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Protection of Mitochondrial Genetic System against Aflatoxin B_1 Binding in Animals Resistant to Aflatoxicosis[1]

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ABSTRACT

Administration of a single dose of aflatoxin B_1 (AFB_1) (6 mg/kg) to Sprague-Dawley rats results in a high level of modification of hepatic mitchondrial DNA (2.1 nmol of AFB_1 adducts per μmol DNA-phosphate) and long-term inhibition of mitochondrial transcription and translation activities (N. Bhat *et al.*, Cancer Res., *42:* 1876–1880, 1982). Similar doses of AFB_1 given to ICR mice and Syrian golden hamsters result in negligible to very low levels (0–0.6 nmol) of adducts in hepatic mitochondrial DNA. Intact mitochondria from rat liver can metabolize significant amounts of AFB_1 (0.29 nmol/mg of protein) without externally added reduced nicotinamide adenine dinucleotide phosphate, and the metabolic activity is stimulated nearly 3-fold by Kreb's cycle intermediates (glutamate and malate), which support intramitochondrial reduced nicotinamide adenine dinucleotide phosphate production. Intact mitochondria from mice and hamsters, on the other hand, metabolize negligible or very low levels of AFB_1 (0–0.1 nmol of AFB_1 per mg of protein) even when intramitochondrial reduced nicotinamide adenine dinucleotide phosphate production is stimulated by the addition of Kreb's acids. Detergent-solubilized mitoplasts containing less than 1% microsome contamination from all three sources can catalyze the metabolic activation of AFB_1 to electrophilic reactive forms as determined in an *in vitro* DNA binding assay at comparable levels (1.2–2.2 nmol of AFB_1 bound per μmol of cytochrome P-450), suggesting that the low levels of AFB_1 metabolism by intact mouse and hamster mitochondria and the relative resistance of macromolecular synthesis in these particles to added AFB_1 may be due to mitochondrial membrane impermeability. In support of this possibility, AFB_1 transported into mouse liver mitochondria through a liposome delivery system causes about 80% inhibition of protein synthesis.

INTRODUCTION

Varied lipophilic compounds including direct acting alkylating agents and those requiring metabolic activation have been known to accumulate in the mitochondrial membranes of treated cells (1–6). A number of studies have shown that structurally different carcinogens such as nitrosamines (7–9) and PAHs[3] (10–14) including AFB_1 preferentially modify mt DNA at frequencies severalfold higher than the nuclear DNA. In the yeast *Saccharomyces cerevesiae*, a wide range of carcinogenic agents induces cytoplasmic petite mutation (15). In rats treated with AFB_1, the high level of adducts in hepatic mt DNA persists possibly due to inefficient excision repair in these organelles, resulting in a long-term inhibition of mitochondrial transcription and translation (13, 14) and also varied metabolic functions (16, 17). Recent experiments in our laboratory have also shown the presence of multiple forms of inducible cytochrome P-450 enzymes in hepatic mitochondria which can metabolize structurally diverse PAHs and nitrosamines (18–20). Despite observations that mitochondria are the direct and possibly preferential targets for attack during experimental carcinogenesis, the precise role of mitochondrial injury in cellular carcinogenicity and cytotoxicity remains unclear.

It is now well known that many animal species like hamsters and mice are resistant to the fungal toxin AFB_1 (21, 22). In the present study we have used this interspecies variation insensitivity to AFB_1 toxicity and carcinogenicity to investigate the relationships, if any, between hepatic mitochondrial injury and the carcinogenic/cytotoxic potential. Our results show that the mitochondrial genetic system of the target tissues in AFB_1-resistant mouse and hamster is protected against the injurious effects of AFB_1. A major source of protection in these animals appears to involve impermeability of mitochondrial membranes to AFB_1.

