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Metabolites of Ochratoxins in Rat Urine and in a Culture of *Aspergillus ochraceus*

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We studied the metabolic profile of ochratoxin A (OA) in rats and in a culture of OA-producing *Aspergillus ochraceus*. Ochratoxin α (Oα), ochratoxin β (Oβ), 4-*R*-hydroxyochratoxin A (4-*R*-OH OA), 4-*R*-hydroxyochratoxin B (4-*R*-OH OB), and 10-hydroxyochratoxin A (10-OH OA) were isolated from a culture of *A. ochraceus* and structurally characterized by ^{1}H nuclear magnetic resonance spectroscopy, mass spectrometry and high-pressure liquid chromatography. 4-*R*-OH OA and Oα were consistently produced and were the dominant biotransformed metabolites in the fungal culture and in rats treated with OA and ochratoxin C (OC), while the formation of 10-OH OA was conditional in the fungal system. Green fluorescent biomacromolecules were isolated by detergent extraction of the fungal culture followed by cold-acetone precipitation and gel filtration. Acid hydrolysis of the fluorescent macromolecules resulted in the release of several ochratoxins, including Oα (80%), OA (2%), and OC (5%), and other unidentified fluorescent compounds but not OB and Oβ. Cross-reactivity studies of the natural macromolecule conjugates of OA with anti-OA polyclonal antibodies indicated that they were covalently linked to the macromolecules via a group other than the carboxyl group. These studies demonstrated that a fungus can produce some of the same metabolites of OA as the rat and that Oα, OA, and OC may be covalently linked to fungal macromolecules.

Ochratoxin A (OA) (7-carboxyl-5-chloro-8-hydroxyl-3,4-dihydro-3*R*-methylisocoumarin linked through its 7-carboxyl group to L-β-phenylalanine) is a highly toxic secondary metabolite of *Aspergillus ochraceus*. It is hepatotoxic, nephrotoxic, carcinogenic, and immunosuppressive to animals and possibly to humans. The toxicity of OA may be the results of three major effects: (i) inhibition of ATP production; (ii) inhibition of protein synthesis; and (iii) promotion of membrane lipid peroxidation (13). Other studies have suggested that OA toxicity may involve the formation of macromolecule adducts of OA. Covalent DNA adducts of OA were isolated from OA-treated mice and from the urine of patients with urinary tract tumors (14–16). Recently, a study reported that the toxicity of OA may involve the formation of an OA phenoxide radical and a thiol-derived conjugate of OA (12). Previous structure-activity relationship studies have suggested that the toxicity of OA is apparently associated with its lactone moiety (26). It was therefore proposed that the toxicity of OA may be the result of the covalent modification of target enzymes by a reaction involving the lactone carbonyl group in the isocoumarin moiety.

Although the metabolism of OA in animals (13) has been studied by several researchers, very little information on the metabolism of OA in the fungal system has been reported (5, 10). Metabolic studies of OA in the mammalian system indicated that OA is hydrolyzed to much less toxic products (ochratoxin α [Oα] and phenylalanine) by mammalian carboxypeptidase A (6) and by some microorganisms in the gastrointestinal tract (9, 11, 17, 28, 29) and is hydroxylated to 4-*R*-hydroxyochratoxin A (4-*R*-OH OA), 4-*S*-hydroxyochratoxin A (4-*S*-OH OA), and 10-hydroxyochratoxin A (10-OH OA) by the microsomal mixed function oxidases (8, 18–22). Studies on the profile of OA production by *A. ochraceus* and by *Penicillium viridicatum* in barley indicated that OA gradually disappears after its maximum production phase (21 days of fermentation at 20°C) (5), suggesting that the toxin-producing fungi are able to further metabolize the toxin over time. The metabolic profile of OA in the fungal system, however, remains uncharacterized. The objectives of the current study were to compare the metabolism of OA with regard to its hydroxylation and hydrolysis profile in rats and in a fungal system (a possible model for animals) and to determine if OA interacts covalently with fungal macromolecules to form adducts.

MATERIALS AND METHODS

Chemicals and reagents. Bovine serum albumin (BSA) and ovalbumin were from Sigma, St. Louis, Mo. OA, ochratoxin B (OB), and ochratoxin α (Oα) were prepared as described by Xiao et al. (27). Ochratoxin β (Oβ) was prepared by acid hydrolysis of OB (1), and the ethyl ester of OA (OC) was prepared from OA and absolute ethanol (23, 24). Anti-OA rabbit serum was obtained from a rabbit (weighing 2 kg) immunized with 500 μg of OA-BSA in 1 ml (4). The covalent conjugates (OA-BSA and OA-ovalbumin) were prepared by cross-linking the carboxyl group of OA to the free primary amine of lysine in BSA and ovalbumin (25). 6D-dimethyl sulfoxide (100 atom% deuterium) was from Aldrich (Milwaukee, Wis.).

Instrumentation. The high-pressure liquid chromatography (HPLC) system for the preparatory purification of the metabolites included a system controller and a delivery system (no. 600E; Waters, Milford, Mass.), a preparatory reverse-phase column (C_{18} coated; 25 by 2.2 cm; particle size, 8 μm; at 42°C) (Bio-Rad Mississauga, Ontario, Canada), and a photo-diode array UV-visible detector with a computer-derived real-time three-dimensional chromatogram (no. SPD-MGA; Shimadzu, Kyoto, Japan). An isocratic mobile phase (flow rate, 10 ml/min) containing 60% distilled water (pH 2.1, adjusted with 6 N HCl), 36% methanol, and 4% isopropanol was used to elute the compounds. The analytical HPLC system included a Waters 712 WISP sample autoinjector, an HPLC controller (no. 2156; LKB, Uppsala, Sweden), a no. 2150 LKB HPLC pump, a no. 2155 LKB HPLC column oven, a Waters Novo-Pak column (C_{18}; 0.46 by 25 cm; particle size, 4 μm, at 42°C), a Shimadzu RF-535 fluorescence detector (excitation, 330 nm; emission, 450 nm), and a Shimadzu CR501 integrator. The analytes were eluted at a constant flow rate (1.5 ml/min) with gradient I or II. Gradient I, containing solvent A (methanol-isopropanol, 9:1) and solvent B (acidified distilled water with HCl [pH 2.1]), was programmed to deliver 38 to

