# Effects of Chronic Ingestion of Ochratoxin A on Blood Levels and Excretion of the Mycotoxin in Sheep

RALF BLANK,*,† JAN-PETER ROLFS,† KARL-HEINZ SÜDEKUM,†
ANDREW A. FROHLICH,‡ RONALD R. MARQUARDT,‡ AND SIEGFRIED WOLFFRAM†

Institute of Animal Nutrition, Physiology, and Metabolism, Christian-Albrechts-University,
24098 Kiel, Germany, and Department of Animal Science, University of Manitoba,
Winnipeg, Manitoba, Canada R3T 2N2

Ruminants are relatively resistant to the acutely toxic effects of ochratoxin A, due to extensive degradation of ochratoxin A to its less toxic metabolite ochratoxin α by rumen microorganisms. However, most estimates of the degradation capacity for ochratoxin A in ruminants are based on in vitro studies. In the current study, the metabolism of ochratoxin A was investigated over a period of 29 days, feeding various doses of the mycotoxin (0, 9.5, 19.0, and 28.5 $\mu$g ochratoxin A/kg body weight) to sheep. Animals were fed diets consisting of 70% concentrates and 30% grass silage. Significant concentrations of undegraded ochratoxin A were detected in serum of sheep at all levels of ochratoxin A tested. Serum concentrations of ochratoxin A slightly accumulated with time of exposure and were linearly dependent on the administered dose of ochratoxin A. Furthermore, a constant proportion (6–8%) of the dose was excreted in the urine. The results of this study indicate that even at moderate to low levels of ochratoxin A in the diet, considerable amounts of the mycotoxin are absorbed by ruminants and may accumulate in tissues. Therefore, feeding of ochratoxin A-contaminated feedstuffs to ruminants does not seem to be a reliable means for using these feedstuffs.

KEYWORDS: Ochratoxin A; ochratoxin α; mycotoxin; sheep; ruminants; metabolism; excretion

## INTRODUCTION

Ochratoxins, of which ochratoxin A (**Figure 1**) is the most prevalent, are secondary fungal metabolites of some toxigenic species of *Aspergillus* and *Penicillium* (*1*). The natural occurrence of ochratoxin A in food and feedstuffs is widespread especially in temperate areas (*2*). The average contamination level of feedstuffs with ochratoxin A is in the range of 100–500 $\mu$g/kg but can be more than 5000 $\mu$g/kg under unfavorable conditions (*3*).

Ochratoxin A has been shown to be nephrotoxic, hepatotoxic, teratogenic, and carcinogenic to single-stomached animals (*3*). In several in vitro studies using ruminal fluid collected from cows and sheep, and also following administration of ochratoxin A into the rumen of calves, sheep, and goats (*4*–*7*), it has been well-documented that ochratoxin A is hydrolyzed by rumen microorganisms, yielding the isocoumarin moiety ochratoxin α (**Figure 1**) and L-phenylalanine. Ochratoxin α has been shown to be nontoxic or far less toxic than ochratoxin A (*8*, *9*). The observation that calves with an intact rumen are more tolerant to orally administered ochratoxin A as compared to preruminant calves (*10*) yields further evidence that ruminal hydrolysis of
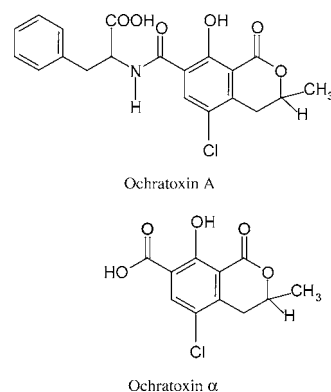


**Figure 1.** Structures of ochratoxin A and ochratoxin α.

ochratoxin A to ochratoxin α might be an important detoxification process in ruminants. One reason for the lower toxicity of ochratoxin α seems to be that it is eliminated 10 times faster from the blood as compared to ochratoxin A (*11*) because it does not readily bind to serum albumin (*12*).

These findings have led to the hypothesis that feeding ochratoxin A-contaminated feedstuffs to ruminants may be an efficient means for using these feedstuffs and to minimize the toxic effects and the carry over of ochratoxin A in the food chain. From in vitro fermentation studies with ruminal inoculum,

* To whom correspondence should be addressed. Tel: +49 431 880 2962. Fax: +49 431 880 1528. E-mail: blank@aninut.uni-kiel.de.
† Christian-Albrechts-University.
‡ University of Manitoba.

it has been estimated that ruminants are able to degrade up to 12 mg of ochratoxin A/kg contaminated feed (*4*). In another study (*13*), it was concluded from an experiment with a single animal that the capacity of a dairy cow to degrade ochratoxin A in the rumen is in the range of 33–72 mg/day and that sheep should be able to degrade 3–7 mg of ochratoxin A/day. However, in vitro studies usually do not include dynamic processes such as passage of digesta out of the rumen into the lower gastrointestinal tract with subsequent absorption from these segments and therefore may not reflect the true in vivo situation. However, in a recent study with sheep (*14*) that were fed doses of 22 and 55 µg ochratoxin A/kg bodyweight over a period of 30 days, considerable amounts of intact ochratoxin A were detected in blood serum. Similarly, concentrations of 10–40 ng ochratoxin A/L in cow's milk were detected in two recent surveys (*15, 16*) pointing to an incomplete detoxification of ochratoxin A by rumen microorganisms, at least under certain conditions.

The present data on metabolism of ochratoxin A in ruminants indicate that at least at high intake levels of ochratoxin A, detoxification to ochratoxin α is not complete; thus, the aim of the present experiment was to investigate the effects of chronic feeding of moderate levels of ochratoxin A on concentrations of ochratoxin A and ochratoxin α in ruminal fluid and blood serum as well as elimination of ochratoxin A and ochratoxin α via feces and urine and on nutrient digestibility in sheep in vivo.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Ochratoxin A-contaminated wheat was produced by inoculation of wheat with *Aspergillus ochraceus* NRRL 3174. Crystalline ochratoxin A was produced by isolation and purification from contaminated wheat. Ochratoxin α was obtained by hydrolysis of ochratoxin A with hydrochloric acid according to the procedure described by Xiao et al. (*17*). For the feeding trial, the high-contaminated wheat (3 mg ochratoxin A/g) was diluted to a concentration of 11.5 µg ochratoxin A/g. All other chemicals were derived from the following sources: chloroform (Walter, Kiel, Germany), 2-propanol (Mallinckrodt Baker, Deventer, Holland), magnesium chloride (Sigma-Aldrich, Steinheim, Germany), hydrochloric acid (Merck, Darmstadt, Germany), methanol, and *ortho*-phosphoric acid (Carl Roth, Karlsruhe, Germany).

