# EFFECT OF AFLATOXIN ON MITOCHONDRIAL TRANSCRIPTION AND TRANSLATION

by

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| | |
|---|---|
| 3'Me-DAB | 3'-methyl-dimethylaminoazobenzene |
| 2-Me-DAB | 2-methyl-dimethylaminoazobenzene |
| $AFB_1$ | aflatoxin $B_1$ |
| $AFB_{2a}$ | aflatoxin $B_{2a}$ |
| $AFM_1$ | aflatoxin $M_1$ |
| $AFP_1$ | aflatoxin $P_1$ |
| $AFQ_1$ | aflatoxin $Q_1$ |
| $AFH_1$ | aflatoxin $H_1$ |
| $AFB_2$ | aflatoxin $B_2$ |
| PPO | 2,5-diphenyloxazole |
| HEPES | N-2-hydroxyethylpiperazine-N - 2 - ethanesulfonic acid |
| HVPE | high voltage paper electrophoresis |
| HPLC | high pressure liquid chromatography |
| i.p. | intraperitoneal |

# INTRODUCTION

The number of toxic and carcinogenic compounds to which man is exposed present a serious health hazard. If we are to be able to develop effective means of treating the health problems resulting from exposure to these compounds, it is necessary to have a basic understanding of their action at the cellular, subcellular and molecular level.

This dissertation examines only a very small portion of this problem, the effect of aflatoxin at the mitochondrial level. Aflatoxin is a very potent hepatotoxin and carcinogen, and although its biochemical effects have been studied in many laboratories very little is known about its effects on the mitochondrion. The following work examines the effect of aflatoxin $B_1$ and its metabolites on mitochondrial RNA and protein synthesis.

## LITERATURE REVIEW

### Mitochondrial Transcription and Translation

The presence of DNA in isolated mitochondrial fractions was noted as early as 1951 (1, 2) but was generally attributed to nuclear contamination. It was not until the 1960's that convincing evidence for the existence of mtDNA was obtained (3, 4). Since then mtDNA has been isolated and characterized in several laboratories (5). It has also been shown that replication, transcription and translation occur in mitochondria and that these processes are distinct from the analogous processes in the nucleus and cytoplasm (see reviews 5-10).

Mitochondrial Transcription - RNA polymerase activity in isolated mitochondria was demonstrated several years ago (11); however, this enzyme has only recently been purified. Küntzel and Schäfer (12) first isolated mitochondrial RNA polymerase from Neurospora crassa. Enzymes have now been purified from mitochondria of yeast (13, 14), Blastocladiella emersonii (15), Xenopus laevis ovaries (16), rat liver (17-19) and Ehrlich ascites tumor cells (20). Several problems have been encountered in these purifications. The polymerase is tightly bound to an inner membrane-DNA complex and solubilization requires destruction of the membrane (19, 21, 22). The solubilized enzyme is quite labile (22, 23) and its activity is sometimes masked by endonucleases (12). In addition, proteolysis and oxidation of mitochondrial lipids sometimes interfere with the purification (24).

A striking feature of the purified enzymes is that they appear to consist of only low molecular weight subunits. Enzymes from N. crassa (12), yeast (25, 26) and rat liver (17, 19, 27) all show only one polypeptide with a molecular weight of 63,000-68,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme from X. laevis has even smaller subunits with a molecular weight of 46,000 daltons (16). These enzymes also have several other properties in common. They readily form aggregates in the absence of monovalent cations (12, 16, 25, 26), are drastically inhibited at low concentrations of ammonium sulfate (20mM) (16, 17, 25, 28) and are insensitive to high concentrations of $\alpha$-amanitin ($10^{-5}$-$10^{-4}$M) (12, 16, 17, 19, 25, 28). In addition, they require a DNA template, all four ribonucleoside triphosphates and $Mg^{2+}$ for activity, although $Mn^{2+}$ can sometimes replace $Mg^{2+}$ (16, 17, 19, 28). The enzymes so far tested also show a preference for a mtDNA template (12, 16, 26, 28).

The mitochondrial enzymes differ from each other in their sensitivity to rifampicin, an inhibitor of procaryotic transcription (29). In early studies using whole mitochondria, differences in sensitivity were frequently attributed to differences in permeability of the mitochondria (8). However, now that purified enzymes have been examined it appears that these differences are more complex than originally assumed. For example, a sensitive enzyme has been isolated from yeast in one laboratory (25) while an insensitive enzyme has been isolated from the same organism in other

laboratories (26, 28). The same is true of the enzyme from N. crassa (12 vs. 28) and liver (17–19 vs. 28). Whether the sensitive and insensitive activities actually represent two different RNA polymerases in mitochondria or two forms of the same enzyme is not clear.

The properties which the mitochondrial RNA polymerases hold in common also serve to distinguish them from the other eucaryotic enzymes. All other eucaryotic RNA polymerases known, like the procaryotic enzymes (10) are composed of two high molecular weight subunits and several smaller subunits (30). The nucleolar enzyme is stimulated at low concentrations of ammonium sulfate (<40mM) and is equally active with $Mg^{2+}$ and $Mn^{2+}$. The nucleoplasmic enzyme is greatly stimulated at higher concentrations of ammonium sulfate (100-120mM) and shows a marked preference for $Mn^{2+}$ over $Mg^{2+}$ (30, 32). While the nucleolar polymerase, similar to the mitochondrial enzyme, is not inhibited by α-amanitin, the nucleoplasmic enzyme is inhibited at very low concentrations ($10^{-9}$-$10^{-8}$M). A third eucaryotic RNA polymerase found in the cytoplasm is also sensitive to α-amanitin, but only at higher concentrations ($10^{-5}$-$10^{-4}$M) (10).

In contrast to the reports of similar mitochondrial RNA polymerases from different sources discussed above, Criddle's group has reported an enzyme from yeast which has a marked resemblance to nuclear enzymes (24, 33). The native enzyme has a molecular weight of about 500,000 daltons and consists of three major subunits with molecular weights of 150,000,

200,000 and 260,000 daltons. This enzyme also differs from other mitochondrial enzymes in its divalent metal ion requirement, preferring $Mn^{2+}$ over $Mg^{2+}$. These workers also report that using the methods of Scragg (13), they are still unable to obtain an enzyme from yeast mitochondria similar to that isolated by Scragg (13) or Rogall and Wintersberger (26). Further investigation is required to determine if a complex mitochondrial RNA polymerase does exist or if the enzyme reported by Eccleshall and Criddle (24, 33) is a nuclear contaminant.

The only products of mitochondrial transcription which have been identified with certainty are rRNAs and tRNAs. Mitochondria contain large and small rRNA species which are components of the large and small subunits of mitochondrial ribosomes (5, 34). In animal mitochondria, these rRNAs sediment at about 16-17S and 12-14S while the corresponding rRNAs from lower eucaryotes are larger, 21-23S and 14-16S (34). Hybridization saturation studies indicate that there is one cistron for each of these rRNAs per mtDNA molecule (35-38).

The mechanism of production of rRNA in N. crassa has been studied by Kuriyama and Luck (39) and resembles the processing of eucaryotic cytoplasmic rRNAs. A 32S precursor molecule is cleaved to yield the two rRNAs with a loss of 20% of the precursor molecule. From studies with a poky mutant of N. crassa which is deficient in mitochondrial small ribosomal subunits, these workers have concluded that methylation of the 32S precursor is

necessary for proper cleavage and production of the rRNAs (40). Whether or not the same type of mechanism exists in animal mitochondria is unknown; however, some investigators have reported these rRNAs to be methylated (41-43), and that the two rRNAs are produced in equimolar amounts (41, 35).

Mitochondria also contain their own specific tRNAs (35, 44-48). These tRNAs can be separated from their cytoplasmic counterparts in various chromatographic systems (49-52) and are acylated by specific mitochondrial aminoacyl-tRNA synthetases (45, 51, 51). In higher eucaryotes hybridization saturation experiments indicate that only 12-15 tRNA cistrons are specified by mtDNA (35, 36). In HeLa cells 12 separate cistrons have been mapped by electron microscopic studies using ferritin labeled tRNA (53). This number is clearly insufficient to support protein synthesis using all 20 amino acids. In addition, recent studies have demonstrated iso-accepting species of tRNA in mitochondria (52, 54) and that some of these iso-accepting species may be coded by separate cistrons (52). Thus, it appears that mitochondria of higher eucaryotes may import tRNA transcribed in the nucleus (55). So far, there is only one report of mitochondrial tRNA hybridizing with nuclear DNA (57). However, considering the much larger size of the nuclear genome, it would be difficult to detect only a few mitochondrial tRNA sequences (52).

In yeast, where the mtDNA is about five times larger and more complex than in the higher eucaryotes (5, 58), hybridization saturation

experiments suggest that there are 20-30 tRNA cistrons (47). Thus, if there is only a limited degeneracy in the tRNA population, mitochondrial protein synthesis may proceed without importing tRNAs coded in the nucleus.

Similar to transcription in the nucleus and in bacteria, the heavy strand of mitochondrial DNA codes for most stable products. Aloni and Attardi have found that the HeLa cell mitochondrial genome is transcribed completely (59) and symmetrically (60) in vivo, but most of the transcript of the light strand is rapidly degraded (60). The heavy strand codes for both of the rRNAs and nine of the tRNAs, while the light strand codes for three tRNAs (36, 53). It has also been shown in rat liver mitochondria that two tRNAs are coded by the light strand (61) but most stable products are complementary to the heavy strand (62-63).

Whereas the stable mitochondrial RNAs have been isolated and characterized in some detail, evidence for the existence of mitochondrial mRNA has mostly been indirect. Chuang and Weissbach (64) have reported that rat liver mtDNA acts as a template in a completely heterologous transcription-translation system from E. coli. However, the pattern of polypeptides produced differs significantly from that of polypeptides synthesized on mitochondrial ribosomes in vivo. In contrast, Küntzel and Blossey (65) have found that N. crassa mtDNA can be transcribed and translated in an E. coli system to yield two polypeptide fractions which appear to be identical to the two polypeptide fractions synthesized in a submitochondrial

system. Furthermore, Scragg (25) has found that transcripts of yeast mtDNA produced by mitochondrial RNA polymerase can be translated in an E. coli system to yield products which can be precipitated with an antiserum against a mitochondrial insoluble protein fraction.

Recently mitochondrial RNA with the properties of mRNA has been isolated. Perlman et al. (66) first isolated such a fraction from HeLa cell mitochondria. This RNA is heterogenous in size, is covalently linked to a poly(A) segment 50-80 nucleotides long, and can be released from mitochondrial ribosomes by puromycin. Ojala and Attardi (67) have confirmed the existence of this poly(A) containing RNA in HeLa cell mitochondria and have separated the fraction into eight distinct components. One of these RNAs has a molecular weight of $9 \times 10^4$ and hybridizes with the light strand of mitochondrial DNA. The other components have molecular weights of $2.6\text{-}5.3 \times 10^5$ and hybridize with the heavy strand. Mitochondrial RNA fractions containing poly(A) have also been isolated from Ehrlich ascites tumor cells (68), rat liver (69) and yeast (70). These data strongly suggest that mitochondrial DNA codes for some mRNA as well as rRNA and tRNA.

Mitochondrial Translation - Protein synthesis inside the mitochondrion takes place on ribosomes which are distinct from cytoplasmic ribosomes both in their physical properties and their response to inhibitors of protein synthesis (34). The mitochondrial ribosomes of higher eucaryotes were originally thought of as "mini-ribosomes" because of their low 55S

sedimentation coefficient (34). However, it has become apparent in recent work that these ribosomes are actually about the same size as bacterial ribosomes (71-74). For example, rat liver mitochondrial ribosomes have a molecular weight of $3.2 \times 10^6$ daltons (72) compared to $2.6\text{-}2.7 \times 10^6$ daltons for E. coli ribosomes (75) and $4.6 \times 10^6$ daltons for rat liver cytoplasmic ribosomes (75). The subunits of these mitochondrial ribosomes, 29S and 39S, also have molecular weights similar to the E. coli ribosomal subunits (72). In addition to their low sedimentation coefficient, these ribosomes also differ from other ribosomes, including those of lower eucaryotic mitochondria, by their low RNA and high protein content, and their low buoyant density (73, 74).

Mitochondrial ribosomes of N. crassa and yeast have generally been found to have sedimentation coefficients of 73-74S (34), but recently Datema, et al. (77) have found that the native ribosome of N. crassa mitochondria is probably an 80S species and that the 73S ribosome is an artifact of isolation. The molecular weight of this ribosome is not yet available to compare with other ribosomes.

The mitochondrial ribosomes of both lower and higher eucaryotes, and the polysomes formed from these ribosomes, have been shown to be active in protein synthesis (70, 76, 78-80). The mechanism appears to be quite similar to that observed in procaryotes. The ribosomes are sensitive to L-chloramphenicol, an inhibitor of bacterial protein synthesis

(37, 82), and insensitive to cycloheximide (37, 83) and emetine (84), inhibitors of cytoplasmic protein synthesis. Initiation of protein synthesis occurs with formyl-met-tRNA (85-87) and elongation requires GTP and the protein factors G and T (88-90). These elongation factors differ from cytoplasmic factors in molecular weight and chromatographic mobility, and cannot be replaced by the cytoplasmic elongation factors (88). On the other hand, E. coli and mitochondrial factors are interchangeable (88, 90).

Proteins synthesized inside mitochondria both in vitro (83, 91, 92) and in vivo (92) are found predominantly in an insoluble, hydrophobic fraction of the inner membrane. Generally these proteins can only be released by detergents that dissolve the membrane or by extreme conditions of alkalinity or acidity which also disrupt the membrane structure (83, 92). Coote and Work (92) have isolated a protein fraction from hamster liver mitochondria labeled in vitro which is insoluble in phosphate buffer at pH 11.5. This fraction contains only 10% of the total mitochondrial protein but 90% of the radioactivity, and could be separated into 20 protein bands by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Ten of these bands were labeled and their molecular weights ranged from 14,000 to 50,000 daltons. Identical patterns were observed when mitochondrial proteins were labeled in vivo using the hamster cell line BHK-21. The relatively simple electrophoretic profile of the proteins synthesized inside mitochondria both in vitro (83, 93) and in vivo (87, 94-96) has been confirmed in several other laboratories.

The hydrophobic nature of mitochondrially synthesized proteins has been further substantiated by the observation that a large portion of these proteins are soluble in chloroform:methanol (2:1) (97, 98). Furthermore, the amino acid compositions of two of these proteins show 76% and 62% non-polar residues (99).

The proteins synthesized inside the mitochondria make up less than 10% of the total mitochondrial protein (7). Most mitochondrial proteins, including those involved in the replication, transcription and translation of mtDNA, are coded for by nuclear DNA and synthesized on cytoplasmic ribosomes (7, 9, 100).

The greatest progress in identifying the proteins which are synthesized on mitochondrial ribosomes has been made with yeast and N. crassa. By in vivo labeling of these organisms in the presence of chloramphenicol or cycloheximide followed by isolation of certain mitochondrial inner membrane enzymes, it has been found that three subunits of cytochrome oxidase (101, 102), four subunits of oligomycin-sensitive ATPase (103, 104) and one subunit of cytochrome b (105) are synthesized inside the mitochondrion and are therefore probably coded for by mtDNA. Whether these proteins play a role in the catalytic function of the enzymes they are associated with or are only structural proteins required for assembly into the mitochondrial inner membrane is not known.

The identity of the proteins synthesized inside the mitochondria of

higher eucaryotes has not yet been determined. The electrophoretic profiles of these proteins from HeLa cells (87, 94), baby hamster kidney cells (92) and rat liver (92) mitochondria are similar to those of yeast (106) and N. crassa (107). This suggests that the mitochondria of higher eucaryotes may synthesize some of the same proteins as the mitochondria of lower eucaryotes.

