

Evaluation of the renal effects of experimental feeding of melamine and cyanuric acid to fish and pigs

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Objective—To determine whether renal crystals can be experimentally induced in animals fed melamine or the related triazine compound cyanuric acid, separately or in combination, and to compare experimentally induced crystals with those from a cat with triazine-related renal failure.

Animals—75 fish (21 tilapia, 24 rainbow trout, 15 channel catfish, and 15 Atlantic salmon), 4 pigs, and 1 cat that was euthanized because of renal failure.

Procedures—Fish and pigs were fed a target dosage of melamine (400 mg/kg), cyanuric acid (400 mg/kg), or melamine and cyanuric acid (400 mg of each compound/kg) daily for 3 days and were euthanized 1, 3, 6, 10, or 14 days after administration ceased. Fresh, frozen, and formalin-fixed kidneys were examined for crystals. Edible tissues were collected for residue analysis. Crystals were examined for composition via Raman spectroscopy and hydrophilic-interaction liquid chromatography–tandem mass spectrometry.

Results—All animals fed the combination of melamine and cyanuric acid developed gold-brown renal crystals arranged in radial spheres (spherulites), similar to those detected in the cat. Spectral analyses of crystals from the cat, pigs, and fish were consistent with melamine-cyanurate complex crystals. Melamine and cyanuric acid residues were identified in edible tissues of fish.

Conclusions and Clinical Relevance—Although melamine and cyanuric acid appeared to have low toxicity when administered separately, they induced extensive renal crystal formation when administered together. The subsequent renal failure may be similar to acute uric acid nephropathy in humans, in which crystal spherulites obstruct renal tubules. (*Am J Vet Res* 2008;69:1217–1228)

In spring 2007, The US FDA received a large number of consumer complaints regarding dogs and cats that developed renal failure, purportedly associated

ABBREVIATION	
M&CA	Melamine and cyanuric acid

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with pet food that was eventually determined to be adulterated with melamine and related compounds.¹⁻⁴ The contamination was traced to wheat gluten and rice protein concentrate imported from China. These products were actually poor-quality wheat and rice products, laced with high concentrations of nitrogen-rich melamine and melamine-related s-triazine compounds such as cyanuric acid, which can be added to increase the apparent concentrations of protein in the ingredients. Isolation and analysis of various particle types from the suspect wheat glutes led to the identification of pure melamine, several pure s-triazine compounds, and melamine-cyanurate complex, the latter of which results from the formation of hydrogen bonds between molecules of melamine and cyanuric acid (Figure 1). When melamine and the s-triazines were identified as possible causative agents, the FDA immediately began to develop chemical methods to detect melamine-related s-triazine compounds in the ingredients of food

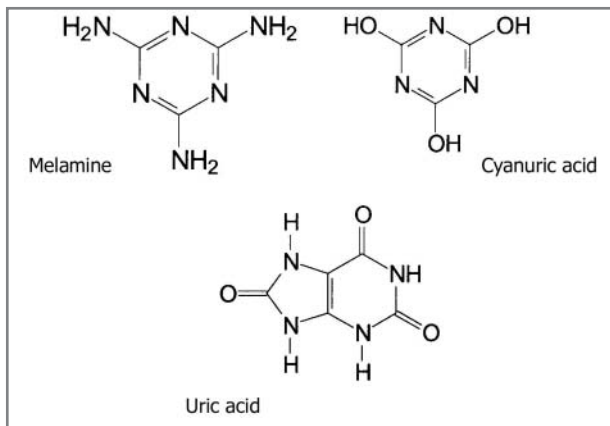


Figure 1—Diagram of chemical structures of some triazines and related compounds. Hydrogen bonds can form between melamine and cyanuric acid, contributing to the crystal formation of melamine-cyanurate. Uric acid can also form crystalline structures that form spherulite aggregates.

for humans and other animals. A method involving gas chromatography in combination with mass spectrometry was developed jointly by several FDA laboratories to analyze flour, wheat gluten, and other food ingredients for adulterants.⁵

The contamination was not limited to pet foods. By-products from pet food manufactured with adulterated wheat flour had been used in chicken and hog feeds.^{3,6,7} Melamine was also detected in fish feeds but at concentrations lower than those detected in pet food.⁸

In response to the discovery that food-producing animals had consumed animal feed that contained melamine and related compounds, the FDA and USDA began a joint effort to develop methods capable of specifically detecting melamine and cyanuric acid in edible animal products that are consumed by the public. These detection procedures were first validated by use of tissues to which the test compounds had been added. Later, however, the new methods needed to be tested on tissues obtained from animals that ingested and metabolized the compounds to ensure that in vivo metabolism would not interfere with the identification of those compounds in edible tissues. Therefore, in May 2007, the FDA Center for Veterinary Medicine conducted a feeding trial in pigs and 4 species of fish to obtain incurred residues of melamine and cyanuric acid in animal tissue for the validation of the newly developed methods. Results of the residue study⁹ are reported elsewhere.

In addition to administering melamine and cyanuric acid to animals and harvesting tissues to validate the newly developed chemical detection methods, our research group examined kidneys from the animals in the FDA study for pathologic changes. This was done because triazine-associated renal failure in pets had been associated with the development of renal and urinary crystals, and crystals in the kidneys of pets that had consumed recalled pet food contained melamine, cyanuric acid, and other triazines.^{10,a} Experiments conducted at the FDA and Procter and Gamble laboratories revealed that melamine solution mixed with cyanuric acid solution generates melamine-cyanurate crystals. The morphology and chemical nature of the resultant

melamine-cyanurate crystals were consistent with the crystals detected in kidneys of cats and dogs with triazine-associated renal failure.

The purpose of the study reported here was to describe renal pathologic effects and crystal formation in fish and pigs that received melamine, cyanuric acid, or a combination of M&CA (1:1 ratio). Another objective was to compare the crystals detected in fresh, frozen, and formalin-preserved kidneys of pigs and fish that received M&CA with those from a cat that was euthanatized after developing renal failure after eating recalled pet food.

Materials and Methods

Animals—Mature tilapia (*Oreochromis* spp; body weight, 350 to 1,550 g), channel catfish (*Ictalurus punctatus*; body weight, 725 to 2,675 g), rainbow trout (*Oncorhynchus mykiss*; body weight, 650 to 1,100 g), and Atlantic salmon (*Salmo salar*; body weight, 1,000 to 2,450 g) were acclimated to 1,892-L tanks in the laboratory for at least 2 months prior to this study. Tilapia and catfish were housed in recirculating systems at $24 \pm 2^\circ\text{C}$, while salmon and trout were maintained at $17 \pm 2^\circ\text{C}$. Concentration of dissolved oxygen and pH were continuously monitored with electronic meters^b and maintained at 8.1 ± 1.2 mg/L and 7.5 ± 0.4 mg/L, respectively. Castrated male Yorkshire-cross pigs (age, approx 16 weeks old; body weight, approx 50 kg) were obtained from a commercial source and acclimated to a research barn in separate pens for 2 weeks. Frozen tissues were submitted to the FDA Center for Veterinary Medicine. The study protocol was approved by the Office of Research Animal Care and Use Committee of the FDA Center for Veterinary Medicine.

