatoxin B_1 in rat liver with similar tlc R_f values as recently described by Friedman and Yin (1973) was probably also aflatoxin Q_1.

Approximately 20% of the substrate was converted to non-chloroform-extractable materials. The loss of substrate could have been due to the formation of aflatoxin B_1 hemiacetal, followed by degradation of this compound in the phosphate-buffered protein solution (Patterson and Roberts, 1970). These materials have not been further characterized as they do not interfere with the production of aflatoxin Q_1.

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Toxic Effects of Oosporein from *Chaetomium trilaterale*

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A toxin-producing strain of *Chaetomium trilaterale* was isolated from moldy peanuts. A red crystalline metabolite, subsequently purified from the fungal culture broth, had a median oral lethal dose of 6.12 mg/kg in day-old cockerels. The toxin also produced plant growth inhibiting and phytotoxic effects. The toxin was conclusively identified as the dibenzoquinone, oosporein (3,3′,6,6′-tetrahydroxy-5,5′-dimethyl-2,2′-bi-*p*-benzoquinone).

Chaetomium spp. have been isolated from various agricultural commodities *including* feeds and food products such as peanuts, rice, flour, spaghetti products, and black and red pepper (Christensen *et al.*, 1968; Kurata *et al.*, 1968; Hanlin, 1973). Christensen *et al.* (1968) reported that 83% of the *Chaetomium* spp. isolated from feeds and foods were lethal to rats. In this study the toxigenic factors were not elaborated. In an unrelated study, Christensen *et al.* (1966) showed that toxicity of moldy corn infected with *Chaetomium globosum* was due to chetomin, an epipolythiadiöxopiperazine compound. Brewer *et al.* (1971) reported that *Chaetomium* spp. were implicated in the growth syndrome of sheep in Nova Scotia.

We wish to report the isolation of a toxigenic isolate of *Chaetomium trilaterale* Chivers from moldy peanuts, and the chemical identification of the major toxic metabolite as oosporein.

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MATERIALS AND METHODS

Isolation of Toxin. *Chaetomium trilaterale* Chivers (ATCC accession number 24912) was cultured in Fernbach flasks (2.8 l.) containing 200 ml of Difco mycological broth (pH 4.8) supplemented with 2% yeast extract and 15% sucrose. After 14-days growth at 28°, the culture broth was acidified to pH 2.0 and extracted three times with ethyl acetate. Toxicity was associated with a red crystalline material that formed after concentration of the