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Received for review January 31, 1977. Accepted June 16, 1977. Technical Paper No. 4459, Oregon Agricultural Experiment Station, Oregon State University, Corvallis, Oregon 97331. This investigation was supported by Food and Drug Administration Grant No. 1R01 FD-00382.

Bovine Liver Metabolism and Tissue Distribution of Aflatoxin B_1

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Studies were undertaken to determine which of the aflatoxin (AF) metabolites may occur as significant residues in edible meat products from cows fed AFB_1. In the first experiment, ^{14}C-labeled AFB_1 was incubated with bovine liver preparations to determine which metabolites could be produced. About 15–22% of the AFB_1 was metabolized to AFQ_1, AFM_1, and two unidentified metabolites; 61–64% of the original AFB_1 was converted to material which was soluble in the aqueous phase. Of the two chloroform-soluble unidentified metabolites, one had chromatographic properties intermediate to those of AFQ_1 and AFM_1; the other was very polar. No AFB_{2a}, AFP_1, or aflatoxicol were found. In the second experiment, AFB_1 was administered twice daily for 14 days to four lactating dairy cows fed concentrate containing either 10, 50, 250, or 1250 ppb AFB_1. Twenty-four hours prior to slaughter, [^3H]AFB_1 was included with the last feeding. Only the animal fed 1250 ppb AFB_1 showed detectable chloroform-soluble metabolites in the edible portions of its carcass. Other than small quantities of AFB_1 and AFM_1 in the kidney and liver, no other identifiable chloroform-soluble metabolites were detectable (<1 ppb) in the edible portions such as brain, heart, and skeletal muscle. On the other hand, based on recoverable radioactivity, skeletal muscle and liver contained approximately 1.7 ppb and 0.3 ppb unidentified, chloroform-soluble metabolites, expressed as "AFB_1 equivalents". Conclusions drawn were (1) that there should be essentially no acute toxicity health hazard for humans who consume meat or edible organs taken from animals fed less than 46 ppb AFB_1. (This latter level was previously reported by this laboratory to be the maximum feed level of AFB_1 giving rise to detectable levels of AFM_1 in milk (Polan et al., 1974); (2) that negligible carcinogenic hazard due to the *chloroform-soluble* AFB_1 metabolites can be assumed from consumption of meat products from animals fed 46 ppb AFB_1; and (3) that more research information is needed before potential hazard due to water-soluble aflatoxin residues in meat products can be assessed.

Aflatoxin B_1 (AFB_1), a potent hepatocarcinogen, is metabolized by the hepatic microsomal mixed function oxidase (MFO) system to a group of hydroxylated derivatives; however, the composition of AFB_1 metabolites is apparently species specific (Masri et al., 1974). For instance, whereas mouse and rat liver produce aflatoxin Q_1 (AFQ_1) (Faris and Hayes, 1975), only rat liver produces significant quantities of aflatoxin M_1 (AFM_1) (Bassir and Emafo, 1970; Faris and Hayes, 1975), and only mouse liver produces significant quantities of aflatoxin P_1 (AFP_1) (Merrill and Campbell, 1968; Faris and Hayes, 1975). Salhab and Hsieh (1975) have shown that monkey and human hepatic microsomal systems produce aflatoxicol H_1 ($AF_{OL}H_1$), which is not produced by either rat or mouse hepatic microsomes.

Another possible source of tissue residues are the water-soluble conjugates of various AFB_1 metabolites. These are a group of poorly defined compounds (Dalezios et al., 1973; Mabee and Chipley, 1973), which may represent a major pathway of AFB_1 metabolism. Since it is possible for these conjugates to be hydrolyzed by enzymes in the digestive tract of humans consuming contaminated tissue, with subsequent absorption of the free aflatoxins, these residues may represent a potential health hazard. Hydrolytic enzymes, such as β-glucuronidase are readily available through gut microflora (Hawksworth et al., 1971).

A third pathway of AFB_1 metabolism may give rise to covalently bound residues. In theory, these may be either the electrophilic aflatoxin 2,3-epoxide, which binds to various nucleophiles (Garner et al., 1972; Swenson et al., 1973), or the aflatoxin hemacetal (AFB_{2a}), which readily forms Schiff bases with the ubiquitous amino group (Patterson and Roberts, 1970; Gurtoo and Campbell, 1974; Ashoor and Chu, 1975).