One 11.5-year-old neutered male domestic short-hair cat from a household of 7 cats, 6 of which developed renal failure after consuming recalled pet food in March 2007, was also included for comparison. The cat had been evaluated by the veterinarian for a 4-day history of anorexia and vomiting. Physical examination revealed the cat had severe gingival and mild tongue ulcerations and was moderately dehydrated. Serum concentrations of urea nitrogen (330 mg/dL; reference range, 14 to 36 mg/dL), creatinine (32.2 mg/dL; reference range, 0.6 to 2.4 mg/dL), and phosphorus (22.1 mg/dL; reference range, 2.4 to 8.2 mg/dL) were high. The cat was euthanatized after becoming anuric following 5 days of IV treatment with fluids. No gross lesions were detected during necropsy.

Experimental exposure of animals to melamine and cyanuric acid—A target dosage of 400 mg of melamine/kg, 400 mg of cyanuric acid/kg, or 400 mg of each compound/kg was administered to trout, salmon, and catfish daily for 3 days via intragastric tubing. Actual dosages calculated on the basis of body weight at necropsy ranged from 390 to 452 mg/kg for trout, 376 to 422 mg/kg for salmon, and 418 to 479 mg/kg for catfish, with the exception of 2 catfish that received only 300 mg of melamine/kg. Because 2 salmon died prior to their scheduled date of euthanasia, a target dosage of 200 mg each of melamine and cyanuric acid/kg was administered to 2 additional salmon. In a follow-up dosing regimen, 3 trout were given 3 daily doses of melamine (20 mg/kg) via intragastric tub-

ing, followed by a 6-day interval, and then 1 dose of cyanuric acid (20 mg/kg), followed by a 3-day interval before euthanasia.

For tilapia, capsules (size 4) were filled with 150 mg of melamine, 150 mg of cyanuric acid, or 75 mg of each compound and embedded in a gelatin-based fish-food nugget that the tilapia had been trained to eat. Actual dosages ranged from 300 to 456 mg/kg for melamine, 147 to 390 mg/kg for cyanuric acid, and 39 to 114 mg/kg for the combination. Fish were observed to ensure they consumed the nuggets; incidents of damaged or rejected capsules were recorded.

The number of fish used at each time point varied depending on the amount of tissue needed to develop the methods for detecting chemical residues in another study⁹ (Appendix). The target dosage was chosen on the basis of concentrations detected in some samples of adulterated pet food and dosages used in preliminary feeding trials in rats.^a Fish received the compounds daily for 3 days to ensure the compounds would reach detectable concentrations in muscle tissue for subsequent analysis. Fish were removed from their acclimation tanks and used as untreated control animals (1 tilapia, 1 catfish, 2 trout, and 2 salmon) or placed in isolated treatment tanks prior to administering the compounds.

For pigs, compounds were mixed into a paste with chocolate pudding. Pigs were given melamine (400 mg/kg; n = 1), cyanuric acid (400 mg/kg; 1), or melamine and cyanuric acid (400 mg of each compound/kg; 1) once a day for 3 days. To obtain control tissues, 1 pig that was from the same cohort as the other pigs but did not receive melamine or cyanuric acid was euthanatized after the acclimation period.

Necropsy and microscopic examination—Fish were euthanatized on days 1, 3, 6, 10, or 14 after dosing ceased by rapid severing of the cervical spine and double pithing. These time points were chosen to provide various concentrations of residues in the muscle tissues for the collaborating chemists involved in assay validation in another study.⁹ Necropsies of all animals were performed to identify any gross abnormalities. Samples of muscle and kidney tissues were frozen at -70°C , pending chemical analysis. Samples of kidney tissues were obtained for microscopic and histologic examination. Wet-mount sections of kidney (2×3 -mm slices compressed between 2 glass slides) were immediately evaluated via light microscopy for presence of crystals.

Pigs were euthanatized 1 day after administration of compounds ceased via captive bolt, followed by exsanguination. Blood samples were collected from control and treated pigs, and serum was stored at -80°C until analyzed for blood concentrations of urea nitrogen and creatinine by use of a veterinary biochemical analyzer.^d All tests were performed in triplicate, except those of pigs that received M&CA, for which tests were performed in quadruplicate. Specimens of muscle and kidney tissues were collected for chemical analysis, and wet-mount sections of kidney specimens were evaluated as described for fish.

Histologic examination—Sections of kidney tissue from fish, pigs, and the cat were stored at -20°C and

fixed in neutral-buffered 10% formalin. Formalin was replaced with 70% ethanol after 24 hours. Other tissues were preserved when lesions were detected. Tissues were processed for routine histologic evaluation, and sections (5 to 6 μm) were stained with H&E or Gomori methylamine silver stain.¹¹ To determine if the crystals would dissolve in formalin, select frozen sections of fish and cat kidneys were cut to widths of 5 to 6 μm and examined unstained. Thicker sections of frozen cat kidney (sliced by hand as for the wet-mount preparations) were also prepared. Unstained slides were photographed and then flooded with neutral-buffered 10% formalin. The same fields were evaluated and photographed at timed intervals up to 70 hours after treatment with formalin.

Analysis of tissue residues—To determine whether measurable residues of melamine or cyanuric acid would be detectable in fish exposed to these compounds and how renal crystal formation affected those residue concentrations, fish muscle tissues were analyzed for the presence of melamine with a method developed for quantitative determination and confirmation of melamine residues in fish.¹² Briefly, residues were extracted from muscle (or muscle with skin in the salmonids) and the kidneys of 3 salmon with a solution of acetonitrile and water (ratio of 50:50) and 1N HCl and purified via solid-phase extraction cartridges. Extracts were subsequently analyzed via liquid chromatography–tandem mass spectrometry with hydrophilic-interaction liquid chromatography and electrospray ionization. Fish muscle tissues were also analyzed for cyanuric acid residues. Residues were extracted from muscle tissue with hot acetic acid, defatted with hexane, and cleaned by use of solid-phase extraction cartridges.^e Extracts were analyzed via liquid chromatography and tandem mass spectrometry by use of a porous graphitic carbon column^f and electrospray ionization in negative ion mode.⁹

Feed analyses—Feeds that fish and pigs had received during routine care were analyzed for melamine and cyanuric acid to determine potential background concentrations. Feed samples were extracted with dilute HCl. Melamine was cleaned via cation-exchange solid-phase extraction, and cyanuric acid was cleaned via solid-phase extraction with graphitized carbon cartridges.^d Extracts were analyzed by use of zwitterionic hydrophilic-interaction liquid chromatography in combination with tandem mass spectrometry, which was a method that was capable of detecting both compounds in 1 run.

Crystal composition—Prior to analyzing the renal crystals from the cat, we grew crystals *in vitro* in controlled conditions to determine if their morphology was similar to the melamine- and s-triazine-containing particles that the FDA had detected in the adulterated wheat glens. First, the pure components melamine, ammeline, ammelide, and cyanuric acid were separately recrystallized from an aqueous solution by spotting a glass slide with the appropriate solution and drying under vacuum at 80°C .

In addition to recrystallization of pure compounds, crystals were grown from various mixtures of melamine

and s-triazines (1:1 ratios), including melamine and cyanuric acid, melamine and ammeline, ammeline and cyanuric acid, and ammelide and cyanuric acid. Appropriate ratios of solutions of pure compounds were placed into 1-mL vials and mixed. Mixed solutions were spotted on glass slides and dried under vacuum at 80°C. Dried crystals were then examined via light microscopy and micro-Raman spectroscopy.⁸

Optical microscopic and micro-Raman spectroscopic analyses were used to determine the composition of the crystals in the kidney from the cat. Raman spectra of embedded crystals in cryosections of the kidney were obtained by generating a Raman chemical map over the entire area of each crystal. Total mapping area was 35 × 41 μm with a step size of 2 μm. Raman spectra were obtained at each sampling point across the crystal by use of a Raman spectrometer⁹ equipped with a 785-nm laser and a charge-coupled device detector. Raman spectra were recorded by use of a 100X-magnification glass objective lens, a grating with 150 lines/mm, and a slit of 200 μm for a spectral resolution of 5 cm⁻¹. The confocal hole was set at 800 μm. The spectral integration time for each sampling point was 10 seconds and was averaged twice. Spectra of the crystals were compared with spectra obtained from the recrystallizations and crystal mixtures. Crystals from a fish and pig that received M&CA were also examined by micro-Raman spectrometric analysis.

