THE FORMATION OF 2,3-DIHYDRO-2,3-DIHYDROXY AFLATOXIN B_1 BY THE METABOLISM OF AFLATOXIN B_1 IN VITRO BY RAT LIVER MICROSOMES

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1. Introduction

The theory has been advanced that aflatoxin B_1 (AFB_1) is activated by microsomal systems to form aflatoxin B_1 2,3-oxide [1,2]. This reactive metabolite has not been isolated and might be expected to be rapidly hydrolysed, either enzymically or nonenzymically, to form 2,3-dihydro-2,3-dihydroxy aflatoxin B_1 (AFB_1-dhd). However, it has been reported that only very low levels of AFB_1-dhd are formed by metabolism of AFB_1 in rat liver microsomal incubations at pH 6.5 (2.9% conversion of AFB_1 using microsomes from phenobarbitone pre-treated rats and 0.6% using control microsomes) and that even smaller amounts of AFB_1-dhd are formed at physiological pH [3]. The latter finding was ascribed to the destruction of AFB_1-dhd at neutral pH. We here present evidence that AFB_1-dhd is a major metabolite of AFB_1 produced by rat liver microsomes at physiological pH but it is removed by binding to microsomal protein. This binding does not occur at pH 6.5. Amines including the buffer tris-hydroxymethyl amino methane (Tris) and both oxidised and reduced glutathione can compete with microsomal protein for binding AFB_1-dhd. Changes in the ultraviolet absorption spectrum of AFB_1-dhd indicating the formation of a dialdehydic phenolate ion correlate with the binding to amines and microsomal proteins at neutral pH and indicate that binding proceeds via the Schiff's base reaction previously suggested for the binding of the hemiacetal aflatoxin B_{2a}.

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2. Materials and methods

2.1. *Chemicals*

AFB_1 was obtained from Makor Chemical Co., Jerusalem. G-[^{3}H]AFB_1 spec. act. 45 Ci/mmol was obtained from Moravek Biochemicals Ltd, City of Industry, CA. AFB_1-dhd was prepared by reaction of AFB_1 with osmium tetroxide [5], [^{3}H]AFB_1-dhd was prepared by the same method using [^{3}H]AFB_1 as the starting material. The preparation of other aflatoxins was as in [6].

Tris, glycine, and oxidised and reduced glutathione were AR grade obtained from Sigma (London) Chemical Co., Ltd, Poole, Dorset. Other chemicals and solvents used were supplied by the sources in [6].

2.2. *AFB_1 metabolism*

Microsomes, prepared from the livers of adult male F344/TIF Lac (Fischer) rats given 0.1% phenobarbitone in the drinking water for 3–8 days, were incubated with AFB_1 and cofactors at 37°C in O_2 with shaking [6]. The buffer used was either phosphate or Tris–HCl (both were 80 mM, pH 7.4). All procedures were carried out in subdued light. Metabolism was stopped after 30 min by the addition of 2 ml ice-cold methanol. After 30 min in ice the incubate was centrifuged at 2000 rev./min and 4°C. The pellet was washed with 2 ml ice-cold methanol and centrifuged. The combined supernatants were blown to dryness at 50°C under a stream of O_2-free N_2. The residue was dissolved in 2 ml of methanol/water (1:1, v/v), centrifuged for 30 min at 2000 rev./min and −10°C to clarify and used for HPLC analysis.

2.3. *High-performance liquid chromatography (HPLC)*

A Du Pont model 830 liquid chromatograph, 833

flow controller, 834 automatic sampler (fitted with a 100 μl injection loop) and 837 variable wavelength spectrophotometer (operated at 365 nm) were used. The fluorescence of the column eluates was monitored using a Kontron Spectrofluorometer SFM 23 fitted with an 8 μl flow cell. The column used was a Partisil-10-ODS (250 × 4.6 mm) obtained from Whatman LabSales Ltd (Maidstone) Kent, and results were displayed on a Hewlett-Packard model 3380S recording integrator. Elution of AFB_1 and its metabolites was achieved using 15% dimethylformamide in 0.01% phosphoric acid at 1.5 ml min^{-1} and a column temperature of 69°C [7].

