Biochemical Pharmacology, Vol. 20, pp. 2825–2831. Pergamon Press, 1971. Printed in Great Britain

AFLATOXIN B_1 INDUCED INHIBITION OF LIVER PROTEIN SYNTHESIS *IN VIVO* AND ITS ROLE IN FATTY LIVER*

K. SURYANARAYANA RAO

Department of Pharmacology, Michigan State University, East Lansing, Mich. 48823, U.S.A.

(*Received* 31 *December* 1970; *accepted* 5 *March* 1971)

Abstract—Effects of single oral doses of aflatoxin B_1 (2 mg/kg) to monkeys *Macaca irus* on total liver protein synthesis and accumulation of liver lipid were investigated. Incorporation of [^{14}C]leucine into total liver protein *in vivo* was inhibited 50 per cent by aflatoxin B_1 3 hr after oral administration. This degree of inhibition was maintained up to 13 hr. Concomitant with this change there was an extensive disaggregation of polysomes in liver preparations. A progressive accumulation of lipid in liver following single dose administration of aflatoxin B_1 was observed. This increase in liver lipid was primarily triglyceride. The role of aflatoxin induced inhibition of hepatic protein synthesis *in vivo* to increase liver lipid is discussed.

THE AFLATOXINS are a group of closely related metabolites produced by the fungus *Aspergillus flavus*. These metabolites have been shown to be acutely toxic to many animal species by virtue of their hepatotoxicity.[1] Aflatoxin B_1 is the most potent of these substances with respect to acute lethality, although there is a considerable species variation in susceptibility. For example, the oral LD_{50} of aflatoxin B_1 in the 1-day-old duckling is 0·6 mg/kg and 5·5 mg/kg in the weanling male rat.[2] Despite this wide variation in species' susceptibility to aflatoxin, there is a high order of specificity with regard to the site of primary toxicity. Most species show hepatotoxicity as the primary, if not the sole, site of toxic effect.

The presence of aflatoxins in a wide variety of foodstuffs together with the knowledge of their toxicity poses an important question about its potential hazard to man. This question has been approached by evaluating the toxic potential of aflatoxins in a primate species.[3] In that study it was shown that Cynomolgus monkeys are acutely susceptible to aflatoxin. The oral LD_{50} in monkeys was 2·2 mg/kg for aflatoxin B_1. The pathological changes observed in monkeys, however, were unlike those observed in other species. In monkeys, a single dose of 2–3 mg/kg aflatoxin produced fatty degeneration of the liver, a response conspicuously absent in rat liver following aflatoxin administration.

Inhibition of protein synthesis is an early feature in the pathogenesis of certain types of experimentally induced fatty liver;[4,5] however, the fatty liver is not always preceded by an inhibition of protein synthesis.[6] Smith[7] reported marked inhibition *in vitro* by aflatoxin B_1 of leucine incorporation into protein of rat and duckling liver slices. This

* This work was supported by GRS Grant No. 71-0728 from Michigan State University, East Lansing, Mich.

was later confirmed in other cell free systems *in vitro*.[8] Despite repeated confirmation of inhibition of [^{14}C]leucine incorporation *in vitro* into liver protein by aflatoxin, Shank and Wogan[9] did not observe any inhibition of incorporation of [^{14}C]leucine into rat liver total proteins *in vivo* by prior treatment of animals with LD_{50} doses of aflatoxin B_1. It therefore became of interest to study whether or not protein synthesis by liver is affected during the early stages of aflatoxin poisoning in monkeys. The present experiments were undertaken to investigate the relationship *in vivo* of liver total protein synthesis to liver lipid accumulation following oral administration of an LD_{50} dose of aflatoxin B_1.

MATERIALS AND METHODS

Eleven cynomolgus monkeys, *Macaca irus*, maintained by the Primate Research Center, Michigan State University, were used. They were approximately 1 year old and weighed 1·5 kg. The monkeys were individually housed in stainless steel cages and were fed Purina Monkey Chow* and water *ad lib.* until the experiment was started. Aflatoxin B_1† was administered to experimental monkeys at a dose of 2 mg/kg. The desired dose of aflatoxin B_1 was dissolved in 0·5 ml of dimethylsulfoxide (DMSO) and administered orally to monkeys, fasted overnight. Control animals were also fasted overnight and given an equivalent amount of vehicle. At various times after administration, two aflatoxin B_1-treated animals and one control animal were injected intraperitoneally with 10 μc of [^{14}C]leucine (26·2 mc/mM; Calatomic, Los Angeles, Calif.) dissolved in 0·5 ml of saline. The animals were sacrificed 30 min later by the intracardiac injection of ethyl ether.

