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SHORT COMMUNICATIONS

Aflatoxin B_1 and phenobarbital inducible aflatoxin-Δ^2-hydration by rat liver microsomes

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THE ISOLATION of aflatoxins B_{2a} and G_{2a} from cultures of *Aspergillus flavus* as well as the structural elucidation and toxicological properties of these aflatoxins were described by Dutton and Heathcote.[1,2] The chemical structures of aflatoxins B_1 and B_{2a} are given in Fig. 1. The enhanced formation of aflatoxins B_{2a} and G_{2a} from aflatoxins B_1 and G_1, respectively, in the presence of rat liver microsomes was observed while studying the 4-hydroxylation of aflatoxins B_1 and B_2[3] and it was decided that these observations merit further investigation. The administration of phenobarbital and aflatoxin B_1 to rats and fractionation of rat livers were performed as described elsewhere.[3]

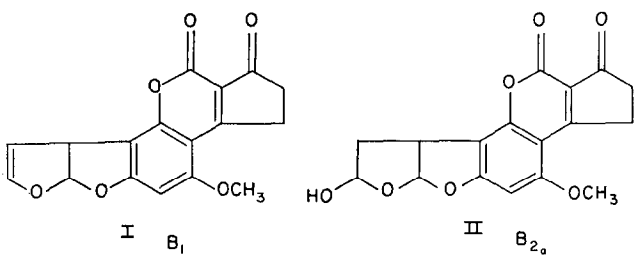

FIG. 1. Chemical structures of aflatoxins B_1 (I) and B_{2a} (II), a 2-hydroxy derivative of aflatoxin B_2.

Incubation mixtures for the determination of aflatoxin-Δ^2-hydrating activity contained 64·3 μM aflatoxin B_1 or G_1 in a final methanol concentration of 8% (v/v), 18·4 mM Tris–HCl buffer (pH 7·4); 2·8 mM $MgCl_2$, and 0·5 ml microsomal protein fraction varying in protein content in a final volume of 5·0 ml. The reaction was initiated by the addition of aflatoxin. The reaction mixtures were incubated at 37° in a waterbath equipped with a shaking device and shaken gently. Aliquots of 1 ml were taken at certain time intervals from the incubation mixtures and extracted as described for aflatoxins M_1 and M_2.[3] Aflatoxins B_{2a} and G_{2a} were identified by thin-layer chromatography by the procedure described for aflatoxins M_1 and M_2.[3] The concentrations of aflatoxins B_1, G_1, B_{2a} and G_{2a} were determined quantitatively by comparing the fluorescence in ultraviolet light of the aflatoxin spots with standards. A Photovolt model 530 densitometer for solid-state fluorescence was employed using filter number 465 (approx. 465 nm). This instrument was used to record the fluorescent peaks corresponding to the different aflatoxin spots, e.g. B_1, B_{2a}, etc. Aflatoxin M_1 and GM_1 (probably a 4-hydroxy derivative of aflatoxin G_1) was also found in these incubation mixtures as could be expected. The concentrations of the aflatoxins were determined from the areas under the peaks as described in the determination of aflatoxin-4-hydroxylation activity.[3] Protein was determined according to the method of Lowry *et al.*[5] Male Wistar rats were used in these studies. Aflatoxins B_{2a} and G_{2a} were produced from aflatoxins B_1 and G_1, respectively, by treatment with cold dilute hydrochloric acid for 30 min, followed by extraction by a chloroform–benzene mixture (1 : 1), evaporation to dryness in a rotary evaporator, separation from aflatoxins B_1 and G_2 on a thin-layer chromatoplate by the method described above, collection of the B_{2a} and G_{2a} containing bands from the chromatoplate, extraction with chloroform and evaporation to dryness. The hydration activity was found to be localized in the microsomal fractions of rat livers. Results obtained from the livers of aflatoxin B_1 and phenobarbital treated rats indicated an induction of the hydrating activity of the microsomal fractions of 4·2 and 3·7, respectively, when aflatoxin B_1 was used as substrate and 1·6 and 1·5, respectively when aflatoxin G_1 was used as substrate (Table 1). A linear relationship between protein concentration and the aflatoxin B_1-Δ^2-hydrating activity of rat liver microsomes was found. A pH optimum of about 7·0 was found for this reaction. No cofactor requirements have been observed. It was evident that after 7 hr of incubation, aflatoxins B_{2a} and G_{2a} concentrations reached a maximum (aflatoxins B_1 and G_1 used as substrates) and then decreased (Fig. 2). According to these results there

