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Metabolism of Ochratoxin A by Rats

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Albino rats were given ochratoxin A (6.6 mg/kg body weight) intraperitoneally or per os. Independent of route administration, 6% of a given dose was excreted as the toxin, 1 to 1.5% as (4*R*)-4-hydroxyochratoxin A, and 25 to 27% as ochratoxin α in the urine. The metabolite (4*S*)-4-hydroxyochratoxin A, which is formed by rat liver microsomes in the presence of NADPH, was not detected. Only traces of ochratoxins A and α were found in feces. Identical experiments were carried out with brown rats, since the K_m value for the formation of the 4*S* epimer was considerably lower when brown rat microsomes were used. About the same ratios of metabolites and metabolite recoveries as those found for albino rats were found for brown rats. Brown rats were also given the two hydroxylated metabolites and ochratoxin α (0.66 mg/kg body weight) intraperitoneally. The three compounds were excreted in the urine; within 48 h, 90% recovery of ochratoxin α and 54 and 35%, respectively, of the 4*R* and 4*S* isomers were observed.

Ochratoxin A, a dihydroisocoumarin derivative linked over a 7-carboxy group to L-phenylalanine by an amide bond, is a secondary metabolite produced by various species of the fungal genera *Aspergillus* and *Penicillium*. It has, among other toxic effects, been shown to cause nephropathy in swine (5). It is also presumed to be involved in a fatal kidney disease which affects people in certain districts of Bulgaria, Romania, and Yugoslavia (6).

When ochratoxin A was incubated with liver microsomes from pig and NADPH, two hydroxylated metabolites were formed in about equal amounts. They were identified as (4*R*)- and (4*S*)-4-hydroxyochratoxin A (Fig. 1); they were also formed when microsomes from human or rat livers were used, but in different proportions (9). These metabolites exhibit similar properties when subjected to thin-layer chromatography (TLC), but they are easily separated by high-pressure liquid chromatography (9). It has been suggested from thin-layer chromatography in five different solvent systems (unknown) and from comparison with (4*R*)-4-hydroxyochratoxin A isolated from cultures of *P. viridicatum* that this hydroxylated compound is excreted after intraperitoneal injection of ochratoxin A (4). The possibility that the urine metabolite is (4*R*)- or (4*S*)-4-hydroxyochratoxin A or a mixture of both has not been determined in previous studies. The ratio of the formation of the 4*R* and 4*S* epimers when albino rat microsomes were used was approximately 8:1 (9). The similar properties of the epimers on TLC explain why the 4*S* metabolite produced in vitro was not discovered in previous studies (4, 10).

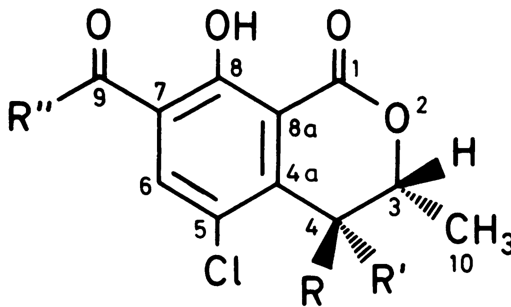
In recent works, a single oral, intravenous, or intraperitoneal dose of ochratoxin A was given to rats (1, 2, 11), and the toxin and ochratoxin α were determined in the urine and feces. In those investigations, only one thin-layer solvent system was used to separate the compounds, and no hydroxylated metabolite was detected.

In this paper we report the excretion of metabolites of ochratoxin A in the urine and feces of rats.

MATERIALS AND METHODS

Chemicals. Ochratoxin A was purchased from Sigma Chemical Co., St. Louis, Mo., and (4*R*)- and (4*S*)-4-hydroxyochratoxin A were isolated from incubation mixtures of ochratoxin A with pig liver microsomes (9). Ochratoxin α was obtained by acid hydrolysis of ochratoxin A (12), and in this work it was characterized by nuclear magnetic resonance spectroscopy. It has mass spectrum and chromatographic properties similar to ochratoxin α isolated from rat urine (see below). The concentrations of the compounds were based either on weight or on the molecular extinction coefficients (M^{-1} cm^{-1}): ochratoxin A, 5,550 at 333 nm (8); ochratoxin α, 5,600 at 338 nm (12); and (4*R*)- and (4*S*)-4-hydroxyochratoxin A, 6,400 at 334 nm (4, 9). Other chemicals were standard commercial high purity materials. Ochratoxin A and metabolites administered to the animals were dissolved in 0.1 M $NaHCO_3$.

