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Formation of (4R)- and (4S)-4-Hydroxyochratoxin A from Ochratoxin A by Liver Microsomes from Various Species

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Two metabolic products were formed from ochratoxin A by human, pig, and rat liver microsomal fractions in the presence of reduced nicotinamide adenine dinucleotide phosphate. They were isolated from the incubation mixture in the presence of pig liver microsomes by extraction, thin-layer chromatography, and high-pressure liquid chromatography. Their structures are suggested to be (4R)- and (4S)-4-hydroxyochratoxin A on the basis of mass and nuclear magnetic resonance spectroscopy. K_m and the maximum velocity for the formation of the two metabolites by human, pig, and rat microsomes were determined. Their formation was inhibited by carbon monoxide and metyrapone. The results indicate that the microsomal hydroxylation system is a cytochrome P-450 and that different species are involved in the formation of the two epimeric forms of 4-hydroxyochratoxin A.

Ochratoxin A, a dihydroisocoumarin derivative linked over a 7-carboxygroup 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 (3). It is also presumed to be involved in a fatal chronic kidney disease which affects people in certain districts of Bulgaria, Romania, and Yugoslavia (4).

(4R)-4-Hydroxyochratoxin A has been identified from cultures of *Penicillium viridicatum* (2). 4-Hydroxyochratoxin A, isolated from rat urine after intraperitoneal injection of ochratoxin A, had identical R_f values with this compound in five different thin-layer chromatographic systems (8). When ochratoxin A was incubated with rat liver microsomes and reduced nicotinamide adenine dinucleotide phosphate, a metabolite identical to 4-hydroxyochratoxin A from rat urine was formed (8).

The present work describes the isolation and identification of (4R)- and (4S)-4-hydroxyochratoxin A from incubation mixtures of pig liver microsomal fractions in the presence of ochratoxin A and reduced nicotinamide adenine dinucleotide phosphate.

MATERIALS AND METHODS

Production and isolation of the metabolites. Pig liver was purchased from the local slaughterhouse. Samples of human liver from kidney transplant donors and of rat liver from male Wistar rats were obtained.

The liver microsomes were prepared in 20 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) containing 0.15 M KCl, and the incubations and the extractions of the metabolites were performed as previously described (8). In the experiments with metyrapone, two additional extractions were performed in order to remove this compound. The chloroform layer from the acidic extraction of the incubation mixtures was extracted three times with 2 ml of 0.1 M $NaHCO_3$. The aqueous phases were combined, acidified with 1 M HCl, and reextracted with chloroform, yielding a solution of ochratoxin A and its metabolites.

Pig liver was used for the preparative purposes, and the incubation time was increased to 2 h with an incubation volume of 500 ml. The amount of microsomal protein was 2 to 2.5 g, and 20 mg of ochratoxin A (Sigma Chemical Co.) was used. The incubation was terminated by the addition of 150 ml of 1 M HCl, 500 ml of water, and 500 ml of methanol-chloroform (2:1). The aqueous phase was extracted five times with 100 ml of chloroform. The combined organic solutions were extracted twice with 250 ml of 0.1 M $NaHCO_3$. The combined aqueous phases were acidified and extracted with chloroform, and the residue obtained upon evaporation was dissolved in a minimal volume of chloroform.

This solution was applied on thin-layer plates with solvent system G (chloroform-diethyl ether-acetic acid [17:5:1]), and each of the two separated bands containing the metabolites was removed and extracted with methanol-chloroform (2:1). The two suspensions were centrifuged at 12,000 × *g* for 10 min. The extracts were washed twice with an equal volume of 0.1 M HCl,

evaporated, and finally dissolved in methanol.