2.4. *Thin-layer chromatography (TLC)*

Silica gel G thin-layer plates (0.25 mm thick) were obtained from Anachem Ltd, Luton, Bedfordshire and were used without activation. Plates were developed in either chloroform:acetone (9:1, v/v) or chloroform:methanol (9:1, v/v) and spots visualized by their fluorescence under long wavelength ultraviolet light.

3. Results and discussion

When aflatoxin B_1 was metabolised by rat liver microsomes in either phosphate or Tris buffer and the metabolites subjected to HPLC analysis and the ultraviolet absorption of the eluate monitored, the chromatograms shown in fig.1a,b were obtained. The only significant difference between these chromatograms is that a major product having a retention time of ~6 min was present in the Tris incubations but was absent from the phosphate incubations. This compound had previously been identified as a degradation product (Tris-diol) obtained when authentic AFB_1-dhd was treated with neutral Tris buffer [6]. The absence of AFB_1-dhd from the phosphate incubations was confirmed by the parallel fluorescence monitoring of the HPLC eluate (excitation (Ex) 372 nm; emission (Em) 446 nm) (fig.1c,d). AFB_1-dhd and its Tris degradation product are more highly fluorescent than aflatoxin B_1 or the other aflatoxin metabolites detected in these studies (fig.1a,c). Despite the absence of the Tris-diol peak when AFB_1 was incubated in phosphate buffer, the overall amounts of AFB_1 remaining unmetabolised and the rates of formation

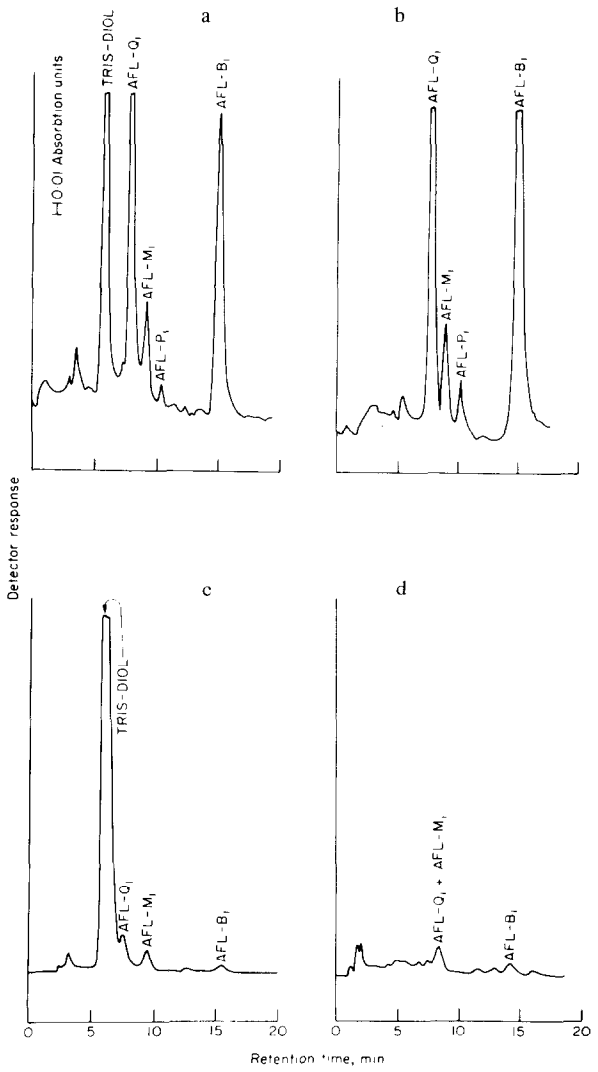

Fig.1. HPLC separations of microsomal metabolites of AFB_1. Incubations, preparations of samples and HPLC carried out as detailed in section 2. (a) Incubations carried out in presence of Tris buffer. Ultraviolet absorption of HPLC effluent monitored at 365 nm. (b) Incubations carried out in the presence of phosphate buffer. Ultraviolet absorption monitored at 365 nm. (c) Incubation carried out in presence of Tris buffer. Fluorescence of HPLC effluent monitored at Ex 372 nm Em 446 nm. (d) Incubation carried out in presence of phosphate buffer. Fluorescence monitored at Ex 372 nm Em 446 nm. Fluorescence measurements at Ex 372 nm Em 446 nm optimal for AFB_1-dhd and Tris-diol. Optima for aflatoxins B_1, M_1 and Q_1 Ex 365 Em 425, but relative fluorescence of these aflatoxin metabolites at optimal settings essentially as in (c).