Animals, housing, and collecting of excreta. Male Wistar (Mol..Wist) and brown rats (BN/Mol), 150 to 170 g, were used. The animals were kept at 23 ± 1°C with a relative humidity of 60 ± 3%. The lighting was



Groups attached	Compound	Found
R = R′ = H, R″ = phe	Ochratoxin A	Urine, feces
R = R′ = H, R″ = OH	Ochratoxin α	Urine, feces
R = H, R′ = OH, R″ = phe	4*R* epimer	Urine, microsomes
R = OH, R′ = H, R″ = phe	4*S* epimer	Microsomes

FIG. 1. Ochratoxin A and its metabolites identified in rat urine and feces and from incubation mixtures of the toxin in the presence of liver microsomal fractions added to an NADPH-generating system. phe, L-phenylalanine.

automatically regulated to provide 12 h of darkness and 12 h of light (7 a.m. to 7 p.m.). The animals were individually housed in metabolism cages to permit controlled food intake and quantitative and separate collection of urine and feces. At approximately 10 a.m. each day, the animals were fed 12 g of a diet containing 12.5% casein, 80% vitamin- and mineral-enriched autoclaved potato starch, 2.5% corn oil, and 5% cellulose. At the same time, urine and feces were collected. The latter were combined for each animal for the whole experimental period. The fractions were stored below 0°C before analysis.

Preparation of subcellular fractions and incubation. Liver microsomes were prepared in 20 mM Tris (pH 7.4) containing 1.15% KCl (10). In some experiments, the rats were given phenobarbital, 1 g/liter, in the drinking water 3 to 4 weeks before sacrifice. Protein was determined by the method of Lowry et al. (7), using bovine serum albumin as a standard.

Isolation of ochratoxin α from rat urine. Urine from rats given ochratoxin A or metabolites intraperitoneally was combined, acidified to pH 2, and immediately extracted three times with the same volume of chloroform. The solvent was evaporated and the residue was dissolved in methanol. This solution was subjected to TLC in solvent system G (chloroform-diethylether-acetic acid, 17:2:1; see below).

The band with blue fluorescence was removed and extracted with methanol-chloroform (2:1). The suspension was centrifuged at 12,000 × *g* for 10 min, evaporated, and dissolved in methanol. This metabolite was further purified by thin layer chromatography on Lichrosorb RP-18 plates in solvent system I (methanol–0.1% phosphoric acid, 80:20; see below) as eluting solvent. The blue metabolite was subjected to high-pressure liquid chromatography (Waters M45, ultraviolet detection at 254 nm) on a Lichrosorb RP-18 column (4.6 by 250 mm; particle size 10 μm) with acetonitrile–0.1% phosphoric acid (25:75) as eluting solvent at a flow rate of 2.0 ml per minute. Fractions (2 ml) were collected, and those containing the metabolite, with the strongest fluorescence, were collected. The metabolite was eluted after approximately 18 ml, and combined fractions containing about 200 μg were extracted twice with chloroform, evaporated, and dissolved in methanol. Ochratoxin α from acid hydrolysis of ochratoxin A (12) was purified by the same procedure.

TLC. TLC plates precoated with 0.25 mm of silica gel containing gypsum and a fluorescent UV (254 nm) indicator (Macherey-Nagel Co., Düren, Germany) and high-performance TLC-plates precoated with Lichrosorb RP-18 (E. Merck AG, Darmstadt, Germany) were used. The solvent systems were (vol/vol): A, toluene-ethyl acetate-formic acid (6:3:1); B, benzene-methanol-acetic acid (90:5:5); C, chloroform-ethyl acetate-formic acid (10:3:1); D, chloroform-acetone-formic acid (6:2:1); E, benzene-ethyl acetate-formic acid (3:1:1); F, benzene-acetic acid (4:1); G, chloroform-diethyl ether-acetic acid (17:2:1); H, methanol–0.1% phosphoric acid (70:30); and I, methanol–0.1% phosphoric acid (80:20).