The metabolites were further purified by high-pressure liquid chromatography (Waters M 45, ultraviolet detection at 254 nm) on a Zorbax-ODS column (4.6 by 250 mm) with methanol-water-acetic acid (300:190:10) at a flow rate of 1.0 ml/min, and 2-ml fractions were collected. The fractions of each metabolite with the strongest fluorescence (see below) were combined. The two metabolites (4*R* and 4*S* isomers of 4-hydroxyochratoxin A, see below) were eluted after approximately 8 and 13 ml, respectively. The solutions were extracted with methanol-chloroform (2:1), dissolved in methanol, and rechromatographed on the Zorbax-ODS column with methanol-water-acetic acid (300:190:10) as the eluting solvent. Finally the residues were dissolved in acetonitrile–0.1% phosphoric acid (35:65) and purified on a Lichrosorb RP-18 column (4.6 by 250 mm; particle size, 10 μm) with the same acetonitrile-phosphoric acid mixture as the eluting solvent at a flow rate of 1.0 ml/min. Fractions (2 ml each) were collected, and the two metabolites (4*R* and 4*S* isomers) were eluted after approximately 17 and 22 ml, respectively. (Ochratoxin A was eluted after 66 ml in this solvent system.) The metabolites were visualized and collected as described above and extracted three times with chloroform. The yield was approximately 500 μg of each metabolite.

Protein was determined by the method of Lowry et al. with bovine serum albumin as a standard (5).

Thin-layer chromatography. The silica gel plates (8) were developed in the following solvent systems (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), and (G) chloroform-diethyl ether-acetic acid (17:5:1).

Visualization and quantitation. Ochratoxin A and its two hydroxylated metabolites were located by ultraviolet 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 μW/cm^2 at a wavelength of 365 nm.

For the quantitative determination of (4*R*)- and (4*S*)-4-hydroxyochratoxin A, the plates were developed in solvent system G and scanned in a Vitatron densitometer LTD 100 equipped with a mercury lamp (excitation at 366 nm and emission at 460 nm). The recorded areas of the spots were compared with standards of (4*R*)-4-hydroxyochratoxin A. The same amounts of these compounds showed equal fluorescent intensity when subjected to fluorescent scanning after thin-layer chromatography. A molecular extinction coefficient of 6,400 M^{-1} cm^{-1} at 334 nm of (4*R*)-4-hydroxyochratoxin A (2) was used to calculate the concentrations of both isomers.

Ultraviolet and fluorescent spectra. After purification on a Lichrosorb RP-18 column (see above), ochratoxin A and (4*R*)- and (4*S*)-4-hydroxyochratoxin A were dissolved in isopropanol (for spectroscopy, E. Merck AG). The ultraviolet spectra were recorded on a Cary 219 spectrophotometer, and the fluorescent spectra were recorded on a Perkin Elmer 650-10S spectrofluorometer.

NMR spectroscopy. The samples were dissolved in ca. 25 μl of $CDCl_3$ and introduced into 1.7-mm nuclear magnetic resonance (NMR) tubes. ^1H and ^{13}C spectra were recorded on a Jeol FX90Q instrument operating in the pulsed-Fourier transform mode. The ^{13}C spectra were obtained with a pulse width of 6.5 μs (45°), a spectral width of 5,000 Hz (8,000 data points), an acquisition time of 0.588 s, and a pulse delay of 0.5 s.

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

RESULTS

Identification of the two metabolites as (4*R*)- and (4*S*)-4-hydroxyochratoxin A. When pig liver microsomal fractions were incubated in the presence of ochratoxin A and reduced nicotinamide adenine dinucleotide phosphate, two metabolites could be detected by thin-layer chromatography in solvent systems C and G (Table 1). Complete separation of the two metabolites was only achieved in solvent system G. One of these metabolites had R_f values identical to 4-hydroxyochratoxin.

The two metabolites exhibited mass spectra similar to the previously recorded spectrum of 4-hydroxyochratoxin A (8), with the most prominent peak at mass/charge ratio (m/z) 91 due to a benzylic cleavage. Diagnostically important pairs of peaks, showing the characteristic isotopic distribution of one chlorine atom, were observed at m/z 227/229, 255/257, and 271/273 (Fig. 1). The peaks at m/z 271/273 are best explained as due to McLafferty rearrangement of the amide carbonyl group. The pair of peaks is 16 mass units higher than the corresponding peaks in the mass spectrum of ochratoxin A, indicating the introduction of one hydroxyl group in the dihydroisocoumarin moiety of ochratoxin A.

