

## Ochratoxin A as the Cause of Spontaneous Nephropathy in Fattening Pigs

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At a number of slaughters nephropathy and high ochratoxin A contents in kidneys have been observed in fattening pigs from two Swedish farms. In one herd the source of contamination was barley grown on the home farm and stored under such conditions that the growth of fungal species (*Penicillium verrucosum* var. *verrucosum*) producing ochratoxin A occurred, with the subsequent formation of the toxin. In this case high ochratoxin A levels in fattening pigs were found during a period of about 18 months. In the second herd, where compounded feed was used, it was impossible to locate the source of contamination. It was presumed that a consignment of feed was damaged by rain during storage at the farm. Ochratoxin A was found in fattening pigs from this herd for a period of about 2 months. Ochratoxin A appeared in the kidneys of all investigated pigs. In some animals the livers, whole blood, and plasma were analyzed, too. The livers contained somewhat lower amounts of ochratoxin A than the kidneys, whereas the content in whole blood and plasma, respectively, was 5 and 13 times greater. Kidneys spontaneously contaminated with ochratoxin A, when stored for 10 months at  $-70^{\circ}\text{C}$ , showed no systematic decrease in toxin content.

The spontaneous appearance of ochratoxin A, a secondary toxic metabolite of several *Aspergillus* and *Penicillium* species (6, 8, 18), has been reported in various cereal products from several countries (6), including Denmark (13) and Sweden (12). Such appearance has occasionally been associated with spontaneous porcine and avian nephropathy (3, 4, 9, 10, 13, 14, 17).

An investigation at slaughter in Sweden regarding ochratoxin A in macroscopically abnormal kidneys from pigs that appeared healthy revealed ochratoxin A contents of  $\geq 2$  ng/g in kidneys from 25% of the animals examined (17). A similar investigation in Denmark (9) showed that 35% of the investigated animals had ochratoxin A contents of  $\geq 2$  ng/g in the kidneys.

During the course of the Swedish investigation, two herds of pigs were discovered in which, in a number of slaughterings, various degrees of nephropathy were found at a high frequency. Ochratoxin A contents in internal organs from such pigs and the conditions in the herds have been studied.

### MATERIALS AND METHODS

**Animals.** The pigs investigated originated from two small herds in the northern part of Sweden.

(i) **Herd 1.** About 150 pigs are produced annually for slaughtering from this herd. The herd is recruited

by the purchase of piglets. Feeding was carried out with barley grown on the home farm and mixed with a purchased pig feed concentrate.

From the autumn of 1975 to the summer of 1977 it was noticed at slaughter that the animals' kidneys were somewhat larger, lighter in color, and firmer than normal. Kidneys, and in some cases livers and blood, from pigs slaughtered during 1976 and the spring of 1977 were removed for investigation of the presence of ochratoxin A.

During the spring of 1976 the herd's owner noticed that for a short period the growth rate decreased and the animals seemed to be somewhat thirstier than normal. Otherwise there had been no symptoms of disease or any mortality.

(ii) **Herd 2.** In this herd approximately 150 to 200 pigs annually are reared for slaughtering, these being recruited from the farm's own breeding. Pigs for slaughter are fed on purchased compounded feed. The feed was delivered in bulk loads about every 2 weeks and stored in a wooden silo. The silo was in poor condition and did not protect the feed from rain.

In the spring of 1977 it was observed during some slaughterings that kidneys of the slaughtered pigs were enlarged, lighter in color, and of a firmer consistency than normal. On two occasions kidneys were removed from slaughtered pigs to investigate any presence of ochratoxin A.

The keeper of the herd had not noticed any symptoms of disease in the pigs. Neither increased thirst nor delayed growth had been observed, and there had been no deaths.

**Organs.** Immediately after slaughtering, the kidneys and livers were cooled and stored at 4 to 5°C until specimens had been removed (usually within 24 h) for histological examination. After this the organs were stored at -70°C until the determination of ochratoxin A could be carried out (within 14 to 45 days). At two slaughters blood from two pigs, originating from herd 1, was analyzed for the presence of ochratoxin A. At the first occasion the blood was immediately frozen and stored at -70°C until analyzed (within 30 days). In the second case heparin was added to the blood. Whole blood and plasma were separated by centrifuging and stored at -70°C until analysis (within 10 days).