There are few reports on the significance of tissue levels of aflatoxin residues present in animals ingesting dietary aflatoxins, although the importance of animal products in human diets deems it essential that more information be gained on possible tissue contamination. Allcroft et al. (1966) found that the blood, liver, and kidneys of sheep fed AFB_1 contained AFM_1. Allcroft and Carnaghan (1963) reported that the liver removed from a cow fed a diet containing 2 ppm aflatoxin for 6 days, elicited no toxic response when fed to ducks. (The general term "aflatoxin" will be used when reference to the literature is made, since in many of the earlier papers it is difficult to know the purity of the aflatoxin preparations utilized.) These workers (Allcroft and Carnaghan, 1963) also showed the presence of AFM_1 in milk, an observation later confirmed by Masri et al. (1969). Purchase (1972) calculated, from Allcroft and Carnaghan's data, that the liver contained less than 40 ppb aflatoxin. Allcroft and Carnaghan (1963)

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reported that the liver from a pig that died of aflatoxicosis was not toxic when fed to ducklings. Keyl et al. (1968) found that steers fed 1 ppm AFB_1 contained no aflatoxin residues, at a detectability limit of 1 ppb, in either muscle, fat, spleen, liver, or kidneys. However, they did report traces of AFB_1 and AFM_1 in the blood. Keyl and Booth (1971) fed pigs a diet containing 810 ppb AFB_1 for 117 days and reported no aflatoxin residues in blood, muscle, fat, spleen, liver, or kidneys. McKinney et al. (1973) fed cows a ration containing a concentration of AFB_1 such that 148.5 mg were consumed over a 14-day period. They reported AFB_1 levels of 0.1 μg/kg or less in liver, a trace in kidney, and none in muscle and heart; for levels of AFM_1, they found 0.1 μg/kg in liver, 0.05–0.30 μg/kg in kidney, less than 0.05 μg/kg in heart, and none in muscle. Platonow and Beauregard (1965) fed White Leghorn hens a meal containing 3.06 ppm aflatoxins for either 2, 4, or 6 weeks and observed no toxicity when either the meat or liver was fed to ferrets for 4 weeks. Abrams (1965) fed 75 ppb aflatoxin to chickens whose tissues were fed to ducklings resulting in no toxic symptoms. Keyl and Booth (1971) reported that broiler chickens fed a ration containing 1.6 ppm AFB_1 for 8 weeks showed no evidence of aflatoxin residues in the muscle, liver, or blood. The same results were reported for White Leghorn hens fed 2.7 ppm AFB_1 for 48 days. Van Zytveld et al. (1970) administered AFB_1 orally to chickens for periods of up to 42 days at daily doses ranging between 0.09–0.61 mg/bird and reported aflatoxin residues in the liver and skeletal muscle in 15 out of 45 chickens. The aflatoxin residues were found mainly in those chickens that elicited a toxic response. Sawhney et al. (1973) fed hens a dose of 11.26 mg of [^{14}C]aflatoxins and found levels of aflatoxin between 3.20 and 40.33 μg/g of tissue (determined by specific activity of the radiolabel) in a wide variety of tissues, with the bile, liver, and reproductive organs showing the greatest concentrations. Mabee and Chipley (1973) found significant levels of radioactivity in blood, liver, heart, gizzard, and muscle from hens intubated with 0.1 mg of [^{14}C]AFB_1/kg of body weight for 14 days. An important finding of these investigators was that aflatoxin conjugates were the predominating form of metabolite produced. That is, they reported that only approximately 19% of an administered dose was found to be extractable with chloroform. Chipley et al. (1974) found that protease treatments of an ethyl acetate phase of tissue extracts from chickens fed [^{14}C]-AFB_1 resulted in the release of 50% of the bound aflatoxins in the form of aflatoxin B_{2a}(AFB_{2a}).

The present study was undertaken to determine the types of AFB_1 metabolites produced by bovine liver and to determine which metabolites are actually found in tissues.