Visualization and quantitation. Ochratoxin A, ochratoxin α, (4*R*)-, and (4*S*)-4-hydroxyochratoxin A were located by UV exposure. The plates or the fractions from the purification of the metabolites by high-pressure liquid chromatography were placed at the filter surface of a transilluminator with an intensity of 6,000 $\mu W/cm^2$ at a wavelength of 365 nm.

For the quantitative determination of the compounds, the silica plates were developed in solvent system B and scanned in a Vitatron LTD 100 densitometer equipped with a mercury lamp (excitation at 366 nm and emission at 460 mm). The recorded areas of the spots were compared with standards of the respective compounds.

Mass spectroscopy. A Micromass MM 70-70 F mass spectrometer was used for mass spectroscopic analysis by the direct inlet method. The ionization voltage was 70 eV, and the ionization chamber temperature was 220°C.

RESULTS

Identification of (4*R*)-4-hydroxyochratoxin A and ochratoxin α. When albino rats were given one dose of 6.6 mg ochratoxin A per kg intra-

TABLE 1. R_f values of (4*R*)- and (4*S*)-4-hydroxyochratoxin A standards and metabolite from rat urine

Compound	R_f in solvent system:		
	C[a]	G[a]	H[b]
4*R* epimer	0.68	0.50	0.37
Metabolite	0.68	0.50	0.37
4*S* epimer	0.71	0.57	0.45

[a] Silica gel.
[b] Lichrosorb RP-18.

peritoneally or per os, only two fluorescent metabolites could be detected in the urine by TLC in solvent systems A through H. One of these metabolites, with green fluorescence, was identical to (4*R*)-4-hydroxyochratoxin A in solvent systems C, G, and H, but had different mobility than (4*S*)-4-hydroxyochratoxin A (Table 1). In addition, the urine metabolite and (4*R*)-4-hydroxyochratoxin A eluted together (17 ml) when subjected to high-pressure liquid chromatography on Lichrosorb RP-18 with acetonitrile–0.1% phosphoric acid (35:65) as eluting solvent (9). (4*S*)-4-hydroxyochratoxin A was eluted after approximately 22 ml.

The second urine metabolite fluoresced blue and cochromatographed with ochratoxin α in solvent systems A through G. In addition, the two substances exhibited equal mobility when subjected to high-pressure liquid chromatography. The second metabolite was identified by its mass spectrum (Fig. 2). The spectrum showed a strong molecular ion with m/z 256 (52.5%) as compared with the base peak m/z 212 (100%). The presence of chlorine in the molecule was evident from a number of ion peak pairs such as m/z 256 and 258, 238 and 240, 223 and 225, 212 and 214, 194 and 196, and 166 and 168, having a ratio of approximately 3:1 (chlorine isotopes 35 and 37). These data strongly indicate that this metabolite is ochratoxin α (molecular weight, 256).

A similar mass spectrum (not shown) was obtained for ochratoxin α obtained by acid hydrolysis of ochratoxin A (see above).

Excretion of ochratoxin A and its metabolites. When ochratoxin A was given to albino rats intraperitoneally or per os, most of ochratoxin A and the metabolites were excreted within 5 to 6 days. The amounts of the excreted metabolites in the urine were determined (Table 2). Only traces of ochratoxins A and α were identified in feces.

Kinetic data showed that the K_m for formation of the 4*S* isomer was considerably lower when microsomes from brown rats instead of albino were used (75 versus 176 μM) (Table 3). Consequently, these animals were given ochratoxin A (6.6 mg/kg body weight) intraperitoneally and per os to determine whether they could form the 4*S* isomer. About the same ratios between urinary metabolites and metabolite recoveries were observed for brown and albino rats. No additional metabolites were detected in either the urine or feces from the brown rats. A total of nine albino and brown rats were used in these experiments.

When three brown rats were given (4*R*)- or (4*S*)-4-hydroxyochratoxin A or ochratoxin α intraperitoneally, the chemicals were mostly excreted in the urine within 48 h (Fig. 3). A 90% recovery of ochratoxin α was observed, whereas 54 and 35%, respectively, of (4*R*)- and (4*S*)-4-hydroxyochratoxin A were recovered. Traces of ochratoxin α could be detected in feces of the rat given this compound. (4*R*)- and (4*S*)-4-hydroxyochratoxin A were only found in the urine.