**Feed.** Analysis for ochratoxin A was done on barley of the 1975 and 1976 harvests, used for feed in herd 1. In the case of herd 2, feed from the storage silo was analyzed.

**Pathology.** After macroscopic examination of the kidneys and livers, specimens were removed and, respectively, frozen or fixed in 10% formaldehyde. Sections from frozen specimens were stained with scarlet red, and sections from fixed, paraffin-embedded material were stained with hematoxylin-eosin and with van Gieson and periodic acid-Schiff stains.

**Ochratoxin A analysis.** Extraction of ochratoxin A was carried out by using the technique described by Nesheim et al. (15), modified for tissue material by Krogh et al. (10). Specimens of 25 g of organ tissue were acidified with 0.1 M phosphoric acid to pH 2 to 3 and homogenized in an Ultra-Turrax homogenizer (11,000 rpm). The homogenate was shaken up with 125 ml of chloroform for 30 min and centrifuged (6,500 × g for 30 min) to obtain the chloroform extract.

The analysis of feed was carried out on 50 g of ground material, to which was added 25 ml of 0.1 M phosphoric acid and 250 ml of chloroform. After shaking for 30 min and filtering through a paper filter, a clear chloroform extract was obtained.

In determining the ochratoxin A content in the chloroform extract the technique described by Hult and Gatenbeck (7) was used. Ochratoxin A was transferred from the chloroform extract via bicarbonate solution to a 0.04 M tris(hydroxymethyl)aminomethane-hydrochloride solution (pH 7.5).

The ochratoxin A was hydrolyzed in the tris(hydroxymethyl)aminomethane-hydrochloride buffer

solution to ochratoxin α and phenylalanine using carboxypeptidase A. The cleavage was followed spectrophotofluorometrically, and the ochratoxin A content was calculated from changes in fluorescence intensity (7).

**Fungi.** Barley from the 1975 harvest, used for feed in herd 1, was cultured for fungi. From 10 g of barley a 10-fold dilution series was made with 0.9% NaCl solution as the diluent. From this series cultures were grown on Sabouraud agar (with the addition of 20 IU of penicillin and 40 μg of streptomycin per ml [pH 7]). Fungi strains growing on the substrate were sent for typing to Centralbureau voor Schimmelcultures, Baarn, Holland.

The ochratoxin A-producing abilities of the isolated fungi strains were investigated with autoclaved barley as a substrate (100 g of barley + 20 ml of sterile water) at 25°C in the dark.

## RESULTS AND DISCUSSION

**Pathology. (i) Kidneys.** Altogether, kidneys from 34 pigs were investigated (22 pigs from herd 1 and 12 from herd 2). With the exception of kidneys from two pigs originating from herd 1, all kidneys showed macroscopic changes consisting of lighter color and a firmer consistency than would be expected from normal kidneys. Many kidneys were also obviously enlarged.

In the kidneys that did not show any macroscopic changes, no histological changes were discovered either. In spite of the absence of demonstrable changes a content of 32 ng of ochratoxin A per g was measured in these kidneys (Table 1). This agrees with earlier observations made in connection with experimental feeding of pigs (10, 11), where similar ochratoxin A contents were found without significant kidney changes.

In the kidneys from all of the remaining 32 pigs investigated, typical changes for ochratoxin A were demonstrated by tubulus degeneration and fibrous interstitial nephritis. The degree of changes varied from slight to severe, the grading being made in relation to the number of fibrous

TABLE 1. Herd 1: occurrence of ochratoxin A in organs and barley

Date of slaughter	No. of pigs	Ochratoxin A (ng/g) <sup>a</sup>				
		Kidneys	Livers	Whole blood	Plasma	Barley
March 1976	6	140 ± 4	— <sup>b</sup>	—	—	—
April 1976	5	100 ± 10	—	—	—	4,300
December 1976	2	49 ± 3	—	—	—	—
January 1977	6 <sup>c</sup>	72 ± 6	65	—	—	20
March 1977	2	73 ± 2	60 ± 0	335 ± 85 <sup>d</sup>	—	—
June 1977	2	32 ± 1	26 ± 7	175 ± 15 <sup>e</sup>	410 ± 20	—
December 1977						<2

<sup>a</sup> Mean with standard error of the mean.