EXPERIMENTAL PROCEDURE

In vitro hepatic metabolism was studied in liver obtained from four young nonlactating cows maintained on adequate diets with no history of previous exposure to AFB_1. Random peripheral samples of the livers were taken immediately after slaughter and maintained at 0–4 °C during the subsequent manipulations. Samples were prehomogenized in a Sorval Omni-Mixer and homogenized in 2 vol of 1.15% KCl containing 20 mM Tris–HCl (pH 7.4) with a Potter-Elvejem homogenizer at 600 rpm using six complete strokes and employing the same homogenizer for all experiments.

The homogenates were centrifuged at 9000*g* for 20 min in a refrigerated centrifuge to remove unbroken cells, nuclei, and mitochondria. The supernatant was decanted through glass wool, and in two cases an aliquot of the filtrate (S-9) was removed for metabolism studies. The S-9 fraction was then centrifuged at 105 000*g* to sediment the microsomes. The microsomal pellet was floated off the glycogen pellet and resuspended in the same KCl–Tris buffer by gentle homogenization to give a suspension equivalent to 1 g of liver/mL.

Incubations were run in triplicate in open 25-mL Erlenmeyer flasks at 37 °C in a Dubnoff metabolic shaking incubator at a shaking rate of 120 oscillations/min for 1 h. Reaction mixtures were comprised of either 0.5-mL microsomal suspension or 1.0 mL of S-9 (each equivalent to 0.5 g of liver), 50 μg ^{14}C-labeled AFB_1, 1.5 mL of 400 mM phosphate buffer (pH 7.4), and 1.0 mL of an NADPH-generating system ($NADP^+$, 4 mM; glucose 6-phosphate, 100 mM; and Torula yeast glucose 6-phosphate dehydrogenase, 2 EU/mL). AFB_1 was added in 5 μL of dimethyl sulfoxide. The reaction mixture was made up to 5 mL by the addition of appropriate amounts of 1.15% KCl. After the reactions were terminated by the addition of 2 mL of saturated NaCl, the incubation mixtures were transferred to extraction tubes and placed on a Virtis Extractomatic. They were extracted four times using 10 mL of chloroform–methanol (4:1) per extraction. The organic phase was filtered through anhydrous sodium sulfate and then evaporated to dryness. One milliliter of chloroform was added to the dry extract and aliquots were spotted on Adsorbosil-5 TLC plates (Applied Science Laboratories, State College, Pa.). The plates were developed in water-saturated chloroform–acetone (88:12). The aflatoxin concentrations were determined by fluorodensitometry using a Photovolt densitometer equipped with an Autolab System I computing integrator. Authentic quantitative standards for AFB_1, AFQ_1, AFM_1, and AFP_1 were employed as reference standards. These spots were then scraped from the TLC plates, along with the areas showing no fluorescence. These scrapings were added separately to a toluene–Triton X-100 counting cocktail and counted.

In order to determine the tissue distribution of chloroform-soluble aflatoxins in the intact animal, AFB_1 was administered twice daily to four lactating dairy cows so that levels of 10, 50, 250, and 1250 ppb of the dietary concentrate were fed daily over a 14-day period. Each cow's allotted concentrate was placed in a rubber feeding tub. AFB_1 (Makor Chemical Co., Ltd., Jerusalem, Israel) was distributed over the concentrate as a chloroform solution, and the chloroform evaporated by placing the tub in front of a fan for a few hours. Cows consumed virtually all of their respective concentrates. Total AFB_1 intakes were 0.5 mg/day for the cow fed 10 ppb, 0.25 mg/day for the 50 ppb cow, 1.34 mg/day for the 250 ppb cow, and 7.31 mg/day for the 1250 ppb cow. Twenty-four hours prior to slaughter, [^{3}H]AFB_1 (1.5 mCi) (New England Nuclear, Boston, Mass.), purified by thin-layer chromatography, was fed as a portion of the regular unlabeled dose.

Tissues were removed from the carcasses immediately after slaughter and subsequently stored at −10 °C until assayed. The method of Pons et al. (1973) was used for the extraction of the various tissues for aflatoxin with the exception that 100 g of tissue were used instead of 100 mL of milk. The residue from the extraction was dissolved in a small quantity of chloroform and an aliquot was spotted on Adsorbosil-1 or Adsorbosil-5 TLC plates and the aflatoxins quantitated as described above.

