CHEMICAL EVIDENCE FOR THE FORMATION OF A REACTIVE AFLATOXIN B_1 METABOLITE, BY HAMSTER LIVER MICROSOMES

R. C. GARNER

Department of Experimental Pathology and Cancer Research, 171 Woodhouse Lane, Leeds LS2 3AR, Yorkshire, England

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1. Introduction

Aflatoxin B_1 is the most potent liver carcinogen known for the rat [1] and is toxic for a wide species range [2]. This has led to the suggestion that this compound does not require metabolic activation to induce cancer, it being carcinogenic per se. There is, however, a considerable body of evidence, such as organ specificity [3], the effect of hypophysectomy [4], binding of ^{3}H-labelled material to cellular macromolecules [5] and species differences [2], to indicate that metabolic activation is a necessary prerequisite for tumour initiation. Furthermore, it is now thought that most chemical carcinogens are reactive electrophilic molecules or must be metabolically converted to such before they can induce cancer [6]. Aflatoxin B_1 does not fit this criterion of extreme reactivity, it only showing a weak association with DNA [7, 8].

A number of metabolites of aflatoxin B_1 have been identified [9–11], but none of these are as active as the parent compound in various biological tests for measuring aflatoxin B_1 toxicity [12–14]. Previous work has provided evidence for a new liver microsomal metabolite of aflatoxin B_1 which is both toxic and mutagenic to bacteria [15, 16] and which reacts covalently with nucleic acids and protein [17]. It was proposed on the basis of structure–activity relationships [15] that this metabolite may be 2,3-epoxyaflatoxin B_1; a possible ultimate carcinogenic form of this compound. This report provides strong evidence for the formation of such an intermediate.

2. Materials and methods

Six replicate flasks, each containing hamster liver microsomes equivalent to 500 mg of fresh liver, 0.7 μmoles aflatoxin B_1 and the necessary cofactors, were incubated together at 37°C for 60 min as previously described [17]. At the end of the incubation period, flasks' contents were combined and the microsomes removed by ultracentrifugation at 100 000 g (av) for 45 min. The supernatant liquid was decanted and extracted 3 times with an equal volume of $CHCl_3$. After solvent extraction the aqueous layer was freeze-dried, the residue redissolved in methanol and purified by preparative thin layer chromatography on 2 mm silica gel layers in $CHCl_3$ methanol (70 : 30). The two main fluorescent bands with R_f values 0.58 and 0.32 were removed and eluted with methanol.

2,3-Dihydrodiolaflatoxin B_1 was prepared as follows: 10 mg aflatoxin B_1 were reacted for 72 hr at room temperature with a 5 M excess of *m*-chloroperbenzoic acid in dichloromethane. The reaction mixture was purified by preparative thin layer chromatography in $CHCl_3$/methanol (97 :3) and the main fluorescent band removed (R_f = 0.45, R_f aflatoxin B_1 = 0.85) and eluted with methanol. Mass spectrum m/e = 484 and 486 (m^+) (3 : 1 ratio of peak heights, indicating the presence of the two chlorine isotopes), 328 ($m^+ - ClC_6H_4COO^-$). Infa-red spectrum (KBr) showed a similar spectrum to aflatoxin B_1 but with new bands at 3400 (–OH), 1732 (chlorobenzoic