The dihydroisocoumarin moiety of ochratoxin A offers four positions, C-3, C-4, C-6, and C-10, which can accommodate a hydroxyl group. The ^1H NMR spectra of both metabolites displayed one-proton singlets in the aromatic region and

TABLE 1. *R_f values of $(4R)^+$- and $(4S)^{2+}$-4-hydroxyochratoxin A formed from ochratoxin A by the pig liver microsomal fraction and the reduced nicotinamide adenine dinucleotide phosphate generating system*

Compound	R_f in given solvent system						
	A	B	C	D	E	F	G
$(4R)^+$	0.65	0.27	0.68	0.95	0.79	0.42	0.50
$(4S)^{2+}$	0.67	0.27	0.71	0.95	0.79	0.42	0.57
Ochratoxin A	0.79	0.60	0.90	0.97	0.90	0.77	0.86

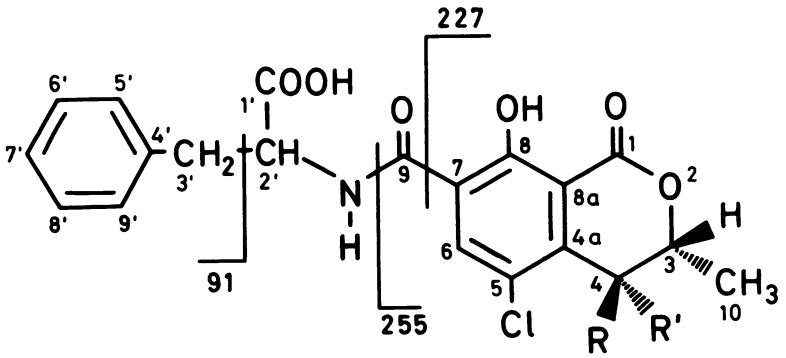

FIG. 1. *Ochratoxin A (R = R' = H) and its two metabolites, (4R)-4-hydroxyochratoxin A (R' = OH, R = H) and (4S)-4-hydroxyochratoxin A (R' = H, R = OH). The numbers 91, 255, and 227 indicate main mass fragmentation.*

three-proton doublets in the 1.3 to 1.7 ppm region, excluding all positions but C-4. The ^1H NMR chemical shifts of the methyl groups of the two compounds differed considerably. Values of 1.71 and 1.30 ppm for the 4*R* (*cis*) and 4*S* (*trans* see below) isomers were observed, but this is in accord with the shifts reported (2) for (4*R*)-4-hydroxyochratoxin A (1.68 ppm in C_5D_5N) and for *trans*-3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin (*trans*-4-hydroxymellein) (1.22 ppm in $CDCl_3$) (1). A value of 1.52 ppm (solvent unknown) has also been reported for the latter compound (7).

These structural assignments were confirmed by comparing their ^{13}C NMR spectra with the ^{13}C NMR noise- and off-resonance-decoupled spectra of ochratoxin A (Fig. 2 and Table 2). Introduction of a hydroxyl group at C-4 in ochratoxin A is expected to induce downfield shifts of 30 to 40 ppm for C-4 and 4 to 8 ppm for C-3 and a slight upfield shift for C-10 (γ effect). The spectra of the two metabolites revealed signals in the regions of 64 to 66 ppm, 79 to 82 ppm, and 16 to 18 ppm in accord with the expected shifts.

