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URINARY EXCRETION OF THE MYCOTOXIN, STERIGMATOCYSTIN BY VERVET MONKEYS

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Abstract—The major urinary metabolite of sterigmatocystin was isolated and identified after oral administration of ^{14}C-labelled sterigmatocystin to vervet monkeys. More than 50 per cent of the radioactivity recovered in the urine was found to be present in the form of a glucuronic acid conjugate of sterigmatocystin.

THE MYCOTOXINS sterigmatocystin and aflatoxin have been described to be of special significance from the point of view of their possible implication in the etiology of liver cancer in Africa.[1] Sterigmatocystin has a chemical structure which resembles that of aflatoxin (Fig. 1) and is produced by several species of fungi.[2] This fungal metabolite has been shown to produce hepatocellular tumours in rats after subcutaneous[3] and oral[4] administration while non-metastasizing hepatomas have been induced in a non-human primate.[5] The possible importance of sterigmatocystin in the etiology of liver cancer is stressed by the fact that it is produced in relatively larger quantities than aflatoxin under laboratory conditions on both synthetic media and on natural products.*

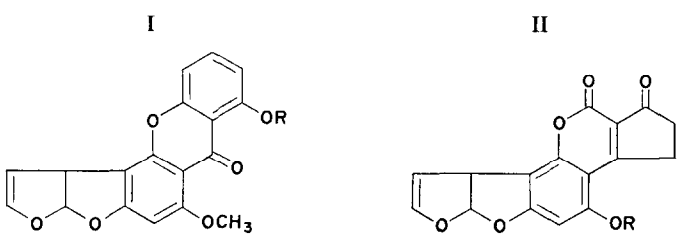

FIG. 1. Chemical structures of sterigmatocystin, aflatoxin and their derivatives. (I) Sterigmatocystin: R=H. Sterigmatocystin glucuronide: R=β-D-glucosiduronyl. (II) Aflatoxin B_1: R=CH_3. Aflatoxin P_1 glucuronide: R=β-D-glucosiduronyl.

To evaluate the toxic and carcinogenic effects of sterigmatocystin on different animals species, a knowledge of the metabolism of sterigmatocystin by the different species is important. This information will enable comparisons of the susceptibility of different species to the toxic and carcinogenic effects on the basis of differing modes and rates of detoxification. Furthermore, a knowledge of the metabolic fate of sterigmatocystin in different animal species will contribute towards a more realistic basis for the assessment of the possible effects on humans.

* C. J RABIE, unpublished data.

It was thus of interest to investigate the urinary excretion of sterigmatocystin by different animal species. Results of such a study on male vervet monkeys are reported here.

MATERIALS

Sterigmatocystin. The fungus, *Aspergillus versicolor* (Vuill.) Tiraboschi was cultured on sterile maize under conditions yielding maximal sterigmatocystin production. The material was dried overnight at 40° in a circulation oven, milled and extracted with an azeotropic mixture of chloroform and methanol. The crude extract was purified on a formamide impregnated cellulose column, eluting the column with a hexane to benzene gradient. The sterigmatocystin was further purified from the appropriate fractions by an initial crystallization from methanol–chloroform (50:50) and subsequent recrystallizations from acetone to an eventual purity exceeding 99·5 per cent based on thin layer chromatography (t.l.c.) and u.v. spectroscopy. This isolation procedure was initially referred to by Holzapfel *et al.*[2] and will be published in more detail elsewhere.

^{14}C-Labelled sterigmatocystin. ^{14}C-Labelled sterigmatocystin was produced by adding acetic acid-1-^{14}C (60 mCi/m-mole) to an actively growing liquid culture of the fungus, *Aspergillus versicolor*. The ^{14}C-labelled sterigmatocystin was extracted and purified by column chromatography and recrystallization as described for the isolation of unlabelled sterigmatocystin. No contaminants could be detected by t.l.c. while the specific activity of the ^{14}C-labelled sterigmatocystin was $3{\cdot}23 \times 10^6$ dpm/mg.

Animals. (1) Unlabelled sterigmatocystin was administered to four male vervet monkeys with respective body weights of 2·31, 3·55, 3·65 and 5·25 kg. (2) Labelled sterigmatocystin was administered to a male vervet monkey (5·43 kg).

METHODS AND RESULTS

Determination of radioactivity. Radioactivity was determined by liquid scintillation counting on a Beckman Liquid Scintillation System using Bray's liquid scintillant[6] and corrected by means of an internal standard of ^{14}C-*n*-hexadecane (Radiochemical Centre, Amersham, England).