RESULTS AND DISCUSSION

Table I illustrates the distribution of chloroform-soluble metabolites produced by the S-9 and the microsomal fractions. AFQ_1, AFM_1, and two fluorescent spots were

Table I. Metabolism of AFB_1 to Chloroform-Soluble Metabolites by Bovine Liver[a]

Radiolabeled fraction	S-9[b]		Microsomes[c]	
	μg/5 mL	μg (mg of protein)$^{-1}$ h^{-1}	μg/5 mL	μg (mg of protein)$^{-1}$ h^{-1}
AFB_1[d]	8.2		8.7 ± 0.4	
AFQ_1	1.2	0.08	1.1 ± 0.2	0.16 ± 0.03
AFX_1[e]	0.5	0.03	0.4 ± 0.3	0.08 ± 0.06
AFM_1	5.0	0.34	1.8 ± 0.0	0.29 ± 0.03
AFX_2 (origin)[e]	4.2	0.28	4.3 ± 0.5	0.64 ± 0.06
$CHCl_3$ solubles	19.1		16.3	
Non-$CHCl_3$ solubles[f]	30.9		33.7	

[a] Incubation mixture extracted with chloroform–methanol (4:1). Quantitation is by quantitative TLC densitometry with specific standards used for all aflatoxins with the exception of "X" and the "origin". [b] Two experiments, each run in triplicate. [c] Four experiments, each run in triplicate (means ± SEM). [d] Unmetabolized AFB_1 (original AFB_1 concentration was 50 μg/5 mL). [e] Quantitated with AFM_1 as standard; was ^{14}C labeled. [f] Contains aqueous-soluble aflatoxins and that which remains bound to protein. Quantity obtained by difference and does not represent all of original AFB_1. Total recoveries were approximately 50% (see text).

radioactive and, therefore, represented AFB_1 metabolites. One spot (AFX_1) migrated between AFQ_1 and AFM_1 and appeared similar to $AF_{OL}H_1$. However, no $AF_{OL}H_1$ standard was available to confirm this observation. Salhab and Hsieh (1975) have shown that $AF_{OL}H_1$ is produced only in the simultaneous presence of both soluble and microsomal enzymes. Since this metabolite is produced independently both by the S-9 and the microsomal fraction, it may not be $AF_{OL}H_1$. The second fluorescent and radioactive spot (AFX_2) remained at the origin and was not identified. No AFB_{2a}, AFP_1, or aflatoxicol were observed. Failure to extract and detect AFB_{2a} does not preclude its production since it may have formed Schiff bases with free amino groups and would have remained unextractable. On the other hand, AF_{OL} has been shown to be a significant metabolic product of AFB_1 by several species (Patterson and Roberts, 1971, 1972a,b; Edwards et al., 1975) and its lack of production by the S-9 fraction was somewhat surprising. The absence of AFP_1 and $AF_{OL}H_1$ is not unusual since they are apparently more species specific (Dalezios and Wogan, 1972; Salhab and Hsieh, 1975).

Microsomal enzyme activities were somewhat higher than the S-9 fraction activities for AFQ_1, AFX_1, and AFX_2, but not for AFM_1. Rates of production of the chloroform-soluble metabolites by bovine liver microsomes were roughly equivalent with rat and mouse hepatic preparations (Faris and Hayes, 1975).

Only 33–39% of the 50 μg of AFB_1 was recovered as chloroform-soluble aflatoxins in these incubation mixtures (Table I). The majority of the aflatoxins, therefore, remained in the aqueous phase. Of that which remained, only a small fraction (about 14%) was found to be associated with the microsomal protein, as measured by counting the microsomal pellet reprecipitated from the microsomal incubation mixture. This left about one-half of the original AFB_1 as unidentified material in the aqueous phase. It is unlikely that this material could be conjugated aflatoxins since conjugate donors were not available in the incubation flasks. Of possible importance is the fact that this material may be the same as that which partially extracts into chloroform but remains at the origin of the TLC plate because of its high polarity. The aflatoxin derived residue at the origin (Table I, AFX_2) was positive for amino groups and sulfur. This compound could result from the binding of AFB_1 epoxide with a small amino acid or peptide containing a sulfhydryl group such as glutathione. Further studies are being undertaken to identify this material.