Collection of tissue samples. Before sacrificing the animals, blood samples were drawn by cardiac puncture and serum obtained after clotting and centrifugation. Following euthanasia the liver and kidneys were rapidly removed and weighed.

Preparation of proteins for radioactivity measurements. Portions of liver and kidney were homogenized in 10 vol. of 10% (weight per volume) trichloracetic acid (TCA). TCA was also added to serum in a final concentration of 10 per cent. After centrifugation, the initial supernatant (acid soluble) fraction was diluted to 10 ml with 5% TCA. A 0·5-ml aliquot was used for radioactivity counting after the addition of 15 ml of Bray's solution.[10] Purified and dried proteins from liver and kidneys were prepared from the TCA precipitates by the procedure of Lombardi and Oler.[11] The purified proteins were dissolved in formic acid and 0·5-ml aliquots containing approximately 5 mg of protein were counted after the addition of 15-ml scintillation solution containing: Naphthalene 125 g, PPO 7 g, POPOP 0·4 g (PPO = 2,5-diphenyloxazole; POPOP = 1,4 bis-2-(4-methyl-5-phenyl-oxazolyl) benzene) and thixotropic gel 25 g/l. Radioactivity was monitored in a Beckman LS-100 Liquid Scintillation System. CPM were converted to DPM using [^{14}C]toluene as internal standard.

Lipids. Total liver lipids were extracted with a 2:1 (v/v) mixture of chloroform:methanol and estimated gravimetrically by the procedure of Folch *et al.*[12] Total liver lipids obtained as described were dried at 40° under N_2, and redissolved in chloroform. Suitable chloroform aliquots were applied to columns of silicic acid (Unicil, Clarkson Chemical Co., Williamsport, Pa.). Neutral lipids were eluted with

* Purina monkey chow, Ralston Purina Co., St. Louis, Mo. 63112.

† Crystalline aflatoxin B_1 (B Grade) was obtained from Calbiochem., Los Angeles, Calif. 90054.

chloroform. Known aliquots of the chloroform eluates as well as aliquots of serum were used for the analysis of triglycerides.[13] Serum and liver total cholesterol were estimated by the method of Bowman and Wolf.[14]

Liver polysome profile. Polysome profiles[15] were determined in samples of liver gently homogenized in 2 vol. of Medium A.[16] Antiferritin grade A (Calbiochem., Los Angeles, Calif.) was added in the proportion of 1–10. The post-mitochondrial supernatant fraction was prepared by centrifuging at 20,000 *g* for 20 min. Deoxycholate was added to give a concentration of 1·3 per cent. In each experiment a constant volume (0·5 ml) of the post-mitochondrial fraction was layered onto a linear 10–40 per cent (w/v) sucrose gradient. The gradients were centrifuged at 25,000 rev/min in a Spinco SW 25·1 rotor for 3·5 hr at 4° using a Beckman Model L-2 Ultracentrifuge. After centrifuging, the sucrose density gradient tubes were punctured at the bottom with a hypodermic needle and the A_{260} of the contents was determined using a flow cell (0·3 cm light path) in a Gilford Spectrophotometer. Results were recorded directly using a Sargent Model SR recorder. A constant flow rate of 5 ml per min was maintained during the determination with a Buchler Polystaltic pump.

The test of significance was calculated using Student's *t*-test and the level of significance was chosen as $P < 0{\cdot}05$.

