CHAPTER II
LITERATURE REVIEW

2.1 Melamine

Melamine was considered by previous working groups, in 1985 and 1987 (IARC, 1999). It is a nitrogen-rich heterocyclic triazine (Figure 1) used primary in the synthetic resins with formaldehyde (Budavari, 1996). Melamine is used in the manufacture of melamine resins, laminates, surface coating resins, plastics, coatings, commercial filters, gypsum-melamine resin mixtures, rubber additives and paper products (National Toxicology Program, 1991). Melamine can also be used as a colorant and as a fertilizer; however, it is not approved for these uses in the USA.

2.1.1 Chemical and physical properties

Chem. Abstr. Name	1, 3, 5-Triazine-2, 4, 6-triamine
IUPAC Systematic Name	Melamine
Synonyms	Cyanuramide; cyanurotriamide; cyanurotriamine; isomelamine; triminotriazine; 2, 4, 6-triaminotriazine; triamino-s-triazine; 2, 4, 6-triamino-1, 3, 5-triazine; 2, 4, 6-s-triazinetriamine; 1, 3, 5-triazine-2, 4, 6 (1H,3H,5H)-triimine
Molecular weight	126.13
Physical form	Crystalline solid (monoclinic colourless prisms)
Boiling point	sublimes

Melting point	354°C
Specific gravity (14°C)	1.574
Vapour pressure	50 mm Hg (315°C)
Vapour density	4.34 (air=1)
Refractive index (20°C)	1.872
Solubility	Slightly soluble in water; very soluble in hot water; very slightly soluble in hot alcohol

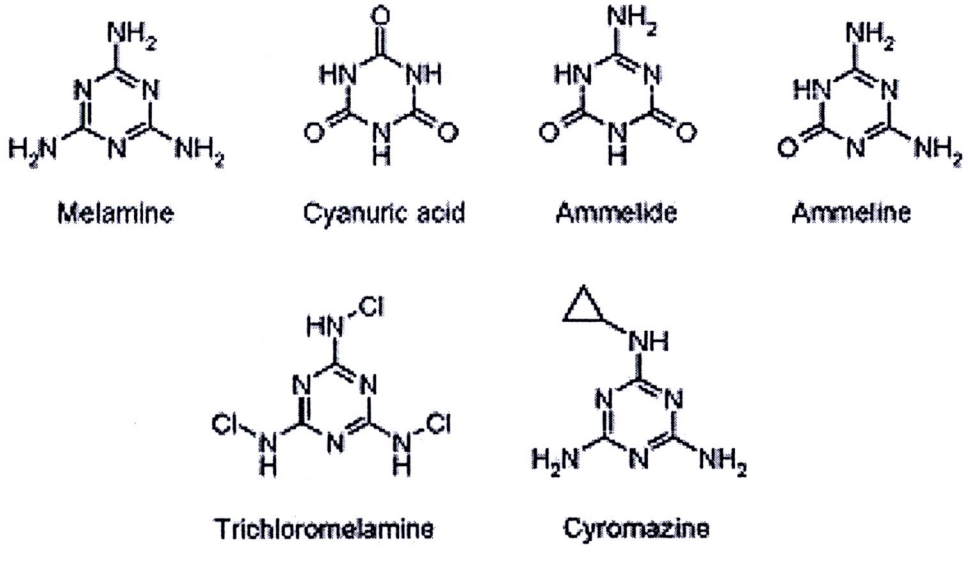


Figure 2.1 Structures of melamine and related triazine compounds

Source : WHO (2009)

Table 2.1 Physical and chemical properties of melamine and analogues

	Melamine	Cyanuric acid	Ammelide	Ammeline
Chemical formula	$C_3H_6N_6$	$C_3H_3N_3O_3$	$C_3H_4N_4O_2$	$C_3H_5N_5O$
Molecular weight (g/mol)	126.12	129.07	128.09	127.10
%nitrogen (w/w)	66.6	32.6	43.7	55.1
Appearance	Fine white crystalline powder	White crystalline solid	White powder Decomposes	White powder Decomposes
Melting point (°C)	345-347 Decomposes	360	76.9	75
Aqueous solubility (mg/l)	3240	2000		
pKa (dissociation constant)	5.35 (25°C)	4.74 (25°C)		9.65 (40°C)

Source : WHO (2009)

Melamine is a metabolite of the pesticide cyromazine. The USEPA (1999) indicated that only 10% of cyromazine is converted to melamine in vivo and has removed melamine from the tolerance limits as a residue of toxicological concern for cyromazine. The USEPA (1999) concluded that only residues of the parent compound, cyromazine, should have a listed tolerance level. However, it is known that cyromazine on the surface of fruits and vegetables is converted to melamine over time (Lim et al., 1990; USEPA, 1999).

Cyanuric acid is an oxytriazine melamine analogue that may be produced as a by-product in melamine synthesis. It is a USFDA-accepted component of feed-grade biuret, a ruminant feed additive, and is also found in swimming pool water as the dissociation product of dichloroisocyanurates used for water disinfection. When used for disinfection purpose in drinking-water, sodium dichloroisocyanurate is rapidly dechlorinated to cyanurate (Brady, Sancier and Sirine, 1963). Cyanuric acid derivatives

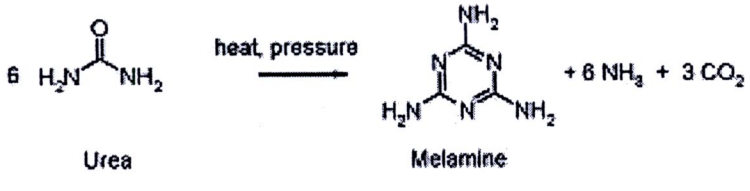
are regulated in the USA as components of sanitizing solutions for use on food-processing equipment, utensils and other food contact articles.

Ammelide and **ammeline** are monoamino- and diaminooxytriazine analogues produced as by-products of melamine synthesis or by the microbial degradation of melamine. Ammeline is used in lubricating greases (USFDA, 2007). No information on the used of ammelide was available.

2.1.2 Manufacture

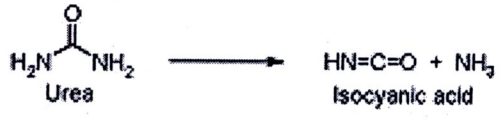
World production of melamine in 2007 was approximately 1.2 million tonnes, with the predominant producers being located in China and Western Europe (Bizzari and Yokose, 2008). Melamine can be produced from three different starting materials: urea, dicyandiamide or hydrogen cyanide. Commercially produced melamine is manufactured using urea as a starting material (Maxwell, 2007; Bizzari and Yokose, 2008). There are differences in the literature regarding the manufacture of melamine from dicyandiamide. Some sources indicate that commercial production of melamine from the thermal condensation of dicyandiamide ceased during the 1980s (Bizzari and Yokose, 2008). However, other sources indicate that this process is still used to manufacture melamine (Ono et al., 1998). It does not appear that production of melamine from hydrogen cyanide is used commercially.