MATERIALS AND METHODS

$NADP^+$, isocitric acid (trisodium salt), isocitrate dehydrogenase type IV, digitonin, D-mannitol, HEPES, bovine serum albumin (Fraction V), Triton N-101, phosphatidyl choline, phenobarbital (disodium salt), and AFB_1 were purchased from Sigma Chemical Company, St. Louis, MO. Native calf thymus DNA was from Worthington Biochemical Corporation, Freehold, NJ. [^{3}H]AFB_1 (16 Ci/mmol) was purchased from Morvak Biochemicals, City of Industry, CA. [^{35}S]Methionine (<800 Ci/mmol) and [^{3}H]UTP (40 Ci/mmol) were from Amersham Radiochemicals Corporation, Arlington Heights, IL.

Animals. Male Sprague-Dawley rats weighing 125–150 g (West Jersey Biologicals, Wenonah, NJ), male Syrian hamsters weighing 100–120 g (Charles River Breeding Laboratories, Inc., North Wilmington, MA), and male ICR mice weighing 25–30 g (Blue Spruce Farms, Altamong, NY) were used in this study. All animals were kept on a 12-h light, 12-h dark cycle, and water and food were made available *ad libitum*. In studies on the intracellular distribution of carcinogen, [^{3}H]AFB_1 (specific activity, 1 μCi/109 nmol) was injected i.p. in dimethyl sulfoxide at a dose rate of 6 mg/kg of body weight as described before (18).

Isolation of Subcellular Fractions. Livers from treated and untreated animals were washed free of blood clots, minced, and homogenized in a buffer containing 1 mM HEPES (pH 7.4), 70 mM sucrose, 220 mM D-mannitol, 1 mM EDTA, and bovine serum albumin (0.5 mg/ml), and mitochondria were isolated using the differential centrifugation method as described before (23). Mitoplasts were prepared using the digitonin method (24). Microsomes were isolated from the postmitochondrial fraction as described before (18). Freshly isolated mitochondria or microsomes were used in all the metabolic activation and biosynthetic studies.

Determination of Cytochrome P-450 Contents of Mitochondrial and Microsomal Fractions. Digitonin-stripped, well-washed mitoplasts and once washed microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 1% (v/v) Triton N-101 at a protein concentration of 2 mg/ml, and the dithionite-reduced CO difference spectra were determined as described by Omura and Sato (25) using a Hitachi 110A dual beam spectrophotometer.

***In Vitro* DNA Binding Assays.** The *in vitro* binding of [^{3}H]AFB_1 to calf thymus DNA was assayed using a system essentially as described before (18, 26). Initially NADPH was generated in a reaction mixture containing 0.1 M potassium phosphate (pH 7.4), 3.3 mM $MgCl_2$, 1 mM $NADP^+$, 17 mM isocitrate, and 0.35 units of isocitrate dehydrogenase by incubation at 37°C for 10 min. Calf thymus DNA (1 mg/ml),

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[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; AFB_1, aflatoxin B_1; mt DNA, mitochondrial DNA; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; DNA-P, DNA-phosphate.

[^{3}H]AFB_1 (25 nmol/ml; specific activity, 10 μCi/25 nmol), and mitochondrial or microsomal protein (5 mg/ml) were added to the reaction mixture, and the incubation was continued at 37°C for 40 min. The reaction was stopped by adding equal volumes of phenol-cresol, and DNA was isolated as described (27). Aliquots of DNA in 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0) were counted with 10 ml of an aqueous scintillation cocktail. DNA was estimated using the extinction coefficient of 6600 cm^{-1} m^{-1} at 260 nm.