Zwitterionic hydrophilic interaction liquid chromatography-mass spectrometry analysis was also conducted on kidney tissues from the cat. The tissue containing melamine-cyanurate complex crystals was homogenized in aqueous 2% formic acid, then diluted and analyzed.

Results

Survival after experimental exposure—Two salmon exposed to M&CA died on days 7 and 11 after administration of M&CA ceased, but tilapia, catfish, and trout survived to day 14, when they were euthanatized. Two additional salmon that received M&CA at a dosage of 200 mg of each compound/kg once a day for 3 days survived to day 14 after exposure. All fish to which cyanuric acid or melamine was administered survived until their scheduled dates of euthanasia (day 1, 3, 6, or 10). All 3 pigs survived to euthanasia (1 day after administration of the compounds ceased).

Clinical and gross pathologic findings—Trout, salmon, and some catfish to which M&CA was administered passed white feces (Figure 2). Necropsy revealed similar material in the lumen of the intestine of all species of fish, particularly the coldwater fish, on days 1 and 3 after administration of M&CA ceased. The salmon necropsied on day 1 had a large amount of white material in its stomach. Petechiae were associated with some of these deposits of white material. Kidneys appeared slightly swollen in some fish to which M&CA was administered. Other organs were unremarkable in appearance. Clinical signs of distress were not detected in any fish. Coldwater fish that had been given cyanuric acid only occasionally passed feces that contained a few grains of cyanuric acid 1 to 3 days after administration

ceased. None of the control fish or fish to which only cyanuric acid or melamine was administered had any clinical signs of distress, nor were any gross lesions detected during necropsies.

The pig that received M&CA developed bloody diarrhea during the first 24 hours of administration, but the pig appeared clinically normal and the diarrhea resolved naturally. Necropsy revealed that this pig had a large amount of edema in the fascia surrounding the kidneys (Figure 2). The kidneys were flaccid and appeared pale, grayish tan, and mottled, with small red foci. Intestines appeared grossly normal. Necropsy did not reveal any gross lesions in either of the pigs to which cyanuric acid or melamine alone was administered. Blood concentrations of urea nitrogen and creatinine were elevated in the pig to which M&CA was administered (BUN, 48 mg/dL [reference range, 6 to 30 mg/dL]; creatinine, 9.8 mg/dL [reference range, 0.5 to 2.1 mg/dL]) but were within reference ranges in the control pig (BUN, 7 mg/dL; creatinine, 1.3 mg/dL) and in the pigs exposed only to melamine (BUN, 16 mg/dL; creatinine, 1.4 mg/dL) or cyanuric acid (BUN, 10 mg/dL; creatinine, 1.4 mg/dL).

Feed analysis—Melamine was detected in the trout (0.5 ppm) and the salmon (6.7 ppm) maintenance feeds. Tilapia, catfish, and pig maintenance feeds contained no detectable concentrations of melamine. Cyanuric acid was not detected in any feed.

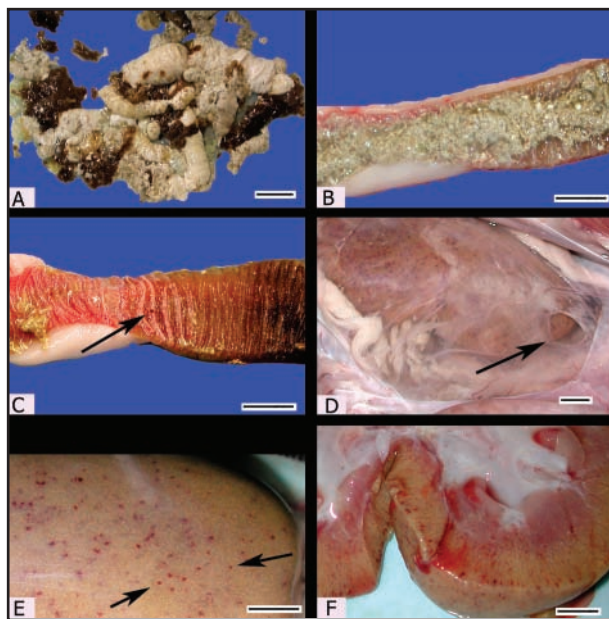


Figure 2—Photographs of gross abnormalities in fish and pigs that received M&CA (400 mg of each compound/kg, q 24 h, for 3 days) in an experiment to assess the pathologic effects of ingestion of the compounds. A—Feces with white, chalky material in casts was removed from a tank containing trout 3 days after the last dose of M&CA was administered. B—Necropsy revealed white, chalky material in the intestinal lumen of a trout 1 day after the last dose of M&CA was administered. C—Removal of the white material in a portion of the intestine shown in panel B revealed hemorrhage (arrow). D—Necropsy revealed edema in the retroperitoneal fascia (arrow) of the kidney of the pig that received M&CA. E—Petechiae (arrows) are evident on the surface of the kidney from panel D. F—Hemorrhagic foci located primarily within the renal cortex are evident on the cut surface of the kidney from panels D and E. Bar = 1 cm.

Microscopic evaluation of kidneys—Wet-mount sections from the kidneys of 25 of 26 (96%) of the fish to which M&CA was administered contained many gold-brown needle-like crystals that had formed radial spheroid aggregates (spherulites; **Figure 3**; **Table 1**). Only 1 salmon, which was euthanatized on day 1 after administration of M&CA ceased, had no crystals detectable via wet-mount or histopathologic examinations. That salmon had a large amount of white precipitate in

