INHIBITION OF *IN VITRO* PROTEIN SYNTHESIS BY AFLATOXIN B_1 DERIVATIVES

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1. Introduction

The highly potent hepatocarcinogen, aflatoxin B_1, is well known for producing biochemical alterations in liver [1, 2]. A single injection of the drug inhibits *in vivo* protein synthesis within 30 min. The mechanism, whereby the carcinogen affects the translational process, is in fact complex. Impairment of protein synthesis beyond 7 hr after dosing may be considered primarily as a consequence of its inhibition of RNA synthesis. However, aflatoxin's short-term effect, evidenced by marked inhibition 2 hr after administration, cannot be explained by a similar mechanism. Since our earlier results suggested direct action by aflatoxin B_1 on polysomes [3], we have sought to further elucidate this point by determining the aflatoxin B_1 effect *in vitro* and *in vivo* on the ability of isolated polysomes to incorporate amino acids.

This paper reports the *in vitro* effect of aflatoxin B_1 and of some aflatoxin-derived metabolites on the synthesizing activity of normal rat liver polysomes in a cell-free system. Under these conditions, crystalline aflatoxin B_1 is completely inactive whereas aflatoxin-derived metabolites obtained by enzymic conversion of the mycotoxin are able to inhibit translation *in vitro*. The present results support the hypothesis of a direct effect of aflatoxin B_1 derivatives on the functional activity of polysomes. Moreover, they raise the problem of the structure of the active compound and its relationship with the derivative inhibiting the translational process.

2. Materials and methods

Male Wistar rats (Commentry strain) weighing about 300 g and fed a balanced semi-synthetic diet were fasted 15 hr before killing by decapitation. Polysomes were isolated by a previously reported modification [4] of the original method published by Blobel and Potter [5]. The polysomal standard system incorporating amino acid owing to its endogenous messenger RNA has been previously described [6]. The determination of incorporated L-[^{14}C]leucine was made using paper disks (Whatman 3 MM) according to Mans and Novelli [7].

Microsomes were isolated from the livers of rats injected intraperitoneally with 3-methylcholanthrene once a day for 3 consecutive days before killing (20 mg/kg on the 1st day, 10 mg/kg on the 2nd and 3rd days). Livers were homogenized in 9 vol of medium R (50 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 0.25 M sucrose) and centrifuged for 20 min at 15,000 rpm (Spinco J 21, rotor JA 20). The postmitochondrial supernatant was removed, then centrifuged for 60 min at 50,000 rpm (Spinco, rotor 50 Ti), in order to spin down the microsomes. The pellets were stored at −50°.

The enzymic activation of aflatoxin B_1 was performed at 37°; the incubation flask routinely contained 500 μg of aflatoxin B_1, 20 mg NADPH, and microsomes equivalent to 4 g of liver in 50 ml of medium R. The flasks were shaken for 45 min. The mixture was extracted 3 times with an equal volume of chloroform. The combined-soluble fractions were dried with Na_2SO_4 and evaporated to dryness. The residue was dissolved in various solvents (Tris-buffer, 1,2-propane diol, acetone).

Table 1
Effect of aflatoxin B_1 and aflatoxin M_1 on *in vitro* protein synthesis.

Aflatoxin	µg/ml of assay	Solvent	Percent of control
B_1	15	Tris-buffer	101
B_1	55	1,2-Propane diol	102
B_1	100	1,2-Propane diol	101
B_1	46	Ethanol	97
M_1	17	Tris-buffer	106
M_1	34	Tris-buffer	100

Incubation mixture contained per ml of standard assay: 0.5 µmoles ATP, 0.1 µmoles GTP, 3 µmoles phosphoenolpyruvate, 25 µg pyruvate kinase, 50 µmoles Tris-HCl pH 7.5, 10 µmoles Mg $(CH_3CO_2)_2$, 100 µmoles KCl, 40 µmoles NaCl, 0.05 µmoles of each 19 ^{12}C-amino acids, 2 µCi L-[^{14}C]leucine, 8 mg pH 5 enzyme, 4.5 A_{260} units rat liver polysomes. The incubation (0.5 ml) was run 60 min at 37°. Each experiment was compared to its own control which contained the same amount of the solvent used to solubilize aflatoxins. Results are means of 6 experiments.

Thin-layer chromatography was carried out on silica gel coated plates (G-HR Macherey-Nagel) using chloroform–acetone (9:1) for development.

Aflatoxin B_1 was purchased from Makor Inc. (Jerusalem, Israel) and uniformly labeled L-[^{14}C]leucine from the Commissariat à l'Energie Atomique (Saclay, France).