The assembly of a functional mitochondrion involves a tightly coupled system of cytoplasmic and mitochondrial protein synthesis. It appears that mitochondrially synthesized proteins are produced in significant amounts only if they are continually combined with cytoplasmically synthesized partner proteins (108, 109). Conversely, integration of the cytoplasmically synthesized subunits of some proteins such as ATPase (110) and cytochrome oxidase (111), into the mitochondrial inner membrane requires proteins made in the mitochondria.

## Mitochondria of Tumor Cells

The high aerobic glycolysis of neoplastic tissue, first described by Warburg in 1923 (112), is a well known and established phenomenon (113). From his early observations, Warburg proposed that cancer originates as a result of injury to the respiratory process (114). This theory has been widely questioned and has been the subject of much debate (113, 115, 116). In spite of the attempts to prove or disprove this theory, the view expressed by R. K. Keilley in 1957 (117) still holds true today: "Although one may

agree that there is still considerable evidence that does not support the view, the basic concept has not been disproved, and continues to challenge the imagination and resources of those engaged in the problem of carcinogenesis."

The rest of this section of the literature review describes some of the characteristics of tumor cell mitochondria with particular emphasis on recent findings.

The mitochondria of tumor cells are more heterogeneous than those of normal tissues and are quite variable in size, shape and density (118). A couple of generalizations about tumor mitochondria can be made. They are usually smaller (119) and more fragile (120) than normal mitochondria, and there are fewer mitochondria per cell in neoplastic tissue than there are in normal tissue (118).

Several studies in the last few years have indicated that there are alterations in the membrane proteins of tumor cell mitochondria. Polyacrylamide gel electrophoresis of the insoluble mitochondrial inner membrane proteins from a rapidly growing Morris hepatoma indicates the absence of a protein band which is present in the mitochondria of host and normal liver. This protein band was present at reduced levels in the more slowly growing "minimal deviation" hepatomas (121). Differences in the electrophoretic patterns of mitochondrial membrane proteins of other Morris hepatomas (122), Zajdela hepatoma (123), Ehrlich ascites tumor cells (123), mouse mammary adenocarcinoma (124) and hamster melanoma (125) have

also been observed. Birkmayer and Balda (125) have found that the electrophoretic profiles of protein fractions from hamster melanoma and liver mitochondria labeled in vitro differ significantly. In contrast, Kuzela et al. (126) have observed no differences in the patterns of mitochondrial proteins of Zajdela hepatoma and liver labeled in vivo in the presence of cycloheximide; however, the pattern of total unlabeled mitochondrial membrane proteins did differ from normal liver (123).

Further investigation is required to determine the nature of the alteration of mitochondrial membrane proteins observed in neoplastic tissues. Considering the close control between cytoplasmic and mitochondrial protein synthesis during the assembly of the mitochondrial inner membrane (9), changes in either or both of these systems in tumor cells could result in an alteration of the mitochondrial inner membrane proteins.

Two inner membrane enzymes which may have some of their subunits synthesized inside the mitochondrion, ATPase and cytochrome oxidase (9), have been examined in tumor cells. Pedersen and co-workers (112, 127) have found that mitochondria from six hepatomas differing widely in growth rate and degree of differentiation, and mitochondria from Ehrlich ascites tumor cells, all have reduced uncoupler-stimulated ATPase activity. Other energy dependent reactions such as oxidative phosphorylation, respiration, ATP-$P_i$ exchange and ATP-supported $Ca^{2+}$ uptake, were normal in these mitochondria. Similar results have been obtained by Kularov et al. (123)

with Zajdela hepatoma.

Sato and co-workers (128, 129) have found that mitochondria of several ascites hepatomas and minimal deviation Morris hepatomas have low levels of cytochrome oxidase, cytochrome b and cytochrome $c_1$, but normal levels of cytochrome c. On the other hand, Schreiber et al. (130) have reported that the cytochrome oxidase content of mitochondria isolated from a rapidly growing Morris hepatoma is higher than that of control mitochondria, and normal levels of cytochrome oxidase have been found in mitochondria isolated from a Morris hepatoma of intermediate growth rate (131). Normal levels of cytochrome oxidase have also been found in melanoma and Novikoff hepatoma mitochondria (132). It is difficult to tell from these studies, conducted in different laboratories by different methods, if there might be a correlation between cytochrome oxidase levels and the rate of tumor growth.

Other mitochondrial enzymes, which are not synthesized in the mitochondrion, have also been reported to be reduced in hamster melanoma and Novikoff hepatoma (132). These include adenylate kinase, monoamine oxidase and succinate dehydrogenase.

Mitochondrial DNA, RNA and protein synthesis are also altered in tumor cells. Smith and Vinograd (133) found an increased frequency of dimeric forms of mtDNA in neoplastic cells. Nass (134) has shown that increased levels of dimeric and oligomeric mtDNA are associated with the

malignant transformation of chick embryo fibroblast cells by an oncogenic Rous sarcoma virus. Studies with a temperature sensitive mutant of the virus which transforms cells at $36^{o}$ but not at $41^{o}$, demonstrated that the increase in dimeric and oligomeric mtDNA levels is associated with cell transformation and not just virus infection and replication. Using the same system, Bosmann et al. (135) have found that a slight elevation of mitochondrial protein synthesis and a two-fold increase in mitochondrial RNA and DNA synthesis are also associated with cell transformation.

The Effect of Chemical Carcinogens on Mitochondrial Functions

Research dealing with the action of carcinogens on mitochondrial biochemical functions has demonstrated a variety of effects. Some of the aminoazo dye carcinogens, particularly 3'-methyl-dimethylaminoazobenzene (3'-Me-DAB), inhibit mitochondrial respiration in vitro (117, 136, 137), however, respiration is also inhibited by the non-carcinogenic aminoazo dye 2-methyl-dimethylaminoazobenzene (136, 137). In vivo administration of 3'-Me-DAB produces a decrease in cytochrome oxidase levels beginning in the 16th week (138). By the 27th week cytochrome oxidase is reduced to the level observed in 3'-Me-DAB induced hepatomas (one-half of normal levels). During this period there is no change in the level of cytochromes b, $c_1$ or c. The effect of 2-Me-DAB on cytochrome oxidase levels was not examined.

Arcos et al. (139) have studied the effect of 3'-Me-DAB feeding on the

ability of mitochondria to undergo active swelling. There is a decreased response to swelling-inducers with a sharp minimum at 4 weeks, the onset of irreversibility of tumor induction. Mitochondria isolated from 3'-Me-DAB induced hepatomas also have a decreased response to swelling-inducers. The authors have suggested that this decrease in sensitivity to swelling inducers may be due to a deletion of inducer receptor sites in the mitochondrial membrane. It was also observed that there is a decrease in the mitochondrial respiratory control ratio with a minimum at 3-4 weeks (137). The P:0 ratios of the isolated mitochondria were not affected during feeding, but a partial or total uncoupling was observed in the tumors. None of these effects were observed with 2-Me-DAB.

Hadler and Daniel (140) have examined the in vitro effect of the N-hydroxyacetylaminofluorenes on mitochondrial swelling. They observed a positive correlation between the carcinogenicity of the various isomers and their ability to induce ATP-energized volume changes in the presence of thiol reagents. These researchers had previously shown that this type of swelling response is produced by inhibitors and uncouplers of oxidative phosphorylation.

Carcinogens have also been found to bind to mitochondrial macromolecules. The N-oxidized derivatives of 2-aminofluorene, N-acetylaminofluorene and N-methyl-4-aminoazobenzene inhibit mitochondrial 5'-endonuclease activity in vitro, whereas the parent compounds are much less

inhibitory (141). The inhibition produced by these compounds was correlated with their binding to mitochondrial proteins. In addition, these compounds did not inhibit nuclear, microsomal or lysosomal ribonuclease activity.

Graffi (142) found that carcinogenic aromatic hydrocarbons accumulate in the mitochondria of animal cells and that N-methyl-N-nitrosourea and dimethylnitrosamine preferentially alkylated mitochondrial DNA over nuclear DNA (143, 144). Wilkinson et al. (145) confirmed this preferential alkylation of mitochondrial DNA by dimethylnitrosamine. Furthermore, they have shown that methyl methanesulphonate, an alkylating agent which does not produce tumors under the conditions used, alkylates mitochondrial and nuclear DNA to about the same extent.

## Biochemical Effects of Aflatoxin

In the 15 years since aflatoxin was identified as the causative factor in "Turkey X" disease, there has been a great deal of research attempting to elucidate the mode of action of these toxins. The toxicity and carcinogenicity of these compounds are well established (145a, 146), and there have been several reviews on the physical, chemical and biological properties of aflatoxin (146-149). This literature review will cover only those aspects which are related to the inhibitory effect of aflatoxin on transcription and translation, and the role of metabolism in mediating these effects.

The Effect of Aflatoxin on Transcription - It has been clearly demonstrated that aflatoxin inhibits in vivo liver RNA synthesis in the rat

(150-152). Clifford and Rees (153) found that a single $LD_{50}$ dose of $AFB_1$ (7mg/Kg body weight) suppressed orotic acid incorporation into rat liver nuclear RNA by 80% within 3 hr. They further demonstrated that there was no inhibition of orotic acid incorporation into the nucleotide pool. Sporn et al. (152) found a similar inhibition of incorporation of $^3$H-cytidine into liver nuclear RNA.

Friedman and Wogan (150, 151) examined the time course of inhibition of RNA synthesis after a single $LD_{50}$ dose of $AFB_1$ (5mg/Kg body weight). Incorporation of $^3$H-cytidine was inhibited 62% within 30 min of dosing, and 93% by 12 hr. An inhibition of 63% was still apparent 5 days after dosing. At lower doses of $AFB_1$ (0.5-1.0 mg/Kg body weight), Lafarge and Frayssinet (154) found the inhibition of RNA and DNA synthesis to be reversible with RNA synthesis reaching normal levels within 24 hr.

Inhibition of RNA synthesis has also been observed in rat liver slices exposed to $AFB_1$ ($3.2 \times 10^{-5}$M) (153, 155); in cultured rat liver cells grown in the presence of $1.6 \times 10^{-4}$M $AFB_1$ (156); and in nuclei (157-159) and nucleoli (160) isolated from livers of rats treated with $AFB_1$. Inhibition of RNA synthesis has not been observed in isolated nuclei treated with $2 \times 10^{-5}$M $AFB_1$ (161) or nucleoli treated with $5 \times 10^{-5}$ to $1.1 \times 10^{-3}$M $AFB_1$ (160). In addition, RNA synthesis is not inhibited by $AFB_1$ in several in vitro transcription systems: calf thymus DNA transcribed by bacterial RNA polymerase (158, 162) or rat testicular RNA polymerase (163); rat liver

deoxyribonucleoprotein (DNA + non-histone protein) transcribed by bacterial RNA polymerase (158). In contrast to these negative results, Portman and Campbell (164) have found a marked inhibition (80% at $1.5 \times 10^{-4}$ M $AFB_1$) of transcription by E. coli RNA polymerase on a rat liver chromatin template, and have commented on the possible involvement of histones. Maher and Summers (165) are the only authors to report in vitro inhibition of transcription on a DNA template by $AFB_1$ (35% at $6.4 \times 10^{-4}$ M). The general lack of in vitro inhibition of RNA synthesis by $AFB_1$ has led several investigators to suggest that a metabolite of $AFB_1$ is responsible for the in vivo inhibition (158, 161, 163).

Recently it has been found that a microsomal metabolite(s) of $AFB_1$ inhibits RNA synthesis in vitro (162, 167, 168); however, the nature of the metabolite(s) involved and the mechanism by which it inhibits RNA synthesis is in question. Moule and Frayssinet (166) reported a stable, chloroform extractable, microsomal metabolite which inhibits RNA synthesis in an E. coli RNA polymerase-calf thymus DNA system. The authors concluded that inhibition was due to a direct effect on the enzyme because addition of excess enzyme eliminated the inhibition, but an excess of template had no effect. Akinrimise, et al. (168) reported a stable microsomal metabolite(s) which inhibits rat liver nucleoplasmic and cytoplasmic RNA polymerase, but has no effect on nucleolar RNA polymerase. This study did not determine if the inhibition was due to a direct effect on the enzyme or a decrease of

template activity. In contrast to these reports of a relatively stable metabolite inhibiting RNA synthesis, Neal (167) found that an unstable microsomal metabolite of $AFB_1$ inhibits RNA synthesis through its interaction with the DNA template. No direct effect on rat liver nucleoplasmic RNA polymerase could be demonstrated. The metabolite(s) of $AFB_1$ which inhibits RNA synthesis has not been identified in any of these studies.

The question of whether aflatoxin inhibits transcription through interaction with RNA polymerase or the DNA template has also been examined using enzyme and template preparations isolated from animals treated with $AFB_1$. Edwards and Wogan (161) found that the template activity of rat liver chromatin isolated from animals 30 min after administration of $AFB_1$ was decreased 28-46%. There was no decrease in the ability of rat liver RNA polymerase isolated from $AFB_1$ treated animals to transcribe either calf thymus DNA or rat liver chromatin from control animals. The RNA polymerase preparation used in these studies was predominantly the nucleoplasmic enzyme. On the other hand, Neal (167) and Akinrimise et al. (168) have both shown a 50% inhibition of the activity of rat liver nucleoplasmic RNA polymerase isolated from animals 2 hr after $AFB_1$ administration. In both cases the nucleolar enzyme was not affected. Neal (167) also found that the template activity of chromatin and DNA isolated from treated animals was decreased 42-60%. Considering these data and the in vitro observations that a stable metabolite of $AFB_1$ inhibits RNA synthesis through direct action

on the RNA polynerase with no apparent effect on template activity (166), and that an unstable metabolite inhibits template activity but has no effect on the polymerase (167), it is possible that aflatoxin exerts its effect on transcription through the complex action of more than one metabolite. An unstable metabolite may be responsible for the inhibition of template activity observed at both 0.5 (161) and 2 hr (167) after in vivo administration $AFB_1$, and a stable metabolite may produce the inhibition of nucleoplasmic RNA polymerase activity observed only at 2 hr (167, 168). Further investigation is required to determine the validity of this hypothesis.

The Effect of Aflatoxin on Translation - Aflatoxin $B_1$ inhibits protein synthesis in vivo (169-172) and in liver slices incubated in vitro (153, 155, 160). It is generally thought that the inhibition is a consequence of altered transcription and disaggregation of polysomes (159, 149).

The disaggregation of polysomes after $AFB_1$ treatment was first reported by Roy (163). An increase in monomers and dimers was observed 3 hr after $AFB_1$ administration and disaggregation was almost complete at 6 hr. This alteration of polysome profiles by $AFB_1$ has been confirmed in other laboratories (170, 171, 173). Disaggregation first appears at about 3 hr after dosing. The effect is reversible with reaggregation beginning at 36 hr and complete by 5 days (173). It is thought that alteration of polysome profiles is due to inhibition of mRNA synthesis (170).

Aflatoxin also affects ribosomes by interfering with the maturation of

rRNA (171). Moule (174) found that $AFB_1$ treatment completely inhibited the appearance of newly synthesized 60S ribosomal subunits in the cytoplasm. It was further shown that 18S rRNA and 40S ribosomal subunits were produced, but no 28S rRNA was found. The 40S subunit produced in the presence of aflatoxin combined with pre-existing 60S subunits in the initiation of protein synthesis and was found in polysomes (175).

