might, in fact, increase the digestibility of peanuts and thereby increase their nutritional value. Investigations are in progress to explore these possibilities.

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Aflatoxin Q_1. A Newly Identified Major Metabolite of Aflatoxin B_1 in Monkey Liver

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Incubation of aflatoxin B_1 with monkey liver microsomal preparations in phosphate buffer (pH 7.4), in the presence of an NADPH regenerating system, resulted in the formation of two main metabolites. One was aflatoxin M_1 (1–3% of the substrate) and the other was a novel metabolite (16–52% of the substrate) which we isolated and identified as an isomer of aflatoxin M_1, with the hydroxyl on the carbon atom β to the carbonyl of the cyclopentenone ring. The name aflatoxin Q_1 was proposed for this newly identified metabolite.

The potent fungal hepatotoxin and hepatocarcinogen aflatoxin B_1 requires metabolic activation to become the immediate carcinogenic agent. This activation occurs with liver microsomal fractions (Goodall and Butler, 1969; Garner *et al.*, 1972; Moule and Frayssinet, 1972; Ames *et al.*, 1973). The difference in susceptibility to aflatoxin B_1 carcinogenesis varies among species and may be related to variation in metabolism (Patterson, 1973). Although susceptibility of man is not clearly known, exposure to aflatoxin appears associated with increased incidence of liver cancer in certain populations (Shank and Wogan, 1972). Comparative metabolic studies in different species including primates are thus relevant to the question of susceptibility in man.

Aflatoxin M_1 is recognized as a major *in vivo* metabolite in mammals (de Iongh *et al.*, 1964; Allcroft *et al.*, 1966; Holzapfel *et al.*, 1966; Masri *et al.*, 1967). Conversion of aflatoxin B_1 to M_1 (1–3%) by rat liver microsomal fractions has been shown (Portman *et al.*, 1968; Masri *et al.*, 1969; Patterson and Allcroft, 1970; Patterson and Roberts, 1971). More recently, aflatoxin P_1, an O-demethylated product of aflatoxin B_1, was shown to be a major *in vivo* metabolite in monkeys given aflatoxin B_1 by intraperitoneal injection (Dalezios *et al.*, 1971). Aflatoxin P_1 appeared in the urine mainly in conjugated form (glucuronide and sulfate) and represented about 20% of the dose, whereas aflatoxin M_1, which was also formed, accounted for only 2.3% of the dose.

Aflatoxin M_1 does not appear to be the immediately active carcinogenic metabolite in a rat microsome mediated bacterial test system for carcinogens (mutagens) (Garner *et al.*, 1972; Ames, 1973). Aflatoxin P_1 was not toxic to the chicken embryo at levels that produce significant mortality when aflatoxin B_1 is used (Stoloff *et al.*, 1972).

We reported the conversion of aflatoxin B_1 by rat and monkey liver postmitochondrial preparations to two main metabolites: aflatoxin M_1 and a newly identified metabolite which we referred to as aflatoxin Q_1 (Masri *et al.*, 1973). We present here evidence of the structure of aflatoxin Q_1 as an isomer of aflatoxin M_1 with the hydroxyl group on the carbon atom β to the carbonyl of the cyclopentenone ring.

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EXPERIMENTAL SECTION

Aflatoxin B_1. This was extracted from cultures of *Aspergillus parasiticus* NRRL 2999. It was purified by col-

