This thesis is dedicated to you dearest grandmother, Sigrid Linnea.

Abstract

Eklund G. 2003. Cadmium in newborns - bioavailability from infant food studied in a rat pup, a piglet and a human intestinal cell line model. Doctor's dissertation.
ISSN 1401-6257, ISBN 91 576 6399 8

Cadmium (Cd) is a well-known nephrotoxic environmental contaminant but there are indications that the developing nervous system might be even more sensitive to Cd than the kidneys in adults. Infants are exposed to Cd from various formulas and infant diets and the gastrointestinal Cd uptake is believed to be higher in newborns than in adults. Cd levels monitored in infant foods ranged between 0.74 and 27.0 µg/kg. Cow's milk formulas had the lowest levels and cereal-based formulas had up to 21 times higher mean levels. The mean weekly Cd exposure from the recommended formula intake was calculated to vary between 0.10 and 3.05 µg/kg body weight.

Rat pups received an oral dose of ^{109}Cd in water or four different formulas. The whole-body Cd retention was higher in the pups than previously reported in adult animals and highest in the water and in the cow's milk formula groups. The small intestinal Cd retention was high, even 9 days after exposure indicating a long absorption period in the newborns. Cd levels in kidney increased still 12 days after exposure in all diet groups. Piglets received low daily doses of Cd in water or wheat/oat/milk-based follow-up formula. The formula reduced Cd uptake in comparison to water, but the distribution of Cd to the kidneys was unexpectedly higher when Cd was given in formula than in water.

Simulated infant digestion of infant foods resulted in lower solubility of Cd compared to adult digestion. In a human Caco-2 cell model, cellular Cd uptake and transport from five different infant food digests was approximately one order of magnitude lower than the solubility and varied between 4-6 % and 1-2 % of the dose, respectively.

Binding of Cd to dietary fibres and phytic acid reduces intestinal Cd retention and probably explains the lower Cd bioavailability from cereal-based formulas compared to water or cow's milk formula. The exposure of Cd is higher from infant formulas than from breast milk and age-specific digestion conditions as well as composition of diets affect both the Cd solubility and bioavailability. The calculated Cd intake from recommended amount of infant formulas is below the established provisional tolerable weekly intake, which however, does not include a safety factor and is based on renal effects in adults.

Keywords: bioavailability, Caco-2 cells, food safety, formula, infant, *in vitro* digestion, metallothionein, piglet, trace element

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Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numeral.

I. Eklund G, Oskarsson A. 1999. Exposure of cadmium from infant formulas and weaning foods. *Food Additives and Contaminants* 16, 509-519.

II. Eklund G, Petersson Grawé K, Oskarsson A. 2001. Bioavailability of cadmium from infant diets in newborn rats. *Archives of Toxicology* 75, 522-530.

III. Eklund G, Tallkvist J, Oskarsson A. A piglet model for studies of gastro-intestinal uptake of cadmium in neonates. *Accepted in Toxicology Letters*.

IV. Eklund G, Lindén A, Tallkvist J, Oskarsson A. 2003. Bioavailability of cadmium from *in vitro* digested infant food studied in Caco-2 cells. *Journal of Agricultural and Food Chemistry* 51, 4168-4174.

Introduction

Cd in the food chain

Cadmium (Cd) is a toxic metal, with a low natural occurrence in soil, often in conjunction with Zn, Pb and Cu ore deposits (WHO, 1992a). There is also an input of Cd to soil from industrial emission sources and application of Cd-contaminated fertilisers on agricultural land (Bergbäck *et al*., 1994; Hedlund *et al*., 1997; Lindén *et al*., 2003). In contrast to other toxic metals, Cd in soil is easily taken up by growing plants through the root systems, and Cd is thereby present in all food (Hedlund *et al*., 1997). The Cd uptake is facilitated by the ongoing acidification of the environment (Andersson, 1981). In Swedish soils, the mean Cd level is 0.23 mg/kg (Eriksson *et al*., 1997). However, the background level is dependent on bedrock and weathering (McLaughlin *et al*., 1999) and levels vary widely in different regions. The highest Cd levels in Sweden are found in Mälardalen and in the area around Lake Storsjön in Jämtland and in the province of Skåne. The atmospheric Cd deposition from the European continent is higher in Skåne than in other parts of the country (Rühling *et al*., 1996) and Skåne soils have a higher plant availability of Cd than most soils in Sweden (Eriksson and Söderström, 1996). The uptake and accumulation of Cd in growing crops are influenced by factors such as Cd concentration in soil (Eriksson *et al*., 1996; Wenzel *et al*., 1996) and plant species and cultivar (Sillanpää and Jansson, 1991; Gray *et al*., 1999; Wenzel *et al*., 1996; Kurz *et al*., 1999). Among the different grains, wheat generally has the highest concentrations and barley the lowest (Jorhem *et al*., 1984, Jorhem and Sundström, 1993; Sillanpää and Jansson, 1991). For example, the mean levels of Cd in samples of winter wheat, oats and barley from 1992 to 1998 were 44, 36 and 19 µg/kg, respectively (Eriksson *et al*., 2000). Since Cd accumulates in the outer part of the grain, including the bran, the highest Cd levels are usually found in wholemeal products, and consumption of such products results in a higher Cd intake (Jorhem and Sundström, 1993). After 20 years of restricted Cd use, Sweden is close to a balance between input and output of Cd in arable soils. However, soil Cd is still increasing by 0.03-0.15 % every year, depending on geographical region and type of farming (Eriksson, 2000). In the long run, this increases the risk of elevated Cd levels in the food chain and until a balance between Cd input and output in the agricultural systems has been accomplished, environmental Cd accumulation remains a continuing problem in our country.