Sarasin and Moule (176) have proposed that aflatoxin inhibits protein synthesis by a direct action on the translational mechanism in addition to its inhibition of transcription. They observed a biphasic inhibition of *in vivo* protein synthesis. Inhibition reached a peak 2 hr after $AFB_1$ administration then declined up to 7 hr. Thereafter, the inhibition progressively increased to about 85% at 48 hr. The inhibition observed in the first two hours cannot be explained by inhibition of RNA synthesis, since there is very little alteration of polysome profiles at this time. Furthermore, Sarasin and Moule (176) have shown that a stable microsomal *in vitro* metabolite of $AFB_1$ inhibits amino acid incorporation by rat liver polysomes in a cell free system. Thus, it appears that the inhibition of protein synthesis by aflatoxin is a complex process with early inhibition due to a direct effect on translation and later inhibition a consequence of impaired transcription.

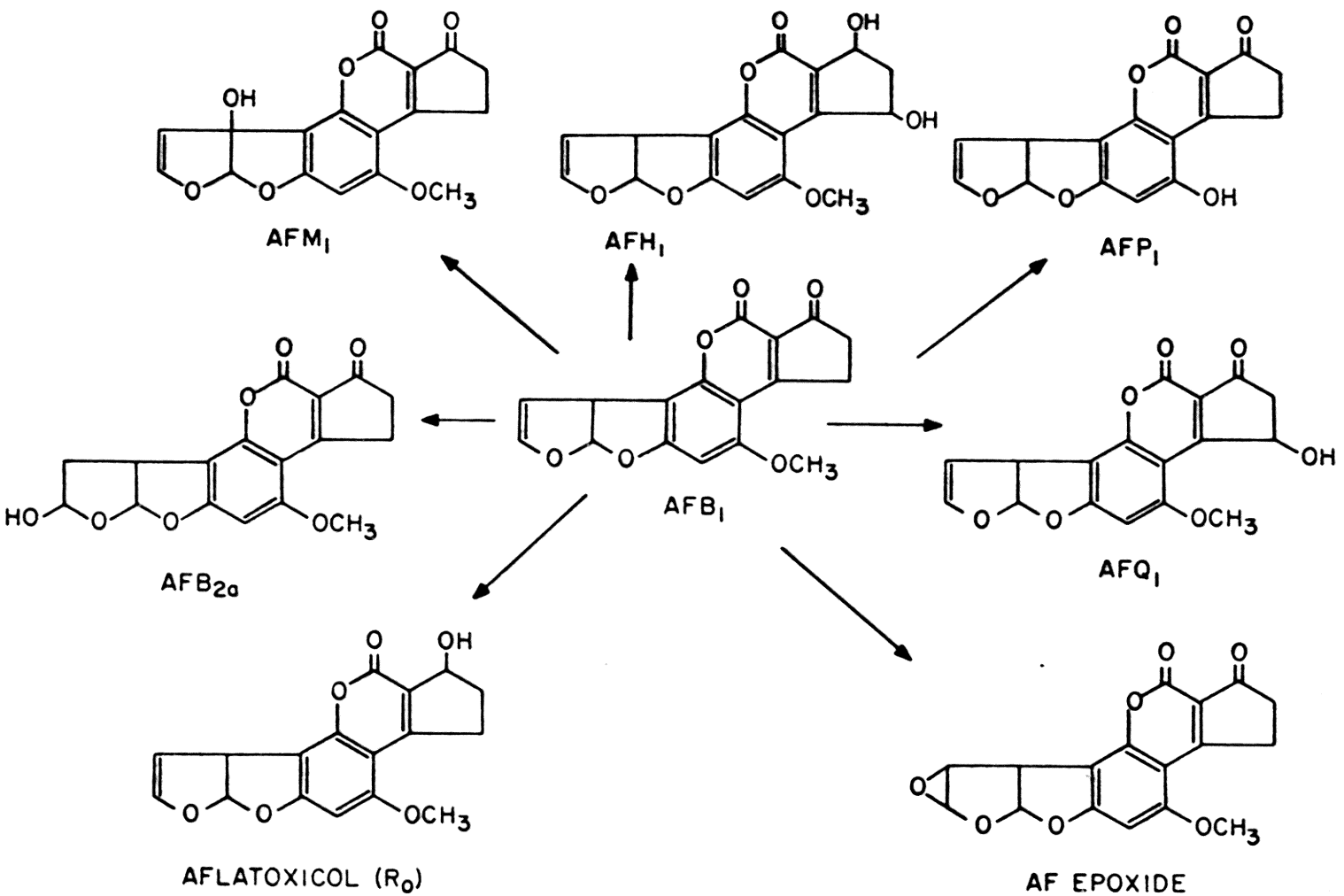
Aflatoxin $B_1$ Metabolites - It has been proposed that $AFB_1$ (177), like many other carcinogens (178) is metabolized to an electrophilic ultimate carcinogen. The metabolite which has received the most attention at this

time is aflatoxin $B_1$-2, 3-epoxide. The first evidence for the existence of such a metabolite was the observation that rat liver microsomes produce an unstable metabolite of $AFB_1$ which is lethal to Salmonella typhimurium (179). This metabolite binds to tissue nucleophiles such as protein, RNA and DNA (180), and mild acid hydrolysis of the $AFB_1$-RNA adduct yields 2, 3-dihydro-2, 3-dihydroxyaflatoxin $B_1$ (177). The $AFB_1$-RNA adduct has a $\lambda_{max}$ at 368 nm and does not exhibit a bathochromic shift in the presence of alkali, indicating that carbon 2 of the bound $AFB_1$ derivative does not bear a hydroxyl (177). These data suggest that the metabolite which binds to RNA is the $AFB_1$-2, 3-epoxide.

Other workers have also shown a microsomal dependent binding of $AFB_1$ to DNA, RNA and protein (181-183). The protein bound metabolite has a $\lambda_{max}$ in the region of 400 nm and degrades to yellow products (183). The spectral properties and degradation of this protein bound metabolite are similar to the properties of protein bound 2, 3-dihydro-2-hydroxy-$AFB_1$ ($AFB_{2a}$) (184, 185).

A number of stable metabolites of $AFB_1$ are known (see Figure 1). Those produced by the microsomal mixed function oxidase system are aflatoxin $M_1$ (186, 187) and aflatoxin $B_{2a}$ (188, 189), aflatoxin $P_1$ (190, 191) and aflatoxin $Q_1$ (192, 193). Aflatoxin $M_1$ is approximately equivalent to $AFB_1$ in acute toxicity (194, 195), but considerably less carcinogenic (196-198) and mutagenic (199). Aflatoxin $B_{2a}$ is essentially non-toxic

Figure 1. Structures of $AFB_1$ and its metabolites


AFM1
AFH1
AFP1
AFB2a
AFB1
AFQ1
AFLATOXICOL (R0)
AF EPOXIDE

(146, 200), however it readily opens to the dialdehyde at physiological pH and binds covalently to protein through Schiff base formation (184). This relatively high reactivity may prevent $AFB_{2a}$ from reaching target molecules after administration at a more distant site (201). Aflatoxin $P_1$ is relatively non-toxic (202, 203) and $AFQ_1$ is 18 times less toxic than $AFB_1$ (203a).

A reduced derivative of $AFB_1$, aflatoxicol, is produced by a soluble enzyme (204, 205), and is 15-18 times less toxic than $AFB_1$ (206). The most recently identified $AFB_1$ metabolite, aflatoxicol $H_1$, is produced by the action of both the microsomal mixed function oxidase system and the cytoplasmic reductase (207). This metabolite is non-toxic.

As mentioned previously, the identity of the $AFB_1$ metabolite(s) which inhibits transcription and translation is unknown. In the study by Edwards and Wogan (161) demonstrating the inhibition of template activity of chromatin isolated from $AFB_1$ treated animals, it was found that $AFB_2$ the 2, 3-dihydro derivative of $AFB_1$, had no effect on template activity. This suggests that 2, 3-unsaturation of the molecule is required for formation of the metabolite which interacts with chromatin. These data and the observation of Neal (167) that the metabolite which inhibits template activity in vitro is unstable, make aflatoxin 2, 3-epoxide and $AFB_{2a}$ the most likely candidate metabolites involved. There is not enough data available to make any speculation as to the metabolites acting on RNA polymerase or the translation process.

The Effect of Aflatoxin on Mitochondrial Functions - The effect of

aflatoxin on mitochondrial functions is not well characterized. Clifford and Rees (155) reported no alteration in the respiratory capacity and P:O ratios of mitochondria isolated from rat liver up to 24 hr after an $AFB_1$ dose of 7 mg/Kg body weight. On the other hand, Svoboda et al. (208, 209) found that phosphorylation and oxygen consumption were decreased in mitochondria isolated from rats dosed with 0.45 mg $AFB_1$/Kg body weight. Brown and Abrams (210) observed an inhibition of respiration in mitochondria from livers of chickens and ducklings dosed with $AFB_1$.

Misra (211) first demonstrated that $AFB_1$ inhibits rat liver mitochondrial respiration in vitro. Doherty and Campbell (212, 213) further characterized this inhibition and found that the site of action of $AFB_1$ is between cytochromes b and $c(c_1)$ in the respiratory chain.

Bababunmi and Bassir (214) have reported that $AFB_1$ induces swelling and activates ATPase in isolated mitochondria.

There have not been any studies on the in vitro or in vivo effect of aflatoxin on the mitochondrial genetic system.

## MATERIALS AND METHODS

### Materials

Animals - Male Sprague-Dawley derived rats weighing 100-250 g were used in all experiments unless otherwise indicated. All rats were obtained from Flow Research Laboratories, Dublin, Virginia, and fed Purina Rat Chow and tap water ad libitum.

Chemicals - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), EDTA, ATP, CTP, GTP, UTP, UDP, UMP, uridine, ADP, $NADP^+$, glucose-6-P, glucose-6-P dehydrogenase, phosphoenolpyruvate, pyruvate kinase, creatine phosphokinase, phosphocreatine, glutamic acid, succinic acid, malic acid, pyruvic acid, β-hydroxybutryic acid, dinitrophenol, oligomycin, actinomycin D, cycloheximide, chloramphenicol, bovine pancreatic ribonuclease, $T_1$ ribonuclease, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Missouri. NCS tissue solubilizer and 2,5-diphenyloxazole (PPO) were obtained from New England Nuclear, Boston, Mass. All amino acids were obtained from Mann Research Laboratories, New York, N. Y. All other chemicals were reagent grade.

Radiochemicals - Uridine 5'-triphosphate (5-$^3$H), tetralithium salt, lot numbers ZR-1032 and XR-2159 were obtained from Schwarz Mann, Orangeburg, N. Y. Uridine 5'-triphosphate (5-$^3$H), tetrasodium salt, lot number 640-232 and uniformly labeled $^{14}$C-L-leucine were obtained from

New England Nuclear. $^{3}H$-$AFB_1$ was obtained from Nuclear Dynamics Inc., El Monte, Calif.

Aflatoxins - $AFB_1$ was obtained from Calbiochem, La Jolla, Calif., and Makor Chemicals, Jerusalem, Israel. $AFB_2$ was obtained from Sigma Chemical Co. Purity of the aflatoxins was checked by measurement of the ultraviolet spectra in methanol or chloroform, and thin layer chromatography on Adsorbosil-5 (Applied Science Laboratories, State College, Penn.) in two of the following solvent systems: water saturated chloroform:acetone (90:10), upper phase of benzene:ethanol:water (46:35:19) and ethyl acetate: chloroform (2:1). All aflatoxins used were better than 95% pure.

Other Materials - Nutrient agar and Trypticase-Soy agar were obtained from Baltimore Biological Laboratory, Baltimore, Md. Blood agar plates were obtained from Flow Laboratories.

## Methods

Preparation of Mitochondrial Fraction for $^{3}H$-UTP Incorporation Studies - Animals were decapitated and the livers were quickly removed, weighed and placed in ice cold isolation buffer consisting of 0.025 M HEPES pH 7.4, 0.002 M EDTA and 0.25 M sucrose. The livers were blotted, minced and homogenized in nine volumes of isolation buffer using a Potter-Elvehjem type homogenizer with a motor driven teflon pestle (600 rpm). To prevent excessive fragmentation of nuclei, only two strokes of the pestle were used. The homogenate was then filtered through four layers of

cheesecloth and the filtrate was centrifuged at 1000 x g for 10 min. This low-speed centrifugation was increased above the usual 600 x g for mitochondrial isolations to insure maximum sedimentation of nuclei and large nuclear fragments. The supernatant was decanted through glass wool and centrifuged again at 1000 x g for 10 min. The supernatant was again decanted through glass wool and centrifuged at 8000 x g for 10 min. The mitochondrial pellet obtained from this last centrifugation was washed by resuspension in isolation buffer followed by centrifugation at 8000 x g for 10 min. For "intact" mitochondria this washed pellet was resuspended in a small volume of isolation buffer or 0.25 M sucrose. For "swollen" mitochondria the pellet was resuspended in 0.1 M potassium phosphate buffer pH 7.4, and incubated at $30^{o}$ for 20 min. The suspension was cooled and centrifuged at 8000 x g for 10 min. This "swollen" mitochondrial fraction was washed once in isolation buffer and resuspended in a small volume of 0.025 M HEPES pH 7.4 containing 0.064 M KCl.

$^{3}$H-UTP Incorporation by Isolated Mitochondria - The incorporation of $^{3}$H-UTP by "swollen" mitochondria was determined by a modification of the method of Saccone, et al. (215). The assay system contained 25 mM HEPES, 64 mM KCl, 3.0 mM $MgCl_2$, 4.0 mM phosphoenolpyruvate, 6 units pyruvate kinase, 1.0mM each ATP, GTP and CTP, 0.1 mM $^{3}$H-UTP (specific activity = 200 or 600 μCi/μmol) and 1-3 mg/ml mitochondrial protein. The pH of the assay system was adjusted to 7.4 with KOH. The reaction

was started with the addition of the mitochondria. At the times indicated 0.1 ml of the reaction mixture was withdrawn and pipeted onto a 23 mm Whatman 3MM filter paper disk. The filters were collected in ice cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate and processed by a modification of the method of Bollum (216). The filters were washed twice with trichloroacetic acid-$PP_i$, once with ethanol:ether (1:1) and once with ether. The filters were dried and placed in scintillation vials and 0.2 ml of NCS:$H_2O$ (5:1) was added to solubilize the precipitated material. After standing overnight, glacial acetic acid was added to neutralize the NCS, 10 ml of 0.4% PPO in toluene was added and the samples were counted in a Beckman LS 133 Liquid Scintillation Counter. The counting efficiency was 35%. The incorporation of $^3$H-UTP by "intact" mitochondria was determined as described above except that the assay system was a modification of that used by Fukamachi, et al. (217) and contained 8.3 mM HEPES, 83 mM sucrose, and 4.0 mM KCl, 7.0 mM $MgCl_2$, 5.0 mM $KH_2PO_4$, 7.5 mM malate, 7.5 mM pyruvate, 7.5 mM succinate, 0.1 mM each ATP, GTP, and CTP, 0.1 mM $^3$H-UTP (specific activity = 200 or 600 $\mu$ Ci/ $\mu$mol) and 1-3 mg/ml mitochondrial protein. The pH of the assay system was adjusted to 7.4 with KOH.

RNase Sensitivity of the Product - The reaction was carried out as described above and stopped by the addition of cold trichloroacetic acid-$PP_i$. The precipitate was washed five times with trichloroacetic acid-$PP_i$, once

with ethanol:ether (1:1), once with ether and air dried. The precipitate was resuspended either in 0.1 M acetate buffer pH 5.0 containing 40 μg pancreatic RNase/ml or 0.05 M Tris-HCl pH 7.5 containing 10 μg $T_1$ RNase/ml and incubated at 37° for the times indicated. After the incubation the samples were cooled and trichloroacetic acid was added to a final concentration of 15%. The samples were centrifuged and the supernatant was counted in triton-scintillation cocktail (4 g PPO + 667 ml toluene + 333 ml triton X-100).