The net reaction for the production of melamine from urea is shown in Equation 1-1:

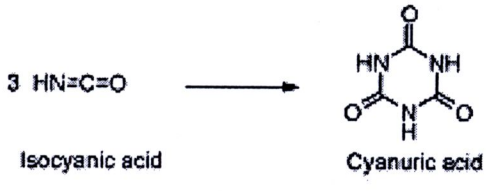


Urea Melamine (1-1)

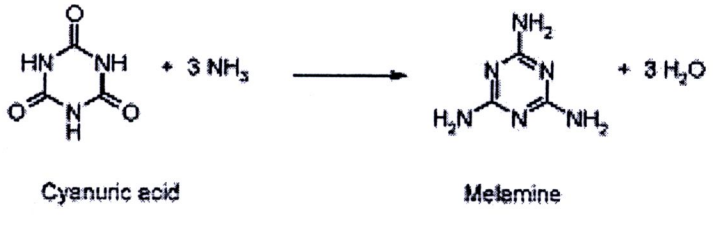
The reaction is typically carried out in one or more stages using either a high-pressure or a low-pressure process. The high-pressure process is performed in the liquid phase without a catalyst, at pressures of 90–150 bar and temperatures of 380–450 °C. In this process, urea is first converted to isocyanic acid (Equation 1-2), which then forms cyanuric acid (Equation 1-3). The cyanuric acid is then reacted with ammonia to form melamine (Equation 1-4).



(1-2)

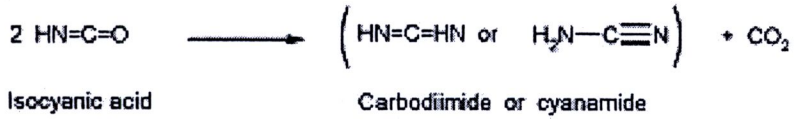


(1-3)

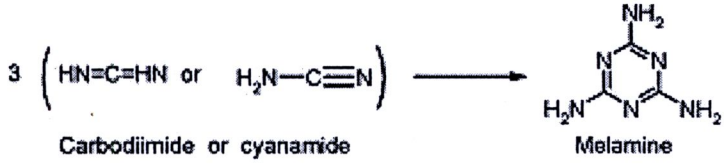


(1-4)

The low-pressure process is carried out in the gas phase in the presence of a catalyst, such as modified aluminium oxide or aluminosilicate, at pressures of 1–10 bar and temperatures of 350–450 °C. As with the high-pressure process, urea is first converted to isocyanic acid (Equation 1-2). In the second stage of the reaction, the isocyanic acid is converted on the catalyst to either cyanamide or carbodiimide (Equation 1-5), which are then converted to melamine (Equation 1-6).



(1-5)



(1-6)

In general, the products from either the low- or high-pressure process are quenched with water or an aqueous mother liquor. With recycling of reaction by-products (ammonia and carbon dioxide), reaction yields can be as high as 95%. Purification can be accomplished by filtration, centrifugation or crystallization. There are also proprietary technologies based on the low-pressure method that yield product purities as high as 99% without further purification (Bizzari and Yokose, 2008).

2.1.3 Degradation of melamine

It has been demonstrated that melamine can be metabolized by at least two strains of bacteria (*Pseudomonas* strain A and *Klebsiella terragena*) into carbon dioxide and ammonia via the pathway shown in Figure 2 (Jutzi et al, 1982; Shelton et al., 1997). As shown in the figure, melamine is metabolized through the successive deamination reactions to form ammeline, then ammelide, then cyanuric acid, with further breakdown to biuret, urea and, ultimately, ammonia and carbon dioxide. Metabolism of melamine is based mainly on enzyme catalyzed hydrolytic reactions (Cook, 1987; Eaton and Karns, 1991[a, b]; Wackett et al., 2002). Deamination is a common pathway of rumen bacteria and protozoa and hence ruminal biotransformation of melamine can be expected. However, detailed studies are not available (Wallace, 1996).

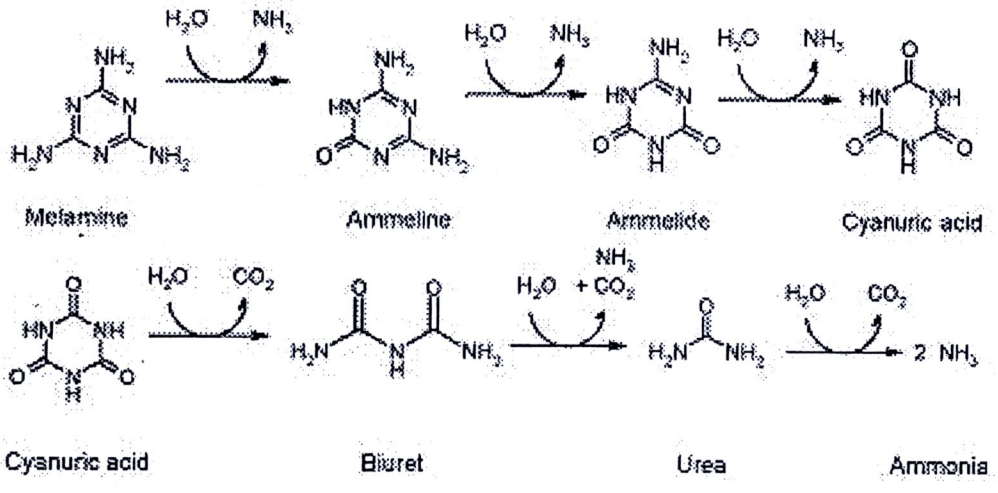


Figure 2.2 Melamine metabolic breakdown pathway attributed to *Pseudomonas* strain A and *Klebsiella terragena*

Source: WHO (2009)

The urinary metabolites recovered after administration of hexamethylmelamine to two patients indicated that the *s*-triazine ring is very stable and does not undergo cleavage *in vivo* (Worzalla et al., 1974). Fifty per cent of a single oral dose of 250 mg/kg bw melamine was recovered from the urine of rats within 6 h (Lipschitz and Stokey, 1945).The urinary metabolites, including melamine, recovered after administration of hexamethylmelamine to rats indicated that the *s*-triazine ring is very stable and does not undergo cleavage *in vivo* (Worzalla et al., 1974).

After administration of a single oral dose of 0.38 mg [^{14}C]-melamine to adult male Fischer 344/N rats, 90% of the administered dose was excreted in the urine within the first 24 h. An elimination half-life of 3 h and a renal clearance 0f 2.5 mL/min were calculated. Most of the radiolabel was concentrated in the kidney and bladder, and negligible amounts were detected in exhaled air and faeces. Virtually no residual radiolabel was observed in tissues after 24 h. Chromatography of the radiolabelled material found in plasma and urine indicated that melamine was not metabolized in rats (Mast et al., 1983).

2.1.4 Physico-chemical properties of melamine and of the melamine: cyanuric acid complex

Melamine can form co-precipitates with uric acid (occurring naturally in urine) or cyanuric acid (excreted into urine when co-exposure occurs). These complexes are very stable and can be hydrolysed at very low or very high pH.

Tolleson et al. (2009) demonstrated that at neutral pH the affinity of melamine for uric acid was 29-fold less than the affinity of melamine for cyanurate. However, at pH 4, a 6.4-fold tighter binding was found for melamine-urate in comparison with the affinity at pH 7, based on the increase in the dissociation constant Kd with increasing pH (140 , 204, 490 and 900 μmol/L at pH 4.0, 5.0, 6.0 and 7.0, respectively). So, in contrast to the melamine-cyanurate complex, the stability of melamine-urate complexes decreases with increasing pH. Grases et al. (2009) stated that in their "in vitro" study melamine and uric acid formed an insoluble compound at urinary pH <5.0. Uric acid and melamine mixed in aqueous media at pH values > 5.5 formed no crystals. Since melamine is soluble in water, and uric acid is in its anionic form at pH values >5.5, formation of insoluble solid can only take place at acidic pH values.