RESULTS

Effects of aflatoxin B_1 on hepatic and serum lipids. Table 1 shows the changes in liver lipids following the administration of a single dose of aflatoxin B_1. A progressive accumulation of triglycerides was noted in liver and this increase was reflected by an increase of total liver lipid. During a 13-hr period, liver triglycerides increased from 6·7 to 51·1 mg/g liver, an 8-fold increase. A significant increase in liver triglycerides was discernible 3·5 hr following the administration of aflatoxin. It is interesting to note that there was no change in the cholesterol content in the liver at any period studied. Concomitant with the increase in liver triglycerides there was progressive fall in the concentration of serum triglycerides (Table 2) and a slight fall in serum cholesterol.

Effects of aflatoxin B_1 on liver protein synthesis in vivo. Previous studies[3] indicated that a single dose of 2·0 mg aflatoxin B_1 per kg body weight was the lowest dose which produced a significant increase in serum hepatic enzyme activity after 48–72 hr. The

TABLE 1. EFFECT OF AFLATOXIN B_1 ON LIVER TOTAL LIPID, TRIGLYCERIDE, AND CHOLESTEROL CONTENT IN MONKEYS AT VARIOUS TIMES AFTER ADMINISTRATION

Treatment	No. of monkeys	(Liver lipids/tissue wet wt.) Total lipid (g/100 g)	Triglycerides (mg/g)	Cholesterol (mg/g)
Control	3	5·40 ± 0·66*	6·76 ± 0·84	4·90 ± 0·93
1 hr after aflatoxin†	3	5·74 ± 0·37	9·86 ± 1·07	4·66 ± 0·35
3·5 hr after aflatoxin†	2	7·33 ± 0·05	15·25 ± 0·85‡	5·80 ± 0·60
13 hr after aflatoxin†	3	10·86 ± 0·88‡	51·20 ± 5·77‡	4·43 ± 0·17

* Mean ± standard error.
† Aflatoxin, 2 mg/kg p.o. in DMSO.
‡ $P < 0{\cdot}05$.

TABLE 2. EFFECT OF AFLATOXIN B_1 IN SERUM LIPID CONTENT IN THE SAME MONKEYS AT VARIOUS TIMES AFTER ADMINISTRATION

Treatment	No. of monkeys	Triglycerides (mg/100 ml)	Cholesterol (mg/100 ml)
Control	3	72 ± 1*	132 ± 5
1 hr after aflatoxin†	3	74 ± 2	136 ± 3
3·5 hr after aflatoxin†	2	57 ± 3‡	122 ± 14
13 hr after aflatoxin†	3	42 ± 2‡	116 ± 3

* Mean ± standard error.
† Aflatoxin, 2 mg/kg p.o. in DMSO.
‡ $P < 0{\cdot}05$.

effect of this dose on the incorporation of [^{14}C]leucine into liver protein *in vivo* was studied in liver, the target organ, and kidney, a non-target organ. The results are presented in Table 3 and demonstrate that the incorporation of [^{14}C]leucine into total liver proteins decreased rapidly to 50 per cent of control values within 3·5 hr after administration of aflatoxin B_1. This inhibition of incorporation persisted up to 13 hr after the administration of aflatoxin. There was practically no change in [^{14}C]leucine incorporation into total kidney proteins of the same animals. Similar levels of ^{14}C were detected in the TCA supernatant solutions (from organs) and the serum of treated as compared to control animals (Table 4) suggesting no effect of aflatoxin on the pool of radioactive amino acids.

TABLE 3. EFFECT OF AFLATOXIN B_1 ON [^{14}C]LEUCINE INCORPORATION INTO LIVER, KIDNEY AND SERUM PROTEINS OF MONKEYS

Treatment	No. of monkeys	Liver (dis./min/mg protein)	Kidney (dis./min/mg protein)	Serum (dis./min/mg protein)
Control	3	311 ± 15*	147 ± 16	54 ± 8
1 hr after aflatoxin†	3	291 ± 11	131 ± 10	55 ± 7
3·5 hr after aflatoxin†	2	169 ± 1‡	113 ± 6	60 ± 8
13 hr after aflatoxin†	3	169 ± 10‡	144 ± 13	43 ± 5

* Mean ± standard error.
† Aflatoxin, 2 mg/kg p.o. in DMSO.
‡ $P < 0{\cdot}05$.