Alkaline Hydrolysis of the Product - The product was prepared as described under "RNase Sensitivity . . .". The precipitate was dissolved in 0.3 N KOH and incubated for 18 hr at 37°. The samples were cooled and trichloroacetic acid was added to a final concentration of 15%. The samples were centrifuged and the supernatant was counted in triton-scintillation cocktail.

High Voltage Paper Electrophoresis of $^3$H-UTP - Analytical electrophoresis was done using Whatman No. 4 paper with 0.025 M citrate buffer pH 5.0 at 2500 volts for 1 hr using a Savant High Voltage Paper Electrophoresis apparatus. A 2.5 cm strip was cut into 0.5-2.5 cm pieces and placed in scintillation vials. The radioactive material was eluted with 0.5-1.2 ml water in the vial and then counted in triton-scintillation cocktail.

Preparative electrophoresis was performed as above using Whatman 3MM paper.

High Pressure Liquid Chromatography of $^3$H-UTP - $^3$H-UTP was

chromatographed on an AS-Pellionex$^{TM}$-SAX anion exchange column using a Varian LCS-1000 as described by Ko, et al. (218). Fractions were collected and counted in triton-scintillation cocktail.

In Vitro Effect of $AFB_1$ on Mitochondrial $^3$H-UTP Incorporation - $AFB_1$ dissolved in dimethylformamide was added to the assay system to give a final concentration of 0.4 mM $AFB_1$. Dimethylformamide was added to control incubations. In some experiments the mitochondrial fraction was incubated with 0.4 mM $AFB_1$ for 15 min at $0^o$ before addition of the mitochondria to the assay system. Control mitochondria were incubated with dimethylformamide.

Effect of In Vivo Administration of $AFB_1$ on $^3$H-UTP Incorporation by Isolated Mitochondria - Rats received an intreperitoneal injection of 5 mg $AFB_1$/kg body weight. The $AFB_1$ was injected in 0.1 ml dimethylformamide. Control animals received dimethylformamide alone. At the times indicated the animals were decapitated and livers removed. The mitochondrial fraction was isolated and assayed for $^3$H-UTP incorporation as described above.

Preparation of Sterile Mitochondrial Fraction for Protein Synthesis Studies - All glassware, solutions, centrifuge tubes, homogenizers and instruments were sterilized by autoclaving or by dry heat ($170^o$ for 2 hr). Rats were decapitated and the abdominal area was shaved and scrubbed with 3% Lysol. The following procedures were carried out in a Laminaire Flow

sterile hood. The abdominal area was again scrubbed with 3% Lysol and a piece of abdominal skin approximately 4 x 8 cm was cut away without penetrating the peritoneum. The peritoneum was rinsed with 77% (v/v) ethanol. Using a second set of instruments, the peritoneum was opened with a V-incision and the upper part of the cavity was held open with hemostats. Using a third set of instruments, the liver was removed without penetrating any part of the gastrointestinal tract. The liver was washed in three 200 ml portions of cold 0.9% NaCl and placed in cold isolation buffer consisting of 0.025 M HEPES pH 7.4, 0.002 M EDTA and 0.25 M sucrose. The pooled livers were minced and rinsed three times with isolation buffer. During the rest of the isolation all transfers were made in a plastic hood equipped with a germicidal UV lamp. Unless otherwise stated, the minced liver was homogenized in nine volumes of isolation buffer. The homogenate was centrifuged at 30 x g for 1 min and then the speed was increased to give 600 x g for 10 min. The supernatant was decanted and centrifuged again at 600 x g for 10 min. The supernatant was decanted and centrifuged at 7000 x g for 8 min. The top of the pellet was rinsed with isolation buffer to remove all of the fluffy layer and the pellet was washed by resuspension in isolation medium and centrifugation at 7000 x g for 8 min. The mitochondrial pellet was washed a second time and resuspended in a small volume of isolation buffer.

Bacterial Contamination - Bacterial contamination was determined

by plating 0.1 ml of the mitochondrial suspension on either blood agar or Trypticase-Soy agar supplemented with 0.5% glucose. Colonies were counted after a 48 hr incubation at 37°. Contamination was routinely found to be less than 300 bacteria/ml of mitochondrial suspension.

Mitochondrial Protein Synthesis - Mitochondrial protein synthesis was determined by a modification of the method of Haldar and Freeman (219). The assay system contained 100 mM sucrose, 2.5 mM HEPES, 50 mM KCl, 20 mM $KH_2PO_4$, 15 mM $MgCl_2$, 10 mM succinate, 1.0 mM ADP, 2 μg/ml of each amino acid except leucine, 1.0 μCi/ml $^{14}$C-leucine and 3 mg/ml mitochondrial protein. The pH of the system was adjusted to 7.4 with KOH and filter sterilized. Incubations were carried out at 30° in a shaking water bath (120 oscillations/min) using sterile test tubes with Morton test tube closures. Unless otherwise stated, the mitochondria were incubated in the assay system minus $^{14}$C-leucine for 5 min prior to the addition of the $^{14}$C-leucine. At 0, 5, 10 and 15 min 0.1 ml aliquots were removed and pipeted onto 23 mm Whatman 3MM filter disks. The filters were treated batch-wise by a modification of the method of Mans and Novelli (220). The filters were placed in cold 5% trichloroacetic acid containing 10 mM leucine and allowed to stand for at least 30 min. The trichloroacetic acid-leucine was replaced and the beaker was placed in a boiling water bath for 15 min. The filters were washed twice with trichloroacetic acid-leucine, dried and counted in 0.4% PPO in toluene.

Counting efficiency was 71%.

In Vitro Effect of $AFB_1$ on Mitochondrial Protein Synthesis - $AFB_1$ was added to the assay system in 0.01 ml dimethylformamide to give the final concentrations indicated. Control incubations contained 0.01 ml dimethylformamide. In some experiments the mitochondrial fraction was incubated with 0.4 mM $AFB_1$ for 20 min at $30^{o}$ and the reaction was started with the addition of mitochondria. Control mitochondria were incubated with dimethylformamide.

Effect of In Vivo Administration of $AFB_1$ on Protein Synthesis of Isolated Mitochondria - Animals were injected with $AFB_1$ as described above and decapitated at the times indicated. Sterile mitochondrial fractions were prepared and assayed for protein synthesis.

Effect of In Vitro Metabolism of $AFB_1$ on Mitochondrial Protein Synthesis - The first 600 x g supernatant of the mitochondrial isolation was incubated under sterile conditions with 0.4 mM $NADP^{+}$, 20 mM glucose-6-P, 5 mM $MgCl_2$, 10 mM succinate and $AFB_1$ or $AFB_2$ dissolved in dimethylformamide. In control incubations aflatoxin was replaced by dimethylformamide. The incubations were carried out in a Dubnoff Metabolic Shaker (120 oscillations/min) at $37^{o}$ for 30 min with an oxygen atmosphere. At the end of the incubation, the flasks were quickly cooled and the isolation of mitochondria was continued. The isolated mitochondria were assayed for protein synthesis.

Determination of the Concentration of Aflatoxin in the Mitochondrial Fraction After In Vitro Metabolism - The in vitro metabolism was carried out as described above using $^{3}$H-AFB$_1$. The final washed mitochondrial pellet was solubilized with NCS, neutralized with glacial acetic acid and counted in 0.4% PPO in toluene.

Determination of Mitochondrial Respiratory Parameters - Mitochondrial respiration, respiratory control ratios and ADP:0 ratios were measured as described by Doherty and Campbell (213).

Effect of In Vitro Metabolism of AFB$_1$ on Mitochondrial Respiration - In vitro metabolism of AFB$_1$ and isolation of the mitochondrial fraction were carried out as described above. Respiratory parameters were determined on the isolated mitochondria.

Other Methods - Protein concentrations were determined by the method of Lowry, et al. (221) using bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

### The Effect of Aflatoxin on Mitochondrial RNA Synthesis

Since the mitochondrial inner membrane is relatively impermeable to the nucleoside triphosphates (222), mitochondria used in the determination of RNA synthesis are frequently treated to increase permeability to the precursor molecules (222-224). Swelling in 0.1 M phosphate buffer pH 7.4 is the most common means of increasing permeability. Repeated washing of mitochondria in 0.25 M sucrose without EDTA and treatment with digitonin have also been shown to increase permeability (222). In the studies reported here, phosphate swelling was employed.

The incorporation of $^{3}$H-UTP into an acid insoluble product by swollen mitochondria was determined by a modification of the method of Saccone, et al. (215). Figure 2 shows that the incorporation was linear for only a very short period of time. The total incorporation for time periods less than 10 min was too low to provide the accuracy and sensitivity needed in this assay. For this reason 10 min assays, which approximate linearity, were employed in most of the studies reported here. As can be seen in Figure 3, the incorporation at 10 min was linear with protein concentration up to at least 6.7 mg/ml.

The sensitivity of the incorporation to actinomycin D is shown in Figure 4. An average of 52% inhibition was observed at 10 min in duplicate

Figure 2. Time dependence of $^{3}$H-UTP incorporation by isolated mitochondria

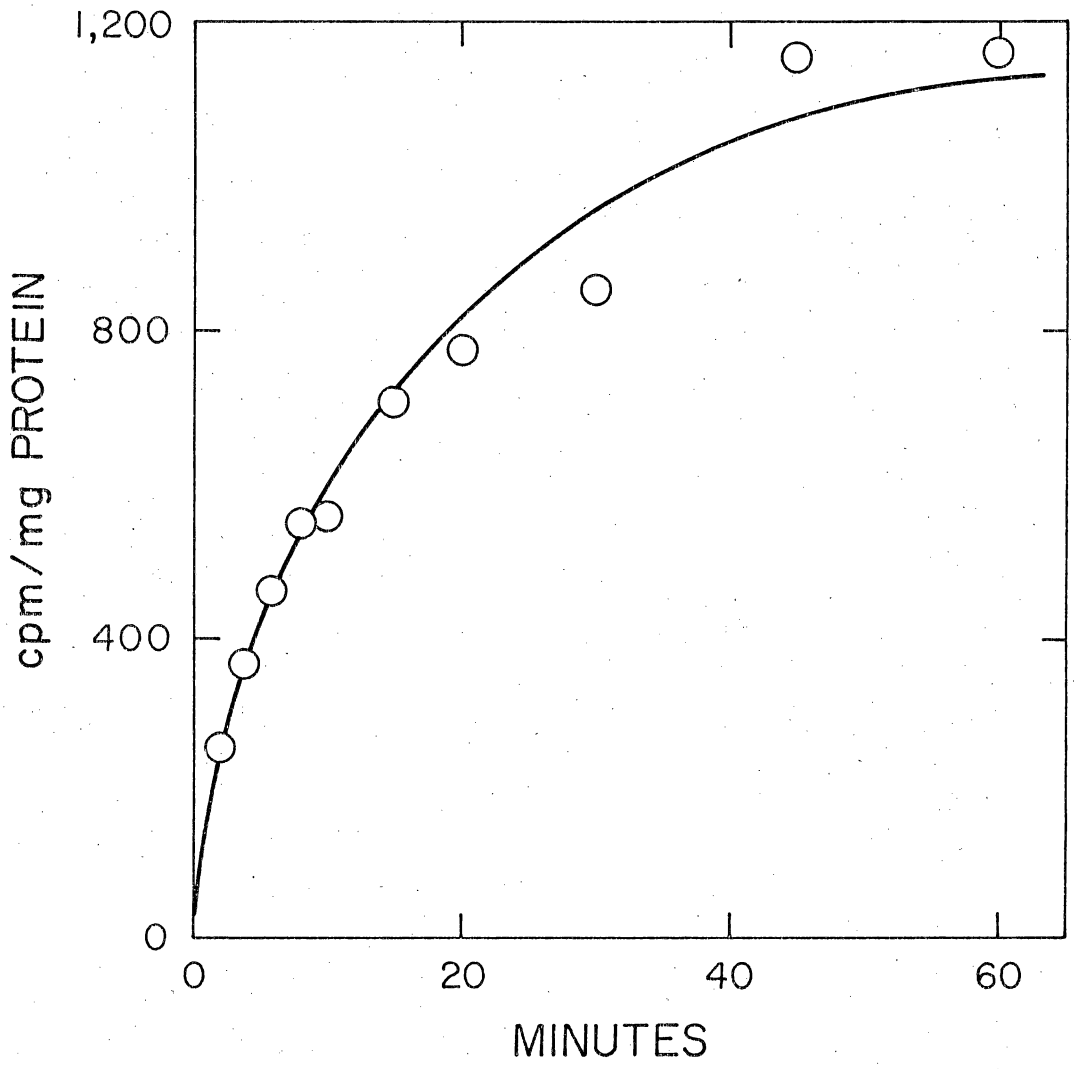

1,200
800
400
0
cpm/mg PROTEIN
0
20
40
60
MINUTES

Figure 3. Protein dependence of $^{3}$H-UTP incorporation by isolated mitochondria