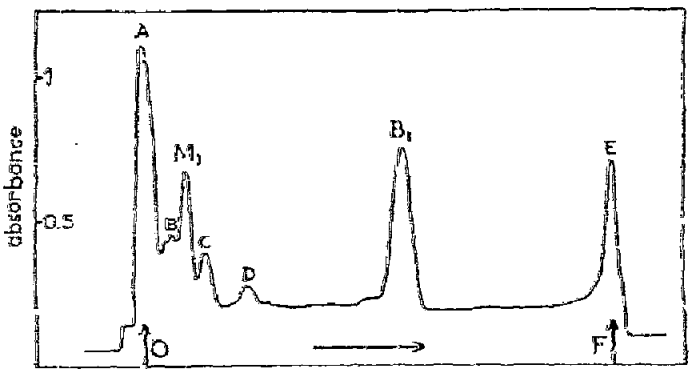

Fig. 1. Fluorodensitometric scanning pattern of thin-layer chromatography plates of aflatoxin B_1-derived metabolites. Thin layer chromatography was carried out on silica gel-coated plates using chloroform–acetone (9:1) for development. Fluorodensitometric determination was made in a recording densitometer equipped for fluorescence emission measurement (Photomètre intégrateur enregistreur Vernon, France). O: origin; F: front of solvent; B_1: aflatoxin B_1; M_1: aflatoxin M_1; A, B, C, D, E: unknown metabolites (see text).

Table 2
Inhibitory activity of aflatoxin B_1 derivatives on *in vitro* protein synthesis.

Aflatoxin B_1-derived metabolites*			
Number of preparations	µg/ml of assay	Solvent	Percent of control
X_2	8	1,2-Propane diol	56
X_4	6	Ethanol	73
X_4	10	Ethanol	23
X_{14}	7.5	Tris-buffer	46
X_{14}	6.2	Tris-buffer	41
E_2	12.6	Tris-buffer	72

* The quantity of aflatoxin B_1-derived compound was estimated by fluorodensitometric measurement using the specific coefficient of aflatoxin B_1. Incubation conditions were identical to those described in table 1. Each experiment was compared to its own control which contained the same amount of the solvent used to solubilize aflatoxin-derived compounds. Results are means of 6 to 9 determinations.

3. Results and discussion

Crystalline aflatoxin B_1 has no effect on amino acid incorporation by the polysomal standard system, whatever the solvent used to dissolve it before testing (table 1). The foregoing result is not too surprising if one considers that many drugs and carcinogens require metabolic transformation to be active *in vitro* as well as *in vivo* [8, 9]. Along this line, it should be noted that pure aflatoxin B_1 also completely fails to inhibit *in vitro* transcription [10]. *In vitro* conversion of aflatoxin B_1 into active derivatives by microsomal-induced enzymes responsible for drug metabolism leads to several compounds: aflatoxin M_1 and components A, B, C, D, E (fig. 1). The aflatoxin-derived compounds inhibit *in vitro* amino acid incorporation by the polysomal standard system (table 2). The inhibitory capacity of so-formed derivatives shows significant differences for successive preparations. The variability may be explained as follows: the quantitative estimation, as indicated in table 2, is determined on the total chloroform-soluble aflatoxin derived

Table 3
Requirements for conversion of aflatoxin B_1 to derivatives inhibiting *in vitro* amino acid incorporation.

In vitro conversion of aflatoxin B_1	Effect on *in vitro* amino acid incorporation
Control*	(100)
Complete system**	41
Minus aflatoxin B_1	103
Minus microsomes	101
Minus NADPH	91
Heated microsomes***	110

* The control for *in vitro* amino acid incorporation was described in table 1.

** The complete system for aflatoxin B_1 conversion contained in 10 ml of medium R: 100 μg aflatoxin B_1, 4 mg NADPH, microsomes equivalent to 0.8 g of liver. Incubations were run 45 min at 37°. Incubation mixtures were extracted with chloroform 3 times. The pooled extracts were evaporated to dryness. The residue was dissolved in Tris-buffer. The addition of the latter was checked on the activity of the polysomal standard system incorporating amino acids. Results are means of 6 determinations.

*** Microsomes resuspended in medium R were heated at 100° for 30 min before adding to the incubation flask.

Table 4
Inhibitory activity of aflatoxin B_1 derivatives on *in vitro* protein synthesis as a function of the solvent used.