Activation of AFB_1 by Intact Mitoplasts. Mitoplasts were suspended in a buffer containing 4 mM HEPES (pH 7.4), 3 mM potassium phosphate (pH 7.4), 1 mM EDTA, 5 mM β-mercaptoethanol, and 0.25 M sucrose at 3-mg/ml concentrations and incubated at 30°C with or without added glutamate and malate as specified in the footnotes. The reaction was initiated by adding 25 nmol of [^{3}H]AFB_1 per ml, and after 10 min of incubation, the reaction was stopped by plunging the tubes into ice. The total AFB_1 including the metabolized forms was recovered by three repeated extractions with $CHCl_3$. The combined $CHCl_3$ extract was exposed to anhydrous Na_2SO_4 for 2 h and was evaporated with a jet of N_2 gas. The residue was dissolved in 50–100 μl of $CHCl_3$ and analyzed by thin-layer chromatography on Silica G plates using a chloroform:isopropyl alcohol (95:5) solvent system as described in an earlier paper (20). The radioactivity in spots corresponding to metabolized and nonmetabolized AFB_1 was measured by scanning the thin-layer chromatography plates in a Bioscan 100 scanner equipped with an HP-85 computer system (20). Appropriate controls without added enzyme and with heat-inactivated enzyme were run. The basal values obtained with these controls were subtracted from all the experimental values.

Preparation of Liposome-encapsulated AFB_1. Multilamellar liposomes were prepared using the procedure described by Van Rooijen and Van Nieuwmegan (28). Phosphatidyl choline (2.5 mg) with or without added AFB_1 (0.7 mg/ml) was dissolved in 1.0 ml of $CHCl_3$:CH_3CH_2OH (3:1) and dried first under a stream of N_2 gas, and then in a vacuum to remove traces of solvents. The thin film of lipid thus formed was suspended in 2.0 ml of 25 mM Tris-HCl (pH 7.4) with mild sonication (10–20 s) and agitated under an N_2 atmosphere for 45 min at room temperature. The suspension was pulse sonicated in bursts of 30 s following a 30-s standing on ice for a total of 3 min using a Branson sonifier (Setting 4). The multilammelar liposomes were pelleted by centrifugation in a Sorvall OTD-2 ultracentrifuge at 150,000 $\times$ g for 30 min. In some experiments AFB_1 encapsulated in liposomes was banded in a sucrose density gradient and then pelleted as described above. The liposomes were suspended in 25 mM Tris-HCl (pH 7.4) and stored in aliquots at −20°C in an N_2 atmosphere until use.

Electrophoresis of Proteins. Mitoplasts labeled with [^{35}S]methionine were dissociated by heating at 95°C in a buffer containing 125 mM Tris-HCl (pH 6.8), 550 mM 2-mercaptoethanol, and 5% sodium dodecyl sulfate, and the solubilized proteins were electrophoresed on an 8–16% gradient polyacrylamide-sodium dodecyl sulfate slab gel (23). The gel was fluorographed using EnHANCE (New England Nuclear), dried, and exposed to Kodak SB 5 X-ray films for a specified length of time.

RESULTS

Interspecies Variations in AFB_1 Binding to Mt DNA. The relative levels of AFB_1 adducts in the nuclear DNA and mt DNA following a single dose of ^{3}H-labeled carcinogen (6 mg/kg) are presented in Table 1. At the peak time of 3 h after AFB_1 administration, rat hepatic mt DNA is modified at 2.1 nmol/μmol of DNA-P, whereas the nuclear DNA is modified at about 0.3 nmol/μmol of DNA-P. After 24 h, however, the adducts in mt DNA remained nearly the same, while those in nuclear DNA are reduced to less than 0.1 nmol/μmol of DNA-P. In the case of hamster liver, the level of AFB_1 bound to nuclear DNA after 3 h of drug administration is nearly the same (0.26 nmol/μmol of DNA-P) as that found in the rat liver system, although the level of modification of mt DNA is about ⅓ the level (0.6 nmol/μmol of DNA-P) of rat hepatic mt DNA. In contrast to the rat liver mitochondrial system, after 24 h of carcinogen administration, the level of adducts in hamster liver mt DNA is reduced by over 50%. These results suggest the possible occurrence of an unknown mechanism in hamster liver mitochondria for the time-dependent removal of AFB_1-DNA adducts. Finally, in the case of mouse liver, the nuclear DNA contains very low levels (0.026–0.04 nmol/μmol of DNA-P) of AFB_1 adducts at 3 and 24 h after carcinogen administration. At each of these time points, however, mouse liver mt DNA contains no detectable AFB_1 adducts (Table 1).