Cd in infant food

Food is the major exposure source for Cd in the non-smoking population (WHO, 2001a). About 50 % of the average dietary intake in adults originates from cereal products (Jorhem and Sundström, 1993). The high contribution from cereal-based food may be of special concern to infants and children, who are introduced to cereal and milk-based formulas and porridges, at an early age. In most foods, Cd concentrations range between 0.01 and 0.05 mg/kg, although higher levels are found in nuts and oil seeds, molluscs and offal, especially in liver and kidney. The current Cd exposure from food in Sweden is about 10 µg/day, ranging from 6 to 26 µg/day (Slorach *et al*., 1983; Becker and Kumpulainen, 1987; Vahter *et al*., 1990;

Berglund *et al*., 1994; WHO, 2001a; Olsson *et al*., 2002). Cd levels in drinking water are generally below 1 µg/l and for most individuals, Cd intake from drinking water is less than 2 µg/day (WHO, 1989). In infants however, Cd intakes are generally higher, on a body weight basis, than that estimated for adults due to a higher energy intake per kg body weight and more uniform food habits in infants compared to adults.

Cd levels in Swedish infant formulas were last analysed in the 1970s (Jorhem *et al*., 1984). Thus, monitoring of Cd in modern infant food in Sweden is necessary in order to obtain data for the exposure assessment of Cd in infants from this type of food. An increase in Cd levels in infant formulas could be expected, due to reports of increased Cd levels in soil (Eriksson *et al*., 1996; Eriksson, 2000) and a doubling of Cd in Swedish winter wheat between 1918 and 1980 (Andersson and Bingefors, 1985). Moreover, in the light of a drastic increase in Cd levels in pig kidneys between 1984 and 1992 (Petersson Grawé *et al*., 1997), the Cd levels in infant formulas are important to follow up, as pigs' feed as well as infant follow-up formulas are based on Swedish grains.

Infant feeding practices

Cd levels in human breast milk are low, generally below 0.1 µg/l (Larsson *et al*., 1981; Dabeka *et al*., 1986; Palminger Hallén *et al*., 1995b). However, higher levels have been reported from Poland, 2.75 µg/l (Plöckinger *et al*., 1996) and from an industrialised area in Croatia, 2.54 µg/l (Frkovic *et al*., 1997). The mammary gland functions like a barrier during the lactation period, restricting transfer of Cd into the mother's milk (Pietrzak-Flis *et al*., 1978; Bhattacharyya *et al*., 1981; Bhattacharyya *et al*., 1982). Low lactational Cd transfer has also been demonstrated in cattle (Smith *et al*., 1991) and in rodents (Andersson *et al*., 1997; Petersson Grawé and Oskarsson, 2000). In Sweden, the official recommendation regarding breast-feeding has recently been changed, from exclusive breast-feeding for the first four months to exclusive breast-feeding for the first six months (National Food Administration, 2003) to harmonise with the latest recommendation from the WHO (2001b). Similarly to other Scandinavian countries, breast-feeding rates are very high in Sweden compared with those in most other Western societies. Among Swedish infants born in 2000, 68 % were exclusively breast-fed at the age of four months and 39 % were still partially breast-fed at the age of six months (National Board of Health and Welfare, 2002). Infants who are not breast-fed receive infant formulas based on cow’s milk or soy proteins. However, soy formula is not a common product in Sweden and only given to infants after recommendations from paediatricians.