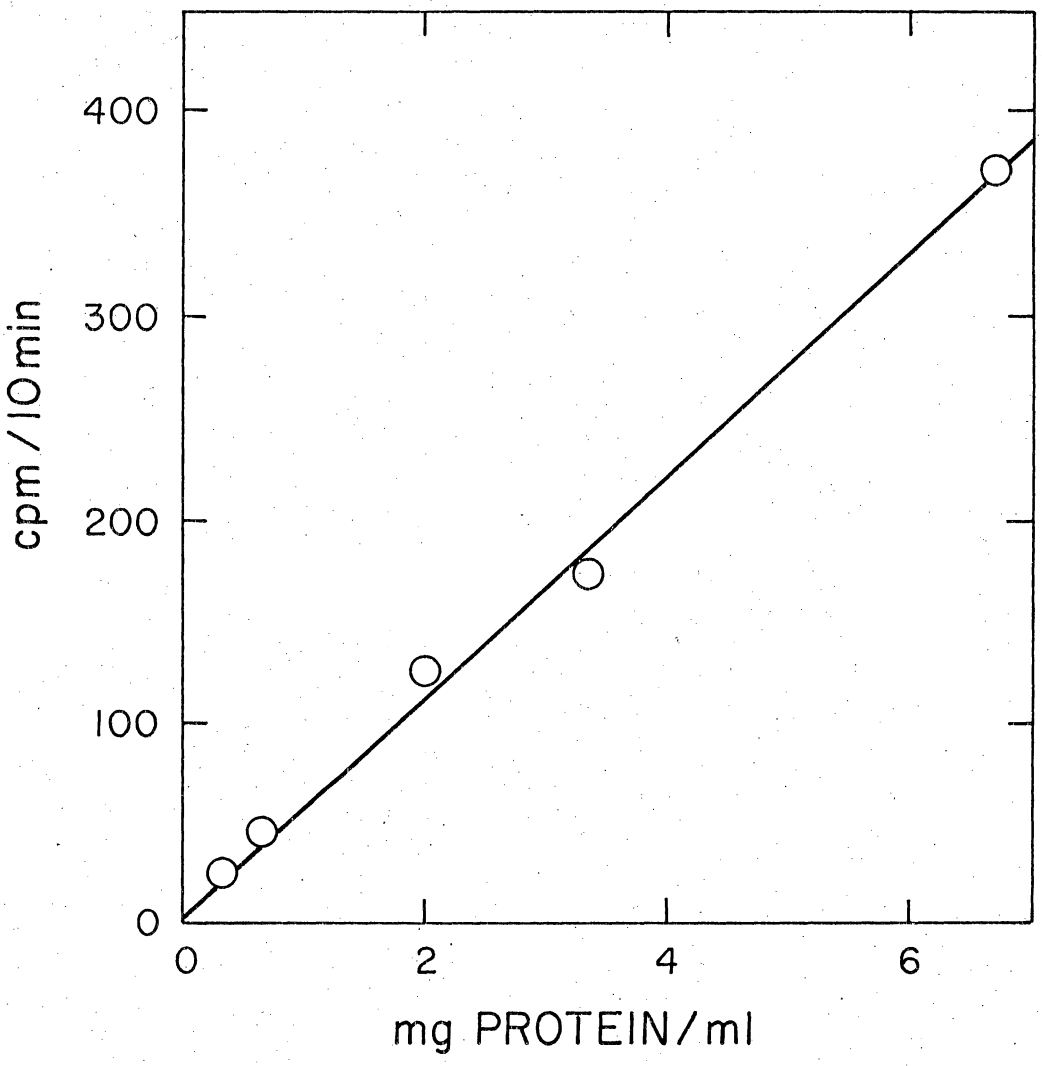
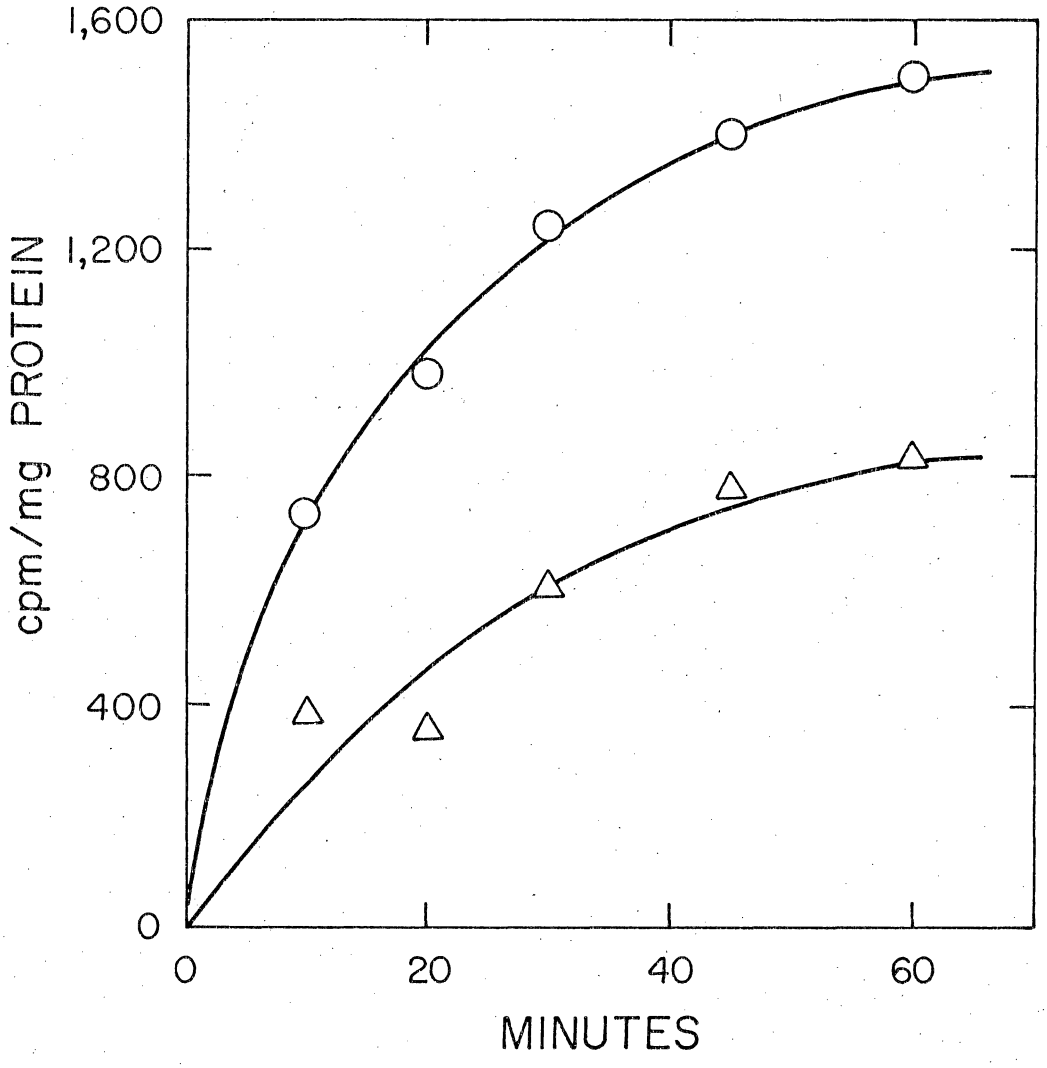

400
300
200
100
0
cpm / 10 min
0
2
4
6
mg PROTEIN / ml

Figure 4. Effect of actinomycin D on mitochondrial $^{3}$H-UTP incorporation

o, control; Δ, actinomycin D (50 μg/ml)


1,600
1,200
800
400
0
cpm/mg PROTEIN
0
20
40
60
MINUTES

experiments using an actinomycin D concentration of 50 μg/ml. This is considerably less than the inhibition reported in the literature. Neubert, et al. (223) observed 85% inhibition with digitonin treated mitochondria at 50 μg actinomycin D/ml, and Saccone, et al. (215) observed 86% inhibition with phosphate-swollen mitochondria at a concentration of 100 μg/ml.

The $^{3}$H-UTP concentration dependence of the incorporation also differed from that reported in the literature. As can be seen in Figure 5, incorporation was linear to 1.0 mM $^{3}$H-UTP. Neubert, et al. (223), using digitonin treated mitochondria, have reported that one-half maximum incorporation occurs at 0.024 mM $^{3}$H-UTP.

In addition to the low actinomycin D inhibition and the unusual concentration dependence of the incorporation, it was found that a 100-fold excess of unlabeled UTP added to the assay system produced only a 54% decrease in the incorporation.

Incorporation of $^{3}$H-UTP was also examined using intact mitochondria and was found to be very similar to that observed with swollen mitochondria (Table I). The rate of incorporation was approximately two-thirds of that observed with swollen mitochondria, and was decreased only 58% by a 100-fold excess of unlabeled UTP. Incorporation was also linear with $^{3}$H-UTP concentration to 1 mM $^{3}$H-UTP.

The low level of dilution with unlabeled UTP suggested that the incorporation may not be UTP, but rather a radiocontaminant. In the

Figure 5. Dependence of mitochondrial $^{3}$H-UTP incorporation on $^{3}$H-UTP concentration

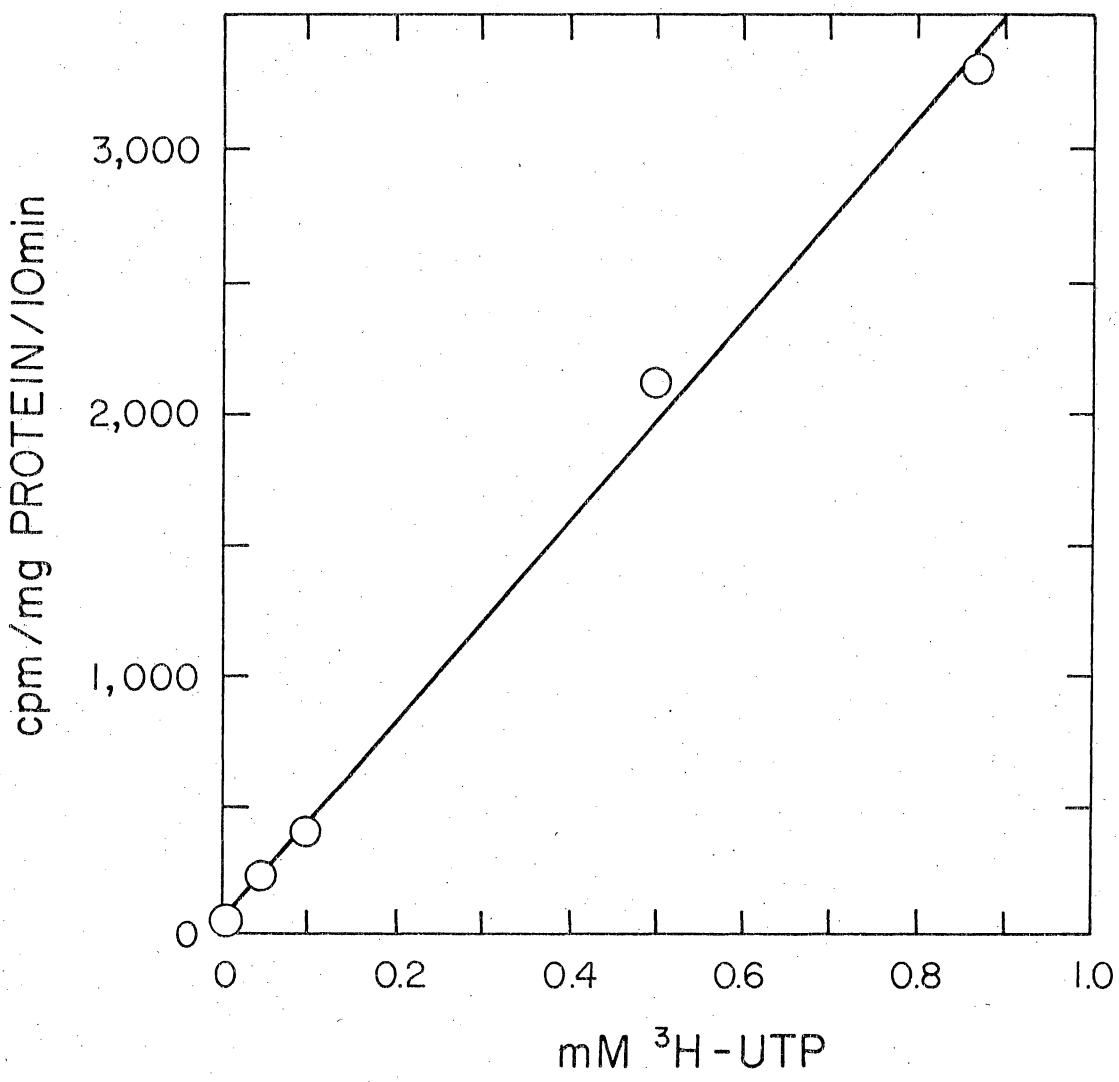

cpm/mg PROTEIN/10min
3,000
2,000
1,000
0
0
0.2
0.4
0.6
0.8
1.0
mM ³H-UTP

TABLE I

$^{3}$H-UTP Incorporation by Swollen and Intact Mitochondria

| | Swollen | Intact |
|---|---|---|
| Incorporation (cpm/mg protein/ 10 min)[a] | 647 ± 85 | 434 ± 28 |
| Isotope dilution (100 fold excess) | 54 ± 1% | 58% |
| $^{3}$H-UTP conc. for 1/2 max. incorporation | <1mM | <1mM |
| Inhibition by Actinomycin D (50 μg/ml) | 52 ± 4% | --- |
| % of product sensitive to KOH | --- | 97% |
| % of product sensitive to pancreatic RNase | --- | 79% |

[a] specific activity = 600 μCi/μmol

experiments reported here only a very small proportion (<0.03%) of the available radioactivity was incorporated into product. Under these conditions even a trace impurity could be responsible for activity of the same order of magnitude as true RNA synthesis (225). Thus, a radiocontaminant could account for the incorporation which was not inhibited by actinomycin D or diluted with unlabeled UTP. The linear increase of incorporation with $^{3}$H-UTP concentration could be due to a linear increase in the concentration of a radiocontaminant.

The radiopurity of the $^{3}$H-UTP was examined by high voltage paper electrophoresis and will be discussed in more detail later. Uridine, UMP and UDP were all found as radiocontaminants. In addition, radioactivity was also found at the origin and in an unknown spot migrating between uridine and UMP.

The possibility that one of these radiocontaminants or some unresolved contaminant was responsible for the low level of dilution by unlabeled UTP was ruled out when it was observed that $^{3}$H-UTP incorporation by other subcellular fractions could be diluted with unlabeled UTP (Table II). These data suggest that the low level of dilution of incorporation in the mitochondrial fraction is due to some property of the mitochondria rather than a contaminant of the $^{3}$H-UTP.[1] In addition, the sensitivity of the product labeled by intact mitochondria to RNase and alkaline hydrolysis (Table I) argues against a radiocontaminant and suggests that the product is RNA.

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[1]See appendix for possible explanation.

TABLE II

Isotope Dilution in Other Subcellular Fractions

| | % Dilution[a] |
|---|---|
| Whole homogenate | 93 |
| 1000 x g pellet | 87 |
| 1000 x g supernatant | 80 |
| Swollen mitochondria | 54 |
| Intact mitochondria | 58 |

[a] 100-fold excess of unlabeled UTP

All of the studies reported above were done with one lot of $^{3}$H-UTP (Schwarz Mann XR-2159). When other lots of $^{3}$H-UTP were used additional problems were encountered. Table III shows some of the characteristics of the incorporation using different lots of $^{3}$H-UTP. Incorporation with the first shipment of Lot 640-232 from New England Nuclear was almost ten times that observed with Lot XR-2159 from Schwarz Mann, and was not inhibited by actinomycin D. The product was, however, hydrolyzed by both $T_1$ and pancreatic RNase, ruling out the possibility of poly(U) synthesis. The second shipment of Lot 640-232 gave an incorporation rate similar to that observed with Lot XR-2159 but dilution with unlabeled UTP was only 24%. Lot ZR-1032 from Schwarz Mann gave a high incorporation rate compared to the Schwarz Mann XR-2159 with intact mitochondria and only 18% dilution with a ten fold excess of unlabeled UTP.

The radiopurity of these lots of $^{3}$H-UTP as determined by high voltage paper electrophoresis (Figure 6) and high pressure liquid chromatography (Figure 7) is shown in Table IV. The purity differed considerably between lots and even between shipments of the same lot in one case. $^{3}$H-UTP purified by these two methods was used in incorporation assays (Table V). The only means by which dilution with unlabeled UTP was increased was to use $^{3}$H-UTP purified by high voltage paper electrophoresis at a very high specific activity and low concentration.

The problem of varying incorporation characteristics with different

TABLE III

Mitochondrial $^{3}$H-UTP Incorporation Characteristics of Different Lots of $^{3}$H-UTP

| Lot No. | Swollen Mitochondria | | | | Intact Mitochondria | | | |
|---|---|---|---|---|---|---|---|---|
| | Incorp. cpm/mg protein/ 10 min | Dilution w/UTP % | Act. D Inhib. % | RNase Sensitivity % | Incorp. cpm/mg protein/ 10 min | Dilution w/UTP % | Act. D Inhib. % | RNase Sensitivity % |
| XR-2159 | 380[a] ±39 | 54 ± 1 | 54 ± 1 | ---- | ---- | 58 | ---- | 79 |
| | 647[b] ±85 | | | | 434[b] ±28 | | | |
| 640-232 1st shipment | 2360[a] ±730 | ---- | 8 ± | 90[c] 86[d] | 4190[a] ±940 | ---- | 0 | ---- |
| 640-232 2nd shipment | 437[a] | 24 | ---- | ---- | --- | ---- | ---- | ---- |
| ZR-1032 | ---- | ---- | ---- | ---- | 3920[b] | 18 | ---- | ---- |

[a] $^{3}$H-UTP specific activity = 200 μCi/μmol

[b] $^{3}$H-UTP specific activity = 600 μCi/μmol

[c] pancreatic RNase, 40 μg/ml

[d] $T_1$ RNase, 10 μg/ml

Figure 6. High voltage paper electrophoresis of $^{3}$H-UTP

Arrows indicate migration of uridine, UMP, UDP and UTP standards.