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52% A from 0 to 8 min, 52 to 70% A from 8 to 26 min, 70 to 90% A from 26 to 28 min, 90% A from 28 to 37 min, 90 to 38% A from 37 to 41 min, and 38% A from 41 to 45 min. Gradient II, containing solvent A and solvent C (0.0025 M phosphate buffer [pH 6.2]), was programmed to deliver 30 to 50% A from 0 to 10 min, 50 to 80% A from 10 to 15 min, 80% A from 15 to 24 min, 80 to 30% A from 24 to 25 min, and 30% A from 25 to 30 min. The UV-visible absorption spectra of the metabolites were recorded with a spectrophotometer (no. DU-8; Beckman, Irvine, Calif.). The mass spectra were obtained with an HPLC-mass spectrometry (MS) system (Hewlett-Packard, Palo Alto, Calif.) including a reverse-phase column (C_{18} coated, 0.46 by 25 cm, 10 μm, at 25°C), a flow rate of 1 ml/min, an isocratic mobile phase (98% acetonitrile, 2% formic acid), and a quadrapole MS detector (ion source temperature, 250°C; negative chemical ionization mode; mass range, 150 to 500) from Food and Agriculture Canada, Winnipeg Research Station, Winnipeg, Manitoba, Canada. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz (no. AM300 spectroscope; Bruker, Karlsruhe, Germany) and at 500 MHz (no. AMX500; Bruker) with tetramethylsilane as the reference (Department of Chemistry, University of Manitoba).

Animal studies. Eight Sprague-Dawley rats (body weight, 200 to 500 g) from the University of Manitoba were used for the metabolic studies of OA and OC. Each toxin (4 mg/kg) was administered intraperitoneally to four adult rats. Each rat was individually housed in a metabolic cage with a funnel for urine collection, and feed and water were provided ad libitum. Urine volume was measured and collected at 24-h intervals after administration of the toxin. Urine samples were stored in separate containers at −20°C until analysis. The experimental procedure was approved by the local animal care committee and followed the procedures outlined by the Canadian Council of Animal Care, Ottawa, Ontario, Canada.

Fungal culture. Twenty Erlenmeyer flasks (500 ml), each containing 30 g of feed-grade wheat and 30 ml of distilled water, were autoclaved for 30 min at 120°C and then inoculated with *A. ochraceus* NRRL 3174. The culture was maintained at 30°C in a dark room. The fermentation was terminated on day 12 of incubation.

Extraction, isolation, and purification of fungal metabolites. The solid matter in each flask was homogenized at 25°C for 2 min with a Polytron homogenizer (1.2 cm; Brinkmann, Rexdale, Ontario, Canada) in 150 ml of a water-methanol (3:7; pH 7.2) mixture. The homogenate was shaken for 30 min and centrifuged at 3,500 × *g* for 10 min at 4°C. The supernatant fraction was transferred to a 4-liter separatory funnel and diluted with an equal volume of distilled water. The solution was mixed with an equal volume of $CHCl_3$ and shaken constantly for 30 min. The $CHCl_3$ fraction was discarded, and the procedure was repeated. The aqueous phase was acidified with 6 N HCl to a pH of 1 to 1.5 and then extracted with an equal volume of $CHCl_3$ for 30 min. The $CHCl_3$ fraction was collected and evaporated to approximately 100 ml with a Rotovapor R110 evaporator (Brinkmann) at 50°C. The solution was transferred into a separatory funnel (500 ml) and extracted with 100 ml of 0.1 M Na_2CO_3 for 30 min. The $CHCl_3$ fraction was discarded, and the Na_2CO_3 fraction was acidified with 6 N HCl to a pH of 1 to 1.5. The acidified aqueous fraction was reextracted with 200 ml of $CHCl_3$ for 30 min. The $CHCl_3$ fraction was collected and dried with the Rotovapor R110 evaporator at 50°C. The residues were reconstituted with 2 ml of methanol. The methanol fraction containing OA, OB, and other fungal metabolites was applied to a flexible preparatory thin-layer chromatography (TLC) plate (20 by 20 cm, silica; Whatman, Clifton, N.J. with the maximum loading being 5 mg per plate. The plate was developed with a solvent mixture of ethyl acetate and acetic acid (95:5). The major metabolites other than OA and OB were well separated, with the R_f values being 0.54 (F5), 0.47 (F4), 0.32 (F3), 0.27 (F2), and 0.14 (F1). The separated metabolites were visualized under long-UV light (300 to 400 nm). Individual bands containing the corresponding metabolite were transferred from the plate to a centrifuge tube and extracted with 5 ml of methanol at 25°C for 30 min (7). The methanol fraction containing each metabolite was dried under N_2 and reconstituted in 1 ml of methanol. Each recovered metabolite, although relatively pure, was further purified by preparatory reverse-phase HPLC, using the procedure described above. Each purified metabolite obtained from each fraction (approximately 150 ml) had a characteristic UV spectrum (λ_{max}, 334 nm for OA metabolites and 323 nm for OB metabolites) as monitored by the Shimadzu UV-visible photo-diode array detector. The fraction containing the pure metabolite was extracted three times with 50 ml of $CHCl_3$. The $CHCl_3$ fractions were then dried with a rotary evaporator, and the residue was reconstituted with 2 ml of methanol. Metabolites used for UV-visible, MS, or NMR characterization were then dried in a vacuum dryer (AS160; Savant, Farmingdale, N.Y.) at 40°C for 24 h. The characterized metabolites were used as standards for HPLC analysis.

Analysis of the metabolites in urine and fungal culture. Samples (1 g) were randomly withdrawn from three flasks of the fungal culture on day 12 of incubation, and each sample was homogenized with a Polytron homogenizer in 10 ml of methanol–0.01 N HCl (7:3) in a 50-ml polyethylene centrifuge tube. The mixture was shaken for 30 min and centrifuged at 2,500 × *g* for 15 min at 4°C. The supernatant fractions (2 ml) were diluted 100-fold with methanol prior to HPLC analysis. Similar extractions were made for fungi that were incubated for 3, 6, and 9 days. Urine samples (500 μl) were acidified to pH 1 to 2 with 6 N HCl, and 1.5 ml of methanol was added. The mixture was extracted for 30 min and centrifuged at 2,500 × *g* for 10 min at 4°C. The supernatant (1 ml) was diluted 10-fold with methanol. Samples from the fungal culture and the rat urine were analyzed (10 μl per injection) by HPLC (gradient II) as described above.