As the child develops and needs additional nutrition, cereal and milk-based follow-up formulas are commonly introduced to Swedish infants at the age of six months (Hörnell *et al*., 2001a). The tradition of feeding cereal and milk-based formulas to infants, as supplement or weaning foods, is very long in Sweden and these diets become transitional foods between a complete liquid diet and solid food. Parents who choose to introduce follow-up formulas at the age of six months, are recommended, by the manufacturers, to gradually substitute meals of breast milk

for servings of formula, for example in the afternoon and in the evening. However, introduction of formula often decrease the breast milk consumption rapidly (Hillervik-Lindquist *et al*., 1990; Hörnell *et al*., 2001b) and the formulas may become major sources of nourishment for the child during early infancy. For example, follow-up formula, together with a main meal, served at mid-day, fully cover the nutritional need of a six months-old infant, according to the manufacturers. Furthermore, the recommended age for introduction of gluten was lowered in Sweden in 1996, from six to four months under the protection of breast-feeding, with the purpose of reducing the risk of coeliac disease (Lindberg, 1996). From the aspect of Cd exposure, early introduction of cereal-based formulas and porridges might result in an earlier and higher Cd exposure compared to breast-feeding.

Gastrointestinal absorption of Cd and kinetics

Besides knowledge of exposure levels of Cd from different foodstuffs, it is essential to know how much Cd that is taken up in the gastrointestinal tract for possible absorption into blood. There is not much data available on bioavailability of Cd in foods, and the WHO (2001a) has pointed out the need for more studies on Cd bioavailability from specific foods. The strict meaning of oral bioavailability of a compound is the fraction of a dose absorbed systemically, determined by comparing the plasma area under curve (AUC) after intravenous and peroral dosing (Medinsky and Valentine, 2001). However, the fractional retention (% of administered dose) of a compound in blood and various tissues is often used as an indicator of bioavailability.

During the passage through the stomach, Cd bound to food compounds might dissociate due to low pH and enzymatic digestion. When the pH rises again in the duodenum, dissociated Cd ions may form new complexes with substances present in the intestinal lumen, or they may bind again to compounds originally present in the food. Several experimental studies on absorption and distribution of oral Cd have been performed by administering Cd in water (Sasser and Jarboe, 1977; Sasser and Jarboe, 1980; Min *et al*., 1991), thus not considering the effects of food composition on Cd bioavailability, and the Cd doses have often been much higher than can be expected to be found in foods (Engström and Nordberg, 1979a; Foulkes and McMullen, 1987; Ohta and Cherian, 1991). A few previous studies have shown that the Cd bioavailability differs with the speciation of Cd in the food (Lind *et al*., 1995) and also that it is dependent on interactions with other nutrients and the nutritional status of the individual. For example, Fe deficiency is known to enhance the gastrointestinal Cd uptake in humans (Flanagan *et al*., 1978; Berglund *et al*., 1994; Olsson *et al*., 2002), and low dietary levels of Ca leads to enhanced Cd absorption in experimental animals (Brzoska and Moniuszko-Jakoniuk, 1998; Reeves and Chaney, 2001). However, none of these studies included newborn individuals, but Sarić and co-workers (2002) have recently demonstrated that Ca supplementation to rat pups reduces the absorption of Cd. In adult mice, the bioavailability of Cd from wheat bran is lower compared to inorganic $CdCl_2$, whereas Cd availability from carrots and sugar-beet fibre is comparable to $CdCl_2$ (Lind *et al*., 1998). Similarly, the fractional retention of Cd was lower in adult rats

on a whole-wheat diet compared to rats on an endosperm wheat diet (Moberg Wing, 1993). Milk diet (Kello and Kostial, 1977a; Engström and Nordberg, 1978) and young age (Kello and Kostial, 1977a; Kello and Kostial, 1977b; Engström and Nordberg, 1979b) have been reported to increase the gastrointestinal Cd absorption. However, the results from some of the previous studies are difficult to extrapolate to human infants as some of the animals were adults and even intraperitoneally exposed to Cd. Furthermore, the Cd absorption in the newborn animals has been estimated by measuring the whole-body retention, which most likely reflects the gastrointestinal retention including contents, rather than absorption. A possible explanation for increased Cd absorption in milk-fed animals could be the much lower intake of dietary Fe compared to the control groups.