Table 1 *Species variations of [^{3}H]AFB_1 binding to hepatic nuclear and mt DNA*

Animals were given injections of [^{3}H]AFB_1 (specific activity, 1 μCi/100 nmol) at a dose rate of 6 mg/kg. At intervals of 3 and 24 h, animals were killed, the livers were removed, and mitoplasts were isolated as described in "Materials and Methods." The mitochondrial circular DNA was isolated by cesium chloride-ethidium bromide banding, and nuclear DNA was isolated by the phenol extraction method also as described in "Materials and Methods."

Animal species	Time (h) after administration	AFB_1 bound to DNA (nmol/μmol DNA-P)	
		Nuclear DNA	Mitochondrial DNA
Rat	3	0.3 ± 0.1[a] (20.7)[b]	2.1 ± 0.2 (16.4)
	24	0.09 ± 0.02 (6.9)	2.1 ± 0.3 (18.3)
Hamster	3	0.26 ± 0.11 (29.6)	0.6 ± 0.06 (13.5)
	24	0.1 ± 0.05 (13.2)	0.3 ± 0.09 (5.1)
Mouse	3	0.04 ± 0.01 (0.5)	0 (0.5)
	24	0.026 ± 0.015 (0.8)	0 (0.5)

[a] Mean ± SD of the average of three separate experiments.
[b] Numbers in parentheses, [^{3}H]AFB_1 (nmol) associated with total mitoplast and total nuclear isolates from 1 g of tissue, the average of two independent estimates.

Results presented in Table 1 also show that the level of AFB_1 in the rat hepatic nuclear fraction is reduced to about 6.9 nmol/g of tissue at 24 h from a relatively high level of 20.7 nmol/g of tissue at 3 h after the drug dose. In the rat mitochondrial fraction, however, the concentration of AFB_1 remains nearly unchanged even after 24 h (see Table 1). In hamster liver mitochondria, on the other hand, the level of AFB_1 is reduced to 5.1 nmol/g of tissue at 24 h from about 13.5 nmol/g of tissue at 3 h after drug administration. These results are indicative of the possible occurrence of an AFB_1 removal system in hamster liver mitochondria which appears to be absent from rat liver mitochondria. Finally, it is seen that mouse hepatic mitochondria contain negligible amounts of AFB_1 at both time intervals after carcinogen administration (see Table 1).

Mitochondrial Cytochrome P-450 Contents and AFB_1 Activation. The hepatic mitochondrial extracts from the three animal sources were assayed for AFB_1 activation in a direct DNA binding system, to determine if monooxygenase activation of the parent carcinogen inside the mitochondrial inner membrane compartment is a factor limiting the levels AFB_1 binding to mt DNA in mouse and hamster systems. Because of the known problems of cross-contamination, digitonin-stripped well-washed mitoplasts with defined purity were used. A typical mitoplast preparation contained 0.5–0.8% of the microsome-specific marker enzymes rotenone-insensitive NADPH cytochrome c reductase and glucose 6-phosphatase (19) and 1–5% outer membrane specific enzyme monoamine oxidase. These digitonin-treated particles contained over 95% of the matrix enzyme carbamyl phosphate synthetase I (29), indicating their structural integrity. As shown in Table 2, hepatic mitoplasts from all three sources contain cytochrome P-450 in the range of 0.18–0.24 nmol/mg, while the microsomal preparations contained 0.9–1.0 nmol/mg of protein. Furthermore, mitochondrial enzymes from these three sources can metabolize AFB_1 into electrophilic forms capable of covalent binding to added calf thymus DNA. Both rat and mouse liver mitochon-

Table 2 *Cytochrome P-450 contents and the level of AFB_1 activation by mitochondrial extracts*

Mitoplasts from rat, mouse, and hamster liver were prepared by the digitonin method and washed 3 times with mitochondrial isolation buffer. Microsomes were once washed with mitochondrial isolation buffer. Determination of cytochrome P-450 contents by CO differential spectra and *in vitro* activation of AFB_1 using a DNA binding assay were as described in "Materials and Methods."