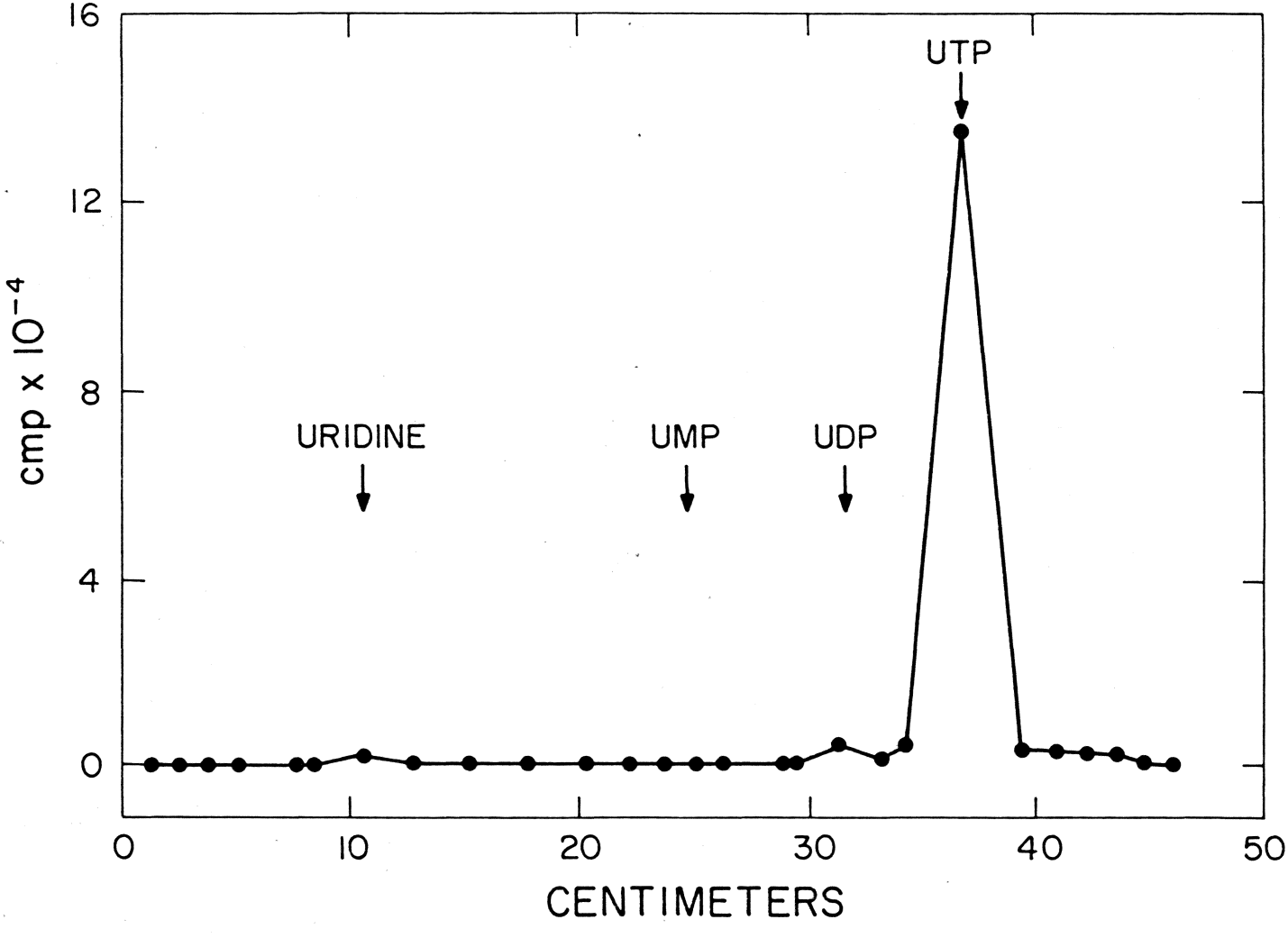

16
12
8
4
0
cmp x $10^{-4}$
URIDINE
UMP
UDP
UTP
0
10
20
30
40
50
CENTIMETERS

Figure 7. High pressure liquid chromatography of $^{3}$H-UTP

The sample was spiked with nucleotide standards: ----, U.V. trace of standards; o, radioactivity.

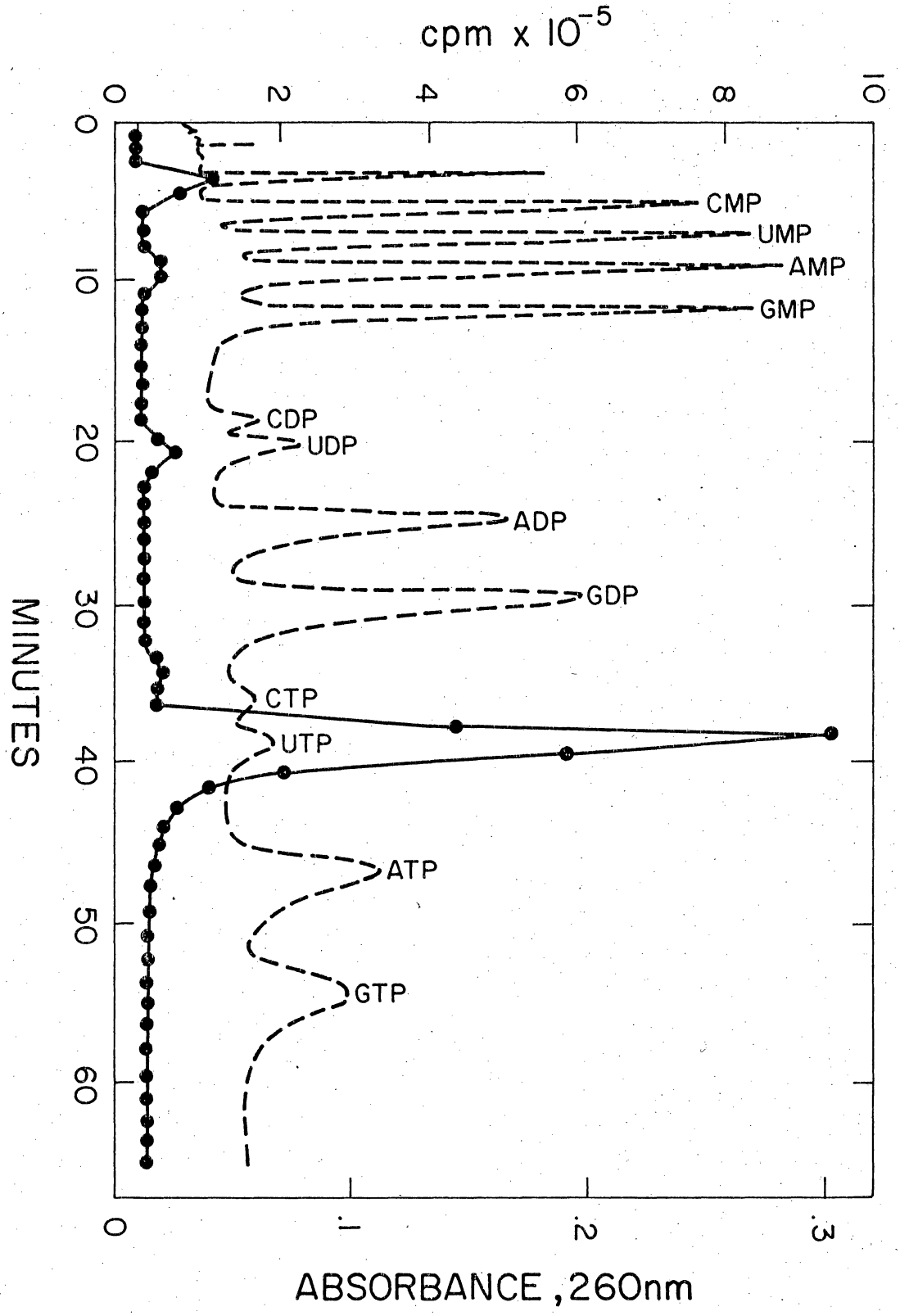

cpm x $10^{-5}$
0
2
4
6
8
10
MINUTES
0
10
20
30
40
50
60
CMP
UMP
AMP
GMP
CDP
UDP
ADP
GDP
CTP
UTP
ATP
GTP
0
.1
.2
.3
ABSORBANCE, 260nm

TABLE IV

Radiopurity of Different Lots of $^{3}$H-UTP

| Lot No. | % of cpm Recovered | | | | | |
|---|---|---|---|---|---|---|
| | Origin + Uridine[a] | Unknown A[b] | UMP | UDP | Unknown B[c] | UTP |
| High Voltage Paper Electrophoresis | | | | | | |
| XR-2159 | 1.34 | 0.16 | 0.16 | 3.30 | ---- | 94.6 |
| 640-232-1st | 4.30 | ---- | 0.90 | 6.40 | ---- | 88.4 |
| High Pressure Liquid Chromatography | | | | | | |
| 640-232-2nd | 10.6 | ---- | 6.60 | 2.70 | 6.7 | 70.3 |
| ZR-1032 | 5.70 | ---- | 2.30 | 3.68 | 4.4 | 76.8 |

[a] or void volume in high pressure liquid chromatography

[b] unknown spot migrating between uridine and UMP on high voltage paper electrophoresis

[c] unknown peak in high pressure liquid chromatography not well resolved from UTP

TABLE V

Incorporation After Purification of $^{3}$H-UTP by High Voltage Paper Electrophoresis and High Pressure Liquid Chromatography

| | Incorporation | % Dilution w/UTP[a] |
|---|---|---|
| Lot 640-232-2nd | | |
| 200 μCi/μmol, 0.1 mM $^{3}$H-UTP | | |
| control | 437 cpm/mg/10 min | 24 |
| HVPE[b] | 77 cpm/mg/10 min | 10 |
| HVPE | 162 cpm/mg/10 min | 0 |
| Lot 640-232-2nd | | |
| 26, 900μCi/μmol | | |
| $1 \times 10^{-6}$ mM $^{3}$H-UTP | | |
| control | 3320 cpm/mg/10 min | 50 |
| HVPE | 1400 cpm/mg/10 min | 92 |
| Lot 640-232-2nd | | |
| 26, 900 μCi/μmol | | |
| $0.03 \times 10^{-6}$ mM $^{3}$H-UTP | | |
| HPLC[c] | 239 cpm[d] | 0 |
| Lot ZR-1032 | | |
| 19, 000 μCi/μmol | | |
| $0.04 \times 10^{-6}$ mM $^{3}$H-UTP | | |
| HPLC | 118 cpm[d] | 3 |

[a] 1.0 mM unlabeled UTP

[b] high voltage paper electrophoresis

[c] high pressure liquid chromatography

[d] due to limited material zero time incubations were not run and 10 min uncorrected values are given

lots of $^{3}$H-UTP remains unresolved. It seems likely that a radiocontaminant is involved, but the nature of this compound is unknown.

All of the studies on the effect of $AFB_1$ on mitochondrial $^{3}$H-UTP incorporation were done using Lot XR-2159 from Schwarz Mann, which was 95% pure by high voltage paper electrophoresis. As reported earlier, the incorporation was inhibited 52% by actinomycin D and the product was hydrolyzed by pancreatic RNase. Although only a 54% dilution of mitochondrial incorporation was observed with unlabeled UTP, incorporation by other subcellular fractions was diluted 87%. All of these data suggest that at least a major part of the incorporation was due to RNA synthesis.

The in vitro and in vivo effects of $AFB_1$ on this incorporation are shown in Table VI. $AFB_1$ added to the assay system at 0.4 mM reduced incorporation only slightly compared to the control incubated only with the dimethylformamide vehicle. Pre-incubation of the mitochondrial fraction with 0.4 mM $AFB_1$ for 15 min at $0^{o}$ did not increase the inhibition. In contrast to this lack of inhibition in vitro, intraperitoneal injection of $AFB_1$ at a dose of 5.3 mg/kg body weight produced 25% inhibition of $^{3}$H-UTP incorporation of mitochondria isolated 4 hr after injection. Similar inhibition was observed at 1 hr after injection.

These data suggest that a metabolite of $AFB_1$ may inhibit mitochondrial RNA synthesis, however, confirmation of these results with a more refined system is necessary.

TABLE VI

Effect of $AFB_1$ on $^3H$-UTP Incorporation by Isolated Mitochondria

| | % Inhibition |
|---|---|
| In Vitro | |
| 0.4 mM $AFB_1$ (3)[a] | 12.0 ± 2.0 |
| mito pre-incubated with 0.4 mM $AFB_1$ (1) | 4.5 |
| In Vivo Administration[b] | |
| 4 hr post injection (4) | 25.3 ± 5.0 |
| 1 hr post injection (1) | 25.7 |

[a] number of experiments

[b] 5.3 mg $AFB_1$/Kg body weight by i.p. injection

## The Effect of Aflatoxin on Mitochondrial Protein Synthesis

Bacterial contamination can sometimes contribute significantly to the rate of protein synthesis with isolated mitochondria (226). For this reason, the work presented here was carried out under sterile conditions. Figure 8 shows that under the assay conditions used here, bacteria did not contribute significantly to mitochondrial protein synthesis at levels less than 100 bacteria/mg protein. With the sterile techniques used here, bacterial contamination was routinely less than 100 bacteria/mg protein and was usually less than 30 bacteria/mg protein.

Mitochondrial protein synthesis was determined in two assay systems. In the first energy was provided through mitochondrial oxidative phosphorylation using succinate as a substrate, and in the second energy was provided by an ATP-generating system. Figure 9 shows that the incorporation of $^{14}$C-leucine into hot acid-insoluble product was linear in both systems for about 15 min, and Figure 10 shows that incorporation was linear with protein concentration to about 3 mg/ml. The optimum $Mg^{2+}$ concentration was 15 mM in both systems (Figure 11), but the ATP-supported assay was less sensitive to changes in $Mg^{2+}$ concentration.