Cd is normally poorly absorbed in the gastrointestinal tract. Only about 5 % of the oral Cd intake is absorbed in adult humans (WHO, 1992b), but data on the gastrointestinal Cd uptake in human infants are virtually non-existent. However, a recent study suggests that it may be as high as 37 % (Crews *et al*., 2000). The fate of intestinal Cd in human infants is unknown, but the gastrointestinal physiological conditions differ from those of an adult. The gastric pH is higher in infants than in adults, and the infant intestinal epithelium is not fully developed. Moreover, the activity of most digestive enzymes is much lower than in adults (Milsap and Jusko, 1994). All mammals, thus far studied, have, at birth, an immature mechanism for intestinal absorption of macromolecules via pinocytosis (Kraehenbuhl and Campiche, 1969; Broughton and Lecce, 1970). However, neonates vary widely with respect to type of macromolecules transported, and length in time of pinocytotic ability. For example, piglets non-selectively absorb a wide variety of macromolecules such as albumin, globulin and enzymes from gut to blood for about 36 hours after birth (Balconi and Lecce, 1966; Hardy, 1969), but the ability to internalise macromolecules by the enterocytes, without further transport into blood, persists in the piglet for an additional two to three weeks (Leary and Lecce, 1976). In mice, the absorption is intermediate selective, as they transport only gamma globulin from the gut into blood for a period of about 17 days postnatally. However, a wide variety of macromolecules may be internalised by the rodent enterocytes (Lecce, 1972). Thus, mammalian neonates are similar in that they non-selectively internalise macromolecules into the intestinal epithelium via pinocytosis, but differ with respect to the macromolecules transported into blood. In human infants, pinocytotic activity has been demonstrated in foetal intestines (Bierring *et al*., 1964), and infants fed cow's milk develop circulating antibodies to cow's milk proteins (Gunther *et al*., 1960; Gunther *et al*., 1962). However, it is generally believed that human infants absorb none (Dixon *et al*., 1959; Morris, 1968; Brambell, 1970) or only moderate amounts (Ganong, 2003) of undigested proteins into the blood from the intestines. In any case, the immature physiological conditions of the infant gastrointestinal tract will most likely affect the bioavailability of Cd from different foodstuffs.

The present knowledge on intestinal uptake of Cd is mainly based on animal experiments with rodents. For example, the site for Cd absorption has been studied in adult mice by Sørensen and co-workers (1993) and Andersen and co-workers (1994) who found a high deposition of Cd in the duodenum, indicating that this is

the major site for Cd absorption. Furthermore, the gastrointestinal Cd uptake has been described, both in adult animals (Foulkes, 1985) and in a human intestinal cell line model, Caco-2 (Jumarie *et al*., 1997; Jumarie *et al*., 1999*)*, as a two-step process, involving a rapid internalisation and a slow basolateral transport of Cd. Recently, Cd uptake into intestinal epithelial cells has also been shown to utilise the divalent metal ion transport system, DMT1 (Divalent Metal Transporter 1) (Picard *et al*., 2000; Tallkvist *et al*., 2001).

Binding of Cd to metallothionein

Once absorbed, Cd is bound to albumin in the blood and transported to the liver. Alternatively, Cd is transported from the gastrointestinal tract in the form of Cd-metallothionein (Cd-MT) directly to the kidney (Groten and van Bladeren, 1994). In the liver, Cd induces the synthesis of MT, a highly conserved metalloprotein with protective properties against Cd toxicity, which lies in its ability to sequester Cd in the cytosol, thereby reducing the amount of free Cd^{2+} in critical organelles (Liu *et al*., 1993). Although MT has been extensively studied and reviewed, (Margoshes and Vallee, 1957; Klaassen *et al*., 1999; Coyle *et al*., 2002) its biological functions are not fully understood. Synthesis is induced by metals and the interaction between Cd and Zn in the induction of MT is well established (Min *et al*., 1991; Cherian 1994; Nordberg and Nordberg, 2000). MT also acts as a free radical scavenger (Thornalley and Vasak, 1985; Klassen *et al*., 1999). There are four known isoforms of MT; MT-1 and -2 have a ubiquitous tissue distribution with particular abundance in the intestine, liver, kidney and pancreas, whereas MT-3 and -4 are found principally in brain and skin (Kägi and Nordberg, 1979; Kägi and Kojima, 1987; Suzuki *et al*., 1993). Cd, Zn, Cu, and Hg bind to MT, with the lowest affinity for Zn and the highest for Cu (Nordberg and Nordberg, 2000).