TABLE 1. INDUCTION OF AFLATOXIN-Δ^2-HYDRATASE ACTIVITY BY PHENOBARBITAL AND AFLATOXIN B_1*

Inducer	Activity 3 different rats ($\mu\mu$moles/min/mg protein) with substrates						Average activity ($\mu\mu$moles/min/mg protein) with substrates	
	Aflatoxin B_1			Aflatoxin G_1			Aflatoxin B_1	Aflatoxin G_1
Phenobarbital	17·5	14·3	16·1	4·6	4·3	4·2	15·9	4·4
Aflatoxin B_1	13·5	12·8	15·5	4·2	4·1	3·9	13·9	4·1
No inducer	3·6	4·1	3·7	2·6	3·1	2·8	3·8	2·8
Controls†	1·8	1·6	1·7	1·4	1·1	1·2	1·7	1·2

* Two groups of three rats weighing 200 $\pm$ 5 g each were dosed intraperitoneally with 75 mg phenobarbital and 500 μg of aflatoxin B_1 per kilogram per day for 5 days, respectively. The groups not receiving any inducer were dosed intraperitoneally with a corresponding volume of the solvent, *viz.* 33·3% dimethylsulfoxide in water (0·3 ml). The assay method is described in the text.

† These are blank values determined in the absence of microsomal protein and expressed in $\mu\mu$moles/min.

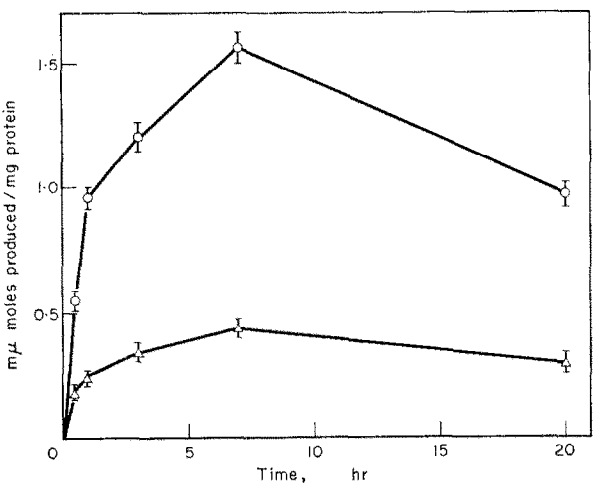

FIG. 2. Effect of incubation time on the producton of aflatoxins B_{2a} (○) and G_{2a} (△) where aflatoxins B_1 and G_1 were used as substrates, respectively. The nmoles of aflatoxin produced per mg protein indicated here, were the averages of values obtained from the liver microsomal fractions of a group of three phenobarbital treated rats and the vertical lines indicate the range in which the values fall. The assay method for the hydratase activity is given in the text. Similar patterns were obtained with aflatoxin B_1 treated and control rats.

is a possibility that aflatoxins B_{2a} and G_{2a} are intermediates in the catabolism of aflatoxins B_1 and G_1 by rat liver microsomes. As for the aflatoxins-4-hydroxylase enzyme system[3] it can be concluded that a specific induction of protein synthesis by aflatoxin B_1 occurred in view of the fact that pre-treatment of the rats with aflatoxin B_1 causes a marked increase in aflatoxin-Δ^2-hydratase activity although it is known to inhibit the induction of other enzymes.[6–9]

