Excretion and Tissue Distribution of Radioactivity from Aflatoxin B₁-¹⁴C in Rats

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SUMMARY

Excretion patterns of ¹⁴C derived from intraperitoneal doses of ring-labeled and methoxy-labeled aflatoxin B₁ were investigated in male rats. It was found that 70-80% of the administered radioactivity from both compounds was excreted during the 24 hours following administration. A major excretory route of the ring-labeled material was through biliary excretion into feces, which accounted for nearly 60% of the administered ¹⁴C. A further 20% was excreted in urine, and only negligible amounts in CO₂. In contrast, approximately 25% of administered radioactivity from the methoxy-labeled compound appeared in CO₂ with a concomitant decrease in feces. These results indicate that O-demethylation represents a significant metabolic pathway for aflatoxin B₁ in the rat.

Radioactivity derived from ring-labeled compound was present at maximum levels in liver and kidneys 0.5 hour after administration, with small amounts present in other organs. The concentration of radioactivity was five to fifteen times greater in liver than in other tissues, and at the end of 24 hours, the liver contained an amount equal to the content of the remainder of the carcass. This finding is associated with the relative tissue specificity of aflatoxin B₁ as a hepatotoxin.

INTRODUCTION

Aflatoxins, metabolites of some strains of Aspergillus flavus, are toxic to most animal species and are presently known to be carcinogenic for the rat (7, 16), trout (6), and duck (11). The potency of aflatoxin B₁ as an acute toxin is illustrated by its LD₅₀ value, 4.3 mg/kg, when administered intraperitoneally to male weanling rats. Hepatomas are induced in rats by diets containing less than 1 mg/kg (1 ppm) and in rainbow trout by as little as 5 µg/kg (18).

Histologic and biochemical manifestations of the toxic action, which appear almost exclusively in liver, persist for prolonged periods following a single dose or a limited number of successive doses of aflatoxin B₁. Thus, Butler (9) observed biliary hyperplasia and parenchymal cell alterations in surviving rats one month following an LD₅₀ dose. We have found liver tryptophan pyrrolase inducibility to be impaired 10 days after administration of a sublethal amount (3 mg/kg) of the compound (19).

It was therefore of interest to determine the excretory and metabolic fate of aflatoxin B₁ as well as its distribution in tissues following a single dose. Quantitative experiments for this purpose became feasible with the availability of radioactive compound obtained from cultures of the mold on media containing ¹⁴C-labeled precursors (1). Selective labeling was achieved by using two precursors, methionine or acetate. Addition to the medium of methionine-methyl-¹⁴C resulted in production of aflatoxin labeled exclusively in the methoxy carbon, as illustrated in Chart 1 (G. Büchi and S. Breechbuehler, personal communication, 1966). The radioactive compound derived from sodium acetate-¹⁴C was labeled only in the ring carbons, although the precise distribution of ¹⁴C within the ring structures has not been determined.

The present report deals with the patterns of excretion of radioactivity and with distribution of radioactivity in liver and other organs of rats during the 24-hour period following single administrations of methoxy-labeled and ring-labeled aflatoxin B₁.

MATERIALS AND METHODS

The aflatoxin B₁-¹⁴C used in these experiments was prepared by submerged culture of Aspergillus flavus (ATCC No. 15517) in liquid media containing either sodium acetate-¹⁴C or L-methionine-methyl-¹⁴C as described by Adye and Mateles (1). The compound was recovered from thin-layer chromatograms of chloroform extracts of culture media. The isolated material moved as a single fluorescent substance on Kieselguhr plates, and contained all radioactivity detectable by autoradiography of the chromatograms on X-ray film. Concentrations of aflatoxin B₁ present in eluates were calculated from extinction values at 363 mμ with a molar extinction coefficient of 2.18 × 10³ (5). The specific activities of various preparations were in the range of 115-155 µc/µmole for the ring-labeled (acetate-derived) compound and 2-3.5 µc/µmole for the methoxy-labeled (methionine-derived) compound.