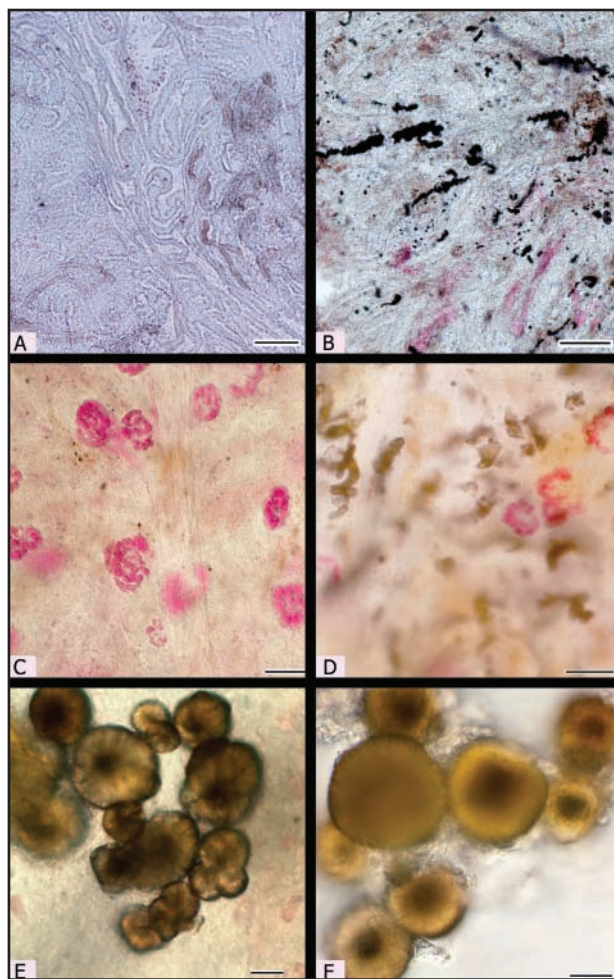


Figure 3—Photomicrographs of unstained wet-mount preparations of kidney sections and urine from fish that received melamine (400 mg/kg) or M&CA (400 mg of each compound/kg) once a day for 3 days in an experiment to assess the pathologic effects of ingestion of the compounds. **A**—Portion of kidney from a melamine-exposed tilapia examined 1 day after administration of melamine ceased. Pale or moderately pigmented tubules with empty lumens are evident. This appearance was similar to that of kidneys of fish that did not receive melamine. Bar = 200 μ m. **B**—Portion of kidney from an M&CA-exposed tilapia examined 1 day after administration of M&CA ceased, revealing many crystals, which appear dark at this magnification, within tubules and collecting ducts. Bar = 200 μ m. **C**—Portion of a kidney from an untreated catfish, revealing pale tubules and prominent glomeruli. Bar = 100 μ m. **D**—Portion of kidney from an M&CA-exposed catfish examined 1 day after administration of M&CA ceased, revealing many crystals lined up within renal tubules. Bar = 100 μ m. **E**—Portion of kidney from an M&CA-exposed catfish examined 3 days after administration of M&CA ceased, revealing gold-brown crystals in radial spheroid aggregates (spherulites). Bar = 20 μ m. **F**—Unstained urine sample from the ureter of an M&CA-exposed salmon examined 11 days after administration of M&CA ceased, revealing gold-brown crystal spherulites. Bar = 20 μ m.

the stomach and may not have absorbed enough of both compounds to produce renal crystals by the time it was euthanatized. Crystals were in kidneys of euthanatized fish even 14 days after administration of M&CA ceased. Crystals also formed in the 2 salmon to which the lower dosage of M&CA (200 mg of each compound/kg) was administered. These 2 fish had been added to the study because the salmon that received a higher dosage died before their scheduled date of euthanasia on day 14. Finally, the 3 trout to which both compounds were administered in sequence (melamine followed 6 days later by cyanuric acid) also developed renal crystals.

The crystals in fish kidneys had the same appearance as those detected in renal tubules of the cat with renal failure and those reported in the dogs and other cats that developed renal failure associated with consumption of adulterated pet food.¹ Many crystals appeared stacked, one after the other, within tubules of the renal cortex and medulla. It was not unusual to detect strings of ≥ 6 crystals within a tubule, with debris occasionally located at 1 end. Crystals were birefringent when examined with polarized light.

Only 2 fish that received cyanuric acid alone had renal crystals; 1 trout had 1 pale gold crystal, not typical of the gold-brown crystals noted before, and 1 salmon (salmon 22) had moderate numbers of the typical gold-brown crystals. Both fish had been fed commercial fish feed in which melamine (0.5 ppm in trout feed and 6.7 ppm in salmon feed) was detected. Salmon 22 was also thin and had a large gonadal lesion. Crystals were not detected in any wet-mount preparations of kidneys from control fish or fish to which only melamine was administered. Wet-mount sections of kidney from the pig to which M&CA was administered had many crystals of the same morphology as the crystals detected in the cat kidney and kidneys from fish that received M&CA. Crystals were not detected in wet-mount sections of kidneys from the pig that received only cyanuric acid and the pig that received only melamine.

Tissue sections from cat and fish kidneys that contained crystals were examined to assess the solubility of crystals in formalin. After flooding of slides from the thick sections of cat kidney, crystals were not visible when examined 60 hours later (**Figure 4**). Crystals in 6- μ m sections of tissue from fish and cat kidneys dissolved within 5 hours after flooding with formalin.

Table 1—Number of fish in which crystals were detected via microscopic examination of wet-mount preparations of kidney sections obtained at various time points after administration of melamine (MEL; 400 mg/kg), cyanuric acid (CYA; 400 mg/kg), or both melamine and cyanuric acid (M&CA; 400 mg of each compound/kg) once a day for 3 days. No crystals were detected in any control fish kidneys.

Type of fish	Melamine	Cyanuric acid	M&CA
Tilapia	0 (6)	0 (6)	6 (6)
Catfish	0 (6)	0 (6)	5 (5)
Trout	0 (6)	0 (6)	8 (8)
Salmon	0 (3)	1 (3)	6 (7)*

Numbers in parentheses are total number of fish in category.
*Half the dosage (200 mg each of melamine and cyanuric acid/kg) was administered to 2 fish because 2 others that received the higher dosage died. The fish that died and the fish that received the lower dosage developed renal crystals.

Histologic evaluation of kidneys—Twenty-five of 26 (96%) kidneys from fish that received M&CA contained many crystals, arranged in the typical radial spherulites, within tubules throughout the kidney (Figure 5). The 1 fish that had no renal crystals was the fish that had a large amount of white precipitate in its stomach. The 2 salmon that died also had crystals in their kidneys.

The radial crystal spherulites ranged in size from 10 to 40 μm in diameter, although a few large (60- to 80- μm wide) crystal spherulites were located within tubules or collecting ducts. As was detected in the slides of fresh tissues, the crystal spherulites were commonly lined up longitudinally within the tubules of fixed tissues, obstructing the lumen. In addition to the crystals, some tubules were dilated, contained necrotic cells, and

had basophilic, regenerative epithelium along the basement membrane. Small, eosinophilic strands of tissue extended from the tubular epithelium to the luminal crystals. Necrotic epithelial cells, sometimes still attached to the basement membrane, were adjacent to crystals. Inflammation was rarely associated with the crystals. Several newly developed nephrons were detected in fish that were euthanatized at day 14, which was considered typical for fish that have sustained tubular necrosis.¹³⁻¹⁵

Some luminal debris and vacuolation of tubular epithelial cells were detected in kidneys of fish that received only cyanuric acid or melamine. Only 1 fish