Animal species	Cytochrome P-450 (nmol/mg protein)		nmol AFB_1 bound to DNA/nmol P-450	
	Mitoplast	Microsomes	Mitoplast	Microsomes
Rat	0.20 ± 0.04[a]	1.0 ± 0.1	1.4 ± 0.3	1.1 ± 0.2
Hamster	0.18 ± 0.05	0.9 ± 0.2	2.2 ± 0.2	1.9 ± 0.2
Mouse	0.24 ± 0.04	0.96 ± 0.2	1.25 ± 0.25	1.2 ± 0.2

[a] Mean ± SD, representing an average of three estimates.

Table 3 *Uptake and metabolic activation of [^{3}H]AFB_1 by intact mitochondria*

Mitochondria were isolated as described in Table 2 except that the digitonin step was excluded. Mitochondria were washed 3 times with a buffer containing 4 mM HEPES (pH 7.4), 3 mM potassium phosphate, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.25 M sucrose and suspended in the same buffer at 3-mg/ml concentration. The suspension was incubated at 30°C for 30 min with added [^{3}H]AFB_1 extraction of AFB_1 with $CHCl_3$, and resolution of metabolites by thin-layer chromatography and quantitation were as described in "Materials and Methods."

Source of mitochondria	Additions	[^{3}H]AFB_1 metabolized (nmol/mg mitochondria)
Rat liver	Control	0.29 ± 0.6[a]
	Glutamate/malate	0.89 ± 0.09
	Glutamate/malate + Microsomes	0.85 ± 0.08
Hamster liver	Control	0.06 ± 0.02
	Glutamate/malate	0.11 ± 0.05
	Glutamate/malate + Microsomes	0.12 ± 0.04
Mouse	Control	0.0
	Glutamate/malate	0.0
	Glutamate/malate + Microsomes	0.0

[a] Mean ± SD, representing an average of three separate estimates.

drial enzymes show comparable activity in the range of 1.2–1.4 nmol of AFB_1 bound to DNA per nmol of cytochrome P-450, while the hamster mitochondrial enzyme shows 50–60% higher activity (2.2 nmol/nmol of cytochrome P-450). Similarly, the microsomal fraction from hamster liver shows 60–70% higher *in vitro* activity (1.9 nmol of AFB_1 per nmol of cytochrome P-450) than similar fractions from mouse and rat liver (1.1–1.2 nmol of AFB_1 per nmol of cytochrome P-450). Results of these experiments demonstrate that the monooxygenase system for the activation of AFB_1 is present in mitochondria from all the three sources under study.

Transport of AFB_1 into Intact Mitoplasts. As shown in Table 3, intact rat liver mitochondria can metabolize added AFB_1 at a significant level of 0.29 nmol/mg. Addition of Kreb's cycle intermediates, glutamate and malate (100 μM each), which are known to support the generation of intramitochondrial NADPH (30), also stimulate AFB_1 metabolism by 3-fold (see Table 3 and Ref. 20). Although not shown here, addition of either NADPH or the isolated microsomal fraction had no effect on the extent of activation by intact mitochondrial particles. It is interesting to note that, under these *in vitro* conditions, hamster liver mitochondria show only a marginal metabolic activity (0.06 nmol/mg), while mouse liver mitochondria show no detectable activity. Addition of glutamate/malate results in a 50% stimulation of activation in hamster liver mitochondria and no effect on mouse liver mitochondria, although intramitochondrial NADPH generation in both of these systems proceeds at rates similar to those in rat liver mitochondria, *i.e.*, 0.5–0.8 nmol of NADPH per mg of mitochondria per min.