**Animals and Experimental Design.** Twelve one year old castrated male sheep (Coburger Fuchsschaf) with a mean body weight (mean ± SD) of 39.3 ± 1.6 kg were used in this experiment. The animals were housed individually in metabolic crates in a temperature-controlled room. Water was freely available from drinking bowls. The sheep were divided into groups of three animals each and assigned to four dietary treatments consisting of a control group (no ochratoxin A) and three experimental groups given dosages of 387, 774, and 1161 µg ochratoxin A/day. The dosages corresponded to an intake of 9.5, 19.0, and 28.5 µg ochratoxin A/kg body weight/day. The sheep were fed twice daily at 08:00 and 18:30. On dry matter basis, the diets consisted of approximately 70% concentrates and 30% grass silage. The energy and protein supply was 1.0 times the requirements for maintenance (*18*). A portion of 100 g of wheat including the necessary amount of contaminated wheat was offered at the beginning of each feeding time to ensure a complete ingestion of the respective dose. Before the start of the experiment, all animals were given a 10 day period to adapt to the toxin-free high concentrate diet.

**Collection of Blood, Urine, Feces, and Ruminal Fluid Samples.** Blood samples were taken from the jugular vein prior to the morning feeding on days 1, 5, 9, 13, 23, and 29. Blood samples were allowed to clot, and after the samples were centrifuged at 3000*g* for 30 min at 2 °C, serum was separated and kept frozen at −20 °C until analysis.

Feces and urine were quantitatively collected over a period of 7 days (day 15 until day 21). At the end of the collection period, the feces were homogenized, and an aliquot was freeze-dried and stored at −20

°C until analysis. For urine, the total volume was recorded and an aliquot was stored at −20 °C until analysis.

Ruminal fluid samples were collected on days 24 and 25 of the experiment. Samples were taken at 1, 3, 7, 10, and 13 (before morning feeding) hours after feeding using an oro-ruminal probe (*19*). After the pH of the rumen fluid was measured, 30 mL of the samples was acidified with 6 mL of 2.2 M *ortho*-phosphoric acid and frozen at −20 °C. Animal care and experimental procedures were conducted according to the German Guidelines and Regulations on Animal Care (Deutsches Tierschutzgesetz, 1986: Durchführung von Tierversuchen) and were approved by the University of Kiel Committee on Animal Care.

**Chemical Analyses of Feeds and Feces.** Feeds and feces were analyzed for dry matter and organic matter according to the methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten (*20*). Feces were mixed, and dry matter was determined by freeze drying and subsequent oven drying at 105 °C overnight. Concentrates and freeze-dried silage and feces were successively ground in mills with 3 and 1 mm screens and, for starch analysis, with a 0.2 mm screen. The organic matter was determined by ashing the samples at 550 °C overnight. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin content were analyzed as described by Van Soest et al. (*21*), except that $NaSO_3$ was not used in the NDF preparation. Starch content was determined by enzymatic hydrolysis of starch to glucose (*22*), employing the heat-stable α-amylase Termamyl 120 L (Novo Industrials, Bagsværd, Denmark).

**Extraction Procedures for Ochratoxin A.** Samples of 1 g of freeze-dried and finely ground feedstuffs (0.2 mm) were extracted with 20 mL of a mixture (80:20 v/v) of methanol and deionized water (pH 2.1, acidified with $H_3PO_4$) according to the procedure described by Clarke et al. (*23*). After the samples were shaken for 30 min on a horizontal shaker, the homogenate was centrifuged at 10 000*g* for 15 min at 2 °C and an aliquot of 1 mL of the supernatatant was removed and diluted with 19 mL of methanol for high-performance liquid chromatography (HPLC) analysis.

Blood serum, feces, and urine samples were extracted according to the method of Hult et al. (*24*) with slight modifications. Briefly, 1 mL of serum was transferred into a 35 mL Nalgene tube followed by the addition of 1.5 mL of saline (0.145 M NaCl) and mixed thoroughly with 10 mL of a solution containing 0.05 M HCl and 0.1 M $MgCl_2$. After 8 mL of chloroform was added, the mixture was shaken for 30 min on a horizontal shaker, centrifuged at 10 000*g* for 15 min at 2 °C, and the upper water layer was removed by suction. The chloroform fraction was transferred into another tube, and 1.5 mL of deionized water was added, shaken for 5 min on a horizontal shaker, and centrifuged at 4000*g* for 10 min at 2 °C. After water was removed, a part of the chloroform layer (5 mL) was transferred into a 6 mL glass tube and evaporated with an AES 1010, Automatic Environmental SpeedVac System (Savant Instruments, Holbrook, NY). Prior to HPLC analysis, the samples were reconstituted with 1 mL of methanol.

For feces and urine, 1 g of freeze-dried sample or 10 mL of urine was transferred into a 50 mL Nalgene centrifuge tube and acidified to a pH of 2.0 with 6 M HCl. After 10 mL of a solution containing 0.05 M HCl and 0.1 M $MgCl_2$ and 20 mL of chloroform were added, the samples were shaken for 30 min on a horizontal shaker and centrifuged for 30 min at 10 000*g* at 2 °C. After the upper water layer was removed, the chloroform layer was transferred into another centrifuge tube, washed with 3 mL of deionized water, and constantly shaken for 5 min before centrifugation at 4000*g* for 10 min at 2 °C. Five milliliters of the chloroform layer was transferred into a glass tube and evaporated. Before HPLC analysis, the samples were reconstituted with 3 mL of methanol and centrifuged at 4000*g* for 10 min at 2 °C, and the supernatant was transferred into HPLC vials.