From the liver, there is a slow release of Cd-MT to the blood and the complex is transported to the kidneys where it is filtered through the glomerulus and subsequently reabsorbed by the proximal tubular cells. It is believed that Cd-MT complexes enter lysosomes in the tubular cells, where they are degraded, thereby releasing Cd^{2+} into the cytosol (Dorian *et al*., 1992). Free Cd^{2+} induce more MT in the tubular cells and Cd is re-bound to MT. The excretion of absorbed Cd is extremely slow and the half-life in the kidney cortex in man, is up to 30 years (WHO, 1992b) leading to increasing Cd concentrations in the kidney with age. Kidney accumulation is continuous up to approximately 50 years of age in humans and falls thereafter, possibly due to age-related changes in the kidney integrity and function. The major excretion routes of Cd include faeces and urine (Nomiyama, 1978; Friberg *et al*., 1985). Minor routes, which make little contribution to the total excretion, are hair (Anke *et al*., 1976), breast milk (Schroeder and Balassa, 1961) and pancreatic fluid (Friberg *et al*., 1985).

Adverse health effects of Cd

Cd is virtually absent in mammals at birth but accumulates selectively in the liver and kidneys with time. Up to 75 % of the total body burden is found in these organs (Friberg *et al*., 1985), whereas the lowest Cd concentrations are found in

brain, bone and fat (Sumino *et al*., 1975; Cherry, 1981). The kidney is considered to be the critical organ following environmental Cd exposure, and renal tubular dysfunction, affecting the ability to reabsorb solutes, such as glucose, phosphate, Ca, amino acids and MT-bound Cu and Zn, from primary urine is the recognised critical effect. However, the glomerular function also seems to be affected, which might be more severe (WHO, 2001a). Cd administered as Cd-MT is distributed more directly to the kidneys whereas inorganic Cd is preferentially distributed to the liver (Cherian, 1983; Sullivan *et al*., 1984a). This pattern of distribution occurs after intravenous, intraperitoneal and oral administration and has been taken as evidence that Cd-MT complexes could be absorbed in the intestine in the intact form (Groten and van Bladeren, 1994). Min and co-workers (1986) have shown that renal damage occurs at lower doses when rats were given intravenous injections of Cd-MT compared to Cd-cysteine or Cd-peptides. Consequently, Cd-MT absorbed in the gastrointestinal tract and distributed directly to the kidneys could have a more pronounced nephrotoxic effect than other forms of Cd. It is believed that the first signs of kidney toxicity appear when the capacity to synthesise MT in the tubular cells is exceeded (Järup *et al*., 1998a; Nordberg and Nordberg, 2000) and an early sign of chronic Cd poisoning is increased urinary excretion of low molecular weight proteins (Kjellström, 1986b; Buchet *et al*., 1990; Staessen *et al*., 1994). The critical Cd concentration of the renal cortex associated with nephropathy in man is 50 to 200 µg/g (Kjellström *et al*., 1984; Kjellström, 1986a; Kjellström, 1986b; WHO, 2001a). Recently, decreased bone density with increased fracture risk have been raised as another possible adverse effect of Cd at environmental exposure levels (Carlsson and Lundholm, 1996; Järup *et al*., 1998b; Staessen *et al*., 1999; Alfvén *et al*., 2000).

Very little is known about adverse effects of early Cd exposure but several studies on Cd toxicity clearly indicate that children might be a risk group for Cd exposure. For example, it has been claimed that the primary period of rapid renal concentration may occur during the early years of life (WHO, 1989). Furthermore, a potential effect of Cd on the immune system has been shown in 5 to 12-year-old children, environmentally Cd exposed (Ritz *et al*., 1998), and similarly, a decrease in antibody production and of antibody forming cells in the spleen have been reported in mice, given Cd in drinking water (Koller *et al*., 1975). In experimental animals, disturbances in the neurotransmitter levels have been reported in rat pups exposed to low levels of Cd via the milk of the dams (Andersson *et al*., 1997) or during the gestation (Antonio *et al*., 1998). Behavioural effects have been demonstrated in offspring, also after maternal Cd exposure during gestation and lactation (Dési *et al*., 1998; Petersson Grawé, 2003) at doses well below those causing renal effects in the mothers (Pelletier and Satinder, 1991). Thus, the developing central nervous system in offspring might be more vulnerable to the toxic effects of Cd than renal damage or osteoporosis in adults.