The *in vitro* transport-coupled intramitochondrial activation of AFB_1 was also studied by way of verifying the effects of added carcinogen on mitochondrial transcription/translation activities. As shown in Table 4, AFB_1 added to rat liver mitoplasts causes 50–80% inhibition of translation activity. Although not shown here, this inhibition is dose dependent. In keeping with the metabolic activation pattern presented in Table 3, AFB_1 inhibits the transcription and translation activities of isolated hamster liver mitoplasts only marginally (10–25%). Furthermore, AFB_1 has no detectable inhibitory effect on *in vitro* transcription/translation by mouse liver mitoplasts. These results show that the mitochondrial genetic system in mouse and hamster liver are protected against the injurious effects of AFB_1 under both *in vivo* and *in vitro* conditions.

Liposome-mediated Transport of AFB_1 into Mitochondria. The results of metabolic activation (Table 3) and effects on transcription/translation (Table 4) in intact mitoplasts, suggesting relative impermeability of mitochondrial membranes to AFB_1 in mouse and hamster systems, were further verified using a liposome delivery system (28, 31, 32). Multilamellar liposomes prepared from phosphatidyl choline were used in this study. As seen from Fig. 1, liposomes alone yield about 50–90% stimulation of [^{35}S]methionine incorporation in mouse liver mitoplasts, possibly by increasing membrane permeability (Fig. 1, *Lane 2*). AFB_1 at 0.65 μmol/ml, added directly, has no significant effect on the protein synthesis pattern (see Fig. 1, *Lane 3*). It is, however, interesting to note that even at a 0.16-μmol/ml concentration, liposome-encapsulated AFB_1 causes about 70–80% inhibition (see Fig. 1, *Lane 4*). The inhibition of protein synthesis by liposomal AFB_1 is dose dependent, and the liposomal AFB_1 also inhibits mitochondrial transcription in both mouse and hamster liver mitochondria (results not presented). These results provide additional support for the possibility that protection of the mitochondrial genetic systems in both mouse and hamster systems may be either partly or completely due to membrane impermeability.

DISCUSSION

AFB_1 is a potential food contaminant affecting many animal species. There is considerable interspecies variation in sensitivity to both toxic and carcinogenic effects of this agent (21, 33). For example, rats are very sensitive to AFB_1 with a high incidence of hepatic tumor, while mice are resistant to its cytotoxic as well as carcinogenic effects (21, 22). Similarly, adult hamsters are considerably resistant to AFB_1, having low tumor and low mortality rates (34). In this study, we have used these three animal species to investigate the relationship between the extent of mitochondrial injury and the sensitivity to

Table 4 *Effects of AFB_1 on in vitro translation by isolated mitoplasts*

Isolation of mitoplasts from rat, hamster, and mouse livers and details of *in vitro* protein synthesis using [^{35}S]methionine were as described before (23). AFB_1 when added was at 0.5 mM, and the concentrations of glutamate and malate were at 100 μM each.

Source of mitochondria	Additions	Protein synthesis (^{35}S cpm/mg protein)
Rat liver	None	1.25 ± 10^6[a]
	AFB_1	0.33 ± 10^6
	AFB_1 + glutamate/malate	0.14 ± 10^6
Hamster liver	None	1.12 ± 10^6
	AFB_1	0.85 ± 10^6
	AFB_1 + glutamate/malate	0.76 ± 10^6
Mouse liver	None	0.90 ± 10^6
	AFB_1	0.94 ± 10^6
	AFB_1 + glutamate/malate	0.89 ± 10^6

[a] Mean ± SD.