For extraction of ruminal fluid samples (*25*), 8 mL of acid-inactivated ruminal fluid was extracted for 20 min with 15 mL of chloroform under constant shaking, followed by centrifugation at 10 000*g* for 30 min at 2 °C in 50 mL polyethylene tubes to separate chloroform and aqueous phases. The upper water layer was removed, and the chloroform fraction was transferred into a 30 mL centrifuge tube containing 2 mL of deionized water, and the mixture was shaken for 5 min, followed by centrifugation at 4000*g* for 10 min at 2 °C. A 5 mL aliquot of the washed chloroform fraction was transferred into a 6 mL glass tube

Effects of Chronic Ingestion of Ochratoxin A

*J. Agric. Food Chem.,* Vol. 51, No. 23, 2003   **6901**

and evaporated. Dry samples were reconstituted in 3 mL of methanol, followed by centrifugation at 4000*g* for 10 min at 2 °C and used for HPLC analysis.

**HPLC Analysis.** The HPLC procedure was similar to that reported by Xiao et al. (*26*). The HPLC system included a Waters 717 plus autosampler, a Waters 600E multisolvent delivery system, a Waters 474 scanning flourescence detector, a Waters in-line degasser, and a Millipore/Waters TCM column oven system (Waters, Eschborn, Germany). An aliquot of 30 $\mu$L of the extracts dissolved in methanol was injected onto a 250 mm × 4.6 mm i.d. Nova-Pak-$C_{18}$ column fitted with a 20 mm × 3.9 mm i.d., 4 $\mu$m Nova-Pak-$C_{18}$ guard colum. The flourescence detector was set at an excitation and emission wavelength of 330 and 450 nm, respectively. All HPLC analyses were performed at a solvent flow of 1.5 mL/min and an oven temperature of 40 °C using one of two solvent gradient elution profiles consisting of deionized water acidified to a pH of 2.1 with $H_3PO_4$ (solvent A) and a mixture of methanol and 2-propanol (90/10 v/v, solvent B). To facilitate the separation of ochratoxin A and ochratoxin $\alpha$ from interfering compounds, two different gradient profiles were used (short gradient: serum, feedstuffs, and ruminal fluid; long gradient: feces and urine). The short gradient was programmed to deliver 50 to 25% A from 0 to 12 min, 25 to 10% A from 12 to 12.1 min, 10% A from 12.1 to 17 min, 10 to 50% A from 17 to 17.1 min, and 50% A from 17.1 to 22 min. The long gradient was programmed to deliver 72% A from 0 to 5 min, 72 to 52% A from 5 to 12 min, 52% A from 12 to 18 min, 52 to 25% A from 18 to 28 min, 25 to 10% from 28 to 29 min, 10% A from 29 to 34 min, 10 to 72% from 34 to 35 min, and 72% A from 35 to 40 min. For preparations of the standards, ochratoxin A and ochratoxin $\alpha$ were dissolved in pure ethanol and calibrated spectrophotometrically, based on their molar absorbance coefficient of 5500/M/cm at 333 nm for ochratoxin A and 6200/M/cm at 335 nm for ochratoxin $\alpha$ (*17*). After the calibration standards were evaporated, the dried samples were reconstituted and diluted with methanol to concentrations of 1−50 ng/mL. Quantification of ochratoxin A and ochratoxin $\alpha$ was based on the peak areas calculated with the Millenium chromatography software (Waters, Eschborn, Germany). Identification of ochratoxin A and ochratoxin $\alpha$ in samples was based on the respective retention times of standards. The retention times of ochratoxin $\alpha$ and ochratoxin A were 4.0 and 11.5 min for the short gradient and 15.6 and 28.9 min for the long gradient, respectively. All samples were analyzed in duplicate. The minimum detectable level for both ochratoxin A and ochratoxin $\alpha$ was 0.2 ng/mL, and recoveries were 94%. Recoveries were estimated on the basis of recovery of ochratoxin A and ochratoxin $\alpha$ from spiked samples of serum, urine, feces, and ruminal fluid.

**Calculations and Statistical Analysis.** The concentrations in serum and ruminal fluid were analyzed using the MIXED procedure of SAS version 6.12 (SAS Inst. Inc., Cary, NC). A spatial covariance model SP(POW) was used, which assumes a reduction in correlation in relation to the power of the distance between time points. The model included dose, time, and dose × time interaction. The subject of the repeated statement was sheep. When a significant dose × time interaction was detected, polynomial regression was used to test for linear, quadratic, and cubic dose effects within time. The area under the curve (AUC) of the concentration−time profiles of ochratoxin A and ochratoxin $\alpha$ in serum and ruminal fluid samples was calculated according to the trapezoidal rule. Elimination half-life for ochratoxin A in the ruminal fluid was calculated using GraphPad Prism version 2.0 (Graph Pad Software, San Diego, CA) assuming a one compartment model with first-order kinetics for ruminal disappearance of ochratoxin A. Data were analyzed for dose effects using the GLM procedure of SAS. The digestibilities were calculated according to the following formula: $D_N = (I_N - F_N)/I_N \times 100$, where $D_N$ is apparent digestibility of a nutrient in the diet (%), $I_N$ the total intake of a nutrient in the diet, and $F_N$ is the total excretion of a nutrient with the feces. For statistical analysis of the digestibilities with the GLM procedures, only treatment effect was included in the model. Data are presented as least squares means. Comparisons among treatment groups were analyzed by Fisher's least significance difference test, and those at $P < 0.05$ were accepted as significant. Additionally, polynomial regression was used to test for linear, quadratic, and cubic dose effects.

**Table 1.** Concentration of Ochratoxin A (ng/mL) in Blood Serum of Sheep after Feeding 0, 9.5, 19.0, and 28.5 $\mu$g Ochratoxin A/kg Body Weight/Day) for a Period of 29 Days

| dose ($\mu$g/kg body weight/day) | ochratoxin A | | | | | | |
|---|---|---|---|---|---|---|---|
| | day 1 | day 5 | day 9 | day 13 | day 23 | day 29 | SEM |
| 0 (*n* = 3) | 0 | $0^A$ | $0^A$ | $0^A$ | $0^A$ | $0^A$ | 1.2 |
| 9.5 (*n* = 2) | $0^a$ | $1.5^{Aa}$ | $3.4^{ABab}$ | $3.3^{Aab}$ | $6.0^{Bb}$ | $3.9^{Bab}$ | 1.4 |
| 19.0 (*n* = 3) | $0^a$ | $4.6^{ABb}$ | $6.0^{Bb}$ | $7.6^{Bc}$ | $9.4^{Bc}$ | $12.4^{Cd}$ | 1.2 |
| 28.5 (*n* = 2) | $0^a$ | $10.6^{Bc}$ | $6.4^{Bb}$ | $12.8^{Cc}$ | $18.2^{Cd}$ | $11.6^{Cc}$ | 1.4 |

$^{a–d}$ Least squares means within a row that do not share a common small letter differ due to time ($P < 0.05$). $^{A,B}$ Least squares means within a column that do not share a common capital letter differ due to dose ($P < 0.05$).