Risk assessment and risk management of Cd

A provisional tolerable weekly intake (PTWI) of Cd has been established, by a WHO/FAO expert group, the Joint Committee of Food Additives and Contaminants (JECFA), that corresponds to 7 µg/kg body weight (WHO, 1989). Excursions above the PTWI may be tolerated provided that they are not sustained for a long period of time. The PTWI is set to avoid Cd concentrations in the kidney cortex exceeding 50 µg/g, after 50 years of dietary Cd intake, in adults. However, recent studies have shown that renal effects can occur at considerably lower renal cortex Cd concentrations (Järup *et al*., 1995; Olsson *et al*., 2002). For example, about 10 % of a Belgian study group, representing the general population, had a slight renal dysfunction with proteinuria and Ca excretion at kidney cortex levels corresponding to 50 µg/g (Buchet *et al*., 1990), and it has been estimated that about 2 % of the Swedish population have Cd concentrations exceeding 50 µg/g kidney cortex (Barregård *et al*., 1999). Thus, the present environmental Cd exposure is close to the level where adverse effects on the renal function starts to develop in adults (WHO, 1989).

JECFA has recently reviewed new data on Cd at the 61st meeting held in June 2003 (JECFA, 2003), after which renal tubular dysfunction still is regarded the critical health outcome with regard to Cd toxicity and the current PTWI of 7 µg Cd/kg body weight was maintained. However, JECFA recognised that data from animal experiments indicate that neurotoxic effects in children could be a more sensitive end-point than renal damage in adults and encourages further studies on the subject. As of April 2002, The Cd levels in food are regulated within the European Union (EEC, 2001; Petersson Grawé, 2001). For example, the maximum Cd levels in cereals are 100 µg/kg, excluding bran, germ, wheat grain and rice, which have a maximum level of 200 µg Cd/kg. These levels are 1.5 to 6 times higher than the average levels found in Swedish cereals and cereal products (Jorhem and Sundström, 1993).

The need for data on Cd exposure and bioavailability in newborns

The pregnant woman and the foetus are well recognised risk groups with a need of special attention in risk assessment of chemical hazards. Compared to the well motivated interests in these established risk groups, relatively little attention has been paid to the susceptibility of the newborn infant. Infancy is characterised by rapid growth and development of tissues and functions, which leave the newborn more vulnerable than adults to harmful substances (Rice and Barone, 2000). A period of rapid synthesis of brain tissue, commonly referred to as brain growth spurt, occurs during the last trimester and continues for about 18 months after birth in humans. This period is characterised for example by formation of synaptic contacts and myelin synthesis (Innis, 1991). Furthermore, kinetics during the neonatal period differs from those of adults, which may put the neonate at special risk (WHO, 1986). Glomerular function in the kidneys develops to adult capacity

during the first six months of life in humans, and adult renal tubular function is reached at approximately one year of age (Alcorn and McNamara, 2002).

JECFA has recognised that the PTWI of Cd will not be uniform with age but claims that the PTWI estimate does take into account the higher Cd intake on a body weight basis by infants and children. However, concerns were expressed about the lack of a safety factor, which should be included when estimating tolerable intakes. JECFA has recommended that, in order to increase confidence in the estimates of the existing PTWI, studies should be conducted on factors that affect Cd bioavailability such as age, health status and dietary nutrients (WHO, 2001a). Also, data on Cd intake and its health effects among the general population in various countries should be examined, and JECFA has stated that levels of Cd in foods should continue to be monitored and should not rise further (WHO, 1989).

Previous studies on Cd toxicity have almost exclusively been focused on the renal effects in adults. However, there are indications of higher gastrointestinal Cd absorption in newborns, and immunotoxic and neurotoxic effects have been demonstrated in experimental animals after perinatal Cd exposure. Still, data for risk assessment of Cd exposure in human infants are practically lacking. To improve the risk assessment for infant Cd exposure, knowledge of the bioavailability of dietary Cd and health effects of early exposure is of paramount importance. However, ethical and practical considerations make it difficult to conduct controlled studies in the human infant and relevant models for bioavailability studies of Cd in food need to be developed.

Aims of the thesis

This thesis aims at investigating the exposure and bioavailability of Cd from infant food in newborns using experimental models with relevance to the human infant. The retrieved data could contribute to a better risk assessment for Cd exposure in infants and children.

The specific aims were:

- to analyse Cd levels in commercial infant diets on the Swedish market and to calculate the Cd intakes in infants consuming these products (paper I).

- to study the impact of infant food compositions on Cd bioavailability in a) newborn rats, given a single oral dose of Cd in water or four different infant formulas (paper II), b) newborn piglets, after ten days of repeated administrations of low Cd doses in water or infant follow-up formula (paper III).

- to estimate the cellular uptake and transport of Cd in a human intestinal cell model by simulating infant digestion of infant foods and exposing Caco-2 cells to the infant food digests containing the soluble Cd fraction (paper IV).