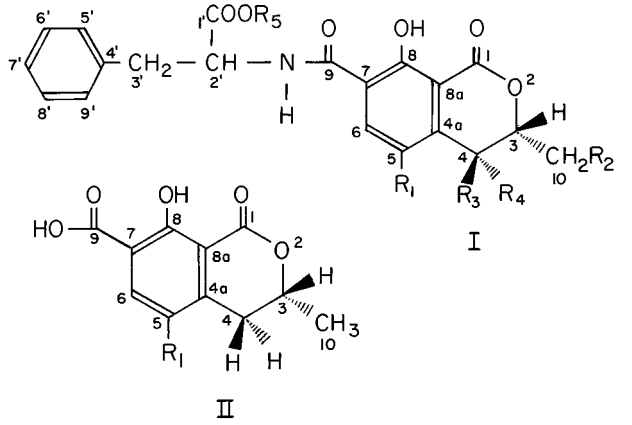

FIG. 1. Structures of ochratoxins used in this study. For identities of R_1 through R_5, see Table 1.

TABLE 1. Structures of ochratoxins used in this study

Structure[a]	Compound	Identity of:				
		R1	R2	R3	R4	R5
I	OA	Cl	H	H	H	H
I	OB	H	H	H	H	H
I	OC	Cl	H	H	H	CH_3CH_2
I	4-*R*-OH OA	Cl	H	H	OH	H
I	4-*S*-OH OA	Cl	H	OH	H	H
I	10-OH OA	Cl	OH	H	H	H
I	4-*R*-OH OB	H	H	H	OH	H
II	Oα	Cl	H	H	H	
II	Oβ	H	H	H	H	

[a] Structure in Fig. 1.

Isolation of natural macromolecular conjugates of OA. The fungal culture that was incubated for 14 days (15 g) was homogenized with a Polytron homogenizer (1.2 cm) at 4°C for 2 min in 15 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.2) containing 2% Tween 20 (PBST). The homogenate was constantly shaken for 30 min and centrifuged at 2,500 × *g* for 10 min at 4°C. The supernatant fraction (approximately 5 ml) was collected and mixed with 15 ml of cold (0°C) acetone. The mixture was centrifuged at 2,500 × *g* for 10 min at 4°C. This procedure was similar to that used previously to remove noncovalently linked OA which is tightly bound to BSA (2) from covalently linked OA-BSA (25). The supernatant containing the soluble metabolites was discarded, and the pellet was reconstituted with 500 μl of PBST followed by centrifugation at 5,000 × *g* for 10 min at 4°C. The new supernatant containing the solubilized macromolecules was collected and applied to a Sephadex G25 (Pharmacia, Uppsala, Sweden) gel filtration column (1 by 15 cm; particle size, 150 to 200 μm) that was equilibrated and then eluted with 0.1 M PBS to remove the small molecules. The void volume (3.5 ml) of the column was determined with blue dextran. The water-soluble macromolecules (1.5 ml) had a strong green fluorescence and were collected with the void volume. The isolated macromolecules (10 μl) were applied to a Whatman TLC plate (silica, 5 by 25 cm) with 10 μl of OA-BSA conjugate (10 mg/ml; OA/BSA molar ratio, 6) as the positive control and 10 μl of free OA (1 mg) in 1 ml of BSA (10 mg) as the negative control. The plate was developed with the mobile phase containing methanol and acetone (1:1) at 25°C for 15 min. The blue and green fluorescent spots that were associated with OA-BSA and the isolated macromolecules remained at the origin, while all of the fluorescence of the negative controls migrated with the solvent (R_f = 0.95).

The fluorescent macromolecule fraction (1.5 ml) collected from the gel filtration column was diluted to 5 ml with PBS and was used for the immunoassay and the acid hydrolysis studies. The diluted macromolecules (500 μl) were hydrolyzed at 25°C following the addition of an equal volume of 1 N HCl. A 100-μl sample was withdrawn at 0.5, 90, and 180 min after mixing with the acid. Each sample was immediately extracted for 2 min with 4 ml of $CHCl_3$. The $CHCl_3$ fraction (2 ml) was transferred into a 5-ml optical vial and dried under N_2 gas at 25°C. The residue was reconstituted with 2 ml of methanol and assayed for OA, OC, OB, Oα, and 4-*R*-OH OA by using the analytical HPLC system (gradient I)

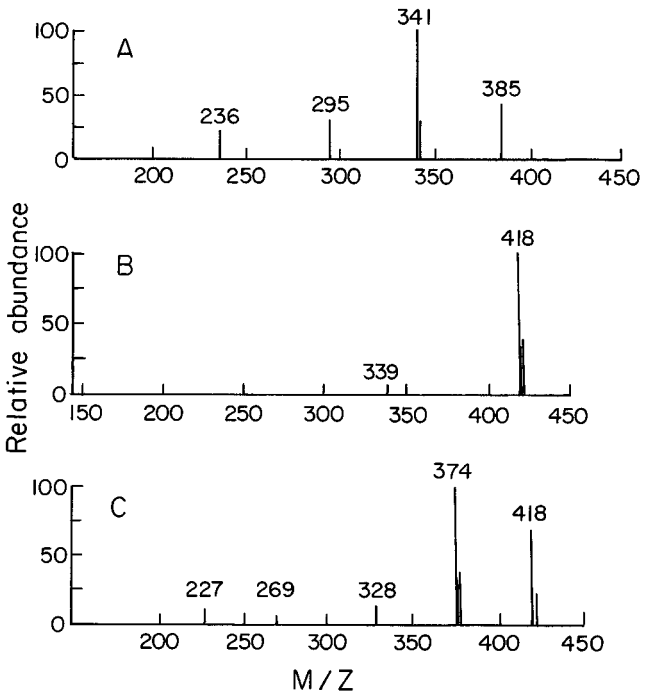

FIG. 2. Negative chemical ionization mass spectra of 4-*R*-OH OB (A), 10-OH OA (B), and 4-*R*-OH OA (C).

as described above. The reactivity of the diluted macromolecules and the negative control with the polyclonal anti-OA antibodies was assayed by an indirect competitive enzyme-linked immunosorbent assay (ELISA) as described by Clarke et al. (4).