Dosage of unlabelled sterigmatocystin. Unlabelled sterigmatocystin was administered to four male vervet monkeys in their food at a dose of 14 mg/kg body weight. The monkeys were placed in metabolism cages and supplied with water and food. Urine was collected in a dark bottle kept in an ice bath for a period of 40 hr after dosage. To obtain sufficient urine metabolites of sterigmatocystin the same vervet monkeys were used repeatedly at intervals longer than 1 week. In this way a total of 720 mg sterigmatocystin was administered to the four male vervet monkeys while the urine excreted in the 40 hr periods following dosage were collected.

Dosage of ^{14}C-labelled sterigmatocystin. ^{14}C-Labelled sterigmatocystin (100 mg, 0·145 mCi) was given *per os* to a male vervet monkey (5·43 kg). Urine was collected in a dark bottle in an ice bath for a period of 40 hr after dosage.

Isolation of conjugates of sterigmatocystin from urine. The urine (510 ml) from the monkey that received ^{14}C-labelled sterigmatocystin was filtered through Whatman No. 1 filter paper and percolated through a column (2·6 × 40 cm) of washed Amberlite XAD-2 resin which is known to absorb conjugates of steroid metabolites and

aflatoxin B_1 quantitatively[7,8]. The column was washed with 1000 ml distilled water and the conjugates were eluted with two volumes of 900 ml methanol. The total radioactivity was determined in the different eluates from the column. Only 0.1 per cent of the radioactivity in the filtered urine was not adsorbed on the Amberlite XAD-2 column under aqueous conditions while 95 per cent appeared in the first methanol fraction and 1·1 per cent in the second methanol fraction.

Each batch of urine, containing unlabelled metabolites of sterigmatocystin, was treated immediately after collection in exactly the same way as the urine containing radioactive metabolites except that the conjugates were eluted from the Amberlite XAD-2 column with 1000 ml methanol.

The methanol eluates containing both ^{14}C labelled and unlabelled metabolites of sterigmatocystin were evaporated to dryness under vacuum, dissolved in distilled water and combined (total volume = 60 ml).

Purification of sterigmatocystin metabolites by ion exchange chromatography. The sterigmatocystin metabolites were purified by ion exchange chromatography in a manner similar to that applied by Dalezios *et al.*[8] to the urinary metabolites of aflatoxin B_1.

The solution containing conjugates of sterigmatocystin was applied to a column (1·6 × 93 cm) of DEAE Sephadex A-25 equilibrated with 0·01 M sodium phosphate buffer (pH 7·0) while the temperature was maintained at 7°. The conjugates were eluted from the column by means of a linear salt gradient [500 ml 0·01 M sodium phosphate buffer (pH 7·0) + 500 ml 0·01 M sodium phosphate buffer (pH 7·0; 0·8 M sodium chloride)] at a flow rate of 28 ml/hr. Fractions (4·6 ml) were collected and radioactivity was monitored in each to determine the position of the radioactive conjugates (Fig. 2). Fractions 170–200 (Fig. 2) containing 3·1 per cent of the radioactivity applied to the column and fractions 207–240 (Fig. 2), containing 74·5 per cent, were combined and lyophilized.

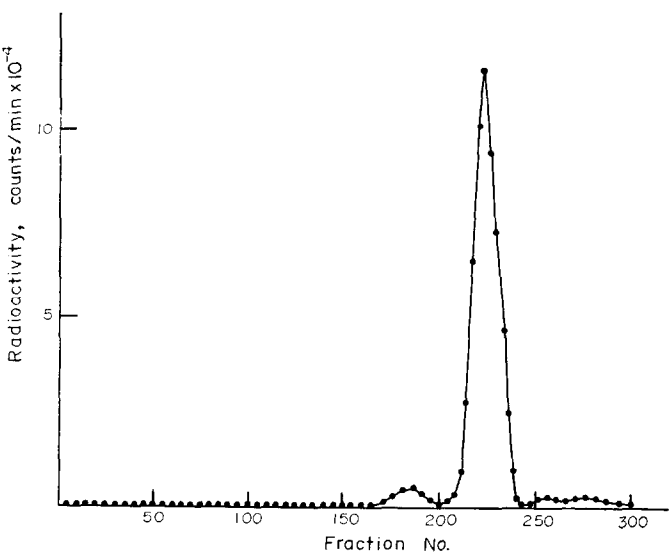

FIG. 2. Elution of radioactive sterigmatocystin metabolites from a DEAE Sephadex column.

Desalination of the radioactive metabolites. The dry material obtained from the combined fractions 207–240 (Fig. 2) was dissolved in 36 ml distilled water and yielded a clear brown solution which was applied to a Sephadex G10 column (2·6 × 74 cm) equilibrated with distilled water. The column was eluted with distilled water at a flow rate of 48 ml/hr while 4·8 ml fractions were collected. The absorbancy at 320 nm, sodium concentration and radioactivity were determined in each fraction (Fig. 3). Fractions 90–125 were combined and lyophilyzed. Only 70 per cent of the radioactivity applied to the column was recovered in fractions 90–125.