Materials and methods

Infant food samples

Infant food from the two dominating manufacturers of infant food in Sweden, Semper and Findus[1], was bought at retailers in Stockholm and Uppsala and analysed for Cd between 1997 and 2000 (Table 1). In paper I, Semper and Findus were referred to as manufacturers A and B, respectively. The infant formulas were based on cow's milk or soy proteins and most of them also contained cereals in the form of flour. Care was taken to get different batches of each brand of formula and therefore at least two packages of each product and brand with different shelf-life were purchased in paper I. In paper IV, two ready-to-use dishes in glass jars, pasta Bolognese and liver casserole, were analysed for Cd. The baby foods were commercial products and therefore the exact proportions of the ingredients are confidential but the ingredients are labelled on the packages in decreasing weight order (Table 1).

Metal analyses (papers I, III and IV)

The infant formulas in paper I were dry-ashed to complete combustion according to a method by the Nordic Committee on Food Analysis No. 139 (1991). In short, infant food samples of 2-10 g were dried at 103° C in a muffle furnace for 48 h, and then ashed for eight days according to an ashing programme (Table 1, paper I), to attain complete combustion. The residues were dissolved in 0.1 M HNO_3 supra pur (s.p.) and transferred to polypropylene tubes. In papers III and IV, a closed-vessel microwave combustion system (Milestone, MLS-1200-Mega) was used for microwave digestion of samples from the piglets and of the supernatants of the infant food digests. These samples were digested in 3 to 5 ml HNO_3 65 % pro analysi (p.a.) until complete combustion and transferred to polypropylene tubes. The samples were diluted with 0.1 M HNO_3 (s.p.) and weighed to obtain an exact sample volume.

A Perkin-Elmer 4100 ZL atomic absorption spectrophotometer with graphite furnace technique (GFAAS) and Zeeman background correction was used for the Cd analyses. However, the faeces samples from the piglets in paper III were analysed by atomic absorption spectrophotometry with flame technique (FAAS) and deuterium background correction (Perkin Elmer 4100), as were all Zn and Fe analyses, also in paper III. Linear calibration with calibration curves made from standard solutions of Cd, Zn and Fe, respectively was used.

In paper III, the Cd blood levels of the piglets were analysed according to a modified method by Stoeppler *et al*., (1978). Briefly, the blood samples were

[1] At the time of infant food sampling, Semper was a subsidiary company to the Swedish dairy association Arla® but is now sold and only part-owned by Semper AB. Furthermore, Findus has changed its name to Nestlé AB.

equilibrated in room temperature for 1 h before adding 500 µl 0.8 M HNO_3 (s.p.) to 300 µl blood and shaken for 30 s on a vortex and stored overnight in 4° C. The following day the samples were re-vortexed for 30 s and centrifuged at 11 500 rpm for 5 min and the supernatants were analysed for Cd with GFAAS technique, as described above.

Quality control of the Cd, Zn and Fe analyses

To prevent metal contamination, all laboratory glass and plastic utensils were soaked in 1.5 M HNO_3 (p.a.) for 24 h and then rinsed individually six times with deionized, distilled water. All samples were analysed in duplicates except the kidney samples from the piglets in paper III, which were analysed as single samples due to the small amount of kidney cortex from each individual. Two replicate determinations were made on each sample solution. The accuracy of the Cd, Zn and Fe determinations was checked on reference samples of similar matrix and with similar Cd, Zn and Fe concentrations, which were run in parallel with the samples in the respective experiments. The analytical results of the reference samples agreed well with the respective certified values (Table 2). Our laboratory has also participated, with satisfactory results (mean Z-score -0.7, n=5), in proficiency tests of trace elements in foods, arranged by the Swedish National Food Administration (Jorhem and Merino, 1997; Jorhem and Engman, 1999; Sundström and Jorhem, 1999; Åstrand and Jorhem, 2000; Åstrand and Jorhem, 2001). Deionized, distilled water was used throughout the studies, whenever water is mentioned. The limit of detection for Cd, calculated as 3 standard deviations of ≥ 20 reagent blanks, was 0.10 µg/l after dry-ashing and GFAAS in paper I, and 0.09 and 0.23 µg/l after microwave digestion and GFAAS in paper III and IV, respectively. For Zn and Fe, the limits of detection were 18 µg/l and 0.04 mg/l, respectively, after microwave digestion and FAAS, in paper III.

Table 1. Description and Cd levels (µg/kg fresh weight) of the infant foods analysed in papers I and IV.