An additional study was undertaken to determine which of these potential metabolites may be present in tissues of animals fed AFB_1. Levels of aflatoxins extracted with organic solvents from the various tissues obtained from a cow receiving 1250 ppb dietary AFB_1 and determined by quantitative thin-layer chromatography are reported in Table II. Only the kidney and liver contained both AFB_1 and AFM_1. AFB_1 alone was found in the spleen and bile, whereas the mammary gland contained only AFM_1. Lack of free AFM_1 in the bile may be related to the biliary excretion of AFM_1 in the conjugated form (Mabee and Chipley, 1973). No known aflatoxin residues were found in lung, brain, heart, pancreas, kidney fat, omental fat, or skeletal muscle. Also, no AFQ_1 was found in any tissue. This latter observation is unusual with respect to the in vitro metabolism studies which indicated that AFQ_1 is a significant chloroform-soluble metabolite produced by bovine liver. Perhaps, this metabolite becomes conjugated, rendering it insoluble in the organic solvents.

When tissues from a cow fed 250 ppb AFB_1 were analyzed, no chloroform-soluble, identifiable aflatoxins were found with these procedures, although the tissues did contain significant radioactivity. Tissues from the cows fed 50 ppb AFB_1 and 10 ppb AFB_1 were therefore not analyzed.

Table II. Tissue Levels of Aflatoxin B_1 and M_1 from Cow Fed 1250 ppb Aflatoxin B_1

Tissue	Aflatoxin[a]	μg/kg of tissue	μg/total organ
Kidney	B_1	0.22 ± 0.05	0.33 ± 0.08
	M_1	0.72 ± 0.13	1.07 ± 0.02
Liver	B_1	0.09 ± 0.02	0.75 ± 0.17
	M_1	0.16 ± 0.06	1.31 ± 0.51
Spleen	B_1	0.17 ± 0.02	0.12 ± 0.01
	M_1	ND	ND
Lung	B_1	ND	ND
	M_1	ND	ND
Mammary gland	B_1	ND	ND
	M_1	0.27 ± 0.06	2.21[b]
Brain	B_1	ND	ND
	M_1	ND	ND
Heart	B_1	ND	ND
	M_1	ND	ND
Pancreas	B_1	ND	ND
	M_1	ND	ND
Kidney fat	B_1	ND	ND
	M_1	ND	ND
Omental fat	B_1	ND	ND
	M_1	ND	ND
Skeletal muscle[d]	B_1	ND	ND
	M_1	ND	ND
Bile	B_1	0.26 ± 0.06	ND[c]
	M_1	ND	

[a] Aflatoxins determined by quantitative TLC. Data represent the mean ± SEM. ND = no detectable aflatoxins. [b] Assuming mammary gland weight as 8.20 kg (Olson, 1950). [c] Total organ weight not known. [d] Semitendinosus muscle.

Table III. Radioactivity of Total Chloroform Extracts and Individual Aflatoxins B_1 and M_1 from Cow Fed 1250 ppb [^{3}H]Aflatoxin B_1[a]

Tissue	Total counts in extract		Counts in aflatoxin fractions			
			B_1		M_1	
	DPM/kg × 10^6	DPM/organ × 10^6	DPM/kg × 10^6	DPM/organ × 10^6	DPM/kg × 10^6	DPM/organ × 10^6
Kidney	0.0853 ± 0.0242	0.1280 ± 0.0363	0.0009	0.0012	0.1125	0.1686
Liver	0.0507 ± 0.0104	0.4283 ± 0.0883	0.0057	0.0480	0.0168	0.1428
Spleen	0.0117 ± 0.0016	0.0085 ± 0.0012	0.0051	0.0039	0.0030	0.0021
Lung	0.0104 ± 0.0010	0.0395 ± 0.0040	0.0015	0.0060	0.0030	0.0111
Mammary gland	0.0454 ± 0.0022	0.3720 ± 0.0180	0.0024	0.0197	0.0138	0.1132
Brain	0.0062 ± 0.0014	0.0025 ± 0.0006	0.0042	0.0018	0.0039	0.0015
Heart	0.0180 ± 0.0031	0.0409 ± 0.0122	0.0006	0.0015	0.0036	0.0087
Pancreas	0.0246 ± 0.0160	0.0067 ± 0.0025	0.0042	0.0012	0.0135	0.0036
Kidney fat	0.0256 ± 0.0224	*[b]	0.0063	*	0.0009	*
Omental fat	0.0088 ± 0.0007	*	0.0027	*	0.0036	*
Skeletal muscle	0.1552 ± 0.0412	11.23[d]	ND[e]		ND[e]	
Bile	0.0621 ± 0.0066	*	0.0021	*	0.0051	*