## RESULTS

Two animals showing acute clinical signs of foot rot were withdrawn from the experiment, and the data were discarded from the statistical analysis. None of the animals developed any overt illness or health disturbance related to ochratoxin A treatment. The daily amount of feed offered was always consumed completely during the entire feeding trial.

Increasing dosage of ochratoxin A tended ($P < 0.10$) to decrease nutrient digestibilities (data not shown). Dry matter, organic matter, and NDF digestibilities decreased linearly ($P < 0.05$) with increasing dosage of ochratoxin A from 77.2 to 71.0%, 77.9 to 73.2%, and 65.7 to 54.8%, respectively. The average digestibilities of starch and ADF were 98.7 and 53.8%, respectively, and not influenced by the mycotoxin.

The concentrations of ochratoxin A in serum are shown in **Table 1**. No ochratoxin A was detectable in the serum, prior to initial exposure on day 1 (before morning feeding) of the experiment. Repeated measures analysis revealed effects ($P < 0.05$) of dose, time, and dose × time interaction on the concentrations of ochratoxin A in serum. The respective mean concentrations of ochratoxin A in serum of sheep fed 0, 9.5, 19.0, and 28.5 $\mu$g ochratoxin A/kg body weight/day over the entire experimental period were 0, 3.0, 6.7, and 9.9 ± 0.9 ng/mL. Polynomial regression analysis for sampling day showed a linear increase ($P < 0.05$) in the concentration of ochratoxin A in the serum with increasing intake of ochratoxin A, except for day 5 where a significant linear and quadratic response ($P < 0.05$) was obtained. The area under the concentration−time profile for ochratoxin A in serum increased linearly ($P < 0.05$) with increasing dosage. The respective AUC values were 0, 102.1, 186.8, and 337.9 ± 25.4 ng/mL × day. Furthermore, the results show that chronic feeding of ochratoxin A resulted in an ochratoxin A accumulation, which is reflected by an increase in its serum concentration over time.

Beside ochratoxin A, small amounts of ochratoxin $\alpha$ were detected in the serum (**Table 2**). As for ochratoxin A, repeated measures analysis revealed effects of dose ($P < 0.05$) and time ($P < 0.05$), but only a trend for a dose × time interaction ($P < 0.10$) on the concentrations of ochratoxin $\alpha$. The mean concentrations of ochratoxin $\alpha$ in serum over the entire period for the four experimental groups were 0, 0.5, 1.0, and 1.1 ± 0.2 ng/mL with a linear ($P < 0.05$) dose response on days 5−29. The area under the concentration−time curve for concentration of ochratoxin $\alpha$ in serum also increased linearly ($P < 0.05$) with increasing dose. The AUC values were 0, 13.9, 22.6, and 31.5 ± 5.3 ng/mL × day. Furthermore, the results show that chronic feeding of ochratoxin A increased the serum concentration of ochratoxin $\alpha$ over time.

**Table 3** summarizes the excretion of ochratoxin A and ochratoxin $\alpha$ in the feces and urine. The excretion of both

**6902** *J. Agric. Food Chem.,* Vol. 51, No. 23, 2003

Blank et al.

**Table 2.** Concentration of Ochratoxin α (ng/mL) in Blood Serum of Sheep after Feeding 0, 9.5, 19.0, and 28.5 μg Ochratoxin A/kg Body Weight/Day for a Period of 29 Days

| dose (μg /kg body weight/day) | ochratoxin α | | | | | | |
|---|---|---|---|---|---|---|---|
| | day 1 | day 5 | day 9 | day 13 | day 23 | day 29 | SEM |
| 0 (n = 3) | 0 | $0^A$ | $0^A$ | $0^A$ | $0^A$ | $0^A$ | 0.3 |
| 9.5 (n = 2) | 0 | $0.5^{AB}$ | $0.8^{AB}$ | $0.4^{AB}$ | $0.5^{AB}$ | $0.7^A$ | 0.4 |
| 19.0 (n = 3) | $0^a$ | $0.9^{Bb}$ | $0.9^{Bb}$ | $1.0^{Bb}$ | $0.7^{ABab}$ | $2.3^{Bc}$ | 0.3 |
| 28.5 (n = 2) | $0^a$ | $1.4^{Bbc}$ | $1.6^{Bc}$ | $0.7^{ABab}$ | $1.3^{Bbc}$ | $1.4^{ABbc}$ | 0.4 |

$^{a-c}$ Least squares means within a row that do not share a common small letter differ significantly due to time ($P < 0.05$). $^{A,B}$ Least squares means within a column that do not share a common capital letter differ significantly due to dose ($P < 0.05$).

**Table 3.** Concentration (ng/mL) of Ochratoxin A and Ochratoxin α in Feces and Urine and Total Excretion of Ochratoxin A and Ochratoxin α (μg) via Feces and Urine after Feeding 0, 9.5, 19.0, and 28.5 μg Ochratoxin A/kg Body Weight/Day for a Period of 29 Days

| | dose (μg/kg body weight/day) | | | | |
|---|---|---|---|---|---|
| | 0 | 9.5 | 19.0 | 28.5 | SEM |
| | feces | | | | |
| Oα (ng/g DM) | $0^A$ | $212.4^{AB}$ | $396.0^B$ | $809.9^C$ | 82.7 |
| OA (ng/g DM) | $0^A$ | $30.8^{AB}$ | $57.2^B$ | $123.1^C$ | 16.7 |
| Oα (total in μg) | $0^A$ | $279.3^{AB}$ | $556.0^B$ | $1357.9^C$ | 144.6 |
| OA (total in μg) | $0^A$ | $40.7^{AB}$ | $80.1^B$ | $206.5^C$ | 26.6 |
| | urine | | | | |
| Oα (ng/mL) | $0^A$ | $158.6^B$ | $283.4^C$ | $227.3^{BC}$ | 37.5 |
| OA (ng/mL) | $0^A$ | $28.4^{AB}$ | $48.4^{AB}$ | $113.0^B$ | 25.9 |
| Oα (total in μg) | $0^A$ | $958.5^{AB}$ | $1881.0^B$ | $1875.6^B$ | 325.6 |
| OA (total in μg) | $0^A$ | $167.8^B$ | $322.8^C$ | $724.2^D$ | 43.2 |

$^{A-C}$ Least squares means within a row that do not share a common supercript differ ($P < 0.05$).