RESULTS

Isolation of OA and its metabolites. The structures of the compounds studied and their abbreviations are outlined in Fig. 1 and Table 1. Metabolites from the fungal culture were readily extracted with $CHCl_3$ at a low pH and were partitioned from the $CHCl_3$ fraction into an aqueous fraction in a basic solution (0.1 M $NaHCO_3$). Preparatory silica TLC with a mobile phase containing a mixture of ethyl acetate and acetic acid (95:5) readily resolved five of the predominant fluorescent metabolites, with the R_f values being 0.54 (F5), 0.47 (F4), 0.32 (F3), 0.27 (F2), and 0.14 (F1). These fractions, as discussed below, were identified as 4-*R*-OH OA, 10-OH OA, 4-*R*-OH OB, Oα, and Oβ, respectively. After TLC, further purification with preparatory reverse-phase HPLC was necessary before each compound could be characterized by NMR and MS.

F1 and F2 were identified as being Oβ and Oα, because they had identical R_f values (silica TLC), HPLC (C18 reverse phase) retention times, and UV λ_{max} (335 and 324 nm, respectively) as the standards which were prepared by acid hydrolysis of OA and OB, respectively.

F3 had a UV λ_{max} at 323 nm and exhibited a blue fluorescence, which was similar to OB. The HPLC mass spectrum of F3 (Fig. 2A), however, indicated that it had a molecular ion mass of 385 Da. The results suggested that F3 may be a derivative of OB. The difference in molecular weight between OB (369) and F3 (385) was 16, suggesting that F3 had an extra oxygen atom compared with OB. The ^{1}H NMR (300 MHz, in 6D-dimethyl sulfoxide) spectrum of F3 (Fig. 3) compared with that of OB (27) revealed a difference between the two compounds, since the doublet of doublet (dd) protons at the C-4 position of OB was not observed in the spectrum of F3. Also, the spectrum of F3 had a proton resonance doublet of doublets at 4.56 ppm (J = 2.1 Hz) and a doublet proton (d) at 5.86 ppm (J = 6.4 Hz) which were not observed in the spectrum of OB.

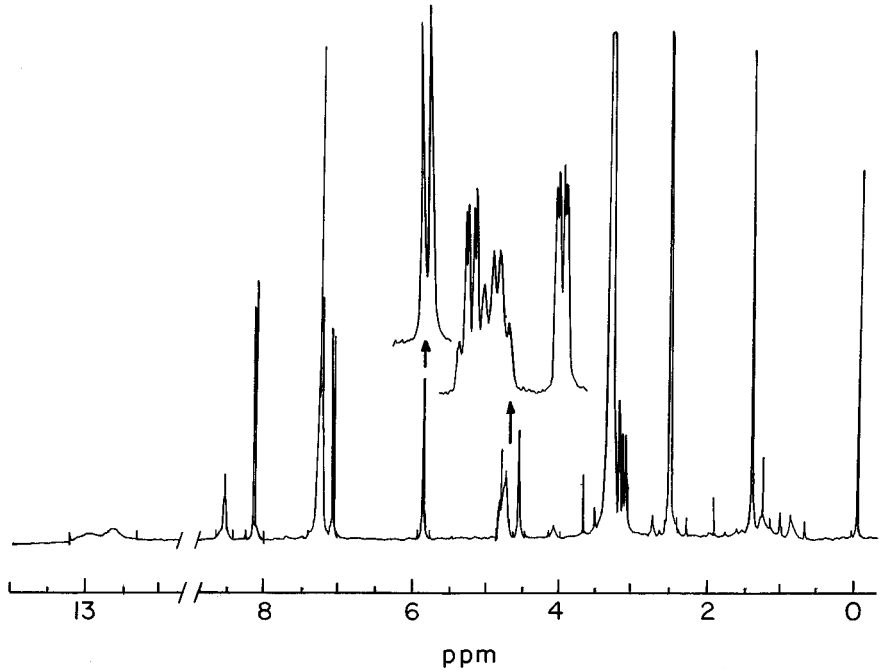

FIG. 3. ^{1}H NMR (300-MHz) spectrum of 4-*R*-OH OB in 6D-dimethyl sulfoxide. Peaks are expanded at arrows. The decoupling experiment, irradiation at 1.41 ppm, resulted in collapse of the doublet of quartets at 4.80 ppm into a doublet (J = 2.1 Hz), and irradiation at 4.56 ppm resulted in collapse of the doublet of quartets at 4.80 ppm into a doublet (J = 6.3) and of a doublet at 5.86 into a singlet.

In CD_3OD, the doublet at 5.86 ppm disappeared and the doublet of doublets at 4.56 ppm become a doublet (J = 2.1 Hz), suggesting that the proton at 5.86 ppm was exchangeable and the two protons were coupled. The coupling constant (J = 2.1 Hz) of the proton at 4.56 ppm was identical to that for the one at 4.80 ppm (J = 6.3 and 2.1 Hz), which corresponds to the C-3 proton of OB. The data suggest that the proton at 4.56 ppm may be located at the C-4 position while the proton at 5.86 ppm is an exchangeable hydroxyl proton which is also located at the C-4 position. This assignment was further confirmed by the decoupling study, as irradiation of F3 with a frequency of 4.56 ppm (4-H) resulted in collapse of the doublet at 5.86 ppm (4-OH) into a singlet(s) and of the double quartets (dq) at 4.80 ppm (3-H) into a doublet (J = 6.31 Hz). Irradiation of F3 with a frequency of 1.41 ppm (d, J = 6.54 Hz [Fig. 3]), which corresponded to the C-3 methyl protons of OB, resulted in the collapse of the double quartets at 4.80 ppm (3-H) to a doublet (J = 2.1 Hz). Overall, the results indicate that the protons at 1.41 ppm (10-H) are coupled to the one at 4.80 ppm (3-H) and that the proton at 3-H (4.80 ppm) is coupled to the proton at 4.56 ppm (4-H), which is also coupled to the proton at 5.86 ppm (4-OH). The coupling constant (J = 2.1 Hz) between the 4- and 3-H protons suggests that 4-H is *cis* oriented with the 3-H and that the 4-OH group should be on the same side as the 3-*R*-CH_3 group. The coupling constant between 4-H and 3-H of 4-*R*-OH OA is 1.9 Hz, which is also analogous to this compound, as discussed below. These data demonstrated that F3 is 4-*R*-OH OB. The spectral proton NMR assignment is as follows: δ 1.41 (3H, d, $J_{10,3}$ = 6.54 Hz, 3-CH_3), 3.10 (1H, dd, $J_{3',3'}$ = 13.78 Hz, $J_{3',2'}$ = 7.8 Hz, 3′-*S*-H), 3.20 (1H, dd, $J_{3',3'}$ = 14.1 Hz, $J_{3',2'}$ = 4.75 Hz, 3′-*R*-H), 4.56 (1H, dd, $J_{4,4\text{-OH}}$ = 6.32 Hz, $J_{4,3}$ = 2.1 Hz, 4-*S*-H), 4.75 [1H, m (multiplets), 2′-H], 4.80 (1H, dq, $J_{3,4}$ = 2.1 Hz, $J_{3,10}$ = 6.56 Hz, 3-H), 5.86 (1H, d, $J_{4\text{-OH},4}$ = 6.4 Hz, 4-*R*-OH), 7.08 (1H, d, $J_{5,6}$ = 7.9 Hz, 5-H), 7.23 to 7.31 (5H, m, 5′-9′-H), 8.14 (1H, d, $J_{6,5}$ = 7.9 Hz, 6-H), 8.54 (1H, d, $J_{9\text{-NH},2'}$ = 7.1 Hz, 9-NH), and 12.5 to 13.0 (2H, broaden, 1′-COOH and 8-OH). It is not known if this compound is the same as 4-OH OB which was isolated by Stormer et al. (20) from a microsomal fraction following incubation with OB, because these researchers did not establish the structure of this metabolite.