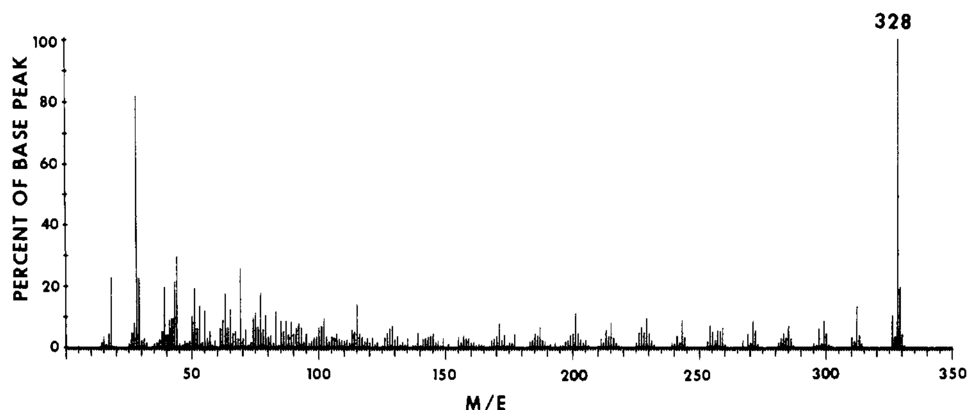


Figure 1. Mass spectrum of aflatoxin Q_1 taken at 70 eV and source temperature 210°

umn chromatography on silica gel and recrystallization from chloroform–methanol (Masri, 1970). Its identity and purity were checked by tlc on silica gel HR, and by ultraviolet, nmr, and mass spectra.

Incubation Extraction and Isolation Procedures. These will be described in detail elsewhere (Hsieh *et al.*, 1974). Briefly, aflatoxin B_1 dissolved in dimethylformamide was incubated with the 9000*g* or 30,000*g* liver supernatant fraction in 0.1 *M* phosphate buffer (pH 7.4) for 1 hr at 37° in air in the presence of the NADPH regenerating system. The metabolites were extracted with chloroform after an equal volume of methanol was added to the incubation mixture. The residue in the chloroform extract was examined by tlc, and the metabolites were quantitated (Hsieh *et al.*, 1974; Masri, 1970). The remainder of the residue was chromatographed on silica gel columns, using chloroform and then chloroform containing 1–3% methanol for elution (Masri, 1970). Positions of the aflatoxins on the column were visualized by long-wavelength ultraviolet light. Aflatoxin Q_1, which has intense yellow-green fluorescence, emerged between aflatoxins B_1 and M_1. It was isolated from the appropriate eluate as colorless needles from methanol–chloroform or hot acetonitrile, mp 295° dec.

The livers used for isolation and structure proof of aflatoxin Q_1 were from a *Macaca irus* monkey (one animal, one experiment) and from two *Macaca mulatta* (Rhesus) monkeys (two separate experiments). Also, pooled livers from male albino rats were used in one experiment to isolate a small amount (about 1 mg) of aflatoxin Q_1 to prove identity with the monkey metabolite.

Instruments. The following spectrometers were used. Ultraviolet spectra were obtained on a Cary Model 14, infrared spectra on a Cary Model 90, nmr spectra on a Varian HA-100 (TMS as internal standard), and mass spectra on a CEC Model 21-110 spectrometer (70 eV, direct introduction).

RESULTS AND DISCUSSION

Aflatoxin Q_1 gave a crystalline *O*-acetyl derivative with acetic anhydride in pyridine. The reaction was complete overnight as revealed by tlc; the acetylated derivative also had yellow-green fluorescence under ultraviolet light and an R_f value only slightly higher than that of aflatoxin B_1. The mass and nmr spectra of *O*-acetyl Q_1 are discussed below. No further data on physical properties of *O*-acetyl Q_1 were obtained, owing to the limited amount that was available.

The ultraviolet spectrum of aflatoxin Q_1 in methanol had λ_{max} 366 (ϵ 17,500), 267 (ϵ 11,450), and 223 nm (ϵ 19,030). *O*-Acetyl Q_1 had similar ultraviolet absorption maxima.

The infrared spectrum (KBr) of aflatoxin Q_1 was similar to that of B_1 but had absorption bands at 3460 and 3550 cm^{-1} (hydroxyl).