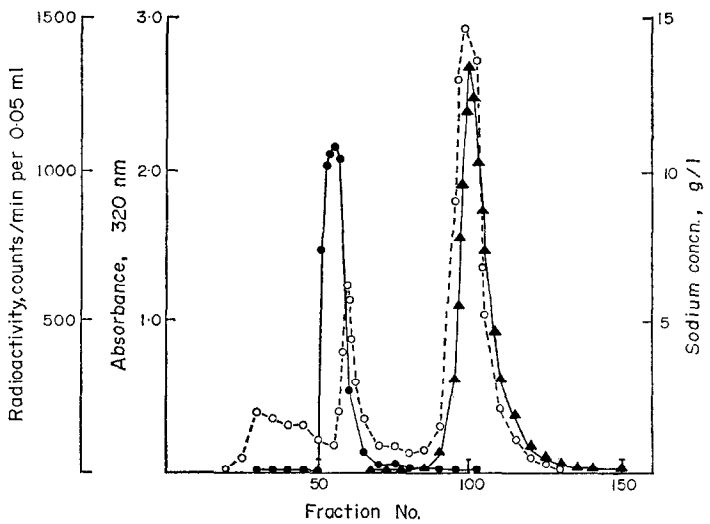

FIG. 3. Desalination of radioactive metabolites of sterigmatocystin on a Sephadex G10 column. (▲) Radioactivity; (○) absorbance (320 nm); (●) sodium concentration.

Identification of radioactive metabolites: treatment with β-glucuronidase. The dry material (fractions 90–125, Fig. 3) was dissolved in 10 ml distilled water. Two 2 ml aliquots were incubated separately for 24 hr at 37° in 0·1 M sodium acetate buffer (pH 5·0), while 0·05 ml β-glucuronidase (Boehringer, Mannheim) was added to the one reaction mixture at the start of incubation. After 24 hr each reaction mixture was extracted three times with 10 ml chloroform. The chloroform and water phases were separated, evaporated to dryness and dissolved in specific volumes of chloroform (5 ml) and water (50 ml), respectively. The water phase from the treatment without β-glucuronidase contained 99·4 per cent of the total radioactivity while 95·2 per cent of the total radioactivity could be recovered in the chloroform phase from the treatment with β-glucuronidase.

Thin layer chromatography of chloroform extracts. The chloroform extracts from the treatments with and without β-glucuronidase were chromatographed on thin layer plates (precoated silica gel, Merck) using four different solvent systems:

(1) 10% acetone in chloroform,
(2) 10% acetone in carbon tetrachloride,
(3) 10% acetone and 1% formic acid in carbon tetrachloride,
(4) *n*-hexane–ether (50:50).

In all four solvent systems the chloroform extract from the treatment with β-glucuronidase contained a red fluorescent spot visible under u.v. light, with a R_f value and colour corresponding to authentic sterigmatocystin. This red spot, similar to sterigmatocystin, turned yellow upon spraying with a 20 per cent ethanolic solution of aluminium chloride and heating at 80°. Under the same conditions no trace of sterigmatocystin could be detected in the extract not treated with β-glucuronidase.

The solvent system consisting of 10 per cent acetone and 1 per cent formic acid in carbon tetrachloride yielded a well-defined spot without any tailing for sterigmatocystin. A thin layer chromatogram, to which fifteen spots (each containing 5 μl of the chloroform extract) were applied, was developed with this solvent system. The area containing the red spots, corresponding to sterigmatocystin, was scraped off the t.l.c. plate. The silica gel was extracted with 15 ml 30 per cent acetone in chloroform, containing 1 per cent formic acid. The solvent was evaporated in a scintillation vial, scintillation fluid was added and the total radioactivity in the extract was determined. By this procedure it was shown that 96 per cent of the radioactivity in the chloroform extract from the treatment of radioactive metabolites of sterigmatocystin with β-glucuronidase would be recovered from the red fluorescent spot with a R_f value corresponding to that of authentic sterigmatocystin.

This red fluorescent spot was confirmed to be sterigmatocystin by comparing the acetylation product with authentic sterigmatocystin monoacetate by means of t.l.c. on silica gel using 10 per cent acetone in chloroform as solvent[9,10]. The monoacetate derivative of sterigmatocystin can be detected as a blue fluorescent spot with a R_f value lower than that of sterigmatocystin. After acetylation of the chloroform extract, the red fluorescent spot corresponding to sterigmatocystin disappeared from chromatograms while a blue fluorescent spot could be detected having the same R_f value as the monoacetate derivative of authentic sterigmatocystin.