Assuming that the two metabolites possess the same 3*R* chirality as has been suggested for ochratoxin A (9), the absolute configurations at C-4 are proposed on the basis of a comparison of their ^{13}C NMR chemical shifts with corresponding data for *trans*- and *cis*-2-methylcyclohexanol (6). Somewhat higher δ values were found for the C-1, C-2, and methyl carbon signals of the *trans* isomer than were found for the *cis* isomer: 76.9, 40.0, 19.1 and 71.4, 36.1, 16.5, respectively. The metabolite exhibiting signals for C-3, C-4, and C-10 at a slightly higher field than the other is ascribed the 4*R* configuration (Table 2 and Fig. 2). This metabolite corresponds to (4*R*)-4-hydroxyochratoxin A previously isolated from *P. viridicatum*, which was identified by infrared, ultraviolet, NMR, and mass spectroscopy (2).

The two metabolites were also formed from ochratoxin A in the presence of human and rat liver microsomes. They could not be separated (cochromatography) from (4*R*)- and (4*S*)-4-hydroxyochratoxin A in solvent system G (Table 1).

Ultraviolet and fluorescent spectral properties. The ultraviolet spectral properties (λ_{max} and λ_{min}) for ochratoxin A and (4*R*)- and (4*S*)-4-hydroxyochratoxin A were 331 and 288 nm, 333 and 287 nm, and 333 and 289 nm, respectively. Similarly, emission and excitation maxima for the fluorescent spectra of the three compounds were 335 and 463 nm, 342 and 460 nm, and 344 and 460 nm, respectively.

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 human, pig, and rat liver (normal and phenobarbital treated) (8). Protein concentrations in the incubation mixtures were 2 to 3 mg/ml, and the rate of hydroxylation was found to be linear in this range. (4*R*)-4-hydroxyochratoxin A was formed by rat liver microsomes as previously described (8), and similar kinetics were obtained for the formation of (4*S*)-4-hydroxyochratoxin A (Table 3). K_m and maximum velocity (V_{max}) values for the formation of the two metabolites when human or pig microsomes were incubated with ochratoxin A are presented in Table 3. When the rats were pretreated with phenobarbital, the V_{max} values increased. In particular, V_{max} for the formation of the 4*S* isomer increased from 0.89 to 10.2 (Table 3).

When ochratoxin A was substituted by either of the two metabolites in the incubation mixture, only one spot corresponding to the added compound was observed on the thin-layer plates.