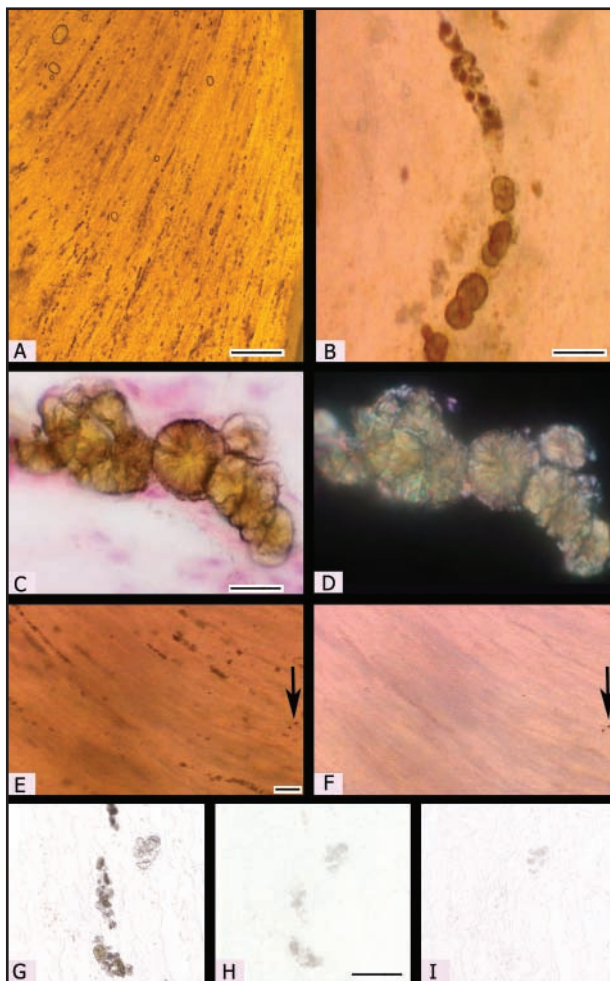


Figure 4—Photomicrographs of wet-mount preparations of a kidney of an 11.5-year-old neutered male domestic shorthair cat that developed renal failure after ingesting recalled pet food suspected to have been adulterated with melamine and related compounds. A—Notice many crystals in linear patterns within renal tubules. Bar = 400 μm . B—Notice the debris caught in the tubule upstream of the crystals (cortex is located on top of photomicrograph). Bar = 50 μm . C and D—Frozen section of kidney revealing high magnification of crystals by bright field (C) and polarized light (D). Bar = 15 μm . E and F—Fresh section of cat kidney prior to the addition of buffered 10% formalin (E) and 60 hours after flooding of tissue with formalin (F). Arrow is pointing to the same 2 spots of debris on the slide. Bar = 200 μm . G, H, and I—Six-micrometer frozen sections of cat kidney at 0, 2, and 5 hours after flooding with formalin. Crystals were paler within 2 hours, and most were no longer visible by 5 hours. Bar = 200 μm .

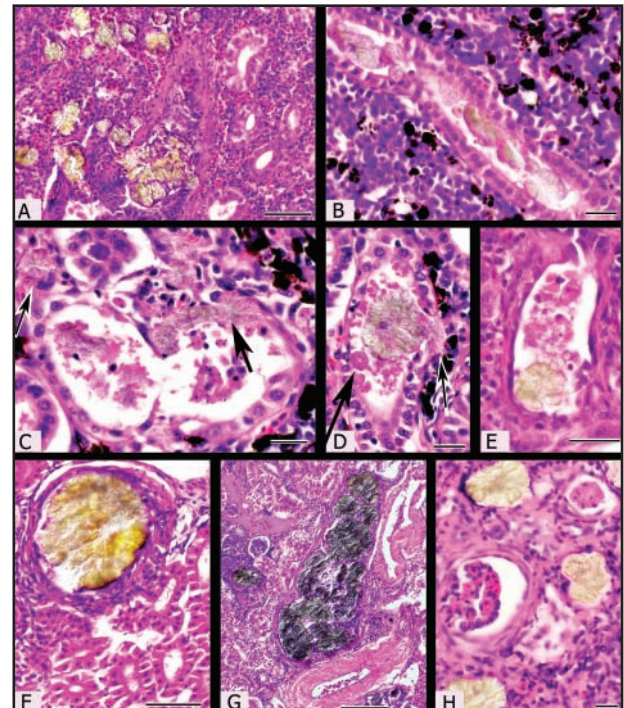


Figure 5—Photomicrographs of histologic preparations (H&E stain) of sections of kidneys from fish that received M&CA (400 mg of each compound/kg) once a day for 3 days and that were obtained 1 (I), 3 (A, B, E, F, and J), and 6 (C, D, G, and H) days after administration of M&CA ceased. A—Many tubules of a catfish kidney are filled with gold-brown crystals. Hematopoietic tissue in the interstitium is normal in fish kidneys. Bar = 50 μm . B—Crystals forming a long chain within a proximal tubule of a trout kidney. Interstitial hematopoietic tissue surrounds the tubule. Melanomacrophages are also normally found in salmonid kidneys. Bar = 20 μm . C—Crystals in the lumen of an injured tubule and closely adhered to basement membrane (black arrow) of a salmon kidney. Notice the necrotic cells and thin tissue strands from the epithelium in the lumen. Some crystals appear to be within the interstitium but could be located in collapsed damaged tubules (black on white arrow). Bar = 20 μm . D—Crystal penetrating through the tubular epithelium to the basement membrane (black on white arrow) of a salmon kidney. An eosinophilic radial crystal (black arrow) is also evident. Bar = 20 μm . E—Tubule of a catfish kidney containing a flattened and basophilic regenerating epithelium. Bar = 20 μm . F—Large, crystalline mass in a collecting duct of a tilapia kidney. A moderate inflammatory response is evident in the surrounding crystal-filled tubule. Renal tubules appear normal. Bar = 100 μm . G—Crystalline masses filling a collecting duct of a tilapia kidney. There is also a slight inflammatory response in the surrounding connective tissue. Renal tubules appear normal. Bar = 50 μm . H—Glomerular and interstitial fibrosis associated with crystalline deposits in tubule remnants of a catfish kidney. Such chronic lesions were seen primarily in fish euthanatized 14 days after administration of M&CA had ceased. Bar = 20 μm .

(1 of 17) that had received cyanuric acid had renal crystals evident in histologic preparations of kidney sections. That salmon had consumed commercial feed that contained melamine (6.7 ppm) during acclimation and also had kidneys in which crystals were detected in wet-mount preparations. None of the kidneys of the control fish and fish to which melamine alone had been administered contained crystals.

Intestines from fish with gross lesions detected at necropsy contained crystalline deposits along the villous tips at sites in which the epithelium had sloughed

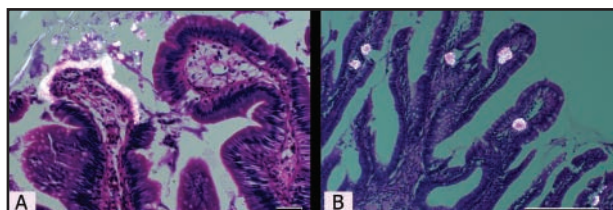


Figure 6—Photomicrographs of histologic preparations of sections of intestines from fish that received M&CA (400 mg of each compound/kg) once a day for 3 days and that were obtained 1 (A) or 3 (B) days after administration of M&CA ceased. A—The intestinal lumen of a trout contains many gold-brown crystals that were birefringent with polarized light, particularly along a portion of intestine denuded of epithelium. B—Crystals are evident in the lamina propria of a salmon intestine (polarized light). H&E stain; bar = 50 μ m.

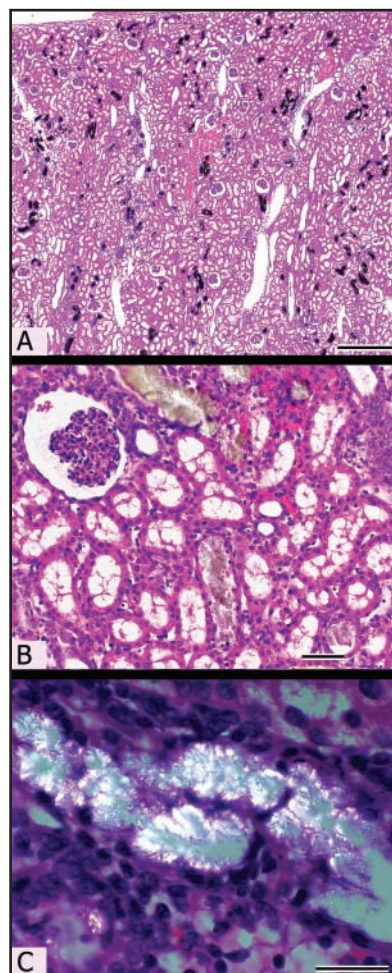


Figure 7—Photomicrographs of histologic preparations (H&E stain) of sections of kidneys from a pig that received M&CA (400 mg of each compound/kg) once a day for 3 days and that were obtained 1 day after administration ceased. A—Crystalline deposits (dark areas) extending from cortex (top) down into the medulla of the kidney. Bar = 100 μ m. B—Dilated glomerulus with tubules containing many gold-brown crystals. Bar = 50 μ m. C—Higher magnification reveals crystals within renal tubules (polarized light). These crystals are arranged more like the needles on the branch of a pine tree than in the typical spherulite pattern. Bar = 20 μ m.

from the basement membrane (Figure 6). Many crystals, similar to those detected in kidneys, were evident in the intestinal lumens. Some crystals were also detected in the submucosa. In the fish that were euthanatized 14 days after administration ceased, crystals were evident in mucosa and submucosa of the stomach, pyloric caeca, and intestines. In a few fish, crystals surrounded by a small infiltrate of lymphocytes were located in the muscularis of stomach, pyloric caeca, or intestines. No lesions were detected in other tissues of any fish.