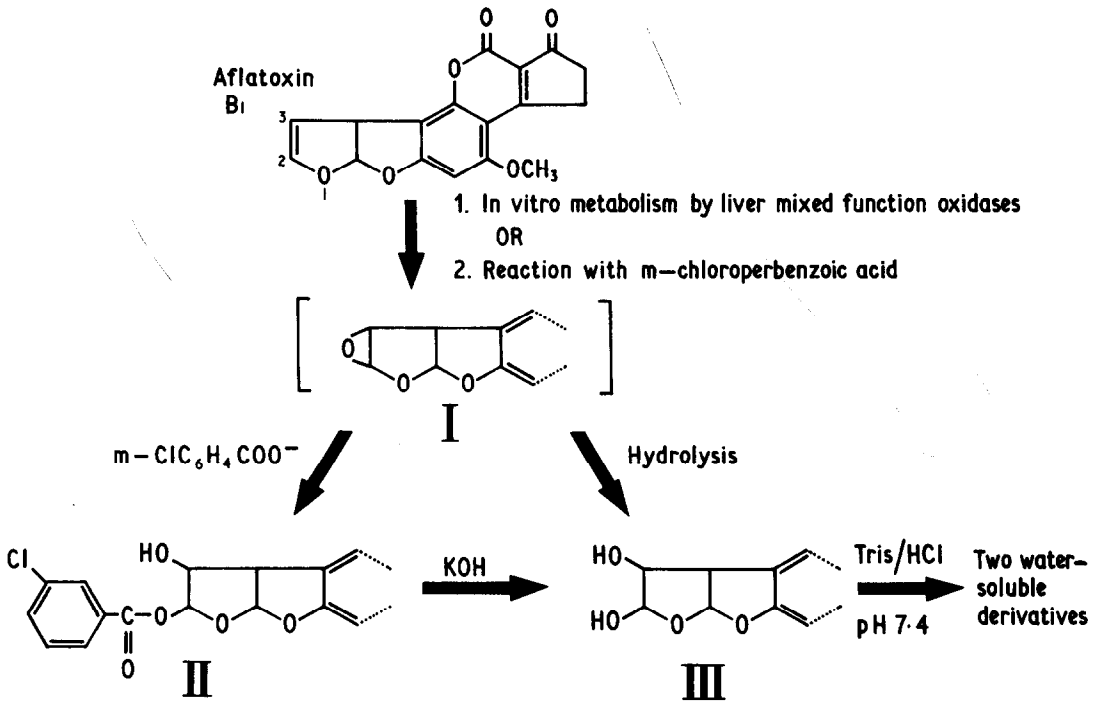

Fig. 1. Enzymatic or chemical reaction sequence for production of the two water-soluble aflatoxin B_1 derivatives.

ester) and 1670 cm^{-1}(phenyl ketone). Ultraviolet spectrum in methanol showed absorption bands at 265 and 367 nm. On additon of alkali the 367 nm band was lost and a new band at 403 nm appeared while the 265 nm absorption moved up to 288 nm. These spectral shifts were reversible on neutralisation. On the basis of these physical characteristics and because *m*-chloroperbenzoic acid will only attack olefinic double bonds, the reaction product was assigned structure **II** (fig. 1). On hydrolysis of **II** at 0°C with 1 M equivalent of methanolic KOH, there was a slow conversion of **II** to a new compound which had a much lower R_f value on thin layer chromatography in $CHCl_3$/methanol (97 : 3). After purification by preparative thin layer chromatography in this solvent system this new product was found to have an identical ultraviolet absorption spectrum to a sample of aflatoxin B_{2a} prepared by the method of Pohland et al.[18] in methanol and alkaline methanol. However the compound was more polar than aflatoxin B_{2a}(R_f in $CHCl_3$/methanol (97 : 3) = 0.06; aflatoxin B_{2a}=0.15; aflatoxin B_1= 0.65). The structure of this compound was adjudged to be that of **III**, 2,3-dihydrodiolaflatoxin B_1.

3. Results and discussion

The 2,3-double bond of aflatoxin B_1 is olefinic in character and is therefore susceptible to peracid attack. During attempts to synthesize the 2,3-epoxide of aflatoxin B_1 using *m*-chloroperbenzoic acid it was noticed that one of the reaction products always predominated with varying molar proportions of acid to aflatoxin, different reaction temperatures or the presence or absence of buffer. On the basis of the physical data set out in Materials and methods, structure **II** was assigned to this product, which could only have been formed by initial formation of 2,3-epoxyaflatoxin B_1 and subsequent attack by *m*-chlorobenzoic acid, as set out in fig. 1. The difficulties in epoxidising vinyl ethers are discussed in ref. [19].

If during the metabolism of aflatoxin B_1 by liver microsomes, a reactive epoxide is formed, one would expect some conversion of this metabolite by hydrolysis to a dihydrodiol. An analogous process has been demonstrated for some epoxides of carcinogenic polycyclic hydrocarbons [20]. 2,3-Dihydrodiolaflatoxin B_1 (**III**) was prepared chemically by alkaline hydrolysis of **II** and its thin layer properties compared

Table 1
Thin layer and spectral properties of the two water-soluble metabolites of aflatoxin B_1.

Metabolite	R_f value			Maximum absorption (nm)
	A	B	C *	
1	0.00	0.58	0.65	402
2	0.00	0.32	0.54	407
Esterified 1	0.65			
Esterified 2	0.24			

Solvents, A = $CHCl_3$/methanol (97 : 3); B = $CHCl_3$/methanol (70 : 30); C = ethyl acetate/methyl ethyl ketone/formic acid/water (5 : 3 : 1 : 1).

with the fluorescent $CHCl_3$ extractable metabolites obtained after a large scale incubation of aflatoxin B_1 with hamster liver microsomes. Microsomes from this species were used because they have high activity in converting aflatoxin B_1 to a reactive form [15]. No equivalent material to the dihydrodiol could be found after aflatoxin B_1 metabolism. However, it was noticed during studies on the metabolism of ^{14}C-ring labelled aflatoxin B_1 by hamster liver microsomes (R.C. Garner, unpublished) that considerable amounts of radioactivity were not $CHCl_3$-extractable from the aqueous incubation medium. On examining the aqueous layer from the large scale incubation of aflatoxin B_1, after solvent extraction, some blue fluorescent metabolites were found which remained at the origin in the standard thin layer chromatographic procedures for separation of aflatoxin B_1 metabolites (table 1).