Kinetics of the formation of (4*R*)- and (4*S*)-4-hydroxyochratoxin A from ochratoxin A. The reaction rates were determined for various substrate concentrations in the presence of microsomal fractions from brown rat liver (normal and phenobarbital treated). Protein concentrations in the incubation mixtures were 2 to 3 mg/ml (9) (Table 3).

TABLE 2. Cumulative urinary excretion of metabolites by albino rats after administration of 6.6 mg ochratoxin A per kg body weight

Metabolite	Urinary excretion[a] after ochratoxin A dose	
	Intraperitoneally	Per os
Ochratoxin A	6.0	6.9
(4*R*)-4-hydroxyochratoxin A	0.8	1.6
Ochratoxin α	25.0	27.2

[a] Given as percent dose administered.

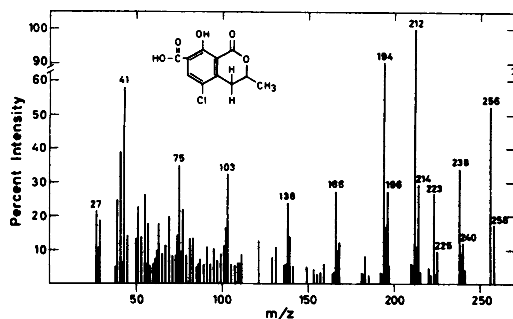


FIG. 2. Mass spectra of ochratoxin α (molecular weight, 256). The formula is inserted. Fragments with intensity less than 2.5% are omitted.

TABLE 3. K_m and V_{max} values for the formation of (4*R*)- and (4*S*)-4-hydroxyochratoxin A by albino[a] and brown rat liver microsomal fraction and NADPH-generating system.

Rat	K_m (μM)		V_{max} (nmol of product formed/mg of protein per h)	
	4*R*	4*S*	4*R*	4*S*
Albino	60.2	175.5	6.8	0.89
Albino[b]	61.3	182.5	23.0	10.2
Brown	48	75	0.77	1.02
Brown[b]	56	71	3.7	8.7

[a] See reference 10.
[b] Phenobarbital induced.

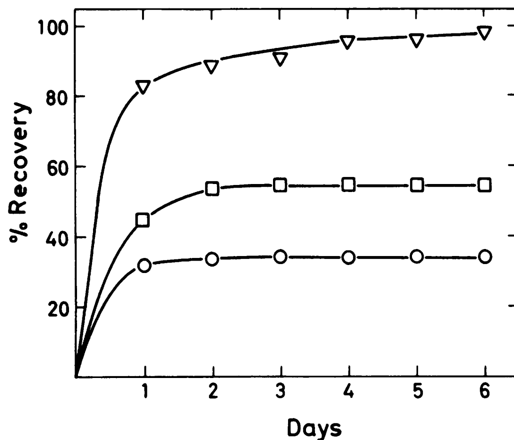


FIG. 3. Cumulative urinary excretion of (□) (4*R*)- and (○) (4*S*)-4-hydroxyochratoxin A, and (▽) ochratoxin α after intraperitoneal injection of 0.66 mg/kg body weight of the respective compounds in brown rats.

K_m and maximum velocity (V_{max}) for the formation of the two metabolites are presented in Table 3, together with the values obtained when microsomes from albino rats were used (9). When the brown rats were pretreated with phenobarbital, the V_{max} values increased 4.8 and 8.5 times, respectively, for the formation of the 4*R* and the 4*S* isomer.

DISCUSSION

The results show that ochratoxin A, ochratoxin α, and (4*R*)-4-hydroxyochratoxin A are excreted in the urine when albino or brown rats are given the toxin intraperitoneally or per os. Only ochratoxin A and ochratoxin α were found in feces. The presence of ochratoxin A and ochratoxin α are in agreement with earlier studies (1, 2, 11). The 4*R* isomer may be identical to "compound IV" described by Galtier et al. (2) based on R_f values. In the present work, about 1 to 1.5% of the administered toxin was recovered as the (4*R*)-hydroxy metabolite, whereas in the previous study 1.1% of the dose was recovered as "compound IV".

Since the 4*S* isomer might have been formed in vivo but further metabolized, the two isomers were given intraperitoneally to brown rats which made these metabolites in about equal amounts when their liver microsomes were incubated with ochratoxin A and NADPH. Both the 4*R* and 4*S* compounds were excreted in the urine at about 54 and 35% within 48 h.