<sup>b</sup> —, Not investigated.

<sup>c</sup> Five kidneys and one liver.

<sup>d</sup> Blood was frozen.

<sup>e</sup> Blood was heparinized.

areas and their spread. Cases with occasional fibrous foci, usually located at the boundary between the cortex and the medullary layers, were described as slight. Severe changes were characterized by a fibrous conversion of the greater part of the kidney's cortex and medullary layers.

In proximal tubuli between fibrous foci, a decrease in the thickness of the brush borders was observed together with slight degenerative changes of the epithelial cells. Within fibrous areas the tubuli were wrinkled and atrophic with periodic acid-Schiff-positive and thickened basal membranes.

In severe cases with widely spread fibrosis, the tubuli had disappeared to a large extent. Residual tubuli often were remarkably dilated (Fig. 1 and 2).

Many of the kidneys in the present investigation showed severe changes that can be assessed as irreversible. However, pigs for slaughter have a life-span of only some 5 to 6 months. This situation, together with the kidneys' large compensatory capacity, explains why the animals' general condition in spite of severe nephropathy is often apparently unchanged or only slightly affected. But should nephropathy strike a herd where the animals are reared for breeding purposes and thus can be considered to have a much longer life than slaughter animals, there is an obvious risk of producing poorer breeding stock.

(ii) **Livers.** In none of the five livers investi-

gated (Table 1) were macroscopic or microscopic changes observed.

**Ochratoxin A.** The ochratoxin A contents in kidneys, livers, blood, and plasma from pigs of the two herds investigated are presented in Tables 1 and 2. The livers examined showed somewhat lower contents than the kidneys; this agrees with the results of earlier investigations in conjunction with experimental feeding (10, 11).

Compared to kidneys, high contents of ochratoxin A were shown in whole blood and plasma (Table 1). The contents in whole blood and plasma were, respectively, 5 and 13 times greater than in kidneys. This agrees with an earlier observation (16) where the ochratoxin A content in pig kidneys was 430 ng/g and, in serum, 6,990 ng/ml (ratio 1:16) after an experimental oral administration of 0.38 mg of ochratoxin A per kg of body weight per day for 8 days. In experiments with rats it is reported that large amounts of ochratoxin A appear in blood (5, 19). Conjugation of ochratoxin A to bovine albumin has been demonstrated in *in vitro* experiments (2), and it has been calculated (5) that rat plasma has a maximal binding capacity of 70,000 ng of ochratoxin A per ml. It thus appears that the greater part of ochratoxin A in the organism is deposited in the form of an albumin complex and that only a smaller part of the total quantity appears in other forms.

In spite of the considerable amounts of ochra-

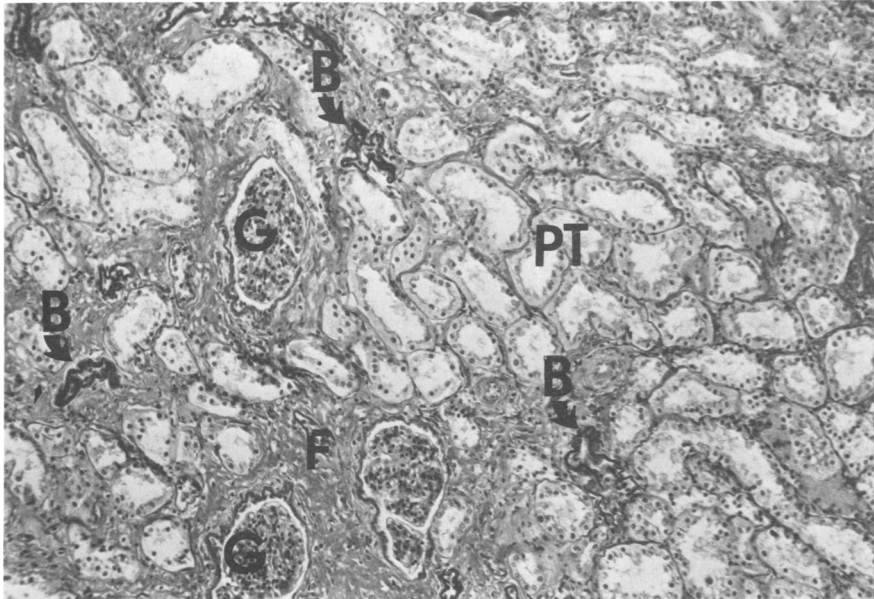


FIG. 1. Moderate nephropathy in a pig kidney (P 1528/76) with an ochratoxin A content of 150 ng/g. Periodic acid-Schiff stain;  $\times 100$ . F, Fibrous foci around glomeruli (G); B, atrophic tubuli with stain-positive and thickened basal membranes; PT, proximal tubuli.