Table VII shows that the incorporation of $^{14}$C-leucine had the characteristics of mitochondrial protein synthesis. Incorporation was inhibited by chloramphenicol, an inhibitor of mitochondrial and procaryotic protein synthesis, but not by cycloheximide, an inhibitor of cytoplasmic

Figure 8. Effect of bacterial contamination on mitochondrial protein synthesis

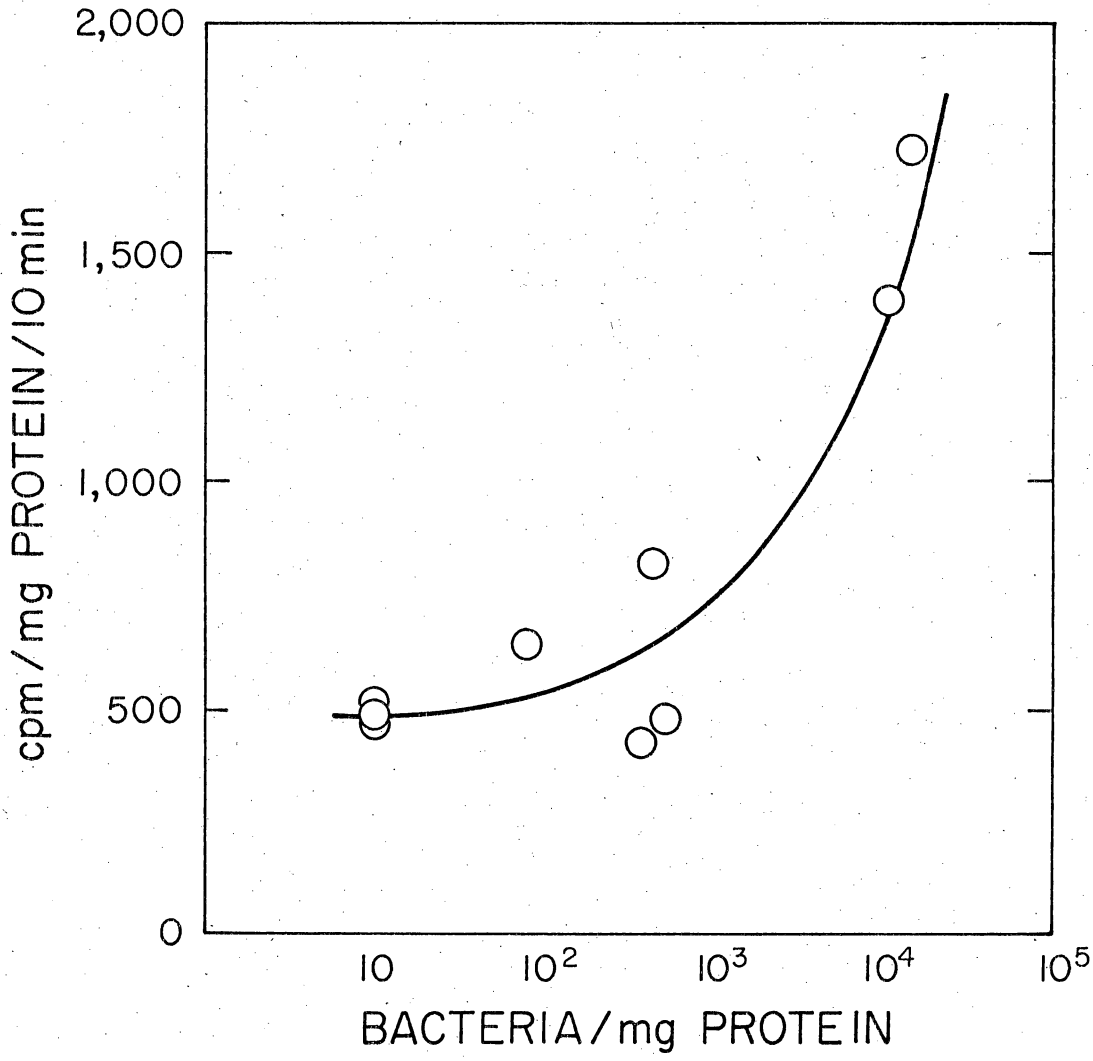

2,000
1,500
1,000
500
0
cpm / mg PROTEIN / 10 min
10
$10^2$
$10^3$
$10^4$
$10^5$
BACTERIA / mg PROTEIN

Figure 9. Time dependence of $^{14}C$-leucine incorporation by isolated mitochondria

o, respiration supported assay;
Δ, ATP generating system.

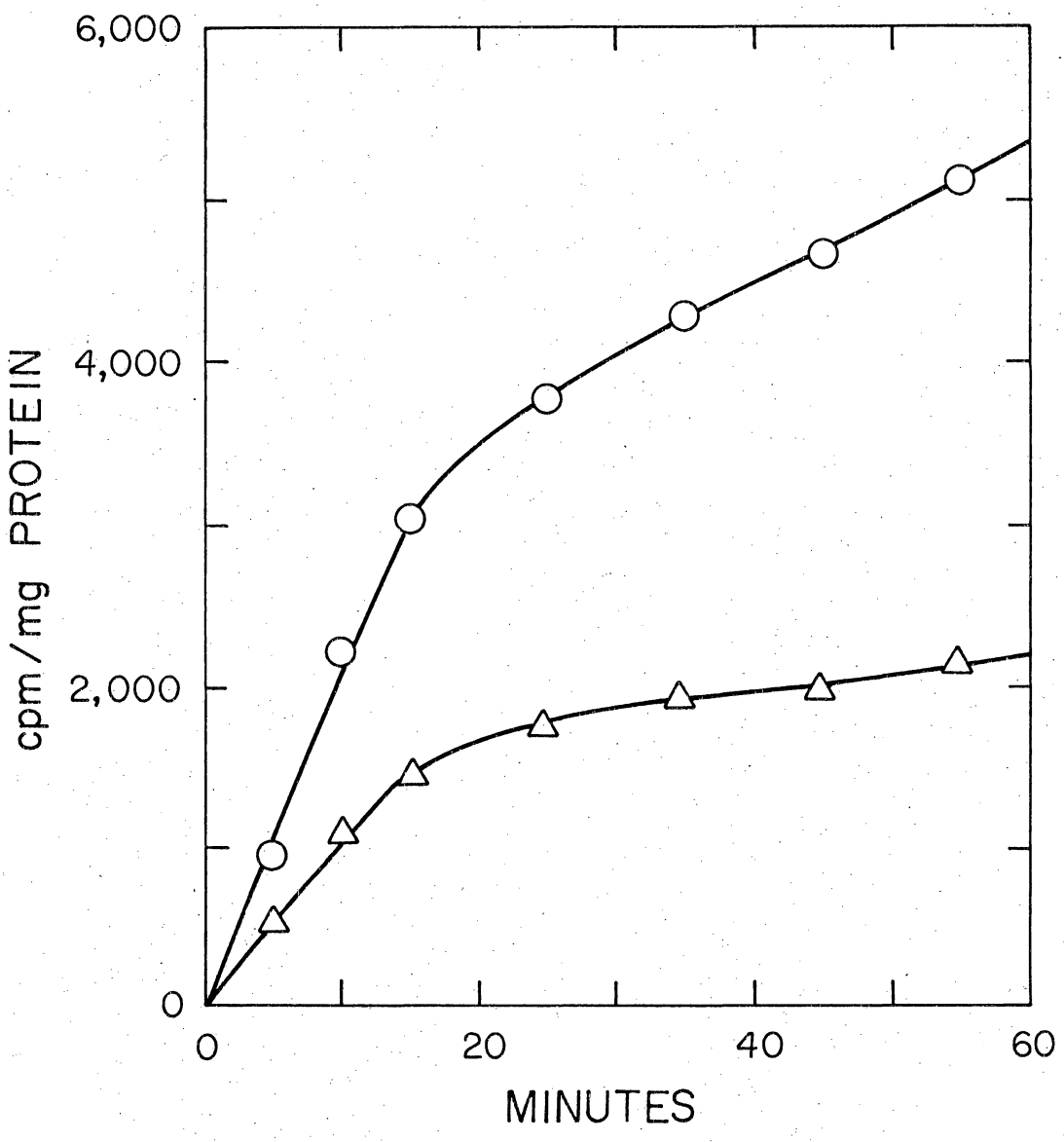

6,000
4,000
2,000
0
cpm/mg PROTEIN
0
20
40
60
MINUTES

Figure 10. Protein dependence of $^{14}C$-leucine incorporation by isolated mitochondria

o, respiration supported assay;
Δ, ATP generating system.

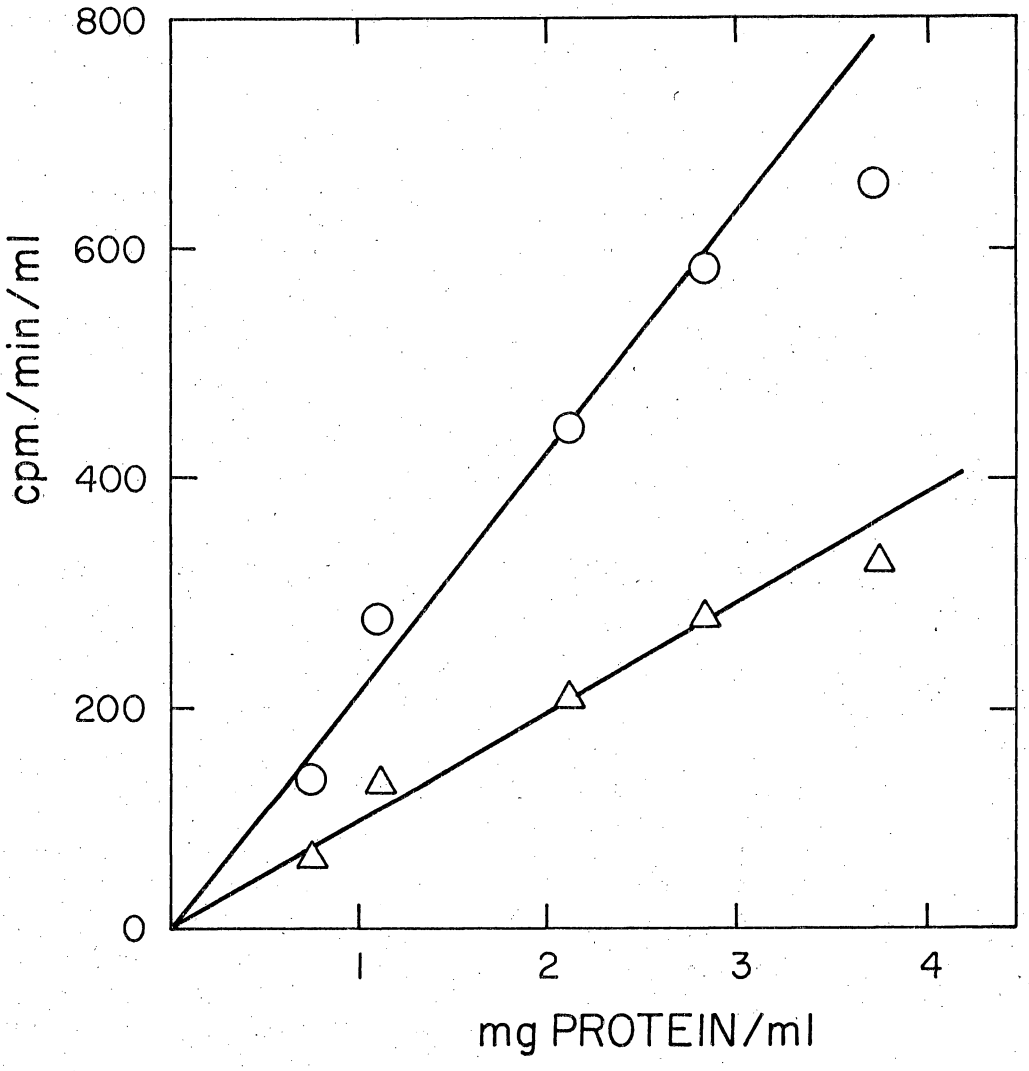

800
600
400
200
0
cpm./min./ml
1
2
3
4
mg PROTEIN/ml

Figure 11. $Mg^{2+}$ dependence of $^{14}C$-leucine incorporation

o, respiration supported assay;
Δ, ATP generating system.

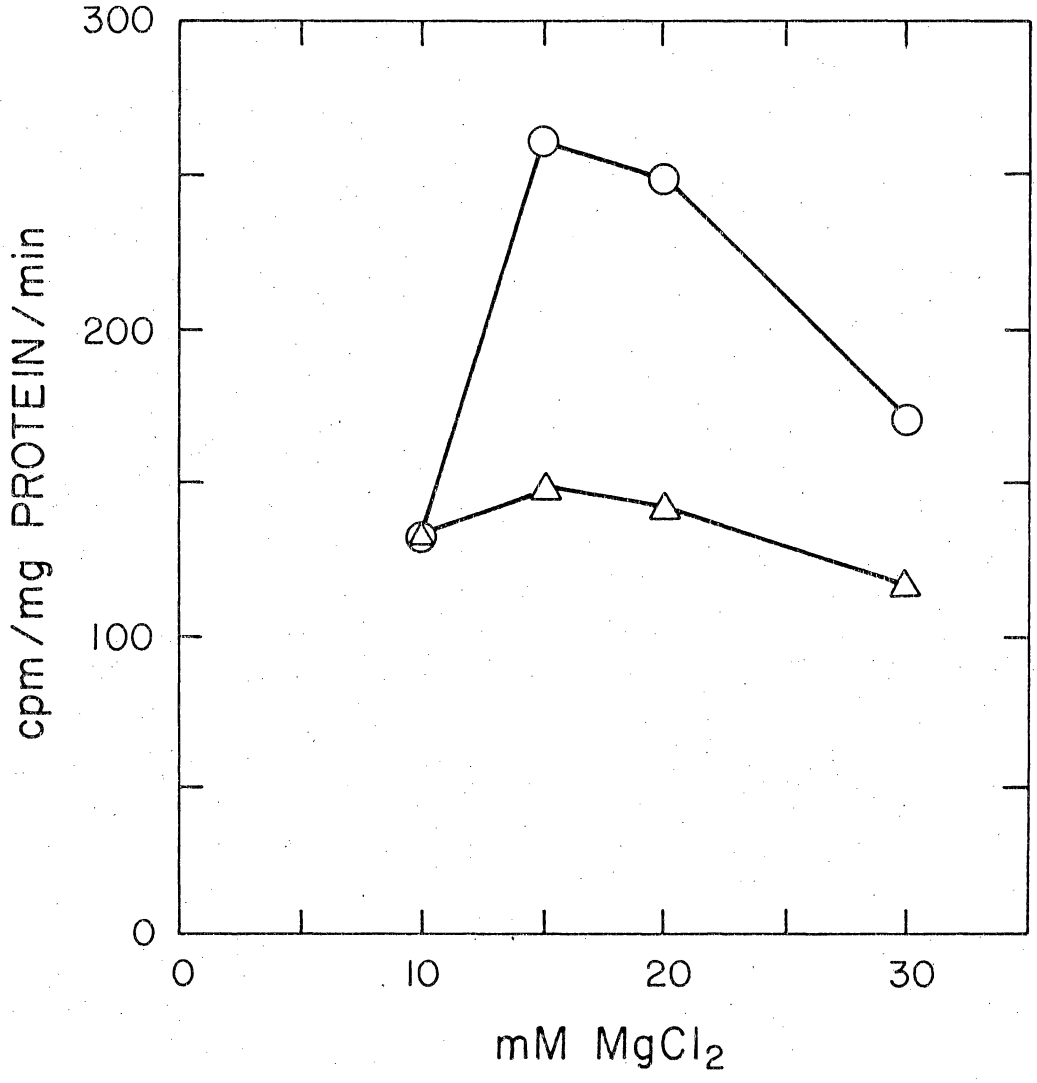

300
200
100
0
cpm/mg PROTEIN/min
0
10
20
30
mM MgCl2

TABLE VII

Characteristics of Mitochondrial Protein Synthesis

| | % of Control[a] |
|---|---|
| Complete System | 100 |
| + cycloheximide (30 μg/ml) | 101 ± 2 |
| +chloramphenicol (100 μg/ml) | 13.6 ± 3.4 |
| +unlabeled leucine (100-fold excess) | 8.3 ± 1.9 |
| +oligomycin (10 μg/ml) | 7.0 |
| +dinitrophenol (0.1 mM) | 1.0 |
| - succinate + ATP generating system[b] | 49.5 ± 2.5 |
| +oligomycin (10 μg/ml) | 53.0 |
| +dinitrophenol (0.1 mM) | 1.0 |

[a] mean ± S. E. for 3 or more experiments

[b] 4.0 mM phosphoenolpyruvate + 1.0 mM ATP + 20 μg/ml pyruvate kinase

protein synthesis. The effects of oligomycin and dinitrophenol are in agreement with those reported by Wheeldon and Lehninger (227) and Beattie and Ibrahim (228). Protein synthesis was inhibited by oligomycin in the respiration supported assay but not when an ATP-generating system was used. On the other hand, dinitrophenol inhibited protein synthesis in both systems. Wheeldon and Lehninger have suggested that inhibition by dinitrophenol in the ATP-supported assay is due to a stimulation of ATPase by dinitrophenol resulting in a decrease in available ATP.