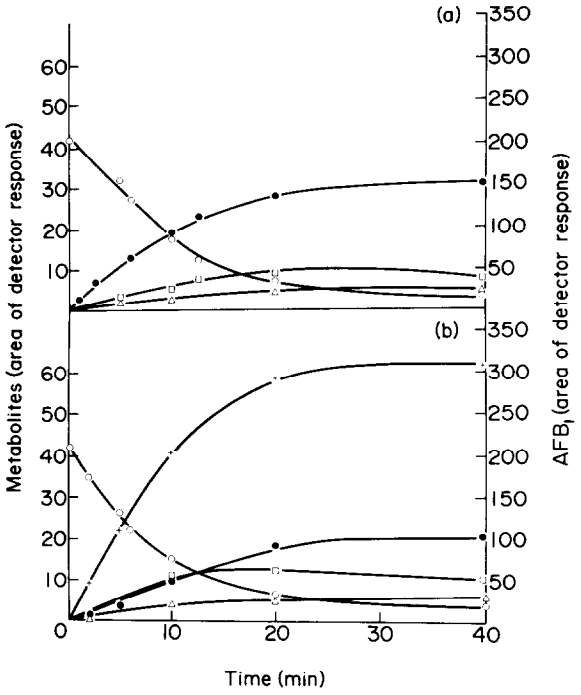

Fig.2. Rate of microsomal metabolism of AFB_1 and production of metabolites in phosphate and Tris buffer. Incubations, preparation of samples and HPLC carried out as detailed in section 2. (a) Phosphate buffer. (b) Tris–HCL buffer. (○) AFB_1; (+) Tris-diol; (●) aflatoxin Q_1; (□) aflatoxin M_1; (△) aflatoxin P_1.

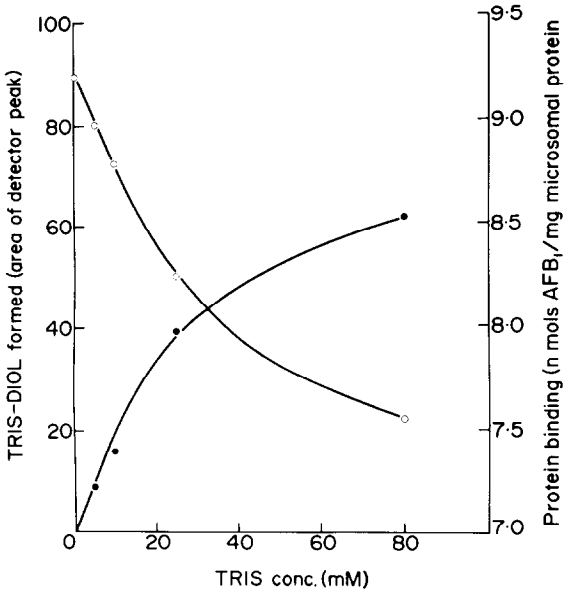

Fig.3. Relationship between Tris-diol formation and protein binding of metabolised AFB_1. Incubations, preparation of samples, HPLC separations and estimation of protein binding as detailed in section 2. (●) Tris-diol formed; (○) AFB_1 bound.

of the other metabolites were similar using the two buffer systems (fig.2). Paralleling the absence of Tris-diol metabolite from the phosphate incubations there was an increased binding of $[^3H]AFB_1$ to the microsomes. That the binding was to microsomal protein was confirmed in some experiments by phenol extraction followed by precipitation of the protein with acetone. Figure 3 shows the inverse relationship between the binding to microsomal protein and the amount of Tris-diol metabolite formed over a range of Tris concentrations.