These water-soluble metabolites were considerably more polar than the dihydrodiol and any other known metabolites of aflatoxin B_1, and might have been formed by further metabolism of the dihydrodiol. This was tested by incubating 100 μg of dihydrodiol with 500 mg fresh liver equivalents of hamster liver microsomes for 60 min at 37°C and extracting any metabolites formed as described in the Materials and methods. Two water-soluble blue fluorescent products were obtained which had identical thin layer and spectral properties to the two water-soluble metabolites found after the large scale incubation of aflatoxin B_1 (table 1). The conversion of the dihydrodiol to these two products was non-enzymatic, since they were obtained even if microsomes were omitted from the incubation medium. Acetyl esters of the two water-soluble aflatoxin B_1 metabolites, obtained by acetic anhydride esterification had identical thin layer properties to the acetyl esters of the dihydrodiol degradation products.

Although these two water-soluble products have as yet not been characterised, because of insufficient material, the fact that they show identical physical characteristics in the properties tested, suggests that the two dihydrodiol degradation products are identical to the two water-soluble aflatoxin B_1 metabolites. This indicates that during the metabolism of aflatoxin by hamster liver microsomes there may be some conversion to the 2,3-epoxide some of which then undergoes hydrolysis to the dihydrodiol and further conversion to the two water-soluble products (see fig. 1).

Evidence has thus been provided for the probable formation of 2,3-epoxyaflatoxin B_1 as an intermediate during aflatoxin B_1 metabolism by hamster liver microsomes. It is likely that it is this metabolite which is lethal and mutagenic to bacteria [15, 16], reacts with nucleic acids [17] and is the ultimate carcinogenic form of this compound. Species differences in sensitivity to aflatoxin B_1 carcinogenicity may well be related to the balance between metabolism via the epoxide pathway and metabolism through the other known pathway i.e. activation versus detoxification.

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References

[1] Wogan, G.N. and Newberne, P.M. (1967) Cancer Res. 27, 2370–2376.
[2] Newberne, P.M. and Butler, W.H. (1969) Cancer Res. 29, 236–250.
[3] Wogan, G.N., Edwards, G.S. and Newberne, P.M. (1971) Cancer Res. 31, 1936–1942.
[4] Goodall, C.M. and Butler, W.H. (1969) Intern. J. Cancer 4, 422–429.

[5] Lijinsky, W., Lee, K.Y. and Gallagher, C.H. (1970) Cancer Res. 30, 2280–2283.
[6] Miller, J.A. (1970) Cancer Res. 30, 559–576.
[7] Clifford, J.I. and Rees, K.R. (1967) Biochem. J. 103, 467–471.
[8] Sporn, M.B., Dingman, C.W., Phelps, H.L. and Wogan, G.N. (1966) Science 151, 1539–1541.
[9] Masri, M.S., Lundin, R.E., Page, J.R. and Garcia, V.C. (1967) Nature 215, 753–755.
[10] Patterson, D.S.P. and Roberts, B.A. (1970) Food Cosmet. Toxicol 8, 527–538.
[11] Dalezios, J., Wogan, G.N. and Weinreb, S.M. (1971) Science 171, 584–585.
[12] Pong, R.S. and Wogan, G.N. (1971) J. Natl. Cancer Inst. 47, 585–592.
[13] Lillehoj, E.B. and Ciegler, A. (1969) Appl. Microbiol. 17, 516–519.
[14] Stoloff, L., Verrett, M.J., Dantzman, J. and Reynaldo E.F. (1972) Toxicol. Appl. Pharmacol. 23, 528–531.
[15] Garner, R.C., Miller, E.C. and Miller, J.A. (1972) Cancer Res. 32, 2058–2066.
[16] Garner, R.C. and Wright, C.M., Brit. J. Cancer, in press.
[17] Garner, R.C. (1973) Chem–Biol. Interactions 6, 125–129.
[18] Pohland, A.E., Cushmac, M.E. and Andrellos, P.J. (1968) J. Assoc. Offic. Anal. Chem. 51, 907–910.
[19] Stevens, C. and Tazuna, J. (1954) J. Am. Chem. Soc. 76, 715–720.
[20] Sims, P. (1973) Biochem. J. 131, 405–413.