The stability of melamine-cyanurate co-crystals under various pH conditions has been discussed by Tolleson (2008). *"Melamine and cyanuric acid are known to form a network of well-ordered intermolecular hydrogen bonds that self-assemble spontaneously. The keto-form of cyanuric acid in equilibrium with the enol-form is the form involved in the hydrogen bonded network with melamine. Solvent pH affects the extent to which both melamine and cyanuric acid exist in their un-ionized forms that are available for hydrogen bonding. Although cyanuric acid is triprotic and melamine is tribasic, their first ionizations (pKa 4.74 and 5.34, respectively) are physiologically relevant within pH 5.0 –7.3 found in the kidney. Below pH 6 melamine is converted from the uncharged free amine form to the melamine ammonium cation, destabilizing hydrogen bonding with the keto-form of cyanuric acid. Similarly, cyanuric acid dissociates above pH 4 to form its conjugate base, also destabilizing the melamine – cyanuric acid complex. Optimal concentrations of free acid and free base forms available*

to form hydrogen bonds can be calculated from the average of the two pKa values, e.g. pH 5.04. At conditions of constant ionic strength similar to plasma (I=0.15) and over a pH range spanning that of the nephron, a solubility minimum was revealed for melamine and cyanuric acid) close to pH 5."

In additional correspondence it was explained that the solubility curves in the figure from Tolleson et al. (2009) reflect the solubility of the melamine-cyanurate complex at different pH. While at low ionic strength the complex of the stability is reduced at pH< 5, the stability is hardly affected at higher pH. However at higher ionic strength (I = 1; reflective of the ionic strength at some parts in Henle's loop) also at pH > 6.5 a decrease in complex stability can be demonstrated (data not shown)16. Dominguez-Estevez et al. (2010) investigated the solubility of melamine alone and in combination with cyanuric acid in urine of healthy human adults and of rats. A strong pH-dependency was observed with the lowest solubility found at pH 5-5.5. At pH 5.5, the solubility of melamine in rat urine was 6.9-fold higher than that in human urine.

2.1.5 Toxicology of melamine and analogues

Melamine is rapidly absorbed from the intestine and attains maximal plasma concentrations in 1 h following a single oral dose in rats (Mast et al., 1983; Sugita and Maekawa, 1991). The plasma half-life is approximately 2.7 h. Melamine is eliminated essentially unchanged by the kidney (Worzalla et al., 1974; Mast et al., 1983). In the pig, the melamine plasma half-life is approximately 4 h, with a clearance of 0.11 l/h per kilogram and a volume of distribution of 0.61 l/kg (Baynes et al., 2008).

Limited information is available on other species. Melamine has been detected in urine of dogs (Lipschitz and Stokey, 1945) and in urine of cats sickned during the pet food episode of 2007 (Brown et al., 2007). In a case-report, melamine has been protein concentrate (Reyers, 2008).

Cyanuric acid is absorbed rapidly and eliminated unchanged in the urine, with a half-life of approximately 1-2.5 h, depending on the dosage administered in rats. Cyanuric acid may be present in the faeces if administered in high doses (500 mg/kg body weight). In dogs, the half-life is 1.5-2 h, and elimination is also via the kidneys. No

metabolites of cyanuric acid were detected in the urine or faeces (Barbee et al., 1984; Hammond et al., 1986).

In human, absorption and excretion of cyanuric acid have been stusied in long-distance swimmers exposed by swimming in pools disinfected with chlorinated isocyanurates and in two volunteers given an unspecified solution of cyanuric acid orally. More than 98% of the administered dose was recovered unchanged in urine after 24 h. The half-life of excretion was about 3 h (Allen et al., 1982).

No information was available for other structural analogues.

2.1.5.1 Acute toxicity

Melamine has low acute toxicity. In male and female rats orally dosed by gavage, a median lethal dose (LD50) of 3161 mg/kg body weight was reported in males (NTP, 1983).

2.1.5.2 Short-term studies of toxicity

Male and female rats in a 14-day study were fed melamine at concentrations of 5000, 10000, 15000, 20000 and 30000 mg/kg in the diet. All animals survived to the end of study. Pale and pitted kidneys were also reported in some highest-dose males.

2.1.5.3 Sub-chronic studies of toxicity

The sub-chronic toxicity of melamine was evaluated in three United States National Toxicicology Program (NTP) 13-week oral studies in Fischer 344 rats. In Study I, males and females were fed melamine at dietary concentrations of 0, 6000, 9000, 12000, 15000 and 18000 mg/kg. Toxicity included reduced body weight gain and body weight in males receiving 6000 mg/kg diet and both sexes receiving 12000 mg/kg diet or more. Study II was performed at lower doses to provide dose estimates for the 2-year carcinogenicity study. Males and females were fed melamine at dietary concentrations of 0, 750, 1500, 3000, 6000 and 12000 mg/kg in the diet. Males at 6000 and 12000 mg/kg diet showed 10% decreases in weight gain compared with control males. All female dosed groups had the same weight gain as the control females. Feed consumption was not

affected in any male or female group. Study III, a 13-week study, male and female mice were fed melamine at dietary concentrations ranging from 6000 to 18000 mg/kg. Body weight gain was reduced in all treated groups (NTP, 1983).

2.1.5.4 Long-term studies of toxicity

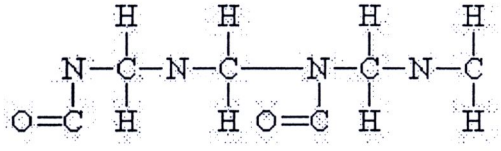
In an NTP carcinogenicity study, rats were fed with melamine at 0, 4500 or 9000 mg/kg in the diet for females and 0, 2250 or 4500 mg/kg in the diet for males for 2 years. There was no significant difference in survival between all groups. In mouse study, melamine fed at 0, 2250 or 4500 mg/kg in the diet. A lowered survival rate in the high-dose males (NTP, 1983).

2.1.5.5 Reproductive and developmental toxicity

There was no evidence of adverse effects on reproductive organs in a study by Melnick et al. (1984), in the 13-week toxicity studies or in carcinogenicity studies described above (NTP, 1983).

2.2 Urea-formaldehyde

Urea-formaldehyde (UF) resins are the most important type of binders for the production of particleboards and other wood-based panels. UF was commonly used when producing electrical appliances casing e.g. desk lamps. It is now mostly replace by melamine resin. UF is also used in agriculture as a controlled release source of nitrogen fertilizer. UF rate of decomposition into CO_2 and NH_3 is determined by the action of microbes found naturally in most soils. The activity of these microbes, and therefore the rate of nitrogen release, is temperature dependent. The optimum temperature for microbe activity is approximately 70-90°F. UF are formed by the reaction of urea and formaldehyde. The molecular structure of urea formaldehyde is shown below Figure 2.3.



Urea Formaldehyde

Figure 2.3 Urea formaldehyde molecular structure
Source: Arnold (1968)

The reaction of urea and formaldehyde is basically a two-step process: usually an alkaline methylolation followed by an acid condensation.

1) Methylolation refers to the addition of up to three (four in theory) molecules of the bifunctional formaldehyde to one molecule of urea to give the so-called methylolureas. Starting the reaction of urea and formaldehyde in the usual molar ratio but under acidic conditions gives methylene-linked ureas which tend to be insoluble in water with *ca* five or six urea units.

$$\underset{\text{(urea)}}{2HNCONH_2} + \underset{\text{(formaldehyde)}}{CH_2O} \xrightarrow[\text{Base catalyst}]{} \underset{\text{(methylolureas)}}{2HNCONHCH_2OH}$$

Methylolureas are used as long-term fertilizers, as neutral fillers and as a white pigment, with some other ideas for industrial and commercial use still being at the development stage.