The rates of formation of the two metabolites

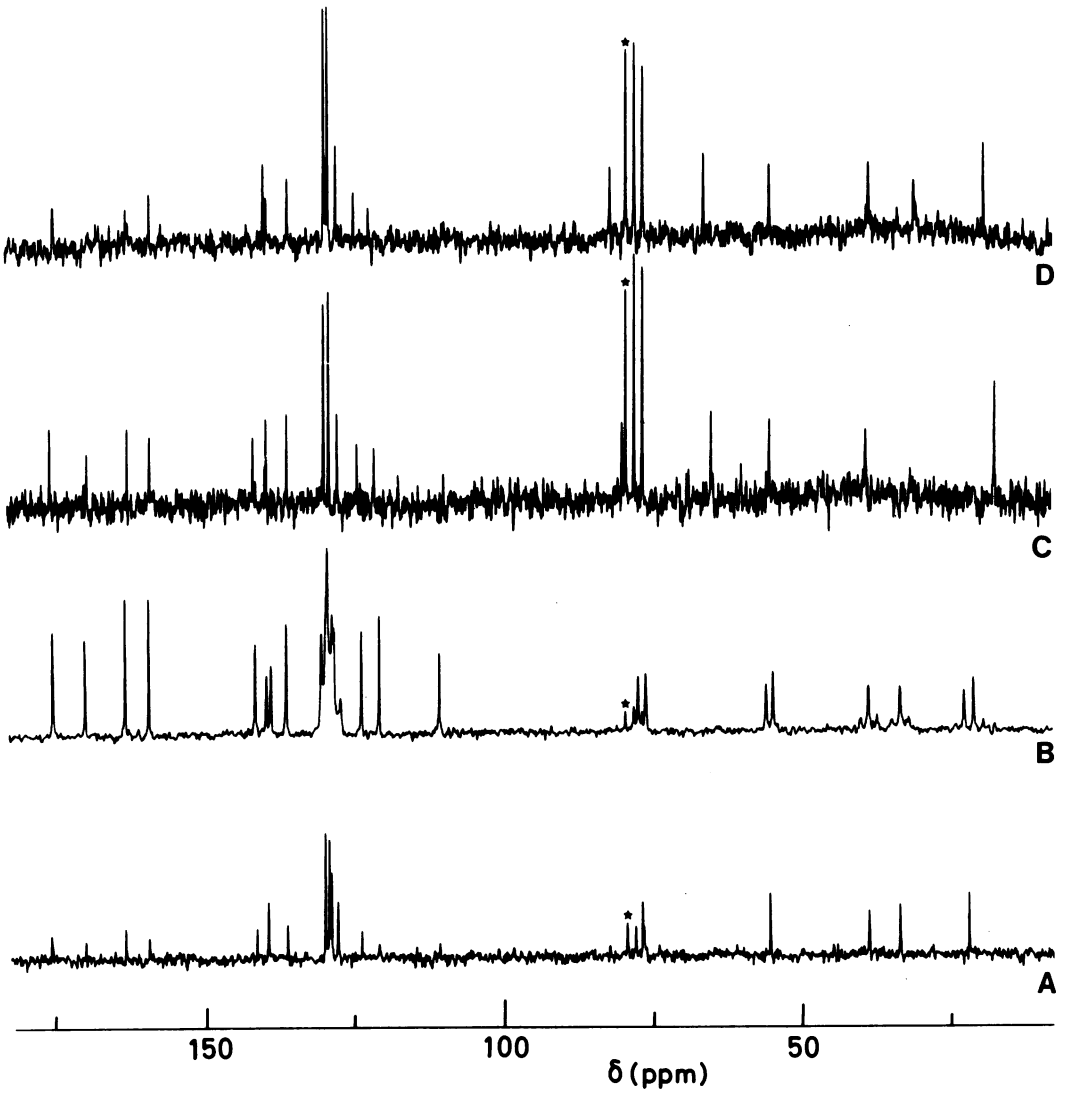

FIG. 2. *^{13}C NMR spectra of ochratoxin A. (A) Noise decoupled, ca. 4 mg, 26,000 pulses, signal at δ 128.3 is due to C_6H_6; (B) off-resonance decoupled, ca. 8 mg, 25,000 pulses, (4R)-4-hydroxyochratoxin A; (C) noise decoupled, ca. 1 mg, 162,200 pulses, (4S)-4-hydroxyochratoxin A; (D) noise decoupled, ca. 1 mg, 136,211 pulses, signal at δ 29.68 is due to an impurity. ★, "Left" signal (78.44 ppm relative to tetramethylsilane [TMS]) of the $CDCl_3$ triplet used as a reference.*

varied from species to species. The major metabolite was the 4*R* isomer in humans and rats but was the 4*S* isomer in pigs.

The formation of both metabolites was inhibited by metyrapone and carbon monoxide (Table 4). In accord with the previous study (8), the results indicate that the microsomal hydroxylating system is a cytochrome P-450.

DISCUSSION

In the present work we have shown that liver microsomal fractions from various animal species catalyze the formation of two hydroxylated metabolites from ochratoxin A. These metabolites have been identified as (4*R*)- and (4*S*)-4-hydroxyochratoxin A. The finding that (4*R*)-4-hydroxyochratoxin A was not converted to the 4*S* isomer during incubation and extraction and vice versa shows that the metabolites are not artifacts of one another. V_{max} values for (4*R*)- and (4*S*)-4-hydroxyochratoxin A increase in different ratios when rats are pretreated with phe-

nobarbital. This indicates that the two alcohols are formed by two different species of cytochrome P-450.