Kidneys of pigs that received M&CA contained many crystals with the same morphology as those detected in kidneys of the fish and cat in the study reported here and those reported in other cats and dogs^{1,16} (Figure 7). Kidneys from the pigs that received only cyanuric acid or melamine contained no crystals or other associated histopathologic lesions.

In the cat kidney, renal tubular dilatation was evident in proximal and distal tubules. Crystals were detected in all uriniferous tubular segments (Figure 8). These crystals were birefringent and positive for Gomori methylamine silver stain. Many renal tubules were severely dilated with obviously attenuated epithelium; others were less dilated and had signs of epithelial regeneration. Fewer crystals were evident in fixed tissue sections versus wet-mount tissue sections.

Analysis of tissue residues—Residues of melamine and cyanuric acid were detected in the muscle tissue of

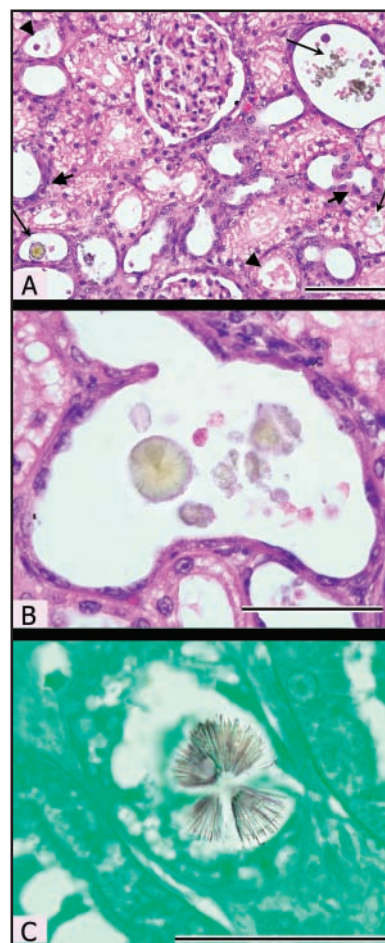


Figure 8—Photomicrographs of histologic preparations of sections of kidneys of a cat that developed renal failure after consuming recalled pet food suspected of having been adulterated with melamine and related compounds. A—Crystals (long arrows) are evident in distal and proximal tubules. Cellular debris is present in dilated tubules (arrowheads), and regenerative epithelium is evident adjacent to the basement membrane of a dilated tubule (short arrows). H&E stain; bar = 100 μ m. B—Higher magnification reveals crystals within a dilated tubule with an attenuated epithelium. H&E stain; bar = 50 μ m. C—Radial arrangement of needle-shaped crystals is evident within a tubule. Gomori methylamine silver stain; bar = 50 μ m.

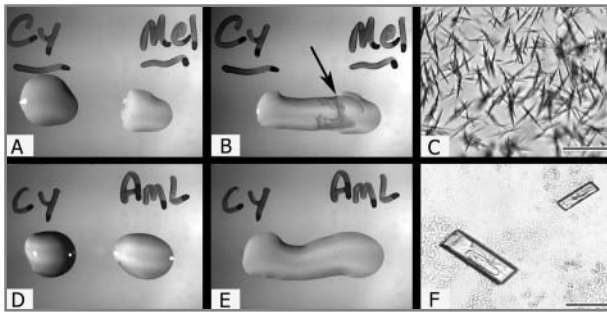


Figure 9—Examples of crystals grown from mixtures of melamine and cyanuric acid (melamine-cyanurate complex; A through C) and ammeline and cyanuric acid (ammeline cyanurate; D through F). A—Photograph of separate drops of cyanuric acid and melamine standards dissolved in water. B—Photograph of crystals (arrow) spontaneously formed when solutions were mixed. C—Photomicrograph of unstained needle-like crystals formed spontaneously from M&CA mixture. Bar = 100 μm . D—Photograph of separate drops of cyanuric acid and ammeline standards dissolved in water. E—Photograph of lack of spontaneous crystal formation when solutions were mixed. F—Photomicrograph of crystals that formed only after mixed solution was dried under vacuum at 80°C. Bar = 20 μm .

fish to which these compounds had been administered. For example, 1 day after administration ceased, 210 mg of melamine/kg of tissue (wet weight) was detected in catfish muscle from a fish given only melamine, and 11 mg of cyanuric acid/kg of tissue was detected in a fish given only cyanuric acid. Muscle of catfish to which M&CA had been administered contained lower concentrations of residues (33 mg of melamine/kg of tissue and 0.14 mg of cyanuric acid/kg of tissue) 1 day after administration ceased.

Melamine residues in kidneys ranged from 31 to 400 mg/kg of tissue in fish given melamine alone and 20 to 480 mg/kg of tissue in fish given M&CA. Surprisingly, melamine was detected in muscle (6.2 mg/kg of tissue) and kidney (32 mg/kg of tissue) of salmon 22, to which only cyanuric acid had been administered but that developed crystals in its kidney. That fish also had a large dysgerminoma in the abdomen. Muscle tissues from control salmon from the same group contained much lower background concentrations of melamine (0.08 to 0.11 mg/kg of tissue). The unexpected pres-