2) Condensation reaction refers to the methylols, urea and free formaldehyde still present in the system react to give linear and partly branched molecules with medium and even higher molar masses. The type of bond between the urea molecules depends on the conditions used: low temperatures and only slightly acidic pH favour the formation of methylene ether bridges ($-CH_2-O-CH_2-$), while higher temperatures and lower pHs lead to the more stable methylene ($-CH_2-$) bridges.

$$2HNCONHCH_2OH \longrightarrow 2HNCONH\text{-}CH_2\text{-}NHCONH_2CH_2OH + H_2O$$

(methylolureas) Acid catalyst (urea-formaldehyde resin)

The most widely aquatic feed (fish and shrimp) used binders are urea-formaldehyde, wheat gluten and gelatin. Gelatinizing the starch is often used to improve water stability. Wheat flour is mostly used as the starch source. However, there is a large price difference between wheat flour and other starch sources. UF is a synthetic binder with no nutritional value. Fish or shrimp cannot digest it. Instead it adds nitrogen (false protein) to the diet that ends up as ammonia in the ponds.

In the process, formaldehyde is bound to the NH_2 group of urea to form a polymer. However, the formaldehyde can also bund to other amine (NH_2) groups in other products such as melamine or amino acids. Formaldehyde is a cross-linking agent which inactivates, stabilizes or immobilizes protein. UF has been shown to react with the amino group of N-terminal amino acid residue and the sidechains of arginine, cysteine, histidine and lysine residues. Furthermore, UF is not permitted either in the EU and the USA, leaving only wheat gluten and gelatin as real options in these regions.

Formaldehyde, a gaseous chemical, is released from formalin as fumes in hatchers to disinfect the environment and prevent the spread of infections (Peckham, 1980). Formalin is highly corrosive, has a pungent odor, and is a strong irritant to the mucous membranes. Due to these characteristics, incorporation of formalin in poultry feed can develop a toxic potential.

Peroral toxicity of formaldehyde has been reported in rats, mice, dogs (Johannsen et al., 1986; Restani and Galli, 1991), and calves (Preston et al., 1960). Formalin at the dose rate of <10 mL/kg fed to broiler chicks decreased feed consumption and BW (Babar et al., 2001). Similar levels of formalin, when fed to Japanese quails (*Coturnix coturnix japonica*), decreased testicular weight and diameter of seminiferous tubules (Anwar et al., 2001). Moreover, decreased BW, egg production and weight, erythrocyte and leukocyte counts, hemoglobin concentration, and hematocrit have also been reported at 10 and 20 mL of formalin/kg of feed in Japanese quail (Khan et al., 2005). However, formalin

mixed up to 5 mL/kg of feed, corresponding to 10 mL/bird of 3% formalin in drinking water by crop tube had no adverse effects on the health and microscopic lesions of White Leghorn cockerels (Khan et al., 2006)

Being a volatile compound, formalin evaporates when mixed in feed; hence, the amount of formalin ingested by the birds is usually lower than that mixed in feed. For this reason, the dose-related toxic effects of different levels of formalin in birds could not be ascertained by mixing it in the feed in the previous studies (Khan et al., 2003).

2.3 Avian urinary system

There are many similarities between birds and mammals in urine formation and elimination. Also, there are many differences. Similarities include the three phenomena of urine formation, glomerular filtration, tubular reabsorption, and tubular secretion. Also, birds are able to modify the concentration of ureteral urine so that it may have an osmolality that is above or below that of plasma. Differences between mammals and birds include in birds the presence of two major nephron types, the presence of a renal portal system, formation of uric acid instead of urea as the major end product of nitrogen metabolism, and postrenal modification of ureteral urine.

2.3.1 Anatomic features

Avian kidneys are paired retroperitoneal structures that are fitted closely to the bony depressions on the dorsal wall of the fused pelvis. Each kidney has cranial, middle, and caudal lobes. Ureters transport urine from the kidneys to the cloaca (mammalian urinary bladder not present). The cloaca is a common collection site, not only for reproductive organs (Figure 4). Each lobe has lobules (Figure 5), and a lobule gives the appearance of a mushroom, with its cortex corresponding to cap of the mushroom and the medulla corresponding to the stem.

Avian kidneys are characterized by having two nephron types, reptilian and mammalian. The reptilian types lack loops of Henle and are located in the cortex.

They are not capable of concentrating urine. Mammalian type nephrons have well-defined loops of Henle that are grouped into a medullary cone (see Figure 5), the part of the lobule that corresponds to the stem of a mushroom. Other structures in the medullary cone are those that would be found in the medulla of a mammalian kidney, the collecting ducts and vasa recta. The medullary structures enter at the wider cortical end of the cone. Osmolality of the medullary ISF increases from its beginning near the cortex to the tip of the cone. The osmotic gradient is estabkished by the loops of Honle and is maintained by the vasa recta as in mammalian kidneys, and permits the excretion of urine that has an osmolality greater than that of plasma. All tubular fluid, whether from nephrons of the reptilian or the mammalian type, is exposed to the osmotic gradient because of the exit of the collecting tubules and ducts through the cone to join the common ureteral branch (see Figure 2.5).

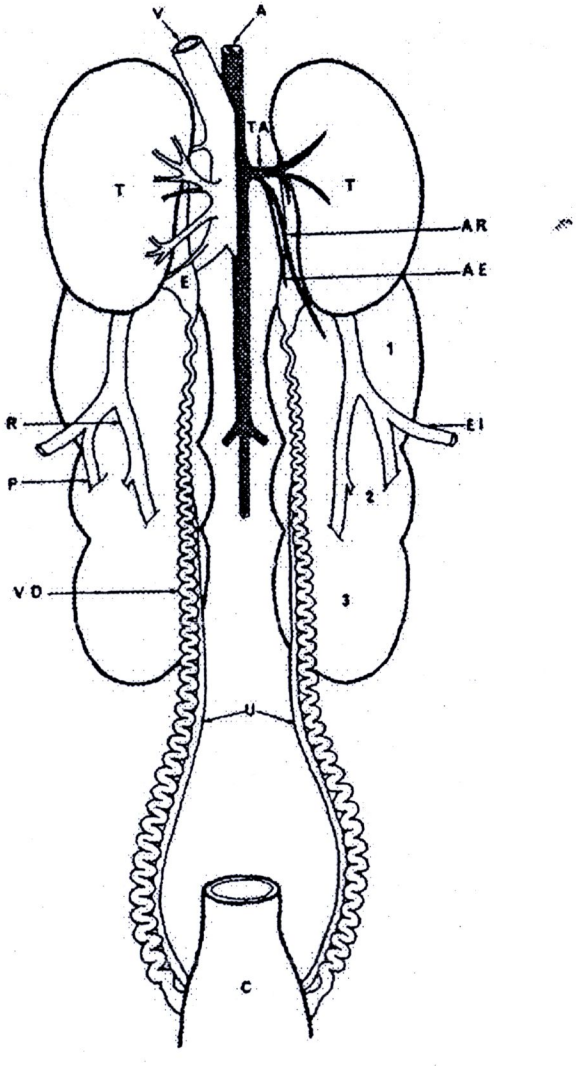


Figure 2.4 Ventral view of organs and associated structures of the dorsal abdominal cavity of a rooster (male chicken). A, abdominal aorta; AE, epididymal artery; AR, cranial renal artery; C, cloaca; E, epididymis; EI, external iliac vein; P, caudal renal portal vien; R, renal vein; T, testis; TA, testicular artery; U, ureters; V, caudal vena cava; VD, ductus deferens; 1, 2, and 3, cranial, middle, and caudal lobes of the left kidney, respectively

Source: William (2005)