When ochratoxin α was given intraperitoneally, about 90% was excreted in the same period of time, indicating that this metabolite is not accumulated in the organism but excreted as soon as it is formed. The total recovery of ochratoxin α in the range of 25 to 27% of given ochratoxin A indicates that the formation of ochratoxin α is the most important mechanism for the removal of ochratoxin A, in addition to the excretion of ochratoxin A itself.

It has previously been shown (3) that isolated hepatocytes from albino rats were able to form (4*R*)-4-hydroxyochratoxin A in significant amount from ochratoxin A, but only a trace of the 4*S* epimer could be detected. This also supports the assumption that only the 4*R* isomer is formed in vivo.

The kinetic data show that there are differences in the microsomal hydroxylation rates in brown and albino rats. K_m values for the 4*R* isomer are similar, but V_{max} for its formation is about 9 times larger in albino rats.

The total excretion of the three metabolites in the urine was in the range of 32 to 36% of dose administered (6.6 mg/kg body weight). In a previous study with rats and an oral dose of 15 mg/kg body weight, 22% recovery was reported (11). Absorption, tissue distribution, and metabolism are likely to be affected by the acute catarrhal enteritis produced by even small doses of ochratoxin A (11). Increasing oral, intravenous, or intraperitoneal administration of the toxin produces further changes in kidneys and liver, and thus might influence the capacity of these organs to metabolize the toxin.

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LITERATURE CITED

1. **Chang, F. C., and F. C. Chu.** 1977. The fate of ochratoxin A in rats. Food Cosmet. Toxicol. **15**:199–204.
2. **Galtier, P., J.-L. Charpenteau, M. Alvinerie, and C. Labouche.** 1979. The pharmacokinetic profile of ochratoxin A in the rat after oral and intravenous administration. Drug Metab. Dispos. **7**:429–434.
3. **Hansen, C. E., S. Dueland, C. A. Drevon, and F. C. Størmer.** 1982. Metabolism of ochratoxin A by primary cultures of rat hepatocytes. Appl. Environ. Microbiol. **43**:1267–1271.
4. **Hutchison, R. D., and P. S. Steyn.** 1971. The isolation and structure of 4-hydroxyochratoxin A and 7-carboxy-3,4-dihydro-8-hydroxy-3-methylisocoumarin from *Penicillium viridicatum*. Tetrahedron Lett. **43**:4033–4036.
5. **Krogh, P., B. Hald, and E. J. Pedersen.** 1973. Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. Acta Pathol. Microbiol. Scand. Sect. B **81**:689–695.
6. **Krogh, P., B. Hald, R. Pleština, and S. Čeović.** 1977. Balkans (endemic) nephropathy and foodborn ochratoxin A: preliminary results of a survey of foodstuffs. Acta Pathol. Microbiol. Scand. Sect. B **85**:238–240.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin reagent. J. Biol. Chem. **193**:265–275.
8. **Nesheim, S., N. F. Hardin, O. J. Francis, Jr., and W. S. Langham.** 1973. Analysis of ochratoxins A and B and their

esters in barley, using partition and thin layer chromatography. I. Development of the method. J. Assoc. Off. Anal. Chem. **56**:817–821.

9. **Størmer, F. C., C. E. Hansen, J. I. Pedersen, G. Hvistendahl, and A. J. Aasen.** 1981. Formation of (4*R*)- and (4*S*)-4-hydroxyochratoxin A from ochratoxin A by liver microsomes from various species. Appl. Environ. Microbiol. **42**:1051–1056.
10. **Størmer, F. C., and J. I. Pedersen.** 1980. Formation of 4-hyroxyochratoxin A from ochratoxin A by rat liver microsomes. Appl. Environ. Microbiol. **39**:971–975.
11. **Suzuki, S., T. Satoh, and M. Yamazaki.** 1977. The pharmacokinetics of ochratoxin A in rats. Japan J. Pharmacol. **27**:735–744.
12. **van der Merwe, K. J., P. S. Steyn, and L. Fourie.** 1965. Mycotoxins. Part II. The constitution of ochratoxins A, B, and C, metabolites of *Aspergillus ochraceus* Wilh. J. Chem. Soc., p. 7083–7088.