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REFERENCES

1. M. F. DUTTON and J. G. HEATHCOTE, *Biochem. J.* **101,** 21 (1966).
2. M. F. DUTTON and J. G. HEATHCOTE, *Chem. Ind.* 418 (1968).
3. J. C. SCHABORT and M. STEYN, *Biochem. Pharmac.* **18,** 2241 (1969).
4. I. F. H. PURCHASE and J. J. THERON, *Int. Path.* **8,** 3 (1967).
5. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
6. G. N. WOGAN and M. A. FRIEDMAN, *Fedn. Proc.* **24,** 627 (1965).
7. M. A. FRIEDMAN and G. N. WOGAN, *Fedn Proc.* **25,** 662 (1966).
8. R. S. PONG and G. N. WOGAN, *Fedn Proc.* **25,** 662 (1966).
9. H. GURTOO, J. C. CAMPBELL, R. WEBB and K. PLOWMAN, *Fedn Proc.* **27,** 551 (1968).

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An improved method for the determination of protohaem in liver microsomes

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OMURA and Sato[1,2] determined the haem content of rabbit liver microsomes and of solubilized microsomal cytochrome, using a modification of the pyridine haemochromogen procedure of Paul, Theorell and Åkeson.[3] The modification consisted of using the difference spectrum (reduced–oxidized) of the pyridine haemochromogen instead of the absolute spectrum of the reduced form. On the basis of these determinations Omura and Sato calculated the molar extinction of cytochrome P-450 to be 91 cm^{-1} mM^{-1} for the increment (450–490 nm) in the CO difference spectrum and showed that they could account for all the microsomal haem as the sum of the cytochromes b_5 and P-450. This value of 91 cm^{-1} mM^{-1} has been used to calculate the cytochrome P-450 content of many materials other than rabbit liver microsomes, apparently without independent confirmation, although the corresponding cytochrome which is induced in the livers of rats treated with 3-methylcholanthrene appears to have a significantly higher molar extinction.[4] The balance of microsomal haem against total cytochromes b_5 and P-450 does not appear to have been confirmed either; indeed Bond and De Matteis[5] reported difficulty in achieving such a balance in rat liver microsomes. Similar difficulties were experienced in some of my experiments and this communication reports the location of the source of the discrepancy and how it can be overcome.

Rabbits were killed by air embolism and rats were killed by cervical dislocation. Livers were excised, cut into 1–2 mm pieces and rinsed three times with ice-cold 0·154 M KCl to remove as much blood as possible. The washed liver was homogenized with 0·154 M KCl (3 ml per gram liver) in a chilled homogenizer with Teflon pestle rotating at 600 rpm in a smooth-surfaced glass tube with 0·25 mm clearance. The use of KCl rather than sucrose in the homogenization medium minimizes the adsorption of haemoglobin.[6] The homogenate was centrifuged in a refrigerated centrifuge for 10 min at 10,000 *g* and the supernatant was centrifuged again for 1 hr at 100,000 *g*. The pellet was resuspended in 0·154 M KCl and centrifuged again for 1 hr at 100,000 *g*. The washed pellet was resuspended in 0·05 M potassium phosphate pH 7·4 to a volume of 1 ml for each gram of liver. An equal volume of 40% v/v glycerol in 0·05 M potassium phosphate pH 7·4 was then added to stabilize the cytochrome P-450.[7] This suspension of microsomes was stored at −20°.

The assays were carried out as described by Omura and Sato,[1] with modifications as described in the text where applicable. Spectra were traced with a Unicam SP800 recording spectrophotometer fitted with the SP850 scale expansion accessory, using cells of 1 cm light path.

Control experiments showed that the addition of glycerol and storage at −20° did not affect the results obtained.

The microsomes used in this investigation were effectively free from contamination with haemoglobin, as shown by the absence of a peak at 420 nm in the CO difference spectrum of reduced microsomes (Fig. 1).