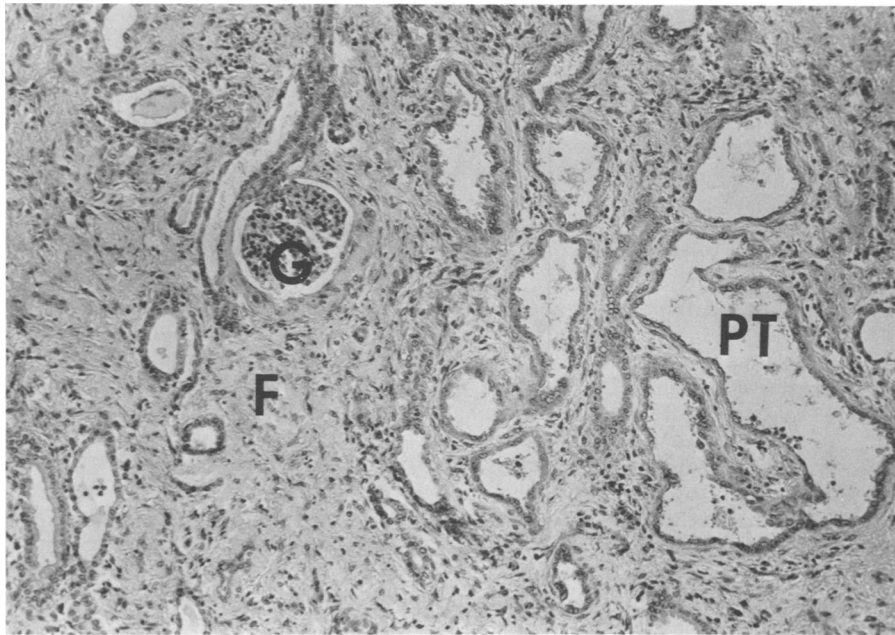


FIG. 2. Severe nephropathy in a pig kidney (P 1531/76) with an ochratoxin A content of 155 ng/g. van Gieson stain;  $\times 100$ . F, Fibrosis; G, glomerulus; PT, dilated proximal tubuli.

TABLE 2. Herd 2: occurrence of ochratoxin A in organs and feed

Date of slaughter	No. of pigs	Ochratoxin A (ng/g) <sup>a</sup>		
		Kidneys	Whole blood	Feed samples
September 1977	6	218 $\pm$ 43	—	—
October 1977	6	91 $\pm$ 16	—	—
December 1977				<2
January 1978	3	—	<2	

<sup>a</sup> Mean with standard error of the mean. —, Not investigated.

toxin A in internal organs and sometimes severe nephropathy shown in pigs from the herds investigated, the clinical manifestation of ochratoxicosis has been amazingly small. This circumstance is a determining factor why herds of pigs whose feed happens to be contaminated with ochratoxin A may repeatedly for a long time deliver slaughter pigs with high contents of this mycotoxin in internal organs. In one of the cases presented here, where one year's whole harvest of home-grown barley intended for the farm's production of pigs was contaminated, high contents of ochratoxin A could be shown in internal organs for a period of about 18 months. With knowledge of the distribution of ochratoxin A in various body tissues of pigs (10, 16), it is apparent that in the present case the mycotoxin

passed into the food chain even though the abnormal kidneys were destroyed.

Tracing of ochratoxicosis in a pig herd nowadays is usually carried out by registering the presence of macroscopically abnormal kidneys at slaughter, this being followed by an investigation of the ochratoxin level in the renal material. Normally, feeding of a herd of pigs can be regarded as uniform. The investigation of renal tissue from only one or two individuals from the same herd can be expected to provide more accurate and more direct information on the relation between the presence of ochratoxin A in the herd's feed and the animals' body tissues than would be provided by an analysis of a comparable number of feed samples. Kidneys have therefore in meat inspection been suggested as indicator tissue for confirmation of the presence of ochratoxin A (11).