**Table 4.** Proportional Excretion (% Applied Amount) of Ochratoxin A and Ochratoxin α in Feces and Urine

| | ochratoxin A dose (μg/kg BW day) | | | | |
|---|---|---|---|---|---|
| | 0 | 9.5 | 19.0 | 28.5 | SEM |
| | feces | | | | |
| OA | | 1.5 | 1.5 | 2.5 | 0.4 |
| $Oα^a$ | | $16.2^A$ | $16.2^A$ | $26.3^B$ | 2.8 |
| sum | | $17.7^A$ | $17.6^A$ | $28.8^B$ | 3.1 |
| | urine | | | | |
| OA | | $6.2^{AB}$ | $6.0^B$ | $8.9^A$ | 0.9 |
| $Oα^a$ | | 55.7 | 54.7 | 36.3 | 7.1 |
| sum | | 61.9 | 60.6 | 45.2 | 6.5 |
| urine + feces | | 79.6 | 78.2 | 74.1 | 3.9 |

$^{A,B}$ Least squares means within a row that do not share a common supercript differ ($P < 0.05$). $^a$ Expressed as OA equivalents = (molecular weight of OA/molecular weight of Oα (403/256) × μg of Oα).

ochratoxin A and ochratoxin α increased according to ingested amounts of ochratoxin A, with ochratoxin α being the predominant compound in feces as well as in urine. The proportional excretions of ochratoxin A and ochratoxin α in feces and urine are given in **Table 4**. The cumulative excretion of ochratoxin α is expressed in terms of ochratoxin A equivalents. The total excretion of ochratoxin A and ochratoxin α was in the range of 74−80% of the respective ochratoxin A intake. The cumulative excretion of ochratoxin A and ochratoxin α in the urine was 2−3-fold higher as compared to the excretion of ochratoxin A and ochratoxin α in the feces. However, for the highest dose of 28.5 μg ochratoxin A/kg body weight/day, the total fecal excretion increased ($P < 0.05$) from 18 to 29% of ochratoxin

A intake, and excretion in the urine showed a trend ($P = 0.1162$) for a decrease from 60 to 45% of ochratoxin A intake.

The pH of the ruminal fluid was only affected by time and showed a pattern typical for meal-fed animals (data not shown). After feeding, the pH declined to a value of 6.2 at 4 h after feeding and increased until the next feeding time to a pH of 7. The relatively high pH of the rumen fluid indicates that the animals were not suffering from a ruminal acidosis, despite being fed a high-concentrate diet.

In ruminal fluid, the average concentration of ochratoxin A (**Figure 3**) decreased with time after feeding. Ochratoxin A completely disappeared in the rumen fluid of the animals receiving 9.5 and 19 μg ochratoxin A/kg body weight/day 10−13 h after feeding, while animals receiving the highest dose still retained a low concentration of ochratoxin A in the rumen fluid until the next feeding. The concentration of ochratoxin A in the rumen fluid revealed a linear dose response ($P < 0.05$) at 1, 4, and 7 h after feeding. This dose effect disappeared at sampling times higher than 7 h. Ochratoxin α could be detected in the rumen fluid from animals given ochratoxin A at all sampling times after feeding (**Figure 3**). Except for the group receiving 9.5 μg ochratoxin A/kg body weight/day, its concentration increased with time after feeding. Within each sampling time, the concentration of ochratoxin α in ruminal fluid increased linearly with the dose of ochratoxin A. The total amount of ochratoxin A and ochratoxin α in the rumen, as determined by the AUC, increased linearly ($P < 0.05$) with increasing ochratoxin A intake. The AUC values were 0, 45.8, 169.6, and 203.0 ± 38.1 ng/mL × h for ochratoxin A and 0, 136.5, 281.3, and 501.6 ± 39.5 ng/mL × h for ochratoxin α. The half-lives for disappearance of ochratoxin A from the rumen of sheep receiving 9.5, 19, and 28.5 μg ochratoxin A/kg body weight/day were 2.60, 3.76, and 3.82 ± 1.38 h and were not affected by dose.

## DISCUSSION

During recent years, a considerable amount of data has been published indicating that ruminants have a high ruminal capacity for hydrolyzing the nephrotoxigenic mycotoxin, ochratoxin A, to the non- or less toxic metabolite, ochratoxin α (**Figure 2**). This seems to be one of the reasons for the relative resistance of ruminants to the toxic effects of ochratoxin A as compared to monogastrics. It should be pointed out, however, that most of the data was mainly derived from in vitro experiments (*4*, *6*) and only reflects the potential for ruminal degradability of ochratoxin A, as the rate of passage and absorption of ochratoxin A are not taken into account. On the other hand, it should be noted that in the few published animal studies, usually short time exposure to ochratoxin A (1−4 days) was investigated (*5*, *25*) or if chronic feeding of ochratoxin A was investigated, much higher doses than those naturally occurring in feedstuffs were fed (*14*). Therefore, the present study was performed to obtain data on the systemic availability and excretion pattern of ochratoxin A in sheep after chronically feeding moderate to low doses of ochratoxin A, as may be found also under practical farming conditions.