Aflatoxin B_1-derived metabolites*			
Number of preparations	μg/ml of assay	Solvent	Percent of control
X_{6a}	5.6	1,2-Propane diol	42
X_{6a}	5.2	Acetone	101
X_{6b}	8.4	1,2-Propane diol	38
X_{6b}	10.4	Acetone	86
E_2	8.2	Acetone	97
E_2	6.0	Ethanol	102
E_2	13.0	Dimethyl sulfoxyde	97
E_2	10.8	1,2-Propane diol	101
E_2	12.6	Tris-buffer	72

The polysomal standard system for amino acid incorporation was described in table 1. Each experiment was compared to its own control which contained the same amount of the solvent used to solubilize aflatoxin derivatives. Results are means of 6 determinations.

* The amount of an aflatoxin-derived compound was estimated by fluorodensitometric measurement using the specific coefficient of aflatoxin B_1.

fraction using arbitrarily the specific coefficient of aflatoxin B_1. This fraction actually contains several compounds; the relative proportion of these compounds varies for the successive batches depending on factors such as the enzymic activity of the microsomes, efficiency of aflatoxin conversion, sensitivity of the molecules to light, stability of the active metabolite in various solvents, etc... These discrepancies will disappear when the activity of the preparations can be expressed in terms of concentration in the true active component (see below). Whatever the variability observed with successive preparations, it must be pointed out that conversion of aflatoxin B_1 is strictly dependent on the presence of microsomes and NADPH (table 3).

The foregoing results clearly demonstrate that aflatoxin B_1-derived metabolites inhibit the incorporation of amino acids by normal polysomes, whereas aflatoxin B_1 is completely inactive. These findings support the hypothesis of a direct and specific action of the drug on protein biosynthesis apart from its effect via the impairment of the transcriptional process. Thus, they may account for the early and strong inhibition observed *in vivo* [3]. Further experiments will attempt to determine the step of the translational process which is affected by the reactive metabolite.

The present results, however, raise several questions. First, it would be of interest to know the structure of the true active metabolite. As of now, we know only what it is not. During *in vitro* conversion of aflatoxin B_1, the formation of 4-hydroxylated derivative (i.e. aflatoxin M_1) predominates; this molecule has no action on *in vitro* amino acid incorporation (table 1). Fluorodensitometric scanning of thin-layer chromatography plates reveals, in addition to residual aflatoxin B_1 and aflatoxin M_1, few minor spots of unknown compounds (A to E in fig. 1). The identification of the ultimate active compound will allow us to determine the absolute concentration of the preparations and to reduce the differences in the activity of successive batches as expressed in table 2. Some preliminary results have been obtained concerning the molecular structure of the active derivative(s). Fractionation of the total chloroform-soluble extract on

a silica gel column in a chloroform–ethanol system (98.5:1.5) shows that the active component is eluted tardily, which indicates an increase in the hydrophilic affinity of the molecule. Moreover, the extent of *in vitro* protein synthesis inhibition depends on the solvent used to solubilize the residue obtained after chloroform evaporation (see Materials and methods). The aflatoxin derivatives soluble in Tris-buffer are more potent inhibitors than those extracted by acetone or ethanol (table 4). These results suggest that the active compounds are more polar than aflatoxin B_1. A similar conclusion may be deduced from Garner's paper concerning the toxicity of aflatoxin B_1 derivatives on *S. typhimurium* TA 1530 [11]. Obtention of the ultimate reactive form of drugs and carcinogens frequently requires a hydroxylation reaction of the initial compound [8, 9]; sometimes an intermediate step involves synthesis of epoxides which are very highly reactive molecules [12]. Thus, activation of aflatoxin B_1 may be thought to proceed through a similar mechanism.

A second question concerns the extent to which the aflatoxin metabolites that inhibit the *in vitro* translational process, coincide with those impairing *in vivo* protein synthesis. There is as yet no answer to this question, owing to the difficulty of detecting this product in isolated polysomes. However, experiments in progress in our laboratory may soon provide information on this problem.

And finally, is the derivative that inhibits *in vitro* translation identical to that which blocks *in vitro* transcription [13]. Preliminary results obtained by comparing the inhibitory activity of different batches of aflatoxin-derived metabolites are not as yet conclusive (results to be published). Further experiments with purified preparations are needed to solve this problem.

The results clearly demonstrate the inhibitory action of aflatoxin B_1 derivatives *in vitro* on polysomal systems. Furthermore, we have evidence demonstrating *in vivo* defects in translational activity of polysomal after aflatoxin B_1 dosing (unpublished reports). Both findings support the hypothesis of a direct action of aflatoxin metabolite(s) on polysomes. Further experiments are now in progress to determine the structure of the active compound and to define the site which is impaired in the translational machinery.

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