F4 had a UV λ_{max} at 334 nm, and its molecular weight was 419 ($M^- - 1$ = 418) as determined by HPLC-MS (Fig. 2B). The ^1H NMR spectrum of F4 (Fig. 4a) was similar to that of 10-OH OA reported by Stormer et al. (22), except that the proton at 12.56 ppm was assigned to the 8-OH instead of to the carboxyl proton. A more detail coupling pattern which was provided by the two-dimensional NMR spectroscopy (COSY, 500 MHz [Fig. 4b]) further confirmed that F4 was 10-OH OA. The proton NMR spectrum of 10-OH OA is assigned as follows: δ 3.16 (1H, dd, $J_{4,4}$ = 17.62 Hz, $J_{4,3}$ = 11.76 Hz, 4-*R*-H), 3.22 (1H, m, 3′-*S*-H), 3.28 (1H, dd, $J_{4,4}$ = 17.46 Hz, $J_{4,3}$ = 4.13 Hz, 4-*S*-H), 3.37 (1H, dd, $J_{3',3'}$ = 14.16 Hz, $J_{3',2'}$ = 4.95 Hz, 3′-*R*-H), 3.90 (1H, dd, $J_{10,10}$ = 12.52 Hz, $J_{10,3}$ = 4.66 Hz, 3-CH_2-), 4.05 (1H, dd, $J_{10,10}$ = 12.53 Hz, $J_{10,3}$ = 3.28 Hz, 3-CH_2-), 4.70 (1H, m, 3-H), 5.02 (1H, m, 2′-H), 7.2 to 7.34 (5H, m, 5′-9′-H), 8.45 (2H, s,broaden, 6-H and 9-NH), and 12.59 (1H, s, 8-OH).

F5 had a UV λ_{max} at 333 nm and a molecular weight of 419 ($M^- - 1$, 418) as determined by HPLC-MS (Fig. 2C). The NMR spectrum of F5 (Fig. 5) agrees with the description of 4-*R*-OH OA reported by Hutchison et al. (10). Its proton assignment is as follows: δ 1.69 (3H, d, $J_{10,3}$ = 6.46 Hz, 3-CH_3), 3.22 (1H, dd, $J_{3',3'}$ = 14.04 Hz, $J_{3',2'}$ = 6.7 Hz, 3′-*S*-H), 3.29 (1H, dd, $J_{3',3'}$ = 14.1 Hz, $J_{3',2'}$ = 5.4 Hz, 3′-*R*-H), 4.68 (1H, dq, $J_{3,4}$ = 1.9 Hz, $J_{3,10}$ = 6.51 Hz, 3-H), 4.90 (1H, d, $J_{4,3}$ = 1.9 Hz, 4-*S*-H), 5.02 (1H, m, 2′-H), 7.18 to 7.30 (5H, m, 5′-9′-H), 8.29 (1H, s, 6-H), 8.56 (1H, d, $J_{9\text{-NH},2'}$ = 7.12 Hz, 9-NH), and 12.73 (1H, s, 8-OH).

OA metabolites in *A. ochraceus* and in rat urine. The isolated and characterized metabolites (4-*R*-OH OA, 10-OH OA, 4-*R*-OH OB, Oα, and Oβ) were used as HPLC reference standards (Fig. 6A) for the identification and quantitation of the compounds in the urine of rats treated with OA (4 mg/kg) or OC (4 mg/kg) and in the fungal culture. HPLC analysis of extracts from a fungal culture (Fig. 6B) or urine (Fig. 6C), using gradient II, which included a mixture of 0.0025 M phosphate buffer (pH 6.2), was able to effectively resolve each metabolite, with the retention times (R_t) being 12.23, 8.78, 8.16, 7.02, 6.03, and 4.51 min for OA, OB, 4-*R*-OH OA, 10-OH OA, 4-*R*-OH OB, and Oα, respectively. The entire assay was completed within a short period, with better resolution of OB and 4-*R*-OH OA than that obtained with gradient I (compare Fig. 6 and 7). The fungal extract contained seven major and several minor peaks. The elution times of six of the seven major peaks were identical to those of the reference standards (compare Fig. 6B and A). The identity of each of the six peaks was confirmed following purification and characterization of each compound as discussed above.

The concentrations of OA and OB ± standard deviation per unit of dry matter in fungal culture after 12 days of incubation were 4.8 ± 0.6 and 0.2 ± 0.1 mg/g, respectively. The amount of 4-*R*-OH OA and 4-*R*-OH OB produced in fungal culture was approximately 2.5% (120 ± 15 μg/g) of the total OA and 13% (25 ± 5 μg/ml) of the total OB produced. Likewise, the total production of Oβ was approximately 4% (7.5 ± 2 μg/g) of that of OB, while the total production of Oα was only 0.1% (4.5 ± 2 μg/g) of the total OA produced. 10-OH OA was also detected at low concentrations (usually less than 0.4% of that of OA [20 μg/g]) and not in all samples, particularly during the early periods of fermentation. These results indicate that the hydrolytic and hydroxylated metabolites of both OA and OB are produced in fungal cultural, with OB appearing to be a better substrate for enzymatic alteration than OA.