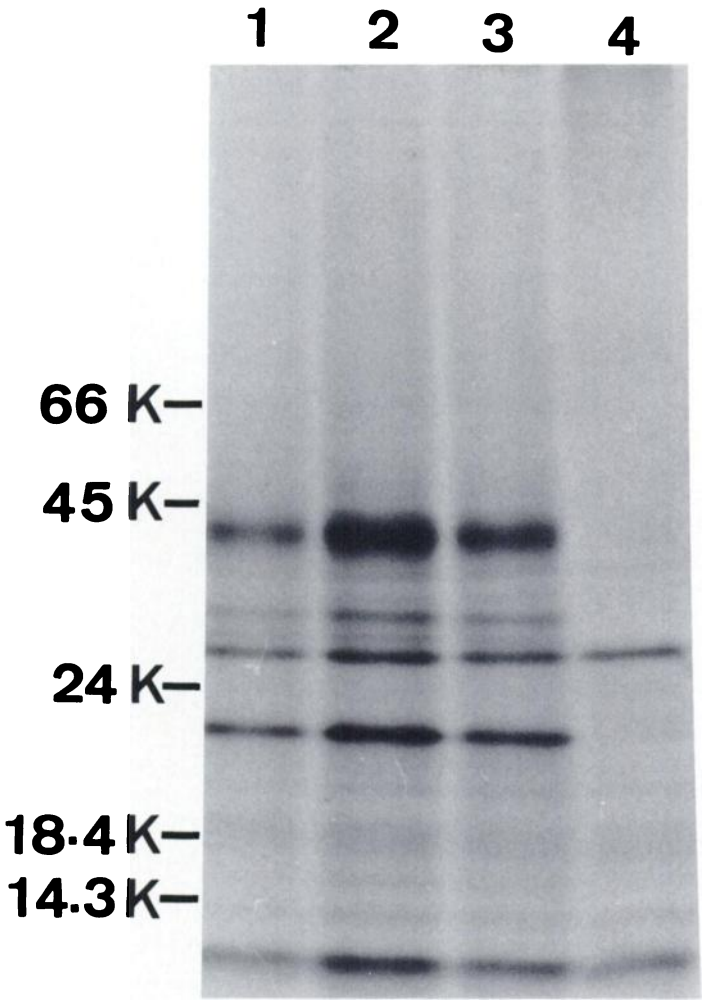

Fig. 1. Inhibition of protein synthesis by liposome-mediated delivery of AFB_1 into mouse liver mitochondria. Mitoplasts were isolated from mouse liver and labeled *in vitro* with [^{35}S]methionine as described in Table 4. Proteins (50 μg each) solubilized in a buffer containing high sodium dodecyl sulfate and 2-mercaptoethanol as described in "Materials and Materials" were electrophoresed on a 8–16% gradient polyacrylamide-sodium dodecyl sulfate gel. Multilamellar liposomes and liposome-encapsulated AFB_1 were prepared using phosphatidyl choline also as described in "Materials and Methods." *Lane 1*, control mitoplasts; *Lane 2*, mitoplasts labeled in the presence of control liposomes (0.1 ml/ml reaction); *Lane 3*, mitoplasts labeled in the presence of AFB_1 (0.65 μmol/ml); and *Lane 4*, mitoplasts labeled in the presence of liposome-encapsulated AFB_1 (0.15 μmol AFB_1 entrapped in 0.1 ml liposome per ml reaction). Details of fluorography and autoradiography were as described in "Materials and Methods."

cytotoxic and carcinogenic effects of AFB_1.

When [^{3}H]AFB_1 was administered to rats, hamsters, and mice at 6 mg/kg, the relative levels of modification of nuclear and mt DNA varied markedly among the three animal species under study. Although rat liver and hamster liver nuclear DNA was modified at comparable levels (0.26–0.3 nmol/μmol of DNA-P), the levels of adducts on mt DNA in these two animal systems varied markedly. Three h after the carcinogen dose, hamster mt DNA was modified at about ⅓ the level (0.6 nmol/μmol of DNA-P) of rat mt DNA (see Table 1). More important, the relative levels of AFB_1 adducts in hamster mt DNA were reduced by about 50% after 24 h, while those in rat liver mt DNA remained unchanged. Comparatively, mouse liver nuclear DNA was modified at very low levels (0.026–0.04 nmol/μmol of DNA-P), and there were no measurable AFB_1 adducts on mt DNA at either time point.