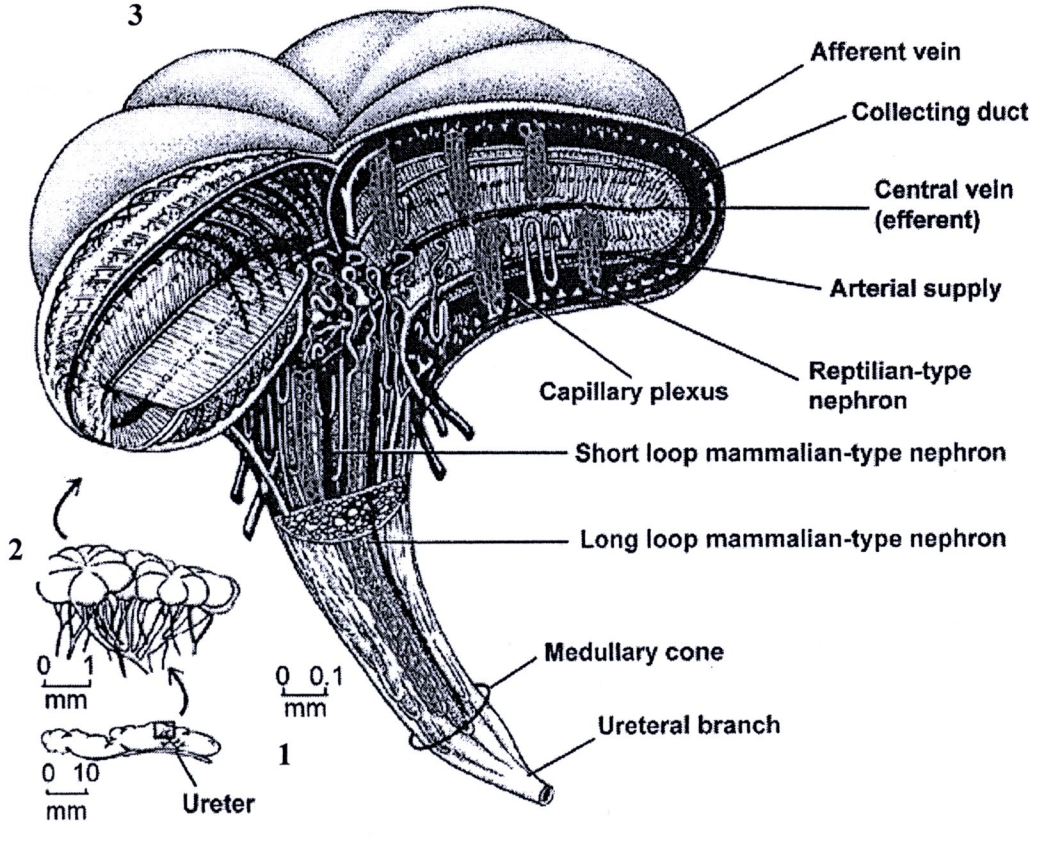


Figure 2.5 Arrangement of reptilian and mammalian nephrons within a lobule. 1) An avian kidney with its three lobes. 2) A number of lobules from a lobe. 3) The inner structure of a lobule. Reptilian nephrons do not have loops of Henle. Mammalian nephron are located near the medullary cone and extend their loops of Henle into the cone. The tubular fluid from both nephron types is received by common collecting ducts that also extend into the medullary cone, where it is exposed to ISF concentration gradients similar to mammalian kidneys. All urine from a lobule leaves by a common ureteral branch

Source: William (2005)

Avian kidneys can alternate between the use of reptilian-type and mammalian-type nephrons, depending on the need for water conservation. Greater use of mammalian-type nephrons would promote greater water conservation. When both nephron types are functional, 25% of the filtrate comes from mammalian-type nephrons and 75% from reptilian-type nephrons.

2.3.2 Excretory system

The kidneys in birds are rather large and elongated and are situated along the fused backbone. Each kidney consists of three lobes (cranial lobe, middle lobe, and caudal lobe). These lobes empty into a ureter leading to the urodeum of the cloaca from which urine is excreted. The primary functions of the kidney are twofold (1) to filter the blood so as to remove water and waste products therefrom, and (2) to reabsorb any nutrients (e.g., glucose or electrolytes) that might be recycled for additional use. Because the kidneys control the absorption and excretion of water and electrolytes, they are the primary control center for maintaining the proper osmotic balance of body fluids.

The urine of birds is cream colored. Much of it consists of a thick, pasty mucoid material that contains uric acid. Unlike mammals, whose urine contains urea primarily, birds excrete uric acid as the primary nitrogen metabolite. Uric acid is synthesized in the liver and is excreted via the urine. It comprises 60 to 80% of the total urinary nitrogen. Birds can, however, produce urea to a limited extent as an end product of purine metabolism and the catabolism of arginine; but the level of urea found in the urine is insignificant as compared to the uric acid content.

Because urine is transported to the cloaca, feces and urine are excreated from the body together. In fact, some urine may pass into the colon where additional water can be reabsorbed.

2.3.3 Uric acid formation and excretion

The metabolism of proteins and amino acids results in the production of nitrogenous end products. Among each of the many different kinds of animals, either ammonia, urea, or uric acid accounts for two-thirds or more of the total nitrogen excreted. Accordingly, animals are divided into three groups depending on whether their main nitrogenous excretory product is ammonia, urea, or uric acid. Because ammonia is a very toxic substance, it must be either excreted rapidly or converted to a substance that is less toxic, such as urea or uric acid. Ammonia excretion is encountered only in animals that are entirely aquatic, in which the ammonia can be quickly discharged into their aquatic environment. The urea excreting group is found among mammals and among amphibians.

In reptiles and birds, uric acid is formed instead of urea because these animals develop in egg shells that are impervious to water. The excretion of urea obligates water excretion (because of its effective osmotic pressure) and, because there is only limited water in eggs, it must be conserved. Uric acid reaches a certain concentration, and it precipitates. As a precipitate (no effective osmotic pressure), there is no water obligated in its excretion. If urea were excreted it would be necessary to eliminate the liquid urine formed, and this is not possible within eggs.

Just as urea is formed in the liver of mammals from ammonia, so is uric acid formed in the liver of birds from ammonia. The kidneys of birds are also a site for the formation of uric acid. Uric acid precipitates in the tubules because the extra blood from the renal portal system that perfuses the tubules leads to greater tubular secretion and consequently greater tubular concentration. The greater amounts in the tubules exceed uric acid solubility, and it precipitates. Uric acid continues in the tubules in its precipitated form and appears in the urine as a white coagulum. Because uric acid is no longer in solution, it does not contribute to the effective osmotic pressure of the tubular fluid, and obligatory water loss is avoided (Figure 6).