The effect of $AFB_1$ on mitochondrial protein synthesis is shown in Table VIII. Very high concentrations of $AFB_1$ (135 nmol/mg protein, 0.4 mM) were required before any inhibition was observed in vitro. Doherty and Campbell (213) previously reported that similar molar concentrations of $AFB_1$ (0.4 mM) inhibited in vitro mitochondrial respiration by 40% and decreased oxidative phosphorylation in the presence of succinate by 13%. However, the ratio of $AFB_1$ to mitochondrial protein (800 nmol/mg protein) was approximately six times higher than the ratio (135 nmol/mg protein) required to inhibit protein synthesis in the respiration supported assay. In addition, inhibition of protein synthesis was also observed when energy was supplied by an ATP-generating system. These data suggest that inhibition of protein synthesis is independent of inhibition of oxidative phosphorylation. It is not known at this time if the difference in the degree of inhibition between the respiration and ATP-supported systems (13% and 25% respectively) is

TABLE VIII

Effect of $AFB_1$ on Protein Synthesis

by Isolated Mitochondria

| | % of Control[a] | |
|---|---|---|
| | Succinate | ATP Generating System |
| In Vitro | | |
| 3.4 nmol/mg protein | 101 ± 4 | 99.6 ± 3.4 |
| 34 nmol/mg protein | 104 ± 8 | 95.0 ± 0.6 |
| 135 nmol/mg protein | 87 ± 2 | 74.8 ± 3.1 |
| In Vivo Administration[b] | 78 ± 4 | |

[a] mean ± 1/2 range of duplicate experiments

[b] 4 hr after i.p. injection of 5.0 mg $AFB_1$/kg body weight

significant. In any case, inhibition in vitro occurs only at extremely high concentrations of $AFB_1$.

In vivo administration of $AFB_1$ (5.0 mg/kg body weight) produced a 22% inhibition of respiration-supported mitochondrial protein synthesis at 4 hr after injection (Table VIII). Similar inhibition was also observed at 2 hr after injection.

Wogan, et al. (229) have studied the tissue and subcellular distribution of aflatoxin after intraperitoneal injection of $AFB_1$. At 4 hr after injection they found that only 10% of the dose remained in the liver, and of that 15% was in the mitochondrial fraction. Using this information and the observation of Cinti and Schenkman (230) that there are 72 mg mitochondrial protein/g rat liver, the concentration of aflatoxin in the mitochondrial fraction after a dose of 0.5 mg per 100g animal (4 g liver) can be estimated at about 0.1 nmol/mg protein. Even much more generous estimates of aflatoxin concentration in the mitochondrial fraction would still place it orders of magnitude less than the concentration of $AFB_1$ required to inhibit mitochondrial protein synthesis in vitro. This would suggest that a more potent metabolite of $AFB_1$ may be responsible for the inhibition of mitochondrial protein synthesis after the in vivo administration of $AFB_1$.

To examine the question of whether or not a metabolite of $AFB_1$ may inhibit mitochondrial protein synthesis, an in vitro system permitting prior metabolism was employed. The first 600 x g supernatant fraction during the

isolation of mitochondria was incubated with $AFB_1$ and an NADPH-generating system. Thus, metabolites of $AFB_1$ were produced by the microsomal mixed function oxidase system in the presence of mitochondria. The mitochondria were subsequently isolated and assayed for protein synthesis. Control preparations were incubated with the dimethylformamide solvent in place of the $AFB_1$. The results of these experiments are shown in Table IX. Mitochondrial protein synthesis was inhibited approximately 40% at an $AFB_1$ concentration of 9.36 nmol/mg of 600 x g supernatant protein. The inhibition was not due to an alteration of mitochondrial respiration, as evidenced by similar inhibition in the ATP-supported assay. In addition, neither mitochondrial respiration nor oxidative phosphorylation were affected at these concentrations of $AFB_1$ (Table X).

The inhibition of mitochondrial protein synthesis was dependent on the presence of NADPH during the incubation of the 600 x g supernatant with $AFB_1$ (Table IX). Furthermore, $AFB_2$ (Figure 12) was inactive in this system (Table IX). From these data it appears that a metabolite(s) of $AFB_1$ inhibits mitochondrial protein synthesis and that 2, 3-unsaturation of the aflatoxin molecule and NADPH are required for production of this metabolite(s). Only two known metabolites of $AFB_1$ fit these conditions, $AFB_{2a}$ and the aflatoxin $B_1$ epoxide. Further investigation is required to determine if either of these aflatoxins is involved in the inhibition of mitochondrial protein synthesis.

TABLE IX

Effect of In Vitro Metabolism of Aflatoxin on

Mitochondrial Protein Synthesis

| Aflatoxin Concentration (nmol/mg supernatant protein) | No. of Experiments | % of Control[a] | |
|---|---|---|---|
| | | Succinate | ATP Generating System |
| $AFB_1$ | | | |
| 0.94 | 1 | 113 | 101 |
| 3.70 | 7 | 76.4 ± 4.1 | 88.7 ± 5.2 |
| 9.36 | 3 | 57.8 ± 4.2 | 60.8 ± 8.5 |
| omit NADPH | 2 | 90.1 ± 4.2 | 98.5 ± 8.5 |
| $AFB_2$ | | | |
| 3.70 | 1 | 97.1 | 97.3 |
| 9.36 | 1 | 96.7 | 104 |

[a] mean ± S.E. or 1/2 range

TABLE X

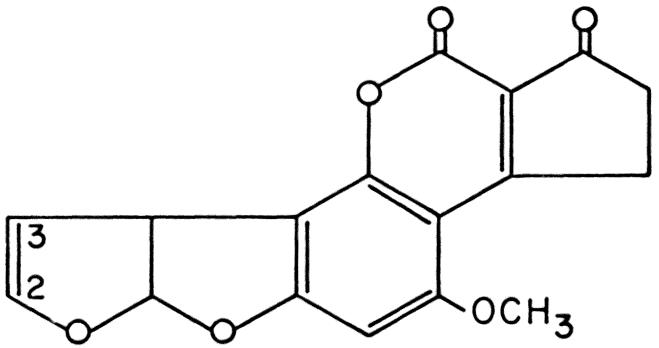
Effect of In Vitro Metabolism of $AFB_1$ on Mitochondrial Respiration

| Substrate | $AFB_1$ Concentration (nmol/mg supernatant protein) | % of Control | | | |
|---|---|---|---|---|---|
| | | State 4 Respiration | State 3 Respiration | RCR | P:O |
| Succinate[a] | 3.70 | 95.4 ± 1.0 | 104 ± 2 | 110 ± 3 | 100 ± 4 |
| | 9.36 | 104 ± 4 | 95.1 ± 4.2 | 90.1 ± 5.3 | 103 ± 3 |
| β-Hydroxybutryate[b] | 9.36 | 109 ± 1 | 110 ± 1 | 99.2 ± 0.2 | 91.6 ± 12.4 |

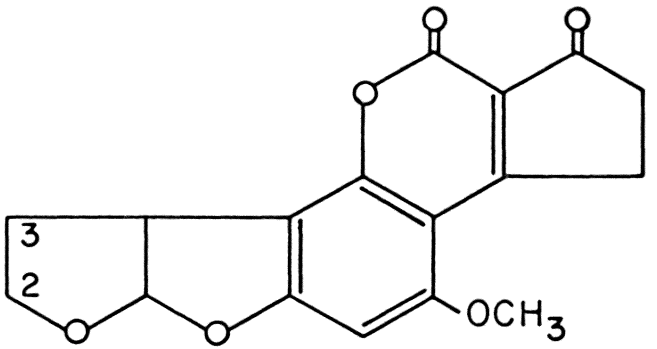
[a] 5.0 mM, data presented as mean ± S. E. for 4 or more experiments

[b] 5.0 mM, data presented as mean ± 1/2 range for duplicate experiments

Figure 12. Structures of $AFB_1$ and $AFB_2$




AFLATOXIN $B_1$




AFLATOXIN $B_2$

The concentration of aflatoxin in the mitochondrial fraction after incubation of the 600 x g supernatant was determined using $^{3}$H-AFB$_1$ (Table XI). At an initial concentration of 7.72 nmol AFB$_1$/mg supernatant protein, the concentration of total aflatoxins (+NADPH) in the mitochondrial fraction was 1.27 nmol/mg protein. Without NADPH, a concentration of 0.81 nmol/mg protein was observed. Assuming that the NADPH was required only for the metabolism of AFB$_1$ to the inhibitory metabolite(s), the difference between these two values gives an estimate of the concentration of the NADPH-dependent metabolite(s) responsible for inhibition of mitochondrial protein synthesis. This value is 0.5 nmol/mg mitochondrial protein and approximates the concentration of aflatoxin expected in the mitochondrial fraction after in vivo administration of AFB$_1$.

TABLE XI

Concentration of Aflatoxin in the Mitochondrial Fraction After In Vitro Metabolism of $AFB_1$

| nmol $^3$H-$AFB_1$/mg supernatant protein | nmol $^3$H-AF/mg mitochondrial protein[a] |
|---|---|
| 3.56 | |
| +NADPH | 0.586 $\pm$ 0.032 |
| -NADPH | 0.132 $\pm$ 0.021 |
| 7.72 | |
| +NADPH | 1.27 $\pm$ 0.07 |
| -NADPH | 0.807 $\pm$ 0.057 |

[a] mean $\pm$ S. E. for 3 groups of animals

## SUMMARY

In studies on the effect of $AFB_1$ on mitochondrial RNA synthesis, two factors were found to interfere with the assay. First, in some lots of $^3H$-UTP there was a radiocontaminant which masked the true incorporation of UTP. Attempts to purify the $^3H$-UTP by high voltage paper electrophoresis and high pressure liquid chromatography were unsuccessful. Secondly, with the lot of $^3H$-UTP in which minimum interference by the radiocontaminant was observed (Lot No. XR-2159), a 100-fold excess of unlabeled UTP reduced the incorporation by whole homogenate 93%, but reduced mitochondrial incorporation only 54%. Thus, properties of both the $^3H$-UTP and the mitochondrial fraction complicated the assay.

The experiments reported on the effect of $AFB_1$ on mitochondrial $^3H$-UTP incorporation were all done with Lot No. XR-2159. Incorporation was inhibited 52% by actinomycin D (50 μg/ml) and the product was hydrolyzed by pancreatic RNase. These data suggested that at least a major portion of the incorporation was due to RNA synthesis. The incorporation determined under these conditions was inhibited only 12% by very high concentrations of $AFB_1$ *in vitro* (0.4mM or 130 nmol/mg protein). On the other hand, 25% inhibition was observed with mitochondria isolated 4 hr after *in vivo* administration of $AFB_1$ (5.3 mg/kg body weight).

The effect of $AFB_1$ on mitochondrial protein synthesis was examined

using the incorporation of $^{14}$C-leucine into hot acid-insoluble product as a measure of protein synthesis. The incorporation had the characteristics of mitochondrial protein synthesis.

In vitro $AFB_1$ inhibited mitochondrial protein synthesis only at very high concentrations (13-25% inhibition at 135 nmol/mg protein). In vivo administration of $AFB_1$ (5.0 mg/kg body weight), which is expected to result in low concentrations of aflatoxin in the mitochondria ( 0.1 nmol/mg protein), produced a 23% inhibition of protein synthesis by the isolated mitochondria.

Using an in vitro system for the metabolism of $AFB_1$ by the mixed function oxidase system in the presence of mitochondria, it was found that a metabolite(s) of $AFB_1$ inhibited mitochondrial protein synthesis 40% at concentrations approaching those expected in vivo. The formation of this metabolite(s) required NADPH and 2, 3-unsaturation of the aflatoxin molecule.

The effect of $AFB_1$ on mitochondrial protein and RNA synthesis is similar to its effect on total cellular protein and RNA synthesis. $AFB_1$ itself does not inhibit these processes, but a metabolite of $AFB_1$ does. However, the inhibition of total cellular protein (170) and RNA (150) synthesis is greater than the inhibition of these processes in the mitochondrion.

The data presented here demonstrates that a metabolite of $AFB_1$ inhibits mitochondrial protein synthesis. However, the relevance of this inhibition to the toxic and carcinogenic effects of $AFB_1$ requires further investigation.

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31. Deleted in proof.

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# APPENDIX

A possible explanation for the low level of dilution of incorporation in the mitochondrial fraction is that even though the mitochondria were treated with phosphate, the inner membrane was not freely permeable to UTP. If this were the case and the mitochondria had an endogenous pool of UTP, a 100-fold excess of unlabeled UTP in the incubation system might not result in a 100-fold decrease in the specific activity of the internal UTP pool. For example, with a 0.05 mM internal UTP pool and a 0.1mM external $^{3}$H-UTP concentration, if only 1% of the external $^{3}$H-UTP entered the mitochondria the relative specific activity of the internal pool would be 1/50. If 10 mM unlabeled UTP was added to the external $^{3}$H-UTP and still only 1% crossed the inner membrane the specific activity of the internal pool would be 1/150, only 33% of the initial specific activity. Thus, only a 66% decrease of incorporation would be observed instead of the 99% expected of the basis of the specific activity of the external UTP.

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# THE EFFECT OF AFLATOXIN ON MITOCHONDRIAL TRANSCRIPTION AND TRANSLATION

by

Judith Ann Belt

(ABSTRACT)

The effect of aflatoxin $B_1$ on RNA synthesis in phosphate-swollen rat liver mitochondria was examined. In the assay system used the incorporation of $^{3}$H-UTP into acid-insoluble product was inhibited 52% by actinomycin D (50μg/ml) and the labeled product was sensitive to pancreatic RNase. Aflatoxin $B_1$ inhibited in vitro mitochondrial $^{3}$H-UTP incorporation only 12% at high concentrations (0.4 mM or 130 nmol/mg protein). On the other hand, 25% inhibition was observed with mitochondria isolated 4 hr after animals had been treated with aflatoxin $B_1$ (5.3 mg/kg body weight by i.p. injection). This suggested that a metabolite of aflatoxin $B_1$ may inhibit mitochondrial RNA synthesis.

The effect of aflatoxin $B_1$ on mitochondrial protein synthesis was examined using sterile mitochondrial preparations and two assay systems. In the first assay mitochondrial respiration served as an energy source, and in the second an ATP-generating system was used. As expected for mitochondrial protein synthesis, the incorporation of $^{14}$C-leucine into hot acid-insoluble product was inhibited 86% by chloramphenicol but was not affected by cycloheximide.

Aflatoxin $B_1$ inhibited *in vitro* mitochondrial protein synthesis only at very high concentrations (13-25% inhibition at 0.4 mM or 135 nmol/mg protein). Treatment of animals with aflatoxin $B_1$ (5.0 mg/kg body weight), which is expected to result in relatively low concentrations of aflatoxin in the mitochondrial fraction (0.1 nmol/mg protein), produced a 23% inhibition of protein synthesis in mitochondria isolated 4 hr after injection. These data suggest that a metabolite of aflatoxin $B_1$ may inhibit mitochondrial protein synthesis.

Using an *in vitro* system for the metabolism of aflatoxin $B_1$ by the mixed function oxidase system in the presence of mitochondria, it was found that a metabolite(s) of aflatoxin $B_1$ inhibited mitochondrial protein synthesis by 40% at a concentration of 0.5 nmol/mg mitochondrial protein. This inhibition was not due to an alteration of mitochondrial respiration, as inhibition was observed in both the respiration and ATP supported assays. In addition, mitochondrial respiration, respiratory control ratios and P:O ratios were not affected. Formation of the inhibitory metabolite(s) required NADPH and 2, 3-unsaturation of the aflatoxin molecule.