Our results are in full agreement with those reported by Höhler et al. (*14*), indicating that the conversion of ochratoxin A to the nontoxic metabolite ochratoxin α in sheep is incomplete and much less than reported in previous in vitro and in vivo studies (*4*, *5*). On the basis of the appearance of intact ochratoxin A in blood and urine, our results demonstrate that a substantial absorption of ochratoxin A into the systemic circulation occurs even at low doses of 9.5 μg ochratoxin A/kg body weight/day
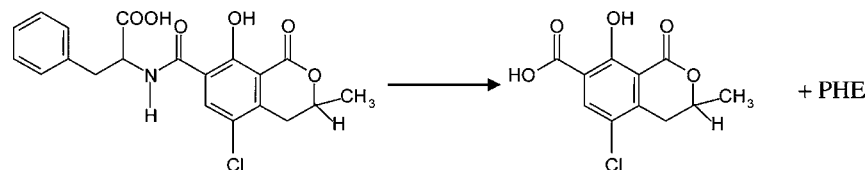
Effects of Chronic Ingestion of Ochratoxin A

*J. Agric. Food Chem.,* Vol. 51, No. 23, 2003 **6903**



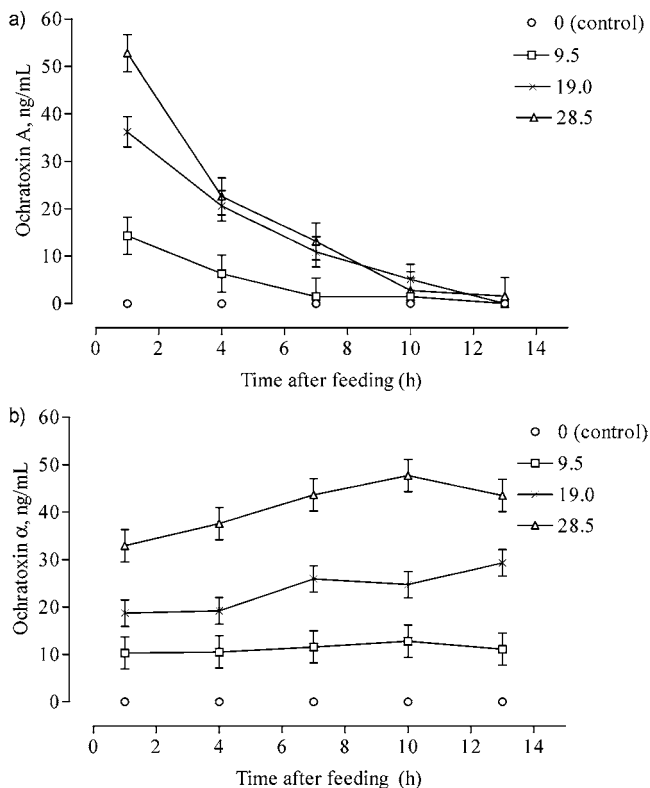**Figure 2.** Conversion of ochratoxin A to ochratoxin α and phenylalanine.



**Figure 3.** Concentrations of (**a**) ochratoxin A and (**b**) ochratoxin α in ruminal fluid (ng/mL) of sheep after feeding 0, 9.5, 19.0, and 28.5 μg ochratoxin A/kg body weight/day at various times after feeding. Each point represents least squares mean ± SEM.

(387 μg ochratoxin A/day). In addition, transfer of ochratoxin A into blood is linearly dependent on the dose of ochratoxin A fed, at least in the concentration range used in this study.

Several factors may be responsible for the differences between the results obtained in this study and those previously reported. First, in the present experiment, ochratoxin A was administered as contaminated wheat whereas previous studies used crystalline ochratoxin A (4−6). However, preliminary in vitro studies with rumen fluid comparing the degradation of naturally occurring ochratoxin A in wheat and in the free crystallized form did not show any differences arising from the source of ochratoxin A (27), confirming similar microbial access and rates of breakdown of the mycotoxin (14). Second, sensitivity of analysis of ochratoxin A has been improved over the past decades. For example, Kiessling et al. (6) fed 2 and 5 mg ochratoxin A/kg diet to sheep for a period of 4 and 2 days, respectively, but did not detect any ochratoxin A in blood at 1 h after feeding. Their detection limit for ochratoxin A was about 75 ng/mL. In contrast, in the current study, the detection limit of ochratoxin A in blood using HPLC analysis with flourescence detection was less than 1 ng/mL.

Differences in the composition and activity of the microorganisms in the rumen, due to the type of diet used, could also partially explain the different results. Conversion of ochratoxin

A into ochratoxin α appears to be mainly associated with rumen protozoa. The ability of the protozoal fraction to degrade ochratoxin A to ochratoxin α is about seven times higher as compared to the bacterial fraction (5); consequently, factors known to influence the activity and population of protozoa in the rumen may as well have effects on the metabolism of ochratoxin A.

High amounts of concentrate in the diet especially in nonadapted animals are well-known to induce ruminal acidosis, accompanied by a reduction or complete disappearance of protozoa from the rumen (28). Xiao et al. (25) compared the metabolism of ochratoxin A in sheep when fed a complete hay or a complete grain diet, which significantly decreased the rumen pH from 6.9 to 5.6. In their study, feeding the complete grain diet resulted in a 4.3-fold increase of the sytemic availabilty of ochratoxin A, delayed the disappearance of the mycotoxin from the rumen, and reduced the hydrolysis rate of ochratoxin A as compared with the hay diet.

In our own study, the rumen pH did not decrease below 6.2, indicating that the activity and number of protozoa may have been only slightly or not impaired. Nevertheless, the half-life of ochratoxin A in the rumen was comparable to the value found by Xiao et al. (25), who fed a 100% grain diet. Müller et al. (6) found a much shorter half-life of ochratoxin A in vitro when using only moderate levels of concentrate. In vitro incubations, however, are usually done with highly buffered solutions; thus, the number and activity of protozoa are unlikely to have changed due to pH shifts. A further factor that may influence the dynamics of ochratoxin A degradation and absorption within the rumen is the magnitude of feed intake. Xiao et al. (25) showed that feeding a complete grain diet at 0.5 kg/day resulted in a rumen pH of 6.5 and a 3-fold lower bioavailability of ochratoxin A as compared to that obtained by feeding a complete grain diet at an intake of 1.5 kg/days; the resulting rumen pH was 5.8. Taking these results into account, it might be presumed that a lower rumen pH due to higher feed intake may further enhance the systemic availabilty of ochratoxin A.

In aqueous medium, ochratoxin A exists in the nonionized and ionized forms because of dissociation of the phenolic hydroxy group in the dihydroisocoumarin ring (p$K$ value = 7.04) and is absorbed in a passive manner in the nonionized form, which occurs at low but not at neutral pH values in rats (29). In rats, absorption of ochratoxin A occurs in the stomach, small intestine, and large intestine, with the highest absorption rates occurring in the upper part of the small intestine (30). There is no data available with respect to absorption from the gastrointestinal tract in ruminants. As ochratoxin A exhibits characteristics of a weak acid, it seems possible that ochratoxin A is already absorbed from the rumen, whereas a decrease in ruminal pH would result in a higher absorption rate. However, in our study, the rumen pH only slightly changed indicating that absorption of ochratoxin A from the rumen would have been low. Therefore, it seems more likely that ochratoxin A bypassed the rumen and was probably absorbed in the intestine.