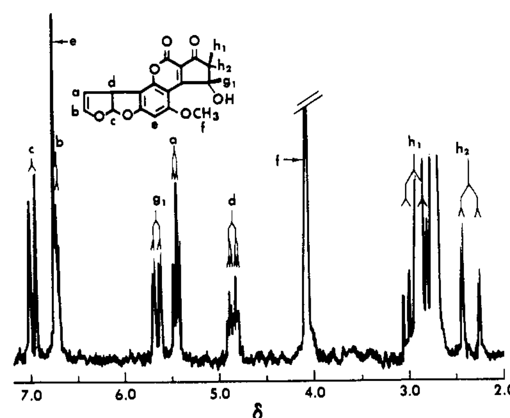


Figure 2. The 100-MHz nmr spectrum of aflatoxin Q_1 in dimethylformamide-d_7 at 65°.

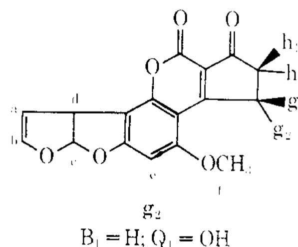
The mass spectrum of aflatoxin Q_1 (Figure 1) indicated a molecular weight of 328 and an elemental composition of $C_{17}H_{12}O_7$ (calcd for $C_{17}H_{12}O_7$, 328.0585; found, 328.0628). *O*-Acetyl aflatoxin Q_1 gave a molecular weight of 370 indicative of a monoacetyl derivative (calcd for $C_{19}H_{14}O_8$, 370.0683; found, 370.0717). The 70-eV fragmentation, as shown in Figure 1, and the temperature of volatilization, 210°, are approximately the same as for previously reported aflatoxin mass spectra (Haddon *et al.*, 1971). However, the relative abundance values of specific peaks, particularly *m/e* 312, 310, and 299, unequivocally distinguish this new metabolite from the isobaric aflatoxins M_1 and G_1 (Haddon *et al.*, 1971).

The 100-MHz nmr spectrum of aflatoxin Q_1 in dimethylformamide-d_7 is very similar to that of aflatoxin B_1 except for signals assigned to protons of the cyclopentenone ring. The aflatoxin Q_1 spectrum is shown in Figure 2, and the shielding values for the two aflatoxins are given in Table I. The assignments given below are consistent with the structure of aflatoxin Q_1 as a hydroxylated metabolite of aflatoxin B_1 with the hydroxyl group on the carbon atom β to the carbonyl of the cyclopentenone ring.

Comparison of the nmr spectra of aflatoxins B_1 and Q_1 shows that the two multiplets (4 H) due to the cyclopentenone protons of aflatoxin B_1 are replaced in the spectrum of aflatoxin Q_1 by three signals (each 1 H) as follows: (1) doublet–split doublet centered at δ 2.35 assigned to proton h_2 with vicinal coupling J_{gh}^{trans} = 1.5 Hz and geminal coupling J = 19 Hz; (2) doublet–split doublet centered at δ 2.96 assigned to proton h_1 with vicinal coupling J_{gh}^{cis} = 6.5 Hz and geminal coupling J = 19 Hz; and (3) split doublet centered at δ 5.67 assigned to proton g_1 with vicinal couplings J_{gh}^{cis} = 6.5 Hz and J_{gh}^{trans} = 1.5 Hz.

The resonance of the hydroxyl proton g_2 was not seen;

Table I. Shielding Values (δ) for Aflatoxins B_1 and Q_1 in DMF-d_7, 31°



	a	b	c	d	e	f	g_1	g_2	h_1	h_2	J_{gh}^{cis}, Hz	J_{gh}^{trans}, Hz	J_{h_1,h_2}, Hz
B_1	5.45	6.76	6.97	4.84	6.74	4.04	3.36		2.53				
Q_1	5.48	6.74	7.01	4.88	6.74	4.08	5.67	(OH)	2.96	2.35	6.5	1.5	19

its presence (aside from being consistent with the interpretation of the above couplings of the cyclopentenone protons) was further corroborated by the 100-MHz nmr spectrum taken in $CDCl_3$ on an acetylated sample of aflatoxin Q_1. Here the significant features were the appearance of a singlet (3 H) at δ 2.08 (*O*-acetyl protons) and the expected shift of 1.07 ppm to lower field of the signal due to a proton on the same carbon as the *O*-acetyl group (*i.e.*, proton g_1).