The great similarities between the ultraviolet and fluorescent spectra for the two isomers and the fact that equal amounts of the two metabolites, based on the molecular extinction coefficient for (4*R*)-4-hydroxyochratoxin A, gave equal fluorescence on the thin-layer plates indicate that the two metabolites have similar molecular extinction coefficients.

TABLE 2. *^{13}C NMR chemical shifts of ochratoxin A and its two metabolites*

Carbon no.	Chemical shifts (ppm)[a] of:		
	Ochratoxin A[b]	4-Hydroxyochratoxin A	
		4*R*	4*S*
1	163.0 (s)	162.5	162.7
3	75.9 (d)	79.1	81.1
4	32.2 (t)	64.0	65.3
4[c]	140.9 (s)	141.4	139.1[d]
5[e]	123.2 (s)	123.8	124.3
6	139.0 (d)	139.2	139.6[d]
7[e]	110.5 (s)	109.4[f]	—[f]
8	159.1 (s)	158.7	158.8
8a[e]	120.5 (s)	120.9	121.9
9	169.7 (s)	169.2	167.7
10	20.6 (q)	16.0	18.0
1′	175.5 (s)	175.5	174.9
2′	54.3 (d)	54.1	54.2
3′	37.4 (t)	37.8	37.3
4′[c]	135.8 (s)	135.6	135.5
5′/9′	129.3 (d)	129.4	129.4
6′/8′	128.7 (d)	128.6	128.7
7′	127.2 (d)	127.1	127.4

[a] Relative to external tetramethylsilane (TMS).

[b] Assignments are based on the splitting patterns observed in the off-resonance-decoupled spectrum of ochratoxin A and on data reported previously (10, 11) for relevant model compounds. (s), Singlet; (d), doublet; (t), triplet; and (q) quadruplet.

[c,d,e] Assignments may be reversed.

[f] Very weak or not observed.

TABLE 3. *K_m and V_{max} values for the formation of (4R)- and (4S)-4-hydroxyochratoxin A by human, pig, and rat liver microsomal fractions in the presence of reduced nicotinamide adenine dinucleotide phosphate*

Species	K_m (μM)		V_{max} (nmol of product formed/mg of protein per h)	
	(4*R*)	(4*S*)	(4*R*)	(4*S*)
Human	77.5	136.0	0.57	0.39
Pig	31.8	68.8	1.30	1.78
Rat	60.2	175.5	6.80	0.89
Rat[a]	61.3	182.5	23.0	10.2

[a] Phenobarbital induced.

TABLE 4. *Effect of carbon monoxide and metyrapone (10 μM) on the hydroxylation of ochratoxin A by human, pig, and rat liver microsomal fractions in the presence of reduced nicotinamide adenine dinucleotide phosphate*

Addition	(4*R*)			(4*S*)		
	Human	Pig	Rat	Human	Pig	Rat
None	100[a]	100	100	100	100	100
Metyrapone	21	10	10	33	44	9
Carbon monoxide	64	60	57	33	50	33

[a] An activity of 100% corresponds to the formation of the following amounts (nanomoles per milligram of protein per hour) of (4*R*)- and (4*S*)-4-hydroxyochratoxin A in humans, pigs, and rats, respectively: 0.28 and 0.18, 0.09 and 0.30, and 0.21 and 0.09. In the absence of carbon monoxide the tubes were flushed with a gas mixture containing 5% O_2, 40% CO, and 55% N_2. The amount of ochratoxin A used in these experiments corresponded to the K_m values given in Table 3.

Because of the low V_{max} values for the formation of the two metabolites, hydroxylation is probably of minor importance for the organisms when exposed to high concentrations of the toxin. The toxic effects of ochratoxin A could be reduced by the formation of the 4*R* and 4*S* isomers during long-term exposure of small amounts of the toxin. (4*R*)-4-Hydroxyochratoxin A appeared to be nontoxic when given to rats at a dose of 40 mg/kg (2). Lack of material has prevented studies of the toxicity of (4*S*)-4-hydroxyochratoxin A.

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