The long persistence of ochratoxin A in the blood of pigs and humans is directly related, in part, to the ability of plasma proteins to bind ochratoxins (31) and to reabsorption of och-

ratoxin A in the nephron (*32*). In the current study, accumulation of ochratoxin A in blood was relatively low as compared to other species. First, this may be attributed to the fact that the binding of ochratoxin A to serum albumin in cattle, pigs, and humans is 2−3 times stronger as compared to sheep, as shown by in vitro studies (*33*). Second, reabsorption in all nephron segments is dependent on the luminal pH, as shown by microperfusion studies in rats, and decreases with increasing urinary pH (*32*). As herbivores such as sheep usually have a higher urinary pH as compared to pigs, this may be one of the reasons for the quite short elimination half-life of 17.3 h in sheep found by Xiao et al. (*25*) as compared to half-lives of ochratoxin A in rhesus monkeys (510 h), pigs (72−120 h), and rats (55−120 h) reported in the literature (*3*).

In the current study, digestibilities of nutrients tended to be negatively affected by ochratoxin A intake. In the study by Höhler et al. (*14*), however, feeding much higher amounts of ochratoxin A had no effect on digestibilities in sheep. On the other hand, several studies indicate that ochratoxin A doses higher than those used in our study may induce diarrhea (*34*), which is usually accompanied by a reduction of digestibilities. However, no signs of diarrhea were observed during the entire experimental period in our study. Although significant results were obtained, our results with respect to the nutrient digestibilities should not be overemphasized due to the limited number of animals used in our study.

The current experiment demonstrates that ochratoxin A is efficiently hydrolyzed in the rumen of sheep, but degradation capacity might be considerably less than described in the literature. Doses of ochratoxin A fed in the current study did not cause signs of ochratoxicosis and may therefore be regarded as safe short-term (29 days) intake levels for sheep. However, Willis et al. (*35*) pointed out that in general long-term feeding of a subacute dose will be more critical than a single high dose with respect to mycotoxin intoxication.

Besides the toxicological aspect, the possible carry over of ochratoxin A to animal products for human consumption is a further point of concern. Previous studies indicate that ochratoxin A may only be transferred into milk from cows (*36*) when fed at levels that are much higher than those naturally occurring. However, in two recent surveys (*15*, *16*), contamination levels of 10−40 ng/L of ochratoxin A were detected in cow's milk. It is unknown, as no effort was undertaken for analysis of the corresponding feed samples, if the contamination was a result of feeding contaminated feedstuffs or by aerogen contamination of milk, a second possible way of contamination. As pointed out by Skaug (*16*), even low concentrations of ochratoxin A in cow's milk can be of importance to consumers of large quantities such as children and infants and may contribute to a significant portion of their total dietary intake. For example, the levels of ochratoxin A in cow's milk found in their investigations would have exceeded the maximum tolerable daily intake of 5 ng ochratoxin A/kg body weight/day for children as recommended by the Nordic Working group on Food Toxicology and Risk Evaluation (*37*).

In conclusion, although the capacity of ruminants to degrade ochratoxin A to its non- or less toxic metabolite ochratoxin α is quite high, the results of this study indicate that even at moderate to low levels of ochratoxin A in the diet, considerable amounts of the mycotoxin are absorbed by ruminants and may accumulate in tissues. Therefore, feeding of ochratoxin A-contaminated feedstuffs to ruminants does not seem to be a reliable means for using these feedstuffs.

## LITERATURE CITED

(1) Krogh, P. Ochratoxins in food. In *Mycotoxins in Food*; Krogh, P., Ed.; Academic Press: London, U.K., 1987; pp 97−121.

(2) Dwivedi, P.; Burns, R. B. The natural occurrence of ochratoxin A and its effect in poultry. A review. *World's Poult. Sci. J.* **1986**, *42*, 32−47.

(3) Kuiper-Goodman, T.; Scott, P. M. Risk assessment of the mycotoxin ochratoxin A. *Biomed. Environ. Sci.* **1989**, *2*, 179−248.

(4) Hult, K.; Teiling, A.; Gatenbeck, S. Degradation of ochratoxin A by a ruminant. *Appl. Environ. Microbiol.* **1976**, *32*, 443−444.

(5) Kiessling, K.-H.; Petterson, H.; Sandholm, K.; Olsen, M. Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. *Appl. Environ. Microbiol.* **1984**, *47*, 1070−1073.

(6) Müller, H.-M.; Lerch, C.; Müller, K.; Eggert, W. Kinetic profiles of ochratoxin A and ochratoxin α during *in vitro* incubation in buffered forestomach and abomasal contents from cows. *Nat. Toxins* **1998**, *6*, 251−258.

(7) Nip, W. K.; Chu, F. S. Fate of ochratoxin A in goats. *J. Environ. Sci. Health B* **1979**, *14*, 319−333.

(8) Creppy, E. E.; Kern, D.; Steyn, P. S.; Vleggaar, R.; Röschenthaler, R.; Dirheimer, G. Comparative study of the effect of ochratoxin A analogues on yeast aminoacyl-tRNA synthetases and on the growth and protein synthesis of hepatoma cells. *Toxicol. Lett.* **1983**, *19*, 217−224.

(9) Föllmann, W.; Hillebrand, I. E.; Creppy, E. E.; Bolt, H. M. Sister chromatid exchange frequency in culture isolated porcine urinary bladder epithelial cells (PUBEC) treated with ochratoxin A and alpha. *Arch. Toxicol.* **1995**, *69*, 280−286.

(10) Sreemannarayana, O.; Frohlich, A. A.; Vitti, T. G.; Marquardt, R. R.; Abramson, D. Studies of the tolerance and distribution of ochratoxin A in young calves. *J. Anim. Sci.* **1988**, *66*, 1703−1711.