[a] Data represent counts in $CHCl_3$-soluble extract, AFB_1 and AFM_1 as separated on TLC plates. Data represent means ± SEM where appropriate. [b] Where total tissue weights are not known, asterisks are shown. [c] Semitendinosus muscle. [d] Assuming skeletal muscle mass as 2.32% of total muscle mass (Orme et al., 1960). [e] Not detectable.

Distribution of the radioactivity in the chloroform-soluble extracts is shown in Table III. Of particular note is the fraction of this chloroform-extractable radioactivity which is represented by AFB_1 and AFM_1 in the various tissues. These percentages are (asterisk indicates tissues which contain very low radioactivity, representing only rough estimates due to the higher experimental errors): kidney (100+%), liver (44%), spleen* (71%), lung* (43%), mammary gland (36%), brain* (100+%), heart* (25%), pancreas* (72%), skeletal muscle (0%), and bile* (12%). Therefore, there appear to be quantities of other chloroform-soluble aflatoxin metabolites for some of these tissues.

The remaining chloroform-soluble radioactivity found on the TLC plates did not cochromatograph with any of the known aflatoxins available as standards and was primarily found either at the solvent front, the origin and/or migrating with a green fluorescent spot observed between AFM_1 and the origin when water-saturated chloroform–acetone (88:12) was used as the developing solvent. Most of the radioactivity in these latter three TLC regions was found to be present in the unknown green-fluorescent spot and the spot at the origin.

AFB_1 has an LD_{50} in the range of 0.5 to 10 mg/kg of body weight for most species tested (Wogan, 1966). The remaining chloroform-soluble metabolites have all been found to be considerably less toxic than AFB_1. The present study, on the basis of two cows, suggests that there is little danger of an acutely toxic response produced by the *chloroform-soluble* aflatoxins occurring as tissue residues in cows consuming as much as 1250 ppb AFB_1 in their concentrate. For example, with kidney, which represents the highest concentration of chloroform-soluble aflatoxin residues, the level is still less than 1 ppb. Therefore, if feed concentrate levels of AFB_1 are maintained at levels below 46 ppb, which do not allow for excretion of AFM_1 in the milk (Polan et al., 1974), the probability of acute aflatoxicosis in individuals consuming tissues should be insignificant.

Although no acutely toxic response from the chloroform-soluble aflatoxins would be expected in humans consuming edible tissue from cows exposed to as much as 1250 ppb AFB_1, the chronic or long-term toxicity presented by the water-soluble AFB_1 metabolites needs to be studied in more depth. The in vitro metabolism studies indicated that large quantities of water-soluble products may be produced by bovine liver preparations; also, preliminary data (Hayes and Campbell, 1975) indicate that similarly large quantities of water-soluble metabolites occur in tissues of cows consuming AFB_1. Upon ingestion, if such products were to be hydrolyzed to free aflatoxins in the gut, a potential hazard may result.