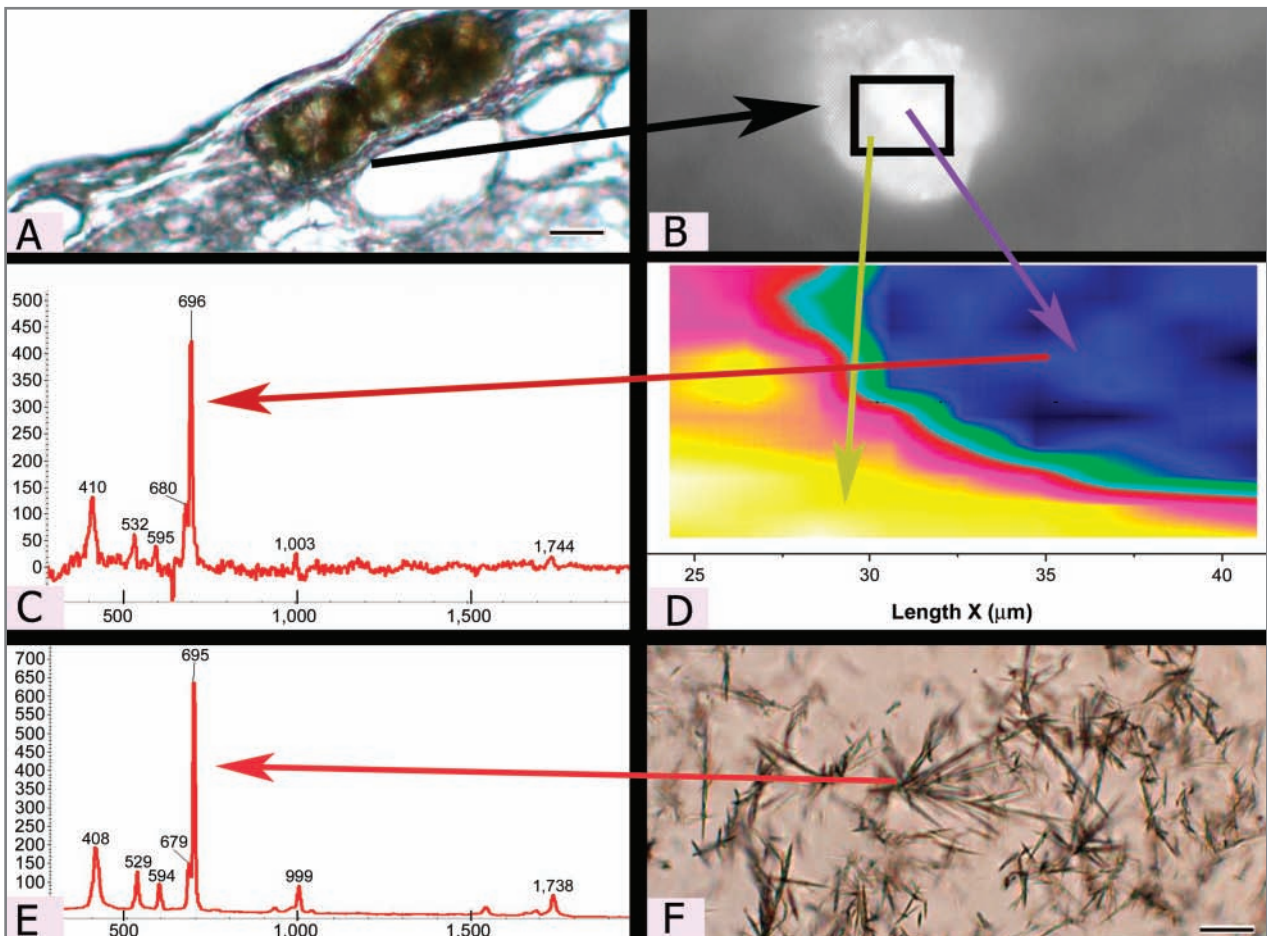


Figure 10—Comparison of Raman spectra of individual points in melamine-cyanurate complex crystals that were formed in vitro with crystal-containing renal tissue from a cat that developed renal failure after consuming recalled pet food suspected of having been contaminated with melamine and related compounds. A—Photomicrograph of frozen section of cat kidney revealing the morphology of the crystal used to generate a Raman map and spectrum. Bar = 15 μm . B—Crystal in the kidney section used for a Raman map; square indicates area mapped was approximately 15 μm (100X objective lens). C—Single point Raman spectrum from map of crystal from panel B. Peaks of comparison were 410, 532, 595, 680, 696, 1,003, and 1,744 cm^{-1} . Units for the y-axis are counts/s. The x-axis represents the Raman shift in cm^{-1} . D—False color Raman map of crystal from panel B. Blue indicates a high concentration of melamine-cyanurate complex; yellow indicates no melamine-cyanurate complex. E—Raman spectrum of melamine-cyanurate complex standard. Peaks of comparison were 408, 529, 594, 679, 695, 999, and 1,738 cm^{-1} . F—Melamine-cyanurate complex crystals formed in vitro by use of solutions containing reagent grade chemicals and viewed by a 20X objective lens. Bar = 45 μm .

ence of melamine in tissues of control salmonids was subsequently attributed to the melamine in the commercial feed consumed by those fish.⁹

Crystal composition—Crystals formed spontaneously when pure solutions of melamine and cyanuric acid were mixed (Figure 9). Spontaneous crystal formation was not detected for any other mixtures that contained melamine, cyanuric acid, or the other s-triazines. Crystal formation for mixtures of cyanuric acid with the other s-triazines (ammeline and ammelide) required vacuum drying.

The optical morphology and Raman spectra of the crystals in the cat kidney sections were consistent with melamine-cyanurate complex crystals. Peak maximum values obtained for the Raman spectra of individual points examined in the crystal-containing tissue were in good agreement with the peak maximum values obtained for the Raman spectrum of melamine-cyanurate complex crystals that were formed *in vitro* (Figure 10). Crystals from fish and the pig had similar spectra.

Discussion

In the past, melamine has been considered a generally nontoxic compound. The chemical is used in the manufacture of plastics, textiles, and glues.^{17,18} It can also be used as a fertilizer¹⁹ or, when combined with cyanuric acid, as a flame retardant.²⁰ Although not acutely toxic, melamine and other s-triazines have been identified as probable carcinogens.^{21–23} One study²³ in rats revealed that, in addition to causing bladder cancers and papillary hyperplasia, consumption of melamine causes renal inflammation, fibrosis, and tubular regeneration. Other adverse effects include hematuria, irregular microcrystals in urinary sediment, and bladder uroliths. Calculi in affected rats are composed of melamine and uric acid (1:1 ratio). Although no crystals were detected via histologic examination of kidneys during that study, uric acid crystals dissolve in formalin,²⁴ and routine processing may have dissolved any melamine-uric acid crystals in the renal tubules.

Melamine-induced crystalluria and consequent death in sheep were detected in a study²⁵ in which investigators evaluated melamine as a possible alternate source of nitrogen. Weight loss and death in sheep fed melamine has also been reported,²⁶ although the cause of death was not determined. Other related high-nitrogen compounds have also been evaluated as nitrogen sources for ruminants, with mixed results. Feeding biuret, a compound formed by the condensation of urea, to ruminants reportedly caused crystalluria and some deaths in 1 study²⁷; however, investigators who compared the effects of urea, biuret, triuret, and cyanuric acid on nitrogen retention in sheep in another study²⁸ reported that biuret was nontoxic.

Cyanuric acid also has low toxicity,^{29,30} although renal lesions can develop in animals that ingest the compound for prolonged periods.³¹ Other researchers reported that orally administered melamine-cyanurate can induce toxic effects in rat kidneys, although renal crystals were not detected in that study.³²

In the study reported here, renal crystals were experimentally induced in fish and pigs via coadminis-

tration of a 1:1 ratio of melamine and cyanuric acid; however, administration of melamine or cyanuric acid alone generally did not result in crystal development. Renal crystals formed in fish that received both compounds administered at the same time and also in fish that received melamine first, then cyanuric acid several days later.

One of the fish, salmon 22, was an apparent exception to this rule. Cyanuric acid alone was administered to this fish, yet the typical melamine-cyanurate complex crystals were detected in its kidney. Subsequent analysis of the commercial maintenance feed the fish had consumed during the months preceding the experiment revealed that the feed contained melamine (6.7 ppm). Tissue analysis revealed melamine in the kidney (32 mg/kg of tissue) and muscle (6.2 mg/kg of tissue) of this fish. The 2 other salmon that received only cyanuric acid did not, however, develop crystals. Their muscle tissues and those of the control fish contained much lower melamine residue concentrations (0.02 to 0.12 mg/kg of tissue). It is possible that renal excretion of melamine in salmon 22 was decreased because of the large gonadal neoplasm and, as a result, melamine was present to form a melamine-cyanurate complex in the kidney.