The urine (48 to 72 h) of rats treated with OC at 4 mg/kg of body weight (Fig. 6C) contained OA (R_t = 12.23 min, peak 6), two known metabolites of OA (4-*R*-OH OA [R_t = 8.19 min, peak 4] and Oα [R_t = 4.49 min]), and two unidentified metabolites (R_t = 6.61 and 7.89 min corresponding to peaks a and b, respectively). Spiking of this urine with 4-*R*-OH OB and 10-OH OA indicated that two unidentified metabolites were not the same as these compounds, because four peaks appeared on the HPLC chromatogram. The HPLC profile for rats given injections of OA, however, was similar to that of rats given OC (data not shown). OC was not detected in the urine, because it was rapidly hydrolyzed to OA in the body (unpublished data). Approximately 3% of OC and 1.5% of OA were excreted in the urine as 4-*R*-OH according to the commutative urinary data. OB, which was not injected, and its metabolites were not detected in the urine of rats given injections of OA or OC. The HPLC profile of the control urine extract from rats showed no interfering fluorescent signal 3 min after injection of the sample (data not shown). This suggests that all of the post-3-min peaks that were observed in the urine of OA-treated rats were formed from OA or from the effects of OA and that they may also be due to metabolites of OA.

Evidence for presence of conjugates in *A. ochraceus*. The final experiment demonstrated that fluorescent compounds were released from a washed detergent extract of *A. ochraceus* in a time-dependent manner following incubation in 0.5 N HCl at 25°C. No OA or its metabolites were extracted from the water-soluble fungal extract when it was acidified for only 0.5

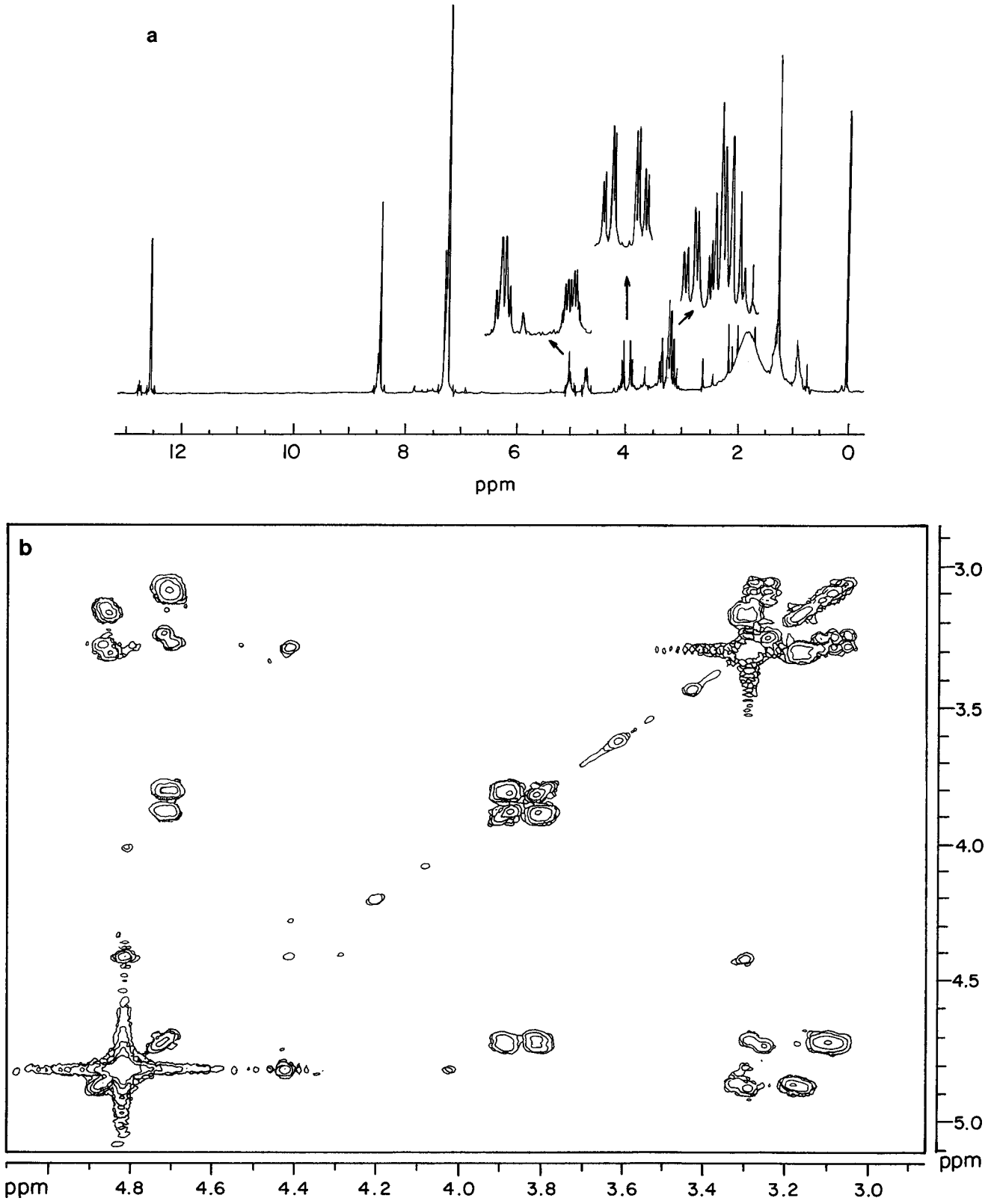

FIG. 4. (a) ^{1}H NMR (300 MHz) spectrum of 10-OH OA in $CDCl_3$. Peaks are expanded at arrows. (b) COSY spectrum (^{1}H, 500 MHz) of 10-OH in CD_3OD. δ 3.08 and 3.26 (2H, 4-H), 3.18 and 3.3 (2H, 3′-H), 3.82 to 3.9 (2H, 10-H), 4.74 (1H, 3-H), and 4.86 (1H, 2′-H).