Thin layer chromatography of the water soluble, radioactive urine metabolites. The compounds dissolved in the water phase obtained after incubation of the water soluble urine metabolites with β-glucuronidase as well as the untreated water soluble urine metabolites were separated on thin layer plates (precoated silica gel, Merck) using chloroform, isopropanol, methanol, 10 N ammonium-hydroxide (40, 40, 20, 8) as solvent system. A schematic representation of the separation is given in Fig. 4.

The major fluorescent component in the isolated, water soluble, urine metabolites showed up as a light blue fluorescent spot with a R_f value of approx. 0·25 (Fig. 4a). Treatment with β-glucuronidase caused the disappearance of this compound from the water phase (Fig. 4b).

Ten spots, each containing 10 μl of the aqueous solution of the isolated radioactive metabolites, were applied to a 20 × 20 cm silica gel plate which was then developed with the above-mentioned solvent system. The areas containing the four spots indicated in Fig. 4a as well as the area containing the point of application were scraped off the plate, and were each eluted with 20 ml distilled water. The extracts were evaporated to dryness in scintillation vials, redissolved in scintillation fluid and the radioactivity determined. Of the radioactivity recovered from the thin layer plate, 94 per cent was found in the light blue fluorescent spot ($R_f = 0{\cdot}25$) which disappeared upon treatment with β-glucuronidase.

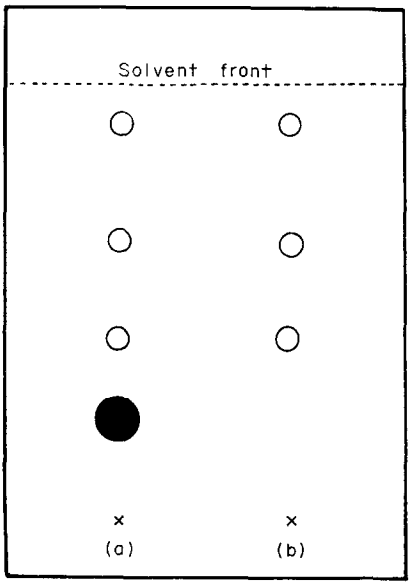

FIG. 4. A schematic representation of a thin layer chromatogram showing the result of β-glucuronidase treatment on the isolated sterigmatocystin conjugate. (a) Untreated, water soluble conjugate. (b) Water phase obtained after β-glucuronidase treatment. (×) Points of application; (●) radioactive sterigmatocystin conjugate; (○) unknown non-radioactive contaminants.

DISCUSSION

The radioactive urinary metabolites of sterigmatocystin were quantitatively adsorbed onto an Amberlite XAD-2 column as indicated by the fact that only 0·1 per cent was eluted under aqueous conditions. This implies that the radioactive metabolites were present in the form of conjugates as this resin is known to adsorb conjugates of steroid metabolites quantitatively[7,8]. Similarly to steroid conjugates, 96·1 per cent of the radioactive metabolites of sterigmatocystin could be eluted from the Amberlite XAD-2 column with methanol.

By purification of the urinary conjugates by salt gradient elution from a column of DEAE Sephadex, 74·5 per cent of the total radioactivity was recovered in a single symmetrical peak which was desalinated by treatment on a Sephadex G10 column from which 70 per cent of the applied radioactivity was recovered. The material isolated therefore represents the major urinary metabolite of sterigmatocystin (> 50 per cent). The fact that sodium was eluted from the Sephadex G10 column ahead of the radioactive metabolites implies that the radioactive conjugates were selectively retarded on the column.

Treatment of the isolated material with β-glucuronidase and subsequent extraction with chloroform recovered the radioactivity almost quantitatively (95·2 per cent) in the chloroform phase, indicating that the major urinary metabolite was a glucuronic acid conjugate of either sterigmatocystin or a derivative thereof. Thin layer chromatography of the chloroform extractable material in four different solvent systems as well as thin layer chromatography of the monoacetate derivative indicated, without doubt, that unaltered sterigmatocystin is conjugated with glucuronic acid

before excretion in the urine (Fig. 1). Unlike aflatoxin B_1 which undergoes *O*-demethylation before conjugation with glucuronic acid[8] (Fig. 1), sterigmatocystin already contains a free phenolic hydroxyl group (Fig. 1) which is available for conjugation. The methoxy group of sterigmatocystin is therefore intact in the major urinary excretion product.

The material isolated from the monkey urine contained four fluorescent compounds (Fig. 4). The radioactivity was, however, concentrated almost exclusively (94 per cent) in the light blue fluorescent compound with a R_f value of 0·25 in the appropriate solvent system.

The fact that this fluorescent compound was shown to be the glucuronic acid conjugate of sterigmatocystin raises the possibility of using thin layer chromatography of the isolated conjugates from human urine as a direct method for assessing exposure of humans to sterigmatocystin. Alternatively, the isolated conjugates can be subjected to the action of β-glucuronidase and sterigmatocystin can be determined according to standard procedures in chloroform extracts of the incubation mixtures.

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