Animals used were male Fisher rats weighing 40-125 gm. Preliminary experiments indicated no significant effect of route
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Immediate injection, the rats were placed into all dissolved in 0.06-0.10 ml dimethylsulfoxide, at doses of 1.6-4.5 X 10^6 dpm per animal. Thus, in most experiments each animal received a total dose of aflatoxin Bi in the order of 0.07 mg/kg of body weight. After injection, at which time liver, kidney, spleen, heart, adrenals, testes, pancreas, thymus, and brain were removed and weighed. The gastrointestinal tract was divided into stomach, small intestine, cecum, and large intestine, and each segment was processed together with its contents. Individual tissues as well as the remaining carcass and feces were thoroughly homogenized in distilled water, the final volume being adjusted to contain 100 mg solids/ml of homogenate. Radioactivity was measured in aliquots of the homogenate after decoloration with 1 N NaOH (100°C, 20 minutes), addition of Hyamine hydroxide, and acidification with 2 N HCl. Urine and 14C solutions were counted without further treatment.

The distribution of radioactivity in subcellular fractions of liver was also studied. For this purpose, livers were homogenized in cold 0.32 M sucrose and fractions collected by successive centrifugation at 800 X g (cell debris and nuclei), 5,500 X g (mitochondria), and 105,000 X g (microsomes); pellets were washed twice at each stage. Radioactivity in whole homogenates, subcellular fractions, and microsomal supernatants was determined by liquid scintillation counting by the techniques used for other tissues.

For biliary excretion studies, male rats weighing 260-310 gm were used. A polyethylene cannula with a metal tip was implanted into the common bile duct of animals anesthetized with sodium 5-allyl-5-(1-methylbutyl)-2-thiobarbiturate (Surital, Parke, Davis and Co.). The anesthetic was administered intravenously in the smallest amount sufficient to produce surgical anesthesia lasting 10-15 minutes. After implantation of the cannula, the laparotomy incision was closed with wound clips and the animals were restrained on their backs. Control bile collections were initiated 10 minutes later when full consciousness had been regained. After a 10- to 15-minute control collection period, aflatoxin Bi-14C was administered intraperitoneally and bile samples were collected successively at 10- to 20-minute intervals for periods up to five hours after aflatoxin was administered.

Because of uncertainties in interpretation of distribution data derived from the methoxy-labeled compound, only the ring-labeled form was used for tissue distribution and biliary excretion studies. Radioactivity in bile and other materials was determined by liquid scintillation counting in the scintillator described by Bruno and Christian (8). The extent of quenching in each sample was estimated by the internal standardization technique using a 14C toluene-standard.

The recovery of radioactivity in all animals exceeded 80% of the administered dose, and in most instances the range was 100 ± 7%. However, as the extent of recovery was not completely uniform, the excretion data have been calculated as a function of the recovered 14C rather than as a proportion of the administered dose. The distribution data were calculated as a percentage of the total radioactivity, and in order to compare tissue radioactivity among various animals, the data have also been expressed as specific activity (dpm/mg fresh tissue) adjusted to 100% recovery.

**RESULTS**

Table 1 shows the results of experiments designed to compare major excretory routes of ring-labeled and methoxy-labeled aflatoxin B1 over the 24-hour period after intraperitoneal injection. These data indicate that the compound or its metabolites are excreted by the rat via urinary and fecal routes. The total excretion of radioactivity from the two forms was not markedly different, nor were the amounts contained in urine, which accounted for approximately 25% of the total excretion. However, marked differences were observed in the excretion of 14C in CO2 and in feces. A substantial proportion of radioactivity from the methoxy-labeled form appeared as 14CO2 in contrast to negligible amounts in animals treated with ring-labeled compound. Fecal excretion accounted for 57% of the administered dose in the latter animals, but for only 22% in those receiving methoxy-labeled aflatoxin. The difference in fecal excretion is largely accounted for by the 14C content of CO2.