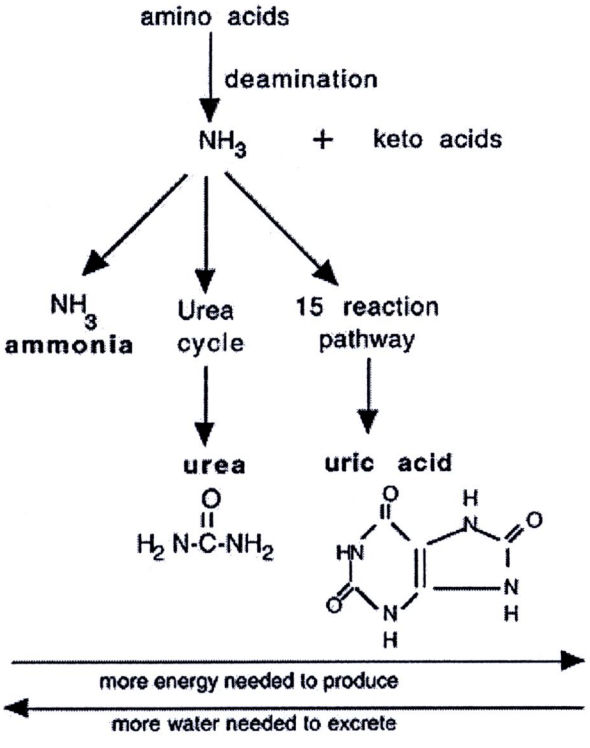


Figure 2.6 Metabolism of amino acids

Source : Champbell and Reece (2002)

2.4 Methods for the analysis of melamine and its analogues

A current analytical method for total protein estimation in proximate composition analysis is the Kjeldahl method, which has been widely used by the food industry. However, the validity of protein determination by the Kjeldahl method has been brought into question due to the aforementioned food safety incidents. The Kjeldahl method measures nitrogen from both protein and nonprotein sources in foods and presumes that all of the nitrogen is contained in protein. Current analytical methods for testing melamine are mainly chromatography based methods (HPLC, GC-MS, etc.) that are time consuming, expensive, and labor-intensive, requiring complex procedures of sample pretreatment, and well trained technicians to operate the instrumentation. Therefore, there is an urgent and increasing need in the food industry and analytical chemistry to develop a simpler, quicker, but sensitive, and cost-effective methods for detection of melamine

and its analogues (e.g., cyanuric acid, melamine cyanurate, ammelide, and ammeline) in food ingredients and processed foods and to develop reliable predictive models for quantitation of melamine in foods.

Ideal characteristics of analytical techniques for melamine detection include the following: (1) high sensitivity or low limit of detection (LOD); (2) high specificity; (3) short detection time; (4) low cost; (5) portable; (6) minimum or no sample preparation; and (7) suitable for measuring melamine in a variety of complex food matrixes (He et al., 2008; Heller and Nochetto, 2008; Huang et al., 2009). A brief discussion of advantages and disadvantages of each method is provided.

1. High-performance liquid chromatography (HPLC)

HPLC techniques include a group of methods using column chromatography to separate, quantify, characterize, and identify samples. A typical HPLC instrument includes a column that holds stationary phase, a pump that pushes the mobile phase(s) through the column, and a detector that detects the retention time and well separated peak, which is affected by the interactions between the stationary phase, the mobile phases (solvents), and sample molecules. HPLC and its hyphenated methods (HPLC-MS, HPLC-MS/MS, HPLC-UV, LC-MS/MS) are among the most reliable and melamine in various food and feed products. It provides high sensitivity and selectivity for a wide range of samples. However, these techniques do not come without limitations. For example, for the HPLC-UV method, the measurement of complex food matrixes such as candy and cookies may give interfering food components that also absorb UV at 240 nm.

2. Gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS)

GC and GC-MS are analytical techniques based on chromatographic principles. The mobile phase is a gas, usually nitrogen, helium, or hydrogen coming from a cylinder of compressed gas through a pressure regulator to the column. The U.S. FDA uses GC-MS method for measuring melamine and its analogues in a variety of food matrices at the

established minimum reporting level (MRL) of 10 μg/g and above. Food matrices that have been tested using this GC-MS method include dry protein ingredients (e.g., wheat gluten, rice protein, corn gluten, and soy protein), wet and dry pet foods, and dry animal feeds. GC-MS has large analytical power due to its wide availability and capability and serves well for analyzing mixtures of samples. However, samples must be volatilizing into a GC and trimethylsilyl (TMS)-derivatives of melamine is mandatory before ready for injection into the system. GC-MS/MS has a slightly better selectivity but lower limit of quantification than GC-MS.

3. Surface enhanced Raman spectroscopy (SERS)

Recently, there has been growing interest in the use of novel vibrational spectroscopic methods such as SERS for detection and identification of chemical and biochemical species. Vibrational spectroscopy provides useful information on the vibrational states or molecular motion of molecules in different ways. For instance, Raman spectroscopy measures inelastically scattered light photons that occurs when an incident laser light hits a sample. This phenomenon is called Raman scattering in which the frequency of scattered light is dependent upon molecular vibrations. However, conventional Raman only generate weak signals because only one out of 1 million photons experience Raman scattering due to low scattering cross sections. Therefore, traditional Raman Spectroscopy works only for bulk samples or concentrated solutions.

4. FTIR and NIR

First reported using near- and mid-inflared spectroscopy (NIR, FTIR-ATR, FTIR-DRIFT) to detect and quantify melamine in infant formula powder. The authors established the PLS models to correlate spectral data to melamine concentration (R^2>0.99). They successfully classified the control with infant formula samples spiked with 1 ppm melamine with a confidence level of 99.99%. The total measurement time is 3 min for NIR and 5 min for FTIR.

5. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a new technique that is used mainly in immunology to detect the presence of an antibody or an antigen in a sample. Garber (2008) tested three commercial ELISA test kits as rapid and reliable screening methods for detection of melamine in foods. The Melamine Plate kit (Abraxis, Warminster, PA, USA) showed a LOD of 9 ng/mL in phosphate-buffered saline (PNS) and about 1 μg/mL for melamine added to dog food, which is better than the other two test kits (Abraxis Atrazine ELISA and EnviroGard Trazine Plate Kit). The author concluded that this Abraxis ELISA for melamine detection is reliable and sensitive and could be a useful alternative to more cumbersome methods. World Health Organization (WHO) also gives a brief description of using ELISA for melamine detection.

The Food and Drug Administration (FDA) has currently stated that a level of up to 2.5 ppm melamine and its analogues in foods (not including infant formula) does not raise public concern. Recently, a threshold of 1 ppm for melamine in infant formula was set by the FDA. The detection limits and total time to detection for a variety of melamine detection methods are summarized in Table 2.

Table 2.2 Summary of published methods for detecting melamine in food products[a]

Method	Food product	Assay time	Total time to detection	Detection limit	Reference
UPLC-ESI-MS/MS	Animal feeds		3+h[b]	10 ppb	Cai et al., 2008
HPLC-DAD	Plant origin protein powders		3+h[b]	10 ppm	Ding et al., 2008
HPLC-MS/MS	Plant origin protein powders		3+h[b]	500 ppb	Ding et al., 2008
HPLC	Rice, wheat, corn flours		3+h[b]	5 ppm	Ehling et al.,2007
ELISA	Solution/buffer		1.5h[b]	9 ppb	Garber, 2008
	Dog food		1.5h[b]	1 ppm	Garber, 2008
SERS	Aqueous solutions[c]		0.5-1h[b]	33 ppb	He et.al., 2008
ZIC-HILIC	Animal feed		2+h[b]	500 ppb	Heller & Nochett, 2008
LTP-MS/MS	Milk powder	25s		6 ppb	Huang et al.,2009
EIA	Pet food		1.5h[b]	20 ppb	Kim et.al.,2008
HPLC-DAD	Pet food	15min	3+h[b]	100 ppb	Kim et.al.,2008
SERS	Wheat gluten		0.5-1h[b]	1000 ppm	Lin et.al., 2008
	Chicken feed		0.5-1h[b]	500 ppm	Lin et.al., 2008
	Cake		0.5-1h[b]	500 ppm	Lin et.al., 2008
	Noodles		0.5-1h[b]	700 ppm	Lin et.al., 2008
HPLC	Animal feed powdered rice protein	8 min	3+h[b]	75 ppm	Muniz et.al.,2008
LC-MS/MS	Dry infant formula		3+h[b]	250 ppb	U.S.FDA, 2009
MS-DART	Pet food	14min	10 min	1 ppm	Vail et.al.,2007
LC-MS	Catfish, pork, chicken, pet food	1-1.5min	30 min[b]	10 ppb	Varelis and Jeskelis, 2008