The spectrum of aflatoxin Q_1 shows the methoxyl protons (f) in Figure 2 as singlet at δ 4.08 (*cf.* at δ 4.04 for aflatoxin B_1). Thus, aflatoxin Q_1 is readily distinguishable from aflatoxin P_1 on the basis of nmr. This is emphasized since both aflatoxins appear to be major metabolites of the same substrate in the same species, but demonstrable in the case of P_1 with the *in vivo* technique, while in the case of Q_1 by means of the *in vitro* technique. The two aflatoxins are also distinguishable by tlc and mass spectra.

Aflatoxin Q_1 is also readily distinguishable from its isomer aflatoxin M_1 by tlc and nmr and mass spectroscopy. We have reported the nmr and mass spectra of aflatoxin M_1 (Masri *et al.*, 1967; Haddon *et al.*, 1971).

In our experiments with liver postmitochondrial fractions, the conversion of aflatoxin B_1 to M_1 with both rat and monkey was about 1-3% of the substrate. The conversion to aflatoxin Q_1 in the rat liver was also about 1-3%. The identity of aflatoxin Q_1 isolated from the rat liver incubation with that from the monkey liver preparations was established by comparison of the nmr and mass spectra. In contrast to the results with the rat, the conversion to aflatoxin Q_1 in the monkey liver was remarkably high. In different experiments using livers from three species of monkeys (three *Saimiri sciureus*, one *Macaca irus*, and ten *Macaca mulatta* monkeys) the conversion to aflatoxin Q_1 ranged from 19 to 52% of the substrate which was used at a level of 0.2-1.0 mg of B_1/g of liver equivalent. Thus, aflatoxin Q_1 is distinctly a major metabolite in these primates *in vitro* and may also be a major metabolite in other primates including man.

In a preliminary experiment using a human liver autopsy specimen, aflatoxin Q_1 was formed from aflatoxin B_1, but the per cent conversion was low with this specimen, which was removed about 6 hr after death. When we used ^{14}C-labeled aflatoxin B_1 in some of the incubation experiments with rat and monkey livers, we observed no evidence that aflatoxin Q_1 undergoes any extensive conjugation (*e.g.*, to glucuronide).

Although in all our experiments with monkey liver, aflatoxin Q_1 was a prominent metabolite (aflatoxin M_1 was also clearly present), a trace of a metabolite with an R_f value slightly lower than that of aflatoxin Q_1, and thus which might have been aflatoxin P_1, was occasionally observed, but the amounts were too small to permit further identification.

These *in vitro* results with the monkey are in contrast to the results of Dalezios *et al.* (1971) which were obtained *in vivo*. These workers reported the appearance of aflatoxins M_1 and P_1 in the urine of Rhesus monkeys given aflatoxin B_1 by intraperitoneal injection, but did not report on a metabolite corresponding to aflatoxin Q_1. If in their experiments, aflatoxin Q_1 was not present in the urine rather than merely overlooked, an explanation could be a rather limited production or a rapid rate of metabolic transformation of aflatoxin Q_1 in the *in vivo* system (such as further hydroxylation, or interaction and intercalation with DNA; in this regard, see Ames *et al.*, 1973). Also, the *in vitro* system permits the use of a relatively much higher concentration of substrate which cannot be achieved with aflatoxin in the whole animal.

The role of aflatoxin Q_1 in hepatocarcinogenesis remains to be answered. Experiments are in progress in collaboration with Professor B. N. Ames of the University of California at Berkeley to test aflatoxin Q_1 using a bacterial system for the detection of carcinogens (mutagens) (Ames *et al.*, 1973). Feeding trials of aflatoxin Q_1 in the trout to test its carcinogenicity are also in progress in collaboration with Professor R. O. Sinnhuber at Oregon State University, Corvallis, Ore.

A ^{13}C nmr study of aflatoxin Q_1 is also planned to confirm the position of the hydroxyl on the carbon atom β and not α to the carbonyl of the cyclopentenone ring.

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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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