Muscle tissues of fish that had received only melamine or cyanuric acid generally contained higher concentrations of residues than muscle tissues of fish that had received M&CA. This finding is consistent with decreased bioavailability attributable to precipitation of the melamine-cyanurate complex in the gastrointestinal tract and kidneys. In addition, liquid chromatography-mass spectrometry analysis of crystal-containing kidney from fish to which M&CA had been administered revealed that the ratio of melamine to cyanuric acid in each sample was slightly greater than the equimolar relationship expected for the melamine-cyanurate complex. Presumably, the excess melamine was attributable to absorption of melamine by kidney tissue via a different process than melamine-cyanurate complex crystallization or to the fact that cyanurate was the limiting compound for crystal formation. Generally, melamine tissue concentrations were an order of magnitude higher than those of cyanuric acid.

The very high dosages of the compounds administered to the fish in the present study may have overwhelmed their capacity to excrete s-triazines. Studies to determine tissue-residue concentrations and depletion times in fish fed lower dosages will help to explore this possibility.

The experimentally induced crystals were similar to renal crystals detected in the cat in the present study and those detected in dogs and other cats that developed renal failure associated with consumption of recalled pet food.^{1,16} Micro-Raman spectroscopy allows for the differentiation of melamine, s-triazines, and melamine-cyanurate complex via the unique Raman spectrum generated for each compound. The application of micro-Raman³³ spectroscopy to the analysis of tissue crystals provided unique chemical data to assist in identification of the causative compounds.

The crystals that formed in the cat and fish in the study reported here and those that formed in dogs and

other cats in other studies^{1,16} were golden brown and generally arranged in a radial pattern. The appearance of these crystals was similar to uric acid crystal spherulites (spherically symmetric, radiating crystal aggregates) that can develop in humans with gout.^{34,35} The crystals were also similar to spherulites of uric acid detected in nephrocytes of the ascidian *Corella inflata*.³⁶ Because uric acid crystals are soluble in formalin, we examined wet-mount tissue sections during necropsy, prior to preserving kidneys in formalin for routine histopathologic examination.

Crystals in fish kidneys were distributed throughout the renal tubules and collecting duct system. Fish have mesonephric kidneys, and the species used in our study have nephrons comprised of glomeruli, neck segment, proximal tubules, and distal tubules. The nephrons empty into small and then large collecting ducts. In the present study, tubular dilatation and epithelial necrosis took place in close association with the crystals. Most of the fish survived the renal damage induced by the melamine-cyanurate complex crystals, probably because fish excrete most of their nitrogenous wastes via the gills. Thus, fish can endure much more extensive renal damage than most mammals.³⁷ However, even fish will die from major renal damage. Two of the salmon that received M&CA in the present study died. Their kidneys contained high numbers of crystals that probably resulted in death caused by a mechanism similar to acute uric acid nephropathy in humans.

In humans, increased purine catabolism can result in the deposition of uric acid crystals within the kidney, causing acute urate nephropathy.³⁸ This precipitation of uric acid is believed to develop because of an increased uric acid concentration in the filtered plasma and increased acidity in the tubular lumen.^{39,40} In humans, urate nephropathy can be caused by rapid tumor lysis, resulting in elevated blood uric acid concentration,^{41–43} or by rhabdomyolysis⁴⁴ or cardiovascular surgery.^{45,46} The mechanism of injury is primarily a physical obstruction of tubules, which causes an increase in intrarenal pressure that compresses the renal vasculature, greatly reducing renal blood flow. The reduction in renal blood flow reduces the glomerular filtration rate, resulting in acute renal failure. Other mechanisms such as renal vasoconstriction and inflammatory mediators may also contribute to the pathogenesis of acute renal failure associated with increased uric acid concentrations.^{47,48}

Uric acid nephropathy has been investigated to some degree in experiments that use animals. Humans and most primates do not have the enzyme urate oxidase (uricase) that, in most other mammals, is responsible for the conversion of uric acid to allantoin. Therefore, most studies^{49,50} of experimentally induced uric acid nephropathy in other mammals use inhibitors of urate oxidase. Investigators in 1 study⁴⁹ administered uric acid to rats in combination with oxonic acid, an s-triazine related to cyanuric acid. They detected many granular birefringent crystals primarily in the medullary tubules but also in the inner and outer cortex of frozen sections of kidneys. Interstitial inflammatory infiltrates, necrotic cellular debris, and regenerative basophilic epithelial

cells were also detected. Similar crystals have been induced in urate oxidase-deficient mice.⁵⁰

The inhibitory function of oxonate on urate oxidase was discovered in an experiment⁵¹ in which an old solution of urate was used to evaluate the kinetics of urate oxidase. As time passed, some of the urate in the old solution oxidized to oxonate, which acted as a strong competitive inhibitor of urate oxidase activity. Other s-triazines, including cyanuric acid (but not melamine), also inhibit uric oxidase.⁵¹

It is not clear whether the cyanuric acid administered to fish in the present study interfered with their uric acid metabolism. Fish have uric oxidase,^{52–54} and their kidneys respond to toxicant injury in much the same way that mammalian kidneys do.^{15,55} Certainly, the crystalline melamine-cyanurate complex precipitates in kidneys account for the tubular damage and obstruction detected in our study. The effect of the melamine-cyanurate complex on urate oxidase needs to be investigated as a possible cofactor in the pathogenesis of renal damage following the ingestion of melamine and cyanuric acid.

The finding that combined ingestion of 2 generally nontoxic compounds can result in such remarkable pathologic effects highlights the need for constant vigilance over animal feeds and human food products. With the globalization of our food supply, it is important that new methods for surveillance be developed to ensure that potential toxicants do not enter the food chain.

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 - c. Sigma Aldrich, St Louis, Mo.
 - d. IDEXX Vet Test Analyzer, IDEXX Laboratories, Westbrook, Me.
 - e. Envi-Carb solid phase extraction cartridge, Sigma Aldrich, St Louis, Mo.
 - f. HyperCarb LC column, Thermo Scientific, Waltham, Mass.
 - g. LabRam HR Raman spectrometer, Horiba Jobin Yvon, Edison, NJ.
 - h. Oasis MCX cartridges, Waters Corp, Milford, Mass.
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Appendix

Number of fish that received melamine (MEL; 400 mg/kg), cyanuric acid (CYA; 400 mg/kg), or melamine and cyanuric acid (M&CA; 400 mg of each compound/kg) once a day for 3 days and were evaluated at various time points after administration ceased to assess the pathologic effects of ingestion of the compounds.

Day after administration	Tilapia			Catfish			Trout			Salmon		
	MEL	CYA	M&CA	MEL	CYA	M&CA	MEL	CYA	M&CA	MEL	CYA	M&CA
1	2	2	2*	2	1	1	2	1	2	1	1	1
3	2	2	2	2	1	1	2	2	2	0	0	1
6	2	2	2*	2	1	1	2	2	2	1	1	1
7												1
10	0	0	0	0	0	0	0	0	0	1	1	0†
11												1‡
14	0	0	2	0	0	2	0	0	2	0	0	2§

*Only 1 fish ingested the dose; therefore, results were only available for that fish. †One fish died on day 7, prior to its scheduled date (day 10) to be euthanized. ‡One fish died on day 11, prior to its scheduled date (day 14) to be euthanized. §Half the dosage (200 mg each of melamine and cyanuric acid/kg) was administered to 2 fish because the other 2 had died.

Fish (1 tilapia, 1 catfish, 2 trout, and 2 salmon) that were housed in the same acclimation tanks as treated fish were used as control animals.