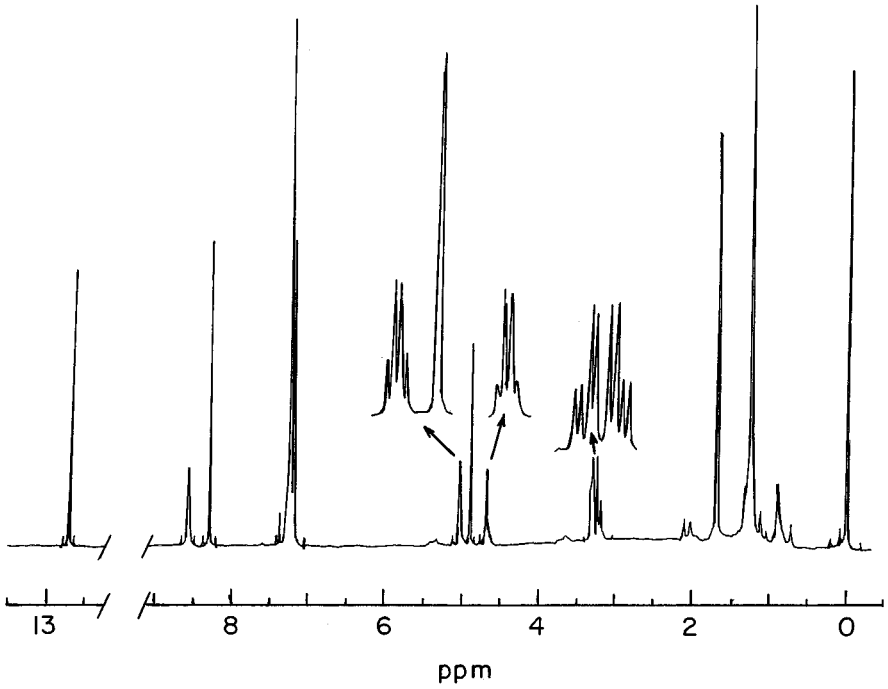

FIG. 5. ^{1}H NMR (300 MHz) spectrum of 4-*R*-OH OA in $CDCl_3$. Peaks are expanded at arrows.

min and extracted with $CHCl_3$ (Fig. 7B). Ochratoxins (OA = 0.8 to 1.3 μg/ml, OC = 0.2 to 4 μg/ml, and Oα = 0.01 to 60 μg/ml), however, were released from the PBS-diluted macromolecules after 90 and 180 min of acid hydrolysis (Fig. 7C and D, respectively). OB and 4-*R*-OH OA were not detected in the hydrolyzed samples. The chemical nature of the macromolecules (proteins, polysaccharides, or polynucleic acids), however, was not established. Studies with control samples demonstrated that all noncovalently bound toxin could be readily removed from a protein matrix during TLC that was developed with acetone-methanol (1:1) following extraction of an acidified matrix with $CHCl_3$ and by gel filtration. Also, TLC studies with the washed detergent extract demonstrated that all of the fluorescent compounds remained at the origin, in contrast to extracts from the crude preparation or pure compounds, which migrated with the solvent as reported above. These studies indicated that the washed detergent extract of *A. ochraceus* did not contain noncovalently bound toxin. Also, studies with pure OA demonstrated that the conditions of hydrolysis did not result in the hydrolysis of the peptide bond linking the phenylalanine and the isocoumarin moiety (Oα). Subsequent studies were carried out to establish the site in OA that was covalently linked to the fungal macromolecules. The ability of the adducts, the OA-OV conjugate, and free toxin in BSA to react with anti-OA antibodies from a rabbit was monitored by competition ELISA. Rabbits were immunized with OA-BSA, prepared by cross-linking of the carboxyl group of OA to BSA. This study demonstrated that the binding of anti-OA serum (1:4,000) to the immobilized OA (OA-ovalbumin) was inhibited with 150 ng of OA in 1 ml of BSA but was essentially not inhibited by the isolated macromolecules that contained an equivalent of more than 500 ng of OA per ml.

DISCUSSION

Metabolites produced in fungi and rats. Although the metabolism of OA in animals has been studied by several researchers, relatively little information has been reported on its metabolism in fungi (5, 10). Hutchinson et al. (10), however isolated OA, OB, and 4-OH OA from *P. viridicatum*. The data from this study demonstrated that *A. ochraceus* is able to produce not only OA and OB but also their respective hydrolytic products, Oα and Oβ, several of their hydroxylated metabolites, including 4-*R*-OH OA, 4-*R*-OH OB, and 10-OH OA, and possibly other metabolites. The production of 10-OH OA appeared to be conditional, because it was not detected in all cultures. *A. ochraceus* also seems to be a better source of the hydroxylated metabolites of OA than the rat, because most of the compounds can be simultaneously and easily isolated from fungal extracts in highly pure form by TLC and HPLC procedures. In the present study, rats given injections of OC or OA also excreted OA, Oα, 4-*R*-OH, and two unknown metabolites not found in fungal culture but did not excrete 10-OH OA. Storen et al. (18) and Stormer et al. (22), however, reported that 10-OH OA was produced by mammalian microsomes in vitro. These comparisons suggest that two very diverse genera, fungi and rats, may have a common mechanism for the metabolism of OA and that information on the metabolism of OA in mammals may be obtained from studies with fungi. It is not clear, however, if Oα and Oβ are synthetic precursors or hydrolytic products of OA and OB in *A. ochraceus*. Oα and Oβ may not be the source of these compounds, because it has been shown that a non-OA-producing fungus, *Aspergillus niger*, is able to efficiently hydrolyze both OA and OB (data not shown). *A. niger* could possibly provide a source of enzymes for the detoxification of OA in contaminated grains or feed, because Oα is much less toxic than OA (3).

Evidence for the presence of OA adducts in fungi. Repeated cold-acetone precipitation of a detergent (PBST) extract of the fungal culture resulted in the isolation of highly fluorescent (green), water-soluble compounds. These compounds were apparently much larger molecules than the parent compounds, because they were eluted from a gel filtration (G-25 Sephadex)