Authentic AFB_1-dhd was found to react with certain compounds having an amino group (Tris and iso propylamine) while a related compound not having an amino group (1,3-propane diol) showed no reaction. Some compounds containing amino-groups, e.g., guanine guanosine and guanylic acid showed no reaction, presumably the pK of the amino group is of importance in determining the possibility of reaction at the pH used. The dependance of both the reaction of AFB_1-dhd with reduced glutathione and the change in ultraviolet absorption spectrum of AFB_1-dhd with pH is shown in fig.4. Oxidised glutathione yielded similar results. The binding of $[^3H]AFB_1$-dhd to microsomal protein was similarly dependent on pH (fig.5).

The results presented here are probably due to the pH-dependent formation of a dialdehydic phenolate ion from AFB_1-dhd which then undergoes Schiff's base reaction with amino compounds (fig.4). It has been suggested that aflatoxin B_{2a} and AFB_1-dhd undergo this pH-dependent rearrangement [4,5]. This hypothesis would be supported by the dependence on pH of binding and the bathochromic shift in ultraviolet absorption spectrum reported in the present study. In the absence of acceptor amine, it is possible to reform the AFB_1-dhd from the dialdehydic phenolate ion by lowering the pH, after reaction with acceptor amine this reversal is not possible (G.E.N., P.J.C., unpublished results). It is probable therefore that the