Because of the significant amounts of radioactivity appearing in CO2, the time course of appearance of 14CO2 was studied in detail in rats injected with the methoxy-labeled compound. The results of two typical experiments are shown in Chart 2, which illustrates the rate of 14CO2 expiration at intervals from 0.25 hour to 24 hours after dosing. In both animals, expired CO2 contained radioactivity at 15 minutes and the peak rate of expiration of 14CO2 was reached 1 to 2 hours after injection. The rate declined rapidly in the succeeding 6 to 7 hours, following which it remained minimal. Detectable quantities of radioactivity were still present 12 to 24 hours after administration. No comparable pattern was discernable in animals treated with ring-labeled compound, in which 14CO2 collected over 24 hours never accounted for more than 0.5% of total radioactivity recovered.

In all subsequent experiments, the ring-labeled form of the compound was used exclusively in order to avoid difficulties in interpretation of excretion data based on radioactivity derived from demethylation of the methoxy-labeled product. The total aflatoxin dose of the former compound used in the comparative

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**CHART 1. Structure of aflatoxin B1-14C labeled in the methoxy carbon.**

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**METHOXY-LABELLED AFLATOXIN B1-14C**

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**RESULTS**

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**TABLE 1.**

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**CHART 2.**

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**CANCER RESEARCH**

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study already described was 0.07 mg/kg. This dose is not known
to cause demonstrable toxic manifestations in rat liver. An
experiment was therefore performed to determine the possible
effects of larger, hepatotoxic doses on the excretion pattern.
Animals were treated simultaneously with 0.07 mg/kg aflatoxin-
14C and sufficient nonradioactive aflatoxin to provide total doses
of 2.13 mg/kg, a toxic but sublethal dose, or 4.95 mg/kg, a lethal
dose. The excretion and intestinal distribution of 14C in these animals
during the 14-hour period after dosing are shown in Table 1. No
significant differences were observed in the total excretion of
radioactivity or in the distribution among excretory routes. The
increased total excretion at 2.13 mg/kg was mainly accounted
for by relatively great urinary excretion. The values in all cases,
however, were within ranges of variation encountered in other
experiments at the lowest dose level.

In order to study the dynamics of distribution and excretory
patterns, animals were killed 0.5, 1, 2, 4, 8, 15, and 24 hours
after administration of radioactive aflatoxin Bi. Chart 3 shows
the excretory pattern of radioactivity at each of these time
intervals. Urine excreted during the first 0.5-hour interval con-
tained significant amounts of radioactivity, and this route ac-
counted for virtually all excretion of 14C until 8 hours after dosing,
at which time approximately 20% of the administered dose
appeared in urine. Feces excreted prior to 8 hours after dosing
did not contain significant quantities of radioactivity. However,
excretion via this route increased rapidly between 8 and 15 hours
and by 24 hours more than 50% of recovered 14C was present in
feces.

The data in Chart 4 indicate that radioactivity appeared in
the small intestine in significant quantity as early as 0.5 hour
after injection, and by 2 hours nearly all of the 14C eventually
excreted in feces was present in this segment of the gut. Con-
versely, significant amounts of labeled material did not appear
in more distal segments, viz., the cecum and colon, until 4 hours