[a]UPLC-ESI-MS/MS, ultraperformance liquid chromatography coupled with electrospray ionization quadrupole tandem mass spectrometry; HPLC-DAD, high-performance liquid chromatograohy with diode array detection; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatograohy; ELISA, enzyme-lonked immunosorbent assay; SERS, surface-enhanced Raman spectroscopy; ZIC-HILIC, zwitterionic hydrophilic interaction chromatography; LTP/MS/MS, low-temperature plasma probe combined with tandem mass spectrometry; EIA, enzyme immunoassay; LC-MS/MS, liquid chromatography-triple-quadrupole tandem mass spectrometry; MS-DART, mass spectrometry using soft ionization by direct analysis in real time; LC-MS, liquid chromatography-mass spectrometry. [b]Estimated total time to detection. [c]Better performing Klarite nanosubstrate used with SERS.

2.5 Effect of melamine in animal diets

2.5.1 Ruminants

Melamine has been suggested as a non-protein nitrogen feed additive for ruminants in the past. This form of nitrogen supplementation was shown to be effective only in low protein feed. The efficacy further decreased as a result of feed intake

reduction and loss of physical condition (e.g. MacKenzie, 1966). It has been studied if melamine would release ammonia in the rumen of cattle *in vitro* and in vivo (Newton and Utley, 1978). Addition of melamine to cattle feed resulted in an increase of rumen ammonium concentration after an adaptation period. A nitrogen balance calculation showed that only ~ 6 % of the melamine nitrogen was retained. The authors speculated that the ring-nitrogens would not be available for protein synthesis. In the *in vitro* study in rumen fluid it was shown that melamineadapted rumen fluid was able to release melamine nitrogen to some (minor) extent. The release of nitrogen from melamine in ruminants was considered to be related to microbial activity. Although melamine has been found in tissues and in milk of several species (for overview see Tolleson et al., 2009), only one systematic study seems to have been published in which carry-over of melamine to milk has been examined. Cruywagen et al. (2009) dosed dairy cows with (nominally) 17.13 g of melamine per day via the feed. The substance was given in 15 kg of a pelleted feed supplement, which contained 1142 mg melamine/kg. Additionally oat hay fodder was provided. Animals were milked twice daily and afternoon milk samples were analysed for melamine (the first sample was taken 8 hrs after the first morning milk; sampling interval time was 24 h). Melamine appeared in the milk within 8 hr following the first feeding and reached a plateau value of approximately 15 mg/kg milk at 56 h. Upon cessation of exposure, milk melamine levels initially declined rapidly (by 39 % at 8 hrs and by 85 % at 32 hrs post exposure) but fell below the detection limit (5 µg/kg of milk) only after 6 days. Apart from normal daily variation, milk yield remained constant during the experiment at approximately 25 kg per day Average total melamine excretion via the milk can be calculated at 15 × 25 = 375 mg per day, during steady-state (Cruywagen et al., 2009). Moreover, reported by Shen et al. (2010) fed melamine at 0-450 mg/d per cow. The levels of melamine used did not affect milk yield or composition, which mean milk melamine concentration increased during the initial 3 d after melamine feeding, and melamine was not detected on d 4 of the clearance period. They suggested that when the daily intake of melamine exceeds 312.7 mg/cow, the milk should not be used to produce infant formula powder.

2.5.2 Monogastric animals

2.5.2.1 Poultry

An experiment was conducted to determine melamine residue levels in the tissues of broiler chickens fed diets containing graded levels of melamine (Lu et al., 2009a). Ten experimental diets were developed to contain 0, 2, 5, 10, 20, 50, 100, 200, 500, and 1,000 mg of melamine/ kg of diet. Each diet was offered in 4 replicate cages (12 male 1-day old broiler chickens per cage) from day 1 to 42, followed by a 7-day feeding of a withdrawal diet that contained no melamine. Throughout the 42-day feeding period, no effect on weight gain, feed intake, feed conversion ratio, and survival of broiler chickens was observed. On days 28, 42, and 49, one bird per replicate cage was killed and tissue samples from the breast meat, liver, and kidney were collected for the determination of melamine residues. Residue levels of melamine in broiler tissues at days 28 and 42 were below the detection limit (LOD 2 mg/kg tissue) when the diets contained ≤50 mg/kg of melamine. At day 28, tissue melamine levels increased ($P < 0.05$) with the increasing levels of melamine in birds fed diets containing 100 mg/kg or more. At day 42, a similar trend of a dose-dependent increase in tissue levels was observed in the kidney, but melamine was detected in breast meat and liver only in birds fed diets containing 500 and 1,000 mg of melamine/kg of diet. Also, the melamine levels in breast meat, liver and kidneys were much lower after 42 days of feeding as compared to the levels observed after 28 days of feeding. The authors postulated that this could be explained by an increased melamine clearance with increasing age, but it may also reflect a decreased exposure to melamine on a mg/kg b.w. per day basis following the feeding behaviour of the growing animals. In addition some of the differences between day 28 and day 42 might be due to variability in time of sampling. If melamine has a similar short half-life in chicken as it has in rats, then the time gap between sampling and feeding is crucial for the determination of tissue levels. However, feed intake and body weight data were not provided so the actual exposure during the study cannot be calculated.

Melamine distribution varied (P <0.05) in different tissues, with the highest concentration in the kidney. A withdrawal period of 7 days was found to clear the tissues of melamine.

Further study have been also experiment by Brand et al. (2009) on the melamine toxicity in young turkey poults fed dietary treatments from hatch to 21 days. Significant mortality was observed in turkeys fed 1.50, 2.00, 2.50 and 3.00% melamine with 27, 63, 93, and 93% mortality, respectively. Due to the high mortality in birds fed ≥2.00% melamine, growth performance could only be evaluated in birds fed 0, 0.50, 1.00, and 1.50% melamine. Compared with controls, feed intake was reduced (P<0.05) in turkeys fed diets containing 1.50% melamine, whereas body weight gain was reduced (P<0.05) in birds fed ≥1.00% melamine. Compared with controls, relative kidney weights were higher (P<0.01) in turkeys fed diets containing ≥1.00% melamine. Relative liver weights were not affected (P>0.05) by dietary treatments. The consistent gross lesions observed in turkeys fed 2.00-3.00% melamine that died were pale and enlarged kidneys. The bile of turkeys that died in these treatment groups contained crystals that were either microscopic (<2 microns) in size or were large white crystals visible to the naked eye. Most of the birds that died on these treatments were still eating food at the time of death with food present in the crop and/or ventriculus. Renal histopathology of birds fed 2.00-3.00% melamine that died were relatively uniform, and could be summarized as moderate to severe tubulointerstitial nephritis with mineralized casts within the collecting tubules and ducts. Data indicate that compared to broilers, turkeys are more susceptible to the toxic effects of melamine. The gross and histopathology findings are compatible with lesions of melamine toxicity reported previously for broiler chicks.