(11) Li, S.; Marquardt, R. R.; Frohlich, A. A.; Vitti, T. G.; Crow, G. Pharmacokinetics of ochratoxin A and its metabolites in rats. *Toxicol. Appl. Pharmacol.* **1997**, *145*, 82−90.

(12) Chu, F. S. A comparative study of the interaction of ochratoxins with bovine serum albumin. *Biochem. Pharmacol.* **1974**, *23*, 1105−1113.

(13) Müller, K. Einfluss der Fütterung und anderer Faktoren auf den Umsatz von Ochratoxin A in Pansenflüssigkeit *in vitro* und *in vivo*. Ph.D. Thesis, Hohenheim University, Stuttgart, Germany, 1995.

(14) Höhler, D.; Südekum, K.-H.; Wolffram, S.; Frohlich, A. A.; Marquardt, R. R. Metabolism and excretion of ochratoxin A fed to sheep. *J. Anim. Sci.* **1999**, *77*, 1217−1223.

(15) Breitholtz-Emanuelsson, A.; Olsen, M.; Oskarsson, A.; Palminger, I.; Hult, K. Ochratoxin A in cow's milk and in human milk with corresponding human blood samples. *J. AOAC Int.* **1993**, *76*, 842−846.

(16) Skaug, M. A. Analysis of Norwegian milk and infant formulas for ochratoxin A. *Food Addit. Contam.* **1999**, *26*, 75−78.

(17) Xiao, H.; Marquardt, R. R.; Frohlich, A. A.; Yang, Z. L. Synthesis and structural elucidation of analogues of ochratoxin A. *J. Agric. Food Chem.* **1995**, *43*, 524−530.

(18) Gesellschaft für Ernährungsphysiologie (GfE). Mitteilungen des Ausschusses für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie: Energie-Bedarf von Schafen; Kirchgeβner, M., Ed. *Proc. Soc. Nutr. Physiol.* **1996**, *5*, 149−152.

(19) Geishauser, T.; Gitzel, A. A comparison of rumen fluid sampled by oro-ruminal probe versus rumen fistula. *Small Rumin. Res.* **1996**, *21*, 63−69.

Effects of Chronic Ingestion of Ochratoxin A

*J. Agric. Food Chem.,* Vol. 51, No. 23, 2003 **6905**

(20) Bassler, R., Ed. *Die chemische Untersuchung von Futtermitteln. Methodenbuch*; VDLUFA: Darmstadt, Germany, 1993; Vol. III, Suppl. 1−3.

(21) Van Soest, P. J.; Robertson, J. B.; Lewis, B. A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583−3597.

(22) Brandt, M.; Schuldt, A.; Mannerkorpi, P.; Vearasilp, T. Zur enzymatischen Stärkebestimmung im Darminhalt und Kot von Kühen mit hitzestabiler Amylase. *Arch. Anim. Nutr.* **1987**, *37*, 455.

(23) Clarke, J. R.; Marquardt, R. R.; Oosterverld, A.; Frohlich, A. A.; Madrid, F. J.; Dawood, M. Development of a quantitative and sensitive enzyme-linked immunosorbent assay for ochratoxin A using antibodies from the yolk of the laying hen. *J. Agric. Food Chem.* **1993**, *41*, 1784−1789.

(24) Hult, K.; Hokby, E.; Hagglund, U.; Gatenbeck, S.; Rutqvist, L.; Sellyey, G. Ochratoxin A in pig blood: Method of analysis and use as a tool for feed studies. *Appl. Environ. Microbiol.* **1979**, *38*, 772−776.

(25) Xiao, H.; Marquardt, R. R.; Frohlich, A. A.; Philipps, G. D.; Vitti, T. G. Effect of a hay and a grain diet on the bioavailability of ochratoxin A in the rumen of sheep. *J. Anim. Sci.* **1991**, *69*, 3715−3723.

(26) Xiao, H.; Marquardt, R. R.; Abramson, D.; Frohlich, A. A. Metabolites of ochratoxins in rat urine and in a culture of *Aspergillus ochraceus. Appl. Environ. Microbiol.* **1996**, *62*, 648−655.

(27) Blank, R.; Münster, Y.; Westphal, A.; Wolffram, S. Comparison of ruminal degradation of ochratoxin A from different sources using the Hohenheim gas test. *Proc. Soc. Nutr. Physiol.* **2002**, *11*, 94.

(28) Abe, M.; Shibui, H.; Iriki, T.; Kumeno, F. Relation between diet and protozoal population in the rumen. *Br. J. Nutr.* **1973**, *29*, 197−202.

(29) Kumagai, S. Effects of plasma ochratoxin A and luminal pH on the jejunal absorption of ochratoxin A in rats. *Food. Chem. Toxicol.* **1988**, *26*, 753−757.

(30) Kumagai, S.; Aibara, K. Intestinal absorption and secretion of ochratoxin A in the rat. *Toxicol. Appl. Pharmacol.* **1982**, *64*, 94−102.

(31) Hagelberg, S.; Hult, K.; Fuchs, R. Toxicokinetics of ochratoxin A in several species and its plasma-binding properties. *J. Appl. Toxicol.* **1989**, *9*, 91−96.

(32) Dahlmann, A.; Dantzler, W. H.; Silbernagl, S.; Gekle, M. Detailed mapping of ochratoxin A reabsorption along the rat nephron *in vivo*: The nephrotoxin can be reabsorbed in all nephron segments by different mechanisms. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 157−162.

(33) Krogh, P. Role of ochratoxin in disease causation. *Food Chem. Toxicol.* **1992**, *30*, 213−224.

(34) Schuh, M.; Schweighardt, H. Ochratoxin A—ein nephrotoxisch wirkendes Mykotoxin. *Übers. Tierernährg.* **1981**, *9*, 33−70.

(35) Willis, R. M.; Mulvihill, J. J.; Hoofnagle, J. H. Attempted suicide with purified aflatoxin. *Lancet* **1980**, *8179*, 1198−1199.

(36) Ribelin, W. E.; Fukushima, K.; Still, P. E. The toxicity of ochratoxin A to ruminants. *Can. J. Comput. Med.* **1978**, *42*, 172−176.

(37) Nordic Working Group on Food Toxicology and Risk Evaluation. *Health Evaluation of Ochratoxin A in Food Products*; Report No. 545; Nordic Council of Ministers: Copenhagen, Denmark, 1991.

JF034547J