AFB_1 is highly tumorigenic. For instance, 50 ppb AFB_1 produced a high yield of tumors in rats after 105 weeks and levels as low as 1 ppb produced significant numbers of tumors (Wogan et al., 1974). Levels as low as 5.8 ppb have produced hepatocellular carcinomas in rainbow trout, which is a very sensitive species, whereas 27.3 ppb AFM_1 was required to produce tumors in the same strain of trout (Canton et al., 1975). Sinnhuber et al. (1970) were able to produce carcinomas in an apparently more sensitive strain of trout fed diets containing 4 ppb AFM_1. Therefore, tissue levels of AFB_1 and AFM_1 from the cow fed 1250 ppb AFB_1 (Table II) were considerably lower than those which are carcinogenic for one of the most sensitive species. On the basis of relative species metabolism of AFB_1 by liver preparations in vitro (Hsieh, 1976), and dietary surveys of aflatoxin ingestion by humans in Kenya (Peers et al., 1973) and Swaziland (Peers et al., 1976), it would appear that the human is one of the most resistant species. If cows are fed concentrate containing AFB_1 levels below that which causes AFM_1 secretion in milk, i.e., 46 ppb (Polan et al., 1974), then it would appear that the carcinogenic potential of the meat of these animals caused by chloroform-soluble aflatoxin residues would be negligible. On the other hand, hazard from the water-soluble aflatoxin residues may be more difficult to estimate. The identification of these residues, characterization of their hydrolytic rates, and carcinogenic potential for tissue sites such as the gastrointestinal mucosa should each be estimated before such conclusions can be drawn.

ACKNOWLEDGMENT

We wish to thank Gail Sullivan and Carol Noble for their technical assistance with this study. Also, we wish to thank R. D. Stubblefield, USDA, ARS, Peoria, Ill., for the generous gift of the aflatoxin M_1 quantitative standards; G. Wogan, Department of Nutrition and Food Science, M.I.T., Cambridge, Mass., for samples of aflatoxin P_1; D. Hsieh, Department of Environmental Toxicology, University of California, Davis, Calif., for aflatoxin Q_1 samples; and A. Ciegler, Northern Regional Research Laboratories, USDA, ARS, Peoria, Ill., for his aid in producing the [^{14}C]aflatoxin B_1.

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Received for review December 21, 1976. Accepted May 2, 1977. This study was supported by Contract No. 72-56 with the Food and Drug Administration, Department of Health, Education, and Welfare.

Nuclear Magnetic Resonance Identification of Versiconal Hemiacetal Acetate as an Intermediate in Aflatoxin Biosynthesis

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An intermediate in aflatoxin biosynthesis, previously identified as "versiconal acetate", was synthesized from labeled acetate using dichlorvos-treated cultures of *Aspergillus parasticus*. ^{13}C and ^{1}H FT NMR studies led to assignment of an alternate hemiacetal structure. Its biosynthetic relationship to aflatoxin is apparent from the labeling pattern found in this compound derived solely from acetate.

The aflatoxin-producing cultures of *Aspergillus parasiticus* when treated with the insecticide dichlorvos (dimethyl 2,2-dichlorovinyl phosphate) produce much less than normal amounts of aflatoxins (Schroeder et al., 1974; Yao and Hsieh, 1974), and instead produce primarily an orange pigment. It has been suggested that this pigment is an intermediate in aflatoxin biosynthesis (Yao and Hsieh, 1974; Singh and Hsieh, 1977); it has been tentatively identified as versiconal acetate (Schroeder et al., 1974). Our preliminary experiments (Yao and Hsieh, 1974) as well as those of Schroeder et al. (1974) could not unequivocally establish the structure of this compound.

We prepared this pigment from [1-^{13}C]-, [2-^{13}C]-, [1,2-^{13}C]-labeled and unlabeled sodium acetate. These samples were subjected to ^{13}C and ^{1}H pulsed Fourier transform NMR analysis in order to firmly assign a structure to this aflatoxin biosynthetic intermediate and to demonstrate its biogenetic relationship to aflatoxins. Our data indicate that the pigment is versiconal hemiacetal acetate (VHA) and is shown as structure **6** (Figure 1).

EXPERIMENTAL SECTION

Materials. [1-^{13}C] and [2-^{13}C]-labeled sodium acetate enriched in ^{13}C by 50–60 and 62%, respectively, were obtained from the International Chemical and Nuclear Corp., Chemical and Radioisotope Division, Irvine, Calif. The [1,2-^{13}C]-labeled sodium acetate 90% enriched was obtained from Merck and Co., Inc., Rahway, N.J. Dichlorvos was a gift from Shell Chemical Co. The

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