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Aflatoxin dose (mg/kg) & Radioactivity (% of recovered 14C) & \\
& Ring-labeled & Methoxy-labeled \\
& 0.07 (four animals) & 2.13 (one animal) & 4.95 (one animal) & 2.8; 4.2 (two animals) \\
\hline
Total excreted & 80.2 (77.4-84.9) & 88.8 & 76.8 & 70.5 (68.2-72.8) \\
Expired CO2 & 0.5 (0.3-0.6) & 0.3 & 0.3 & 26.6 (20.6-32.6) \\
Urine & 22.6 (14.8-32.1) & 34.4 & 21.6 & 20.0 (13.8-26.1) \\
Feces & 57.1 (44.7-69.8) & 54.1 & 54.8 & 23.9 (14.1-33.8) \\
Stomach and contents & 0.1 (0.1-0.2) & 0.1 & 0.1 & 1.5 (0.3-2.6) \\
Intestine and contents & 0.8 (0.5-1.3) & 0.5 & 0.4 & 1.0 (0.7-1.3) \\
Cecum and contents & 2.8 (1.9-4.2) & 0.9 & 8.3 & 6.1 (5.9-6.3) \\
Colon and contents & 1.1 (0.6-1.9) & 0.4 & 0.1 & 4.2 (4.2-4.2) \\
Average recovery of adminis-
tered 14C (%)\textsuperscript{a} & 94.5 (80.0-106.4) & 91.0 & 104.1 & 106.7 (105.8-107.6) \\
\hline
\end{tabular}
\caption{Excretion and Gut Content of Radioactivity 24 Hours following Injection of Ring-labeled or Methoxy-
labeled Aflatoxin Bi}
\end{table}
after injection. Subsequently, the contents of these two segments rose to a maximum at 8 hour and decreased thereafter, reflecting ultimate excretion in feces.

The early appearance of labeled material in the small intestine as well as the relatively large proportions excreted via feces suggested that the compound or its metabolites were excreted in bile. Therefore, the rate of biliary excretion was studied in a group of animals by cannulation of the common bile duct. Typical results from three such animals are summarized in Chart 5, in which rates of $^{14}$C excretion during successive 10-
Metabolism of Aflatoxin Bi-$^{14}$C

Chart 5. Biliary secretion of $^{14}$C after a single i.p. dose of ring-labeled aflatoxin Bi.

Minute collection periods are expressed as functions of the average rate for the entire period of collection (125 minutes) in each animal.

Radioactivity appeared in bile within the first five minutes after injection, and maximum secretion rates were reached within 10–20 minutes. The rates subsequently declined until minimal secretion was established after 90 minutes. Detectable levels were still being excreted after 400 minutes in two rats studied for that length of time. As illustrated in Chart 5, this pattern of biliary secretion appeared to be relatively independent of substantial fluctuations in rate of bile flow that occurred during the experiments.

Chart 6 shows the distribution of radioactivity in the eviscerated carcass, liver, kidney, and other tissues of rats killed at intervals during the 24-hour period following intraperitoneal dosing with radioactive aflatoxin Bi. In this chart, tissue contents of radioactivity are expressed as proportions of the total recovered activity.

At 0.5 hour after dosing, the kidneys contained approximately 5%, the liver 17%, and the eviscerated carcass 27% of the recovered $^{14}$C. During the ensuing 90 minutes, radioactivity in the kidneys and liver decreased rapidly so that 2 hours following dosing, the kidneys contained less than 1% and the liver 10% of recovered radioactivity. Levels of radioactivity in the kidneys showed little further change over the remaining 22 hours. Carcass levels declined less rapidly, reaching a minimum value (8% of recovery) 8 hours after dosing, following which there was little further change. The liver content of radioactivity continued to fall during the interval between 2 hours and 24 hours. However, the rate of decline was low, and at 8, 15 and 24 hours after dosing, the liver contained amounts of radioactivity equivalent to the entire remainder of the carcass (5–8% of recovered $^{14}$C).

Among those tissues other than liver and kidney which were studied individually, none contained more than 0.5% of the recovered activity at any time, and there were no significant alterations during the entire period of study.

In order to permit meaningful comparisons among organs of widely different mass, the relative specific activities (corrected to 100% recovery of $^{14}$C) of several tissues are presented in Chart 7. Values for adrenals and spleen were typical for tissues other than liver and kidneys and are presented for comparative purposes. These results parallel those presented in Chart 6, in

Chart 6. Tissue distribution of $^{14}$C derived following i.p. administration of ring-labeled aflatoxin Bi. Data from 3 animals at 0.5 hr, 4 animals at 24 hr and 2 animals at other intervals.