TABLE 4. TISSUE ACID SOLUBLE ^{14}C AT VARIOUS TIMES AFTER AFLATOXIN B_1 ADMINISTRATION

Treatment	No. of monkeys	Liver (dis./min/g wet tissue)	Kidney (dis./min/g wet tissue)	Serum (dis./min/ml)
Control	3	6230 ± 672*	6913 ± 295	6262 ± 971
1 hr after aflatoxin†	3	7271 ± 354	7233 ± 260	7053 ± 632
3·5 hr after aflatoxin†	2	7367 ± 286	6269 ± 361	7395 ± 1260
13 hr after aflatoxin†	3	8514 ± 401	6938 ± 203	6867 ± 551

* Mean ± standard error.
† Aflatoxin, 2 mg/kg p.o. in DMSO.

Pattern of liver ribosome aggregates. The results of numerous investigations[17] have shown that there is usually a good correlation between amount and size of ribosomal aggregates (polysomes) and the ability of the liver to incorporate amino acids into proteins. Typical polysome profiles of liver from control monkeys and aflatoxin B_1 treated monkeys are depicted in Fig. 1. The control profile shows a single broad polysome peak. One hr after aflatoxin the polysome profile was not different from that of control animals. Three and one-half hr after aflatoxin treatment the polysome

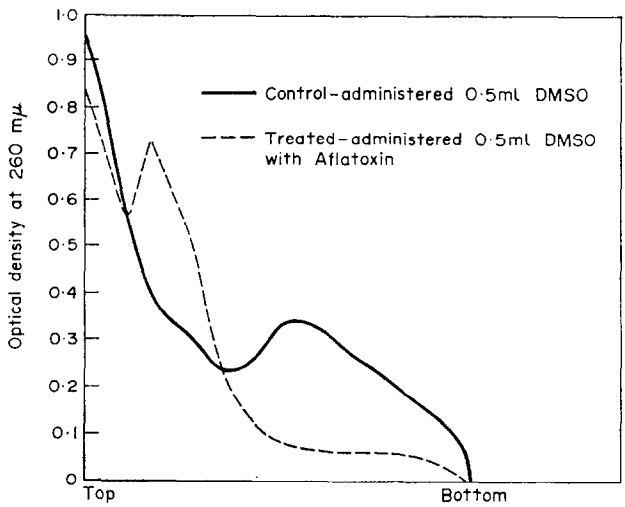

FIG. 1. Monkey liver polysome profiles 3·5 hr after single oral dose of 2 mg/kg of aflatoxin B_1.

portion of the profile was decreased with a corresponding increase in the monomer and dimer areas (Fig. 1). The same pattern was observed 13 hr after toxin administration.

DISCUSSION

The liver cell has a remarkable capacity to maintain structural and functional integrity. Many substances challenge normal metabolic processes of the organ resulting in fatty accumulation and necrosis. Fatty accumulation of the liver may be induced in experimental animals by a wide variety of toxic substances, metabolites or nutritional deficiencies. Despite the heterogeneity of these etiologic factors the lipids accumulating in the liver appear to be predominantly triglycerides.[18] It has been assumed by many workers, either tacitly or explicitly, that the accumulation of lipids in fatty liver is a result or consequence of an inhibition of the secretion of lipids from liver into plasma which, in turn, is due to some defect in protein synthesis. From the evidence available in the literature it is clear that the key pathophysiologic defect in fatty livers[19] in a number of instances of drug-induced toxicity is due to a failure in hepatic triglyceride secretion. However, the underlying molecular biological disturbances which result in failure of triglyceride secretion is no doubt unique for each hepatotoxin.

Depression of hepatic protein synthesis has been invoked as the key to pathological hepatic lipid accumulation.[4] Verbin *et al.*[20] in their studies with cycloheximide showed

that hepatic parenchymal cells in the rat tolerated a considerable inhibition (95 per cent) of protein synthesis activity for at least several hours without significant lipid accumulation. Hence they concluded that fatty liver cannot be attributed simply to interference with protein synthesis in the hepatocyte.