The present investigation has shown that the highest ochratoxin A content is found in the blood. Access to a rapid and sensitive method for the analysis of ochratoxin A in blood should thus make it possible to carry out clinical investigations on live animals when ochratoxicosis is suspected and also comprehensive frequency studies to discover periodically the extent of the presence of ochratoxin A in feed. Work on a method for routine analysis of ochratoxin A in blood is under way, and existing results suggest that such a method may shortly be available.

**Stability of ochratoxin A in kidneys after freezing.** Storage of kidneys for 10 months at  $-70^{\circ}\text{C}$  showed that the values of the remaining ochratoxin A content did not significantly differ from the original values (Table 3). This result seems to be contrary to earlier observations (17) where 54 days of storage at  $-70^{\circ}\text{C}$  produced an approximately 20% decrease in the ochratoxin A content. In the latter case, however, the kidneys had been experimentally contaminated by injections of ochratoxin A. Therefore it seems probable that the ochratoxin A found spontaneously in organs has a greater stability than the ochratoxin A experimentally injected into a dead tissue and thus forming a depot.

**Ochratoxin A in feed.** The occurrence of ochratoxin A in feed from the two herds examined is shown in Tables 1 and 2. Herd 1 was fed on barley grown on the home farm, and the investigation shows that the barley from the 1975 harvest was highly contaminated with ochratoxin A. The surplus barley from the harvest of 1975 was mixed with barley from the 1976 harvest, which meant that ochratoxin A could be found in slaughter pigs from the herd for a long period. It can be presumed that this mixing of grain took place quite often and in varying proportions. The 20-ng/g content measured on one occasion in such a mixture is therefore not representative for the period when barley of both the 1975 and 1976 harvests was fed together. No ochratoxin A ( $<2$  ng/g) was demonstrated in barley from the 1977 harvest.

In herd 2, no source for the appearance of ochratoxin A in pigs for slaughtering could be discovered. Compounded feed was bought from a feed factory for this herd. Feed was delivered from the factory about every 2 weeks and was stored in a wooden silo, which was in a poor condition. It is considered possible that a delivery during the summer of 1977, when there was

a lot of rain, was damaged by water during storage, with resultant growth of ochratoxin A-forming fungi. It is considered less likely that the feed was primarily contaminated when delivered from the factory, since no kidney damage was observed in pigs from other herds also using feed from the same factory during the same period.

Variations in ochratoxin A content in kidneys from slaughter pigs of herd 2 were much greater than in herd 1. This supports the hypothesis that pigs in herd 2 were subjected to a brief and unhomogeneous intake of the toxin. The relatively rapid disappearance of ochratoxin A from internal organs of slaughter pigs in this herd also suggests a temporary appearance of ochratoxin A in the feed.

**Isolation of fungal species.** In barley from the 1975 harvest used for feeding herd 1, an abundance of *Penicillium verrucosum* var. *verrucosum* and *Penicillium verrucosum* var. *cyclopium* were found. Both *Penicillium* species produced similar colonies on Sabouraud agar. After 7 to 12 days of cultivation at  $20^{\circ}\text{C}$ , however, the *P. verrucosum* var. *verrucosum* in agar produced a greenish-yellow fluorescence seen under UV light (350 nm), but this was not the case for *P. verrucosum* var. *cyclopium*. After a 9-day cultivation on barley as a substrate, the *P. verrucosum* var. *verrucosum* had produced 400 ng of ochratoxin A per g. *P. verrucosum* var. *cyclopium* failed to produce this toxin ( $<2$  ng/g).

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TABLE 3. Ochratoxin A content of kidneys from pigs before and after 10 months of storage at  $-70^{\circ}\text{C}$

Kidney	Ochratoxin A (ng/g)		Remainder after 10 months at $-70^{\circ}\text{C}$ (%)
	Before storage	After storage	
P 1528/76	150	155	103
P 1530/76	140	150	107
P 1532/76	130	110	85
P 2292/76	115	140	122
P 1533/76	135	125	93
P 1529/76	140	125	89
P 2293/76	75	80	107
P 2290/76	80	90	113
Mean <sup>a</sup>			102 $\pm$ 5

<sup>a</sup> Mean with standard error of the mean.

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