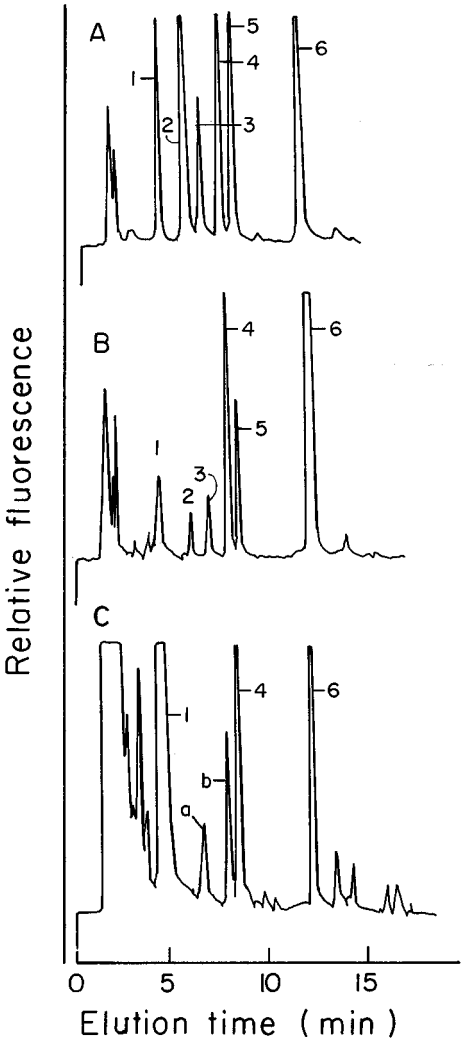

FIG. 6. Typical HPLC profile of ochratoxin metabolites. (A) Standards; (B) fungal extract; (C) rat urine. Each point represent mean ± standard deviation of three replicate determinations. The standard compounds and their elution times with gradient II were as follows: 1, Oα, 4.51 min; 2, 4-*R*-OH OB, 6.03 min; 3, 10-OH OA, 7.02 min; 4, 4-*R*-OH OA, 8.16 min; 5, OB, 8.78 min; 6, OA, 12.73 min. The fungal extract was obtained from a culture of *A. ochraceus* that was incubated for 12 days. The urine extract was obtained from rats (48 to 72 h) given injections of OC (4 mg/kg of body weight). Numbers in panels B and C correspond to the same elution time as the standards. Elution times for two unidentified metabolites (a and b) were 6.61 and 7.77 min, respectively. See Materials and Methods for further details.

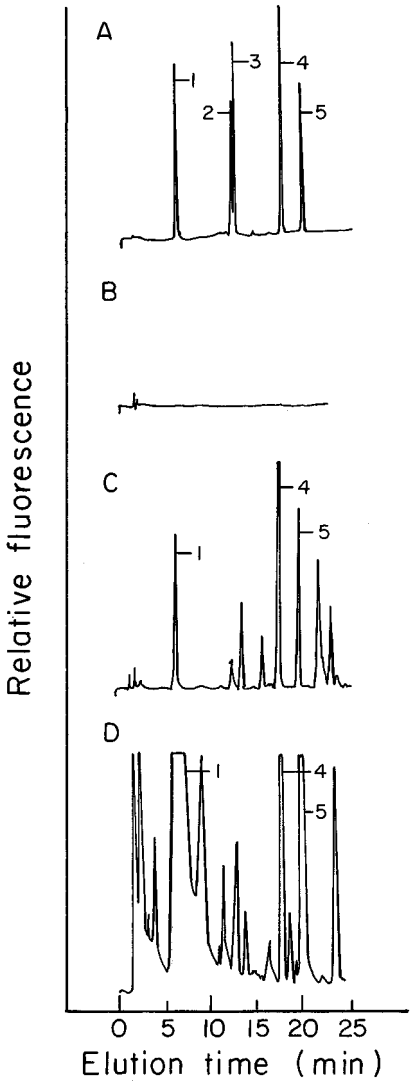

FIG. 7. HPLC elution profile of covalently bound fluorescent compounds following acid hydrolysis (0.5 N HCl) of a detergent extract of a fungal culture of *A. ochraceus*. (B to D) The residual extract was solubilized at 25°C for 1 min (B), 90 min (C), or 180 min (D). The released ochratoxins were extracted into $CHCl_3$ from the detergent extract and assayed by HPLC with gradient I. (A) Elution profile of standard compounds. The peak number, the compound, and the elution times were as follows: 1, Oα, 6.65 min; 2, 4-*R*-OH OA, 11.90 min; 3, OB, 13.18 min; 4, OA, 18.45 min; 5, OC, 20.08 min. See Materials and Methods for further details.

column (1 by 15 cm) at a volume similar to that obtained with blue dextran. The molecular weight of the complex should therefore be greater than 5,000, because this is the exclusion limit of the gel. The fluorescent compounds were also not removed from the macromolecules by TLC (silica) separation and $CHCl_3$ extraction, procedures that have been shown to be highly efficient in the removal of noncovalently bound OA (25). These observations suggested that the detergent-soluble macromolecules contained covalently bound toxin (OA) or its metabolites (Oα or OC) but not bound OB. The inability of the adducts to react with anti-OA antibodies prepared by cross-linking the carboxyl group of OA to BSA suggested that the ochratoxins were linked to the isolated macromolecules via a group other than the carboxyl group, presumably via the phenolic or the lactone carbonyl group of these compounds (26). The isolated conjugates of OA, however, do not appear to be linked through the phenolic group of OA, because they exhibited a green fluorescence. If the linkage involved the phenolic group, the fluorescence should have been abolished in a manner similar to that observed when the phenolate is linked to a methyl group (OM-OA) (27). The observation that the lactone carbonyl group of OA or Oα was highly susceptible to nucleophilic attack (26) and that the adducts, as shown in the present study, were hydrolyzed under mild conditions also indicates that the covalent linkage may be a readily hydrolyzable ester bond. These results suggest that OA is covalently bound to macromolecules and that this may involve the lactone carboxyl group of OA.

Summary. Oα, Oβ, 4-*R*-OH OA, 4-*R*-OH OB, and 10-OH-OA were isolated from a culture of *A. ochraceus* and identified

by various spectroscopic and chromatographic techniques. The 4-*R*-OH ochratoxins, Oα, and Oβ were consistently produced and were the dominant metabolites, while formation of 10-OH OA was conditional in the fungal culture. A similar profile of OA metabolites was detected in the urine of rats given injections of OA or OC, suggesting that the enzymatic systems for hydrolysis and hydroxylation of OA may be similar in both animal and fungal systems. The hydrolytic activity was also observed in a culture of non-OA-producing species of fungus, *A. niger*, 2 days after inoculation with an OA-containing medium. The observations also suggest that *A. niger* may provide a means for the detoxification of OA in contaminated grain products. Natural macromolecule conjugates of OA, OC, and Oα but not OB were isolated from the detergent extract of the fungal culture, suggesting that covalent modification of biomacromolecules by OA may occur in this organism.

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