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that values for all tissues are relatively high 0.5 hour after dosing and fall rapidly during the first 2 hours. It is thought that the initial values in all tissues reflect, at least in part, the presence of radioactivity in entrapped blood. The substantial initial levels in the kidneys also are probably attributable to the presence of large amounts of 14C in urine within the renal structures, as shown by the excretory pattern already described.

Within two to four hours after dosing, radioactivity in all tissues reached a virtually constant level, after which time the rate of disappearance was slow. At all times after dosing, the specific activity of liver exceeded the value for all other tissues and at 24 hours, the liver contained about five times more radioactivity per gm than kidney and nearly ten times more than any other tissues studied.

In order to obtain preliminary information concerning the intracellular distribution of 14C, liver homogenates from animals used in these experiments were fractionated by differential centrifugation. Distribution of total liver radioactivity among the mitochondrial and microsomal fractions and in the microsomal supernatants (cell sap) at intervals following dosing is shown in Chart 8.

As illustrated in Chart 8, most (60%) of the radioactivity present in liver 0.5 hour after injection is in the microsomal supernatant, with 20% associated with the microsomes and about 10% in the mitochondrial fraction. Within the first 2 hours, increasing amounts of radioactivity were found in the microsomes, with a concomitant decrease in the microsomal supernatant. At the end of 24 hours, the microsomes contained...
50\% of liver radioactivity and 28\% was present in the soluble (supernatant) fraction. The mitochondrial fraction contained from 10–15\% of the radioactivity of liver, and showed only small variations during the 24-hour period.

**DISCUSSION**

The results of these experiments indicate that aflatoxin B₁ or its metabolites are excreted relatively efficiently by the rat after administration of a single dose. The data derived from the methoxy-labeled compound reveal that a significant proportion of the administered radioactivity appeared in CO₂. Thus, it is apparent that O-demethylation represents a major pathway in the metabolism of aflatoxin B₁. The failure of ¹³C in the ring carbons to appear in CO₂ also suggests either that ring cleavage does not take place to a significant extent or that the products formed by cleavage are not fully oxidized.

Total excretion of radioactivity from both labeled forms of the compound amounted to 70–80\% of the administered dose in the 24-hour period following administration. Excretion through the urinary route was also similar, with a relatively constant proportion, approximately 20\% of the dose, excreted in urine. The time course was such that 50–60\% of the amount ultimately appearing in urine was present during the first hour after administration. In the case of the ring-labeled material, urine represents the sole route of excretion during the initial eight-hour period. Feces represented a more important pathway of excretion of the ring-labeled compound, as nearly 60\% of administered radioactivity was excreted by this route in a 24-hour period. Only 22\% of the methoxy-labeled form was present in feces, a reflection of the contribution of ¹³CO₂ to the total distribution of radioactivity from this form.

Studies on the kinetics of excretion of ring-labeled aflatoxin B₁ through the intestinal route revealed that the radioactivity ultimately excreted in feces is derived from biliary secretion. The rapidity with which this process takes place is illustrated by the observations that radioactivity appeared in bile within 5 minutes after injection, reached a peak 5–15 minutes later, and rapidly declined over the following hour. Similar conclusions were reached by Falk et al. (14), who studied biliary excretion of aflatoxin by fluorescence technics. These findings correlate well with the pattern of distribution in gut contents. Radioactivity was maximal in the small intestine two hours after injection and passed through the remainder of the intestinal tract during the ensuing 6-hour period. In quantitative terms, it appears that biliary secretion is a primary route of excretion of the ring-labeled compound, since the quantity secreted in bile accounts for essentially the total amount eventually excreted in feces within 24 hours. These data also suggest that there is relatively little enterohepatic recirculation of the compound or its metabolites.