Ledoux et al. (2009) reported the toxicity of melamine (0-3.00%) in young broiler chicks (1- 14 days). There was no difference (P>0.05) in feed intake among controls and chicks fed 0.50 or 1.00% melamine. Feed intake was reduced (P<0.001) in chicks fed diets containing ≥1.50% melamine. Body weight gain decreased significantly in birds fed ≥1.00% melamine, with the greatest decrease in BWG observed in birds fed ≥2.00% melamine. Birds fed 2.50 and 3.00% melamine were less efficient

($P<0.001$) in converting feed to gain compared to all other dietary treatments (average 1.96 vs. 1.18 g:g). Mortality was observed in chicks fed 2.50 and 3.00% melamine as early as day 5 of the study, and by day 14, mortality was 12, 20 and 30%, respectively, in chicks fed 2.00, 2.50 and 3.00% melamine. Gross lesions in necropsied birds were completely uniform with all birds having enlarged pale kidneys and gallbladders containing opaque bile. Microscopic examination of the bile and histopathologic examination of the renal tubules, revealed crystals similar to those observed within the renal tubules of cats with melamine associated renal failure. Results indicate that over a 14 day feeding period, melamine concentrations $\geq$1.00% depressed growth performance, whereas melamine concentrations $\geq$2.00% caused increased mortality, crystal formation in bile, and histopathologic lesions in kidney.

The resulted of the study by Gao et al. (2010) showed the toxicity of melamine and determine the melamine residue in eggs. Ducks were fed melamine-supplemented diet (0, 1, 5, 25, 50, or 100 mg/kg) for 21 days followed by a 21 day withdrawal period. There were no adverse effects of graded levels of melamine in feed on the average egg weight, egg production, feed intake, and feed conversion ($P>0.05$). Supplemental melamine levels $\geq$ 50 mg/kg significantly increased blood urea nitrogen (BUN) levels in serum ($P>0.05$) and relative kidney weight was significantly increased in the group of 100 mg/kg in feed. These were in agreement with Dobson et al. (2008) and Lin et al. (2008) that melamine increased BUN and creatinine CRE) levels and led to kidney damage in cats and rats. Melamine levels in the eggs of laying ducks increased with dietary melamine levels similarly to Lu et al. (2009a) reported that melamine residue in broiler tissues increased with dietary melamine levels. However, during the withdrawal period melamine concentration in the eggs decreased rapidly. Similar Buur et al. (2008) findings melamine in the kidney of pigs decreased rapidly after withdrawal of oral doses.

In a recent study (Chen et al., 2010), the disposition profile of melamine was established in laying hens, with special focus on the transfer of melamine to eggs. In this study, laying hens were given an experimental feed spiked with 0, 5, 25, 50 or 100 mg of melamine per kg of feed. Eggs were collected over a period of 15 days

and analyzed for the presence of melamine by a validated GC/MS method. Melamine concentrations in eggs were 0.16, 0.47, 0.84 and 1.48 mg/kg egg, respectively, in the 0, 5, 25, 50 and 100 mg/kg feed treated groups. There was no evidence for a further accumulation of melamine in the egg yolk following long-term exposure and the calculated transfer rates vary between 1.5 and 3.2 %.

2.5.2.2 Pigs

Toxicokinetic data for melamine in pigs following intravenous bolus administration were obtained by Baynes et al. (2008). Melamine was administered at 6.13 mg/kg b.w. to weanling pigs (n=5) and plasma samples were collected over 24 h post administration. Plasma melamine concentrations could be described with a first-order one-compartment model with a half-life of 4.04 h, a clearance of 0.11 L/h/kg and a volume of distribution of 0.61 L/kg. The data from Mast et al. (1983), Sugita et al. (1991) and Baynes et al. (2008) were used to build a multi-compartment toxicokinetic model which could predict levels of melamine in plasma, liver and kidney tissue in pigs exposed to melamine via the food (Buur et al., 2008). The model consisted of four tissue compartments including kidney, liver, plasma, and carcass, which represents the combination of the remaining tissues (Figure 2.7). Melamine input was modeled as intravenous directly into the plasma compartment. In addition concentration time curves for repeated oral dosing were generated with a model expanded to describe absorption from the GI-tract. Figure 2.7 depicts a schematic diagram of the chronic oral dosing module which consisted of two subcompartments representing the stomach and small intestine (site of absorption). All melamine was assumed to be immediately available in the stomach. Transport into the small intestine was controlled by the gastric emptying time (Kst) and assumed to be first-order. Absorption into the liver compartment was governed by first order kinetics with constant Ka. Implicitly, 100 % bioavailability was assumed, which is in line with the very high renal excretion as reported in the rat (Mast et al., 1983). Physiological constants for organ volume, tissue blood volume, and blood flow for male Fischer 344 rats were obtained from various

literature sources. Based on the experimental data from the rat, hepatic clearance was considered insignificant and total body clearance was assumed to be by renal elimination only. Renal clearance was modeled as first-order excretion with constant Clrenal. Model parameterization and optimization was against data from *in vivo* studies in rats (Sugita et al., 1991) and the model was subsequently validated against the *in vivo* study by Mast et al. (1983).

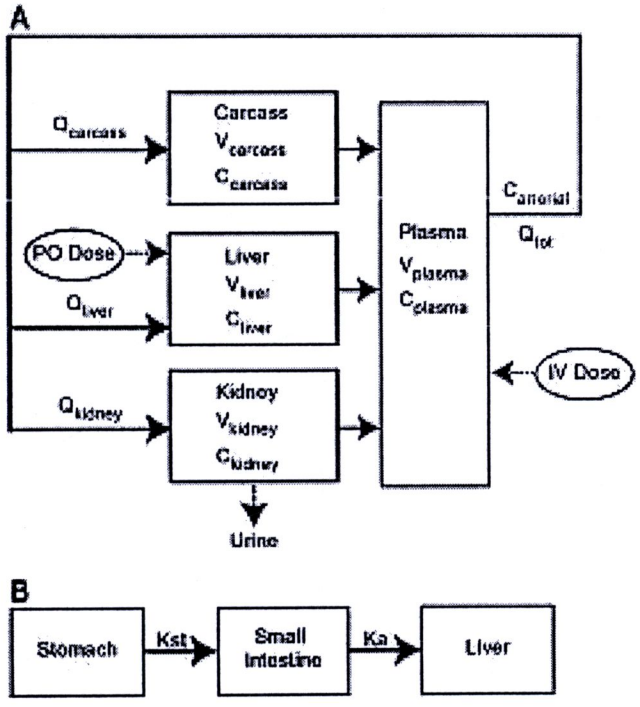


Figure 2.7 Schematic illustration of the physiologically based pharmacokinetic model of melamine (A). This model was used in both rat and porcine species. Arrows represent mass transfer of melamine via blood flow. (B) Schematic representation of the chronic oral dosing regimen. Kst and Ka are rate of absorption, respectively

Source : Buur et al. (2008)

2.5.2.3 Pets

There are no specific data on the toxicokinetics of melamine in pet animals such as cats and dogs, despite the detailed description of the renal pathology from the pet feed contamination incident.

2.6 Effect of urea-formaldehyde in animal diets

Poultry diets are approval pelleted to improve gains or feed utilization. Stilborn et al. (1991) reported that urea formaldehyde resin pelleting binder on productivity, egg quality, and blood parameters of laying hens over an eight week feeding period. These data showed that the addition of up to 0.4% of the urea formaldehyde had no adverse effects on overall performance and blood parameters (hematocrit, white blood cell count, red blood cell count, hemoglobin) of laying hens. Hemoglobin values were similar to those reported by Pilaski (1972). White blood cell numbers are similar to those reported by Lucas and Jamroz (1961). However, differential white cell count, and blood ammonia levels. A wide range of differential white cell counts for adult female hens have been reported (Lucas and Jamroz, 1961; Cook, 1987; Brake and Baker, 1982); therefore it is difficult to ascertain whether present values are considered within a normal range.