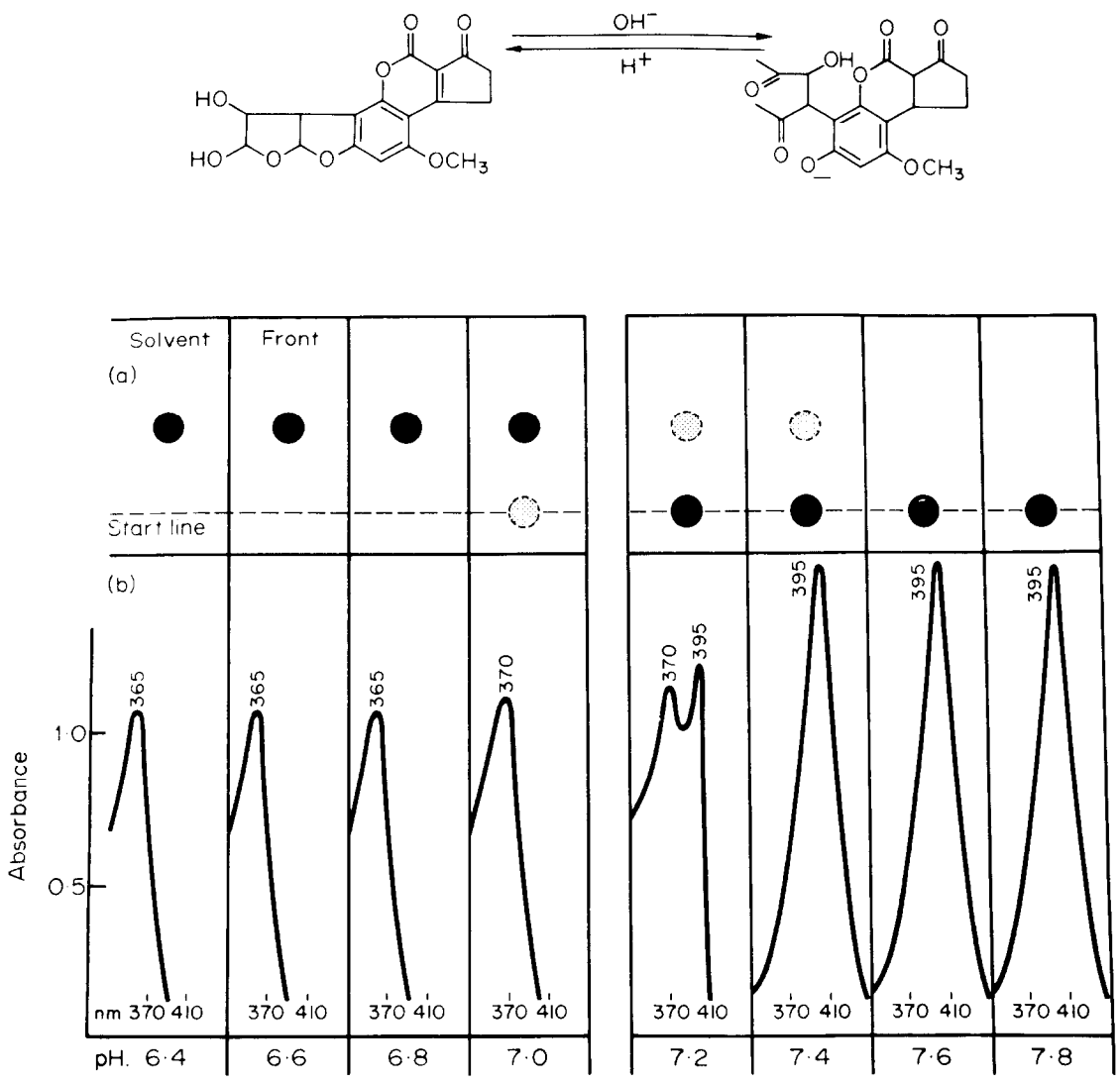

Fig.4. Reaction of AFB_1-dhd with reduced glutathione. (a) TLC of AFB_1-dhd after reaction with reduced glutathione at various pH values. Spots represent areas fluorescent under ultraviolet light. Developing solvent chloroform/methanol 1:1 v/v. (b) Ultraviolet absorption spectra of AFB_1-dhd at several pH values. Suggested pH-dependent molecular rearrangements are also indicated.

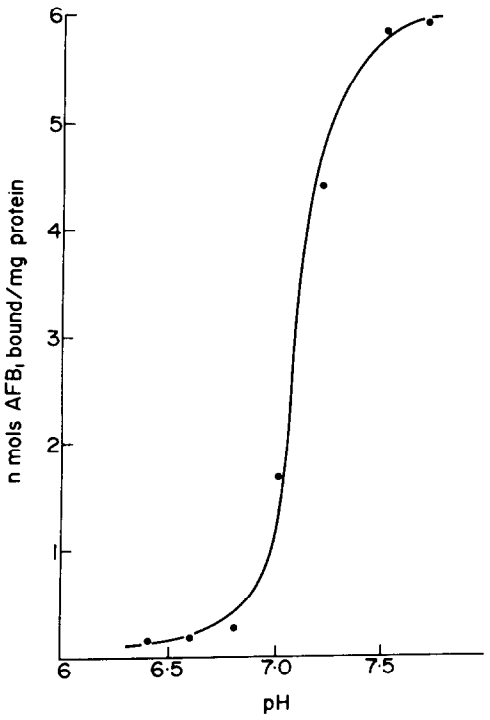

Fig.5. Effect of pH on the binding of AFB_1-dhd to microsomal protein. [^{3}H]AFB_1-dhd 12.5 nmol (10^4 dpm) and 200 μl microsomal suspension in 180 mM KCl (2.1 mg protein) added to 1 ml phosphate buffer at room temperature. After 1 h, diluted 10 × with distilled water and centrifuged at 100 000 × g for 1 h. After washing, pellet dissolved in 2 ml 1 M NaoH at 60°C. Aliquots taken for determination of radioactivity.

product of reaction between Tris and dialdehydic phenolate ion form of AFB_1-dhd is a Schiff's base adduct of these compounds, containing 1 or possibly 2 molecules of Tris per molecule of AFB_1-dhd. The results of this study would indicate a need for caution in ascribing the formation of adducts of metabolites of xenobiotic compounds with reduced glutathione as being solely due to a reaction of an electrophilic metabolite of the xenobiotic compound (epoxide?) with the nucleophilic reduced glutathione. Also care should be exercised when using Tris buffers in such systems.

It is interesting that the reaction of AFB_1-dhd with microsomal protein does not appear to inhibit the mixed function oxidase system. This is shown by the similarities of overall metabolism in phosphate and Tris buffers. Also in other experiments (G.E.N., P.J.C., unpublished) we have found that if microsomal metabolism of AFB_1 is allowed to proceed in duplicate incubations in phosphate buffer one in the presence and the other in the absence of Tris for 15 min, and that Tris is then added to the incubation from which it was previously omitted, the subsequent rate of production of Tris-diol is the same in both incubations.

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