The total excretion and distribution of radioactivity among major excretory routes were not affected over a wide range of aflatoxin doses. Toxic or lethal doses failed to alter these parameters despite hepatic parenchymal cell damage and other histologic alterations which have been reported (9) within 16–24 hours following similar treatment. The apparent insensitivity of the processes involved may be attributable to the rapidity with which excretion takes place during the two-hour period following dosing.

Previous investigations of the excretion of aflatoxin B₁ have necessarily been based upon detection of the compound or its metabolites by toxicity measurements or by fluorescence technics. Thus, Allcroft and Carnaghan (3) found that extracts of milk from cows fed diets contaminated with aflatoxin were toxic to ducklings but contained no detectable aflatoxin B₁. Subsequent experiments by de Jongh et al. (13) revealed the presence of a toxic, fluorescent metabolite of aflatoxin (the so-called "milk toxin") in the milk of cows and rats. Similarly, the urine of sheep dosed orally or parenterally with aflatoxin B₁ were found to contain a substance with similar properties (4) and was toxic to ducklings (2). Recently, the composition of the "milk toxin" has been established by chemical identification of crystalline substances isolated from milk of cattle fed aflatoxin-contaminated feeds (15). Two compounds, referred to as aflatoxins M₁ and M₄, were identified as hydroxylated metabolites of aflatoxins B₁ and B₂, respectively. On the basis of their fluorescence characteristics and chromatographic behavior, these compounds appear to be common metabolites of aflatoxins found in urine, feces, and milk of various animal species.

Results of the tissue distribution studies indicate that the labeled compound is rapidly absorbed, as it was present in tissues studied within 0.5 hour of its administration. Levels of radioactivity were 5 to 15 times greater in liver than in any other tissue. During the 8- to 24-hour interval, the liver contained as much radioactivity as the remainder of the carcass, and at the end of 24 hours retained nearly 10\% of the administered dose. These findings correlate well with numerous observations that even large (lethal) doses of aflatoxins do not result in significant histologic damage in tissues other than liver and would suggest that effective concentrations of the compound do not accumulate in these tissues.

The relative retention of the compound by liver is also of interest in view of the prolonged character of histologic and biochemical effects resulting in this organ after a single dose. The present results indicate a significant residual amount of radioactivity in liver 24 hours after dosing. This finding is somewhat at variance with those reported by Butler and Clifford (10), who studied the aflatoxin content of liver from rats given a single dose of 7 mg/kg. On the basis of fluorescence and chromatographic properties of methanol-chloroform extracts of liver, these investigators concluded that only slight traces of aflatoxins B₁ and M₁ were present 24 hours after dosing. The discrepancy may be attributable to binding of the compound to cellular constituents, with concomitant alterations of solubility in the solvents used, or to conversion to nonfluorescent metabolites.

The liver fractionation experiments indicate that the radioactive compound appears mainly in the microsomal supernatant 30 minutes after the compound is injected, and rapidly becomes associated with the microsomal components during the first two hours. This accumulation presumably is associated with localization in this fraction of enzymes effecting metabolic conversion of the compound. However, the possible solubility of the compound in membrane components of the microsomal fraction or binding to other constituents of the fraction cannot be excluded.

The present results, being based on measurements of radioactivity alone, provide no information on the binding characteristics of aflatoxin B₁ or on the chemical nature of its metabolic products. Although in vitro binding of aflatoxin B₁ to DNA and failure of binding to RNA, histone, and albumin have been re-
ported (12, 17) in vivo binding to cellular constituents has not yet been demonstrated.

Quantitative studies of excretion and distribution of aflatoxins by chemical means has been difficult in the absence of information regarding the extent to which metabolites retain the fluorescent properties of the parent compound. The data described in the present experiments permit quantitative evaluation of the excretory pattern and tissue distribution after a single dose of aflatoxin B1. Although these data provide relatively little information on the nature of the excretory products, the demethylation observed gives rise to a phenolic derivative, which by analogy to compounds containing similar molecular configurations would be expected to be excreted in conjugated form. Experiments are now in progress to characterize these and other metabolites present in excreta and tissues of rats.

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