Results presented here demonstrate that oral administration of aflatoxin B_1 to monkeys at a dose level of 2 mg/kg causes significant accumulation of triglycerides in the liver 3·5 hr following the administration. At the same time there is 50 per cent inhibition in total protein synthesis in liver. However, at earlier times there was no change in protein synthesis. There does not appear to be any temporal relationship between these observations. From such a study it is hazardous to conclude that these two phenomena are unrelated, coexistent biochemical anomalies. The reasons for this are that the inhibition of protein synthesis observed in this study represents the effects of a host of different proteins. The turn-over rates of these proteins vary from a few minutes to days in length. From these considerations, the possibility is not ruled out that protein components of lipoprotein, which form a very small fraction of the total liver proteins, may be more significantly affected at much earlier times. Such an effect may not have been detectable by the technique employed here.

The inhibition of protein synthesis observed in this investigation probably cannot be attributed to differences in pool size, since there was no difference in the amount of recovered radioactivity in the acid-soluble fraction of liver between the control and experimental animals. The observed inhibition *in vivo* of total protein synthesis does not agree with observations of Shank and Wogan[9] in which no significant inhibition in total protein synthesis in rat liver was found. This discrepancy could be attributed to differences in species variability. Furthermore, the inhibition of protein synthesis appears to be specific for liver since no change in the specific activity of kidney proteins was detected at any time period studied.

In the present study the administration of a single dose of aflatoxin B_1 was found to produce a marked disaggregation of polyribosomes 3·5 hr after administration. The effect was still observed at 13 hr. These results on polysome profiles correlate, in general, with electron microscopic observations by Svoboda *et al.*[21] who found that monkey liver ribosomes dissociate from the endoplasmic reticulum after aflatoxin B_1 treatment. Further experimental data will be required in order to elucidate the observed biochemical effects of aflatoxin directly at the subcellular and cellular site and to ultimately correlate these with the toxicity.

REFERENCES

1. P. M. NEWBERNE and W. H. BUTLER, *Cancer Res.* **29,** 236 (1969).
2. G. N. WOGAN, *Bact. Rev.* **30,** 460 (1966).
3. K. S. RAO and P. J. GEHRING, *Toxic. appl. Pharmac.* in press (1971).
4. E. A. SMUCKLER, O. A. ISERI and E. P. BENDITT, *J. exp. Med.* **116,** 55 (1962).
5. D. S. ROBINSON and A. SEAKINS, *Biochim. biophys. Acta* **62,** 163 (1962).
6. P. S. ROHEIM, S. SWITZER, A. GIRARD and H. A. EDER, *Biochem. biophys. Res. Commun.* **20,** 416 (1965).
7. R. H. SMITH, *Biochem. J.* **88,** 509 (1963).
8. J. I. CLIFFORD and K. R. REES, *Biochem. J.* **102,** 65 (1967).
9. R. C. SHANK and G. N. WOGAN, *Toxic. appl. Pharmac.* **9,** 468 (1966).
10. G. A. BRAY, *Analyt. Biochem.* **1,** 279 (1960).
11. B. LOMBARDI and A. OLER, *Lab. Invest.* **17,** 308 (1967).
12. J. FOLCH, M. LEES and G. H. SLOANE STANLEY, *J. biol. Chem.* **226,** 497 (1957).
13. E. VAN HANDEL and D. B. ZILVERSMIT, *J. Lab. clin. Med.* **50,** 152 (1957).

14. R. E. Bowman and R. C. Wolf, *Clin. Chem.* **8,** 302 (1962).
15. J. W. Drysdale and H. N. Munro, *Biochim. biophys. Acta* **138,** 616 (1967).
16. F. O. Wettstein, T. Staehelin and H. Noll, *Nature, Lond.* **197,** 430 (1963).
17. H. Noll, in *Developmental and Metabolic Control Mechanisms and Neoplasma.* A collection of papers presented at the 19th Annual Symposium on Fundamental Cancer Research 1965, p. 67. Williams & Wilkins, Baltimore (1965).
18. R. O. Recknagel, *Pharmac. Rev.* **19,** 145 (1967).
19. B. Lombardi and R. O. Recknagel, *Am. J. Path.* **40,** 571 (1962).
20. R. S. Verbin, P. J. Goldblatt and E. Farber, *Lab. Invest.* **20,** 529 (1969).
21. D. Svoboda, H. Grady and J. Higginson, *Am. J. Path.* **49,** 1023 (1966).