The relative levels of carcinogen adducts in nuclear and mt DNAs of different animal species are consistent with the distribution of AFB_1 in these two cell compartments. As reported before (13, 14), the level of AFB_1 in rat liver mitochondria remains nearly unchanged even after 24 h as opposed to a 50–60% reduction in hamster liver mitochondria (Table 1). The presence of glutathione in hepatic mitochondria has been documented (35), although there is no evidence of glutathione-*S*-transferase activity in mitochondria. It remains to be seen if hamster liver mitochondria contain a carcinogen removal system reminiscent of the glutathione-*S*-transferase system present in the cytoplasm for the detoxification of AFB_1 (36–38).

The observed species specific differences in the relative level of AFB_1-mt DNA adducts could be due to a number of factors, such as transport, scavenging systems (22, 37, 38), or the levels of mitochondrial monooxygenase for activation of AFB_1. It is seen in Table 2 that the hepatic mitochondrial cytochrome P-450 contents in all three animal species are nearly identical. Furthermore, mitochondrial extracts from both mouse and hamster liver can activate AFB_1 as efficiently as the rat liver mitochondrial enzyme in an *in vitro* DNA binding assay system. For these reasons, very low levels of mt DNA adducts in these AFB_1-resistant animal species may not be due to limitations of carcinogen activation in these organelles.

Previous studies from our laboratory showed that AFB_1, added to intact rat liver mitochondria, modifies mt DNA at a high level of 8–12 adducts/10^7-dalton genome and also causes a severe inhibition of mitochondrial transcription/translation activities (13). It was also shown that addition of glutamate/malate increases the oxidative metabolism of AFB_1 by intact mitochondria, suggesting that monooxygenase activation of AFB_1 takes place inside the mitochondrial inner membrane using mitochondrially generated NADPH (20). In the present study, therefore, the level of activation of [^{3}H]AFB_1 by intact mitochondria, its dependence on intramitochondrially generated NADPH, and the biological manifestations of added carcinogen, as reflected in the inhibition of mitochondrial transcription/translation activities, were used as indices of AFB_1 transport into mitochondria. Our results show that both mouse and hamster liver mitochondria are very inefficient in malate/glutamate-dependent as well as independent activation of AFB_1. Similarly addition of AFB_1 caused a negligible inhibition of translation activity in mouse liver mitochondria and only a marginal inhibition in hamster liver mitochondria. Since similar concentrations of AFB_1 inhibit rat liver mitochondrial activity by 70–80%, it is likely that mitochondrial membranes from mouse and hamster livers may be more impermeable to AFB_1 than those from rat liver.

It is well known that the mitochondrial inner membrane presents a barrier to a number of lipophilic and hydrophilic compounds (5, 30). Liposome vesicles have been used for transmembranous delivery of proteins (39, 40) and small molecules like nitrogen mustard (31–33). In the present study, use of this liposome system resulted in a severe inhibition of protein synthesis even at concentrations of 0.2 μmol of AFB_1 per ml, whereas even 4 times that concentration of AFB_1 alone had no effect on the protein synthesis by mouse liver mitochondria. Although not shown, liposome-mediated delivery of [^{3}H]AFB_1 causes a high level (9–16 adducts per genome) of modification of mt DNA in intact mouse liver and hamster liver mitochondria.

The results of this study show that the hepatic mitochondrial genetic systems in mouse and hamster are protected against the injurious effects of AFB_1 under both *in vivo* and *in vitro* conditions. In the case of mouse liver mitochondria, the protection seems to result from the impermeability of the mitochondrial membrane to the carcinogen. In the case of the hamster liver

system, the protective mechanism appears to be more complex and includes both a permeability barrier and the possible occurrence of a scavenging system. Although the nature of this scavenging system remains unknown, preliminary results show that over 40% of AFB$_1$ associated with hamster liver mitochondria is water soluble as against no significant water-soluble component in rat liver mitochondria.

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