Product type	Manufacturer and brands		Intro-duction age	Main ingredients in decreasing weight order	n	Cd	Ana-lysed in paper
	Semper	Findus	(months)			(min-max)	
Soy formula	Soja Semp[a]		0	water, dextrin-maltose, glucose, soy protein	4	1.23-1.64	I
Cow's milk formulas	Välling 1		4	whey powder, skimmed milk powder	2	0.74-0.90	I
		Milkoplus	4	whey powder, skimmed milk powder, corn starch	2	0.89-1.89	I
Follow-up formulas	Majsvälling		6	skimmed milk powder, corn flour, corn starch	2	1.95-2.10	I
		Majsvälling	6	skimmed milk powder, corn starch	3	0.86-1.82	I
	Risvälling		6	skimmed milk powder, rice flour, whey powder,	2	3.58-4.18	I
	Drickfärdig Välling 2[b]		6	whey powder, skimmed milk, oatmeal, wheat flour	2	2.06-2.20	I
	Välling KRAV		6	whey powder, skimmed milk, oatmeal, wheat flour	2	11.3-14.1	I
	Välling 2		6	whey powder, skimmed milk powder, oatmeal, wheat flour	2	12.5-13.0	I
		Plusvälling	6	wheat flour, skimmed milk powder, oatmeal, corn starch	4	17.0-18.6	I
		Plusvälling[c]	6	corn starch, skimmed milk powder, oatmeal, wheat flour	1	11.4	IV
Wholemeal follow-up formulas	Välling 3		8	skimmed milk powder, whey powder, oatmeal, wheat flour, whole-wheat meal, rye flour	4	8.78-11.7	I
	Fullkorns-välling		12		2	8.28-8.42	I
		Mild fullkorns-välling	8	whole-wheat meal, oatmeal, wheat flour, skimmed milk powder, corn starch	2	14.8-17.2	I

		Fullkorns-välling	12	whole-wheat meal, wheat flour, skimmed milk powder, oatmeal, rye flour	4	18.9-24.8	I
Children's porridges	Majs-havregröt		4	whey powder, skimmed milk powder, corn flour, corn starch, oatmeal	3	3.46-6.02	I
		Mild havregröt	4	oatmeal, corn starch, rice flour, skimmed milk powder, wheat flour	3	17.3-19.6	I
	Ananas & Aprikos Risgröt		6	rice flour, concentrate of pineapple juice, skimmed milk powder, apricots	2	5.32-21.5	I
		Päron-Äpplegröt KRAV	6	wheat flour, oatmeal, concentrate of pear juice, concentrate of apple juice, skimmed milk powder	2	17.4-17.9	I
	Fruktgröt		8	skimmed milk powder, whole-wheat meal, concentrate of pear juice, oatmeal, rice flour, wheat flour, banana powder, prunes, corn starch	4	8.26-15.3	I
		Fruktgröt	8	oatmeal, concentrate of pear juice, skimmed milk powder, whole-wheat meal, rice flour, wheat bran, apricot powder, apple powder	4	23.5-25.7	I
		Fruktgröt[c]	8	oatmeal, skimmed milk powder, concentrate of pear juice, rice flour, whole-wheat meal, apricot powder, apple powder, wheat bran	1	10.3	IV
	Mild fullkorns-gröt med hallon KRAV		8	oatmeal, wheat flour, skimmed milk powder, raspberries	2	18.1-22.4	I
		Mild fullkornsgröt	8	whole-wheat meal, oatmeal, skimmed milk powder, wheat flour, corn starch	2	19.9-27.0	I
		Mild fullkornsgröt[c]	8	whole-wheat meal, skimmed milk powder wheat flour, oatmeal	1	12.8	IV
Ready-to-use baby foods		Pastasnäckor i köttfärssås	12	beef, pasta of durum wheat, sweet pepper, green peas, wheat flour, onions	2	7.35-11.7	IV
		Gräddstuvad levergryta	12	potatoes, green peas, beef liver, carrots	2	9.12-14.6	IV

[a]75 % water content
[b]86 % water content
[c]new composition of formula, bought and analysed in 2000

Table 2. Reference material used in papers I-IV for accuracy of Cd, Zn and Fe analyses by atomic absorption spectrophotometry.

	Cd		Zn		Fe				
Reference material	Certified value[a]	Analysed value[b]	Certified value[a]	Analysed value[b]	Certified value[a]	Analysed value[b]	n	Analytical technique	Paper
Wheat Flower GBW 8503[c]	31 ± 2 µg/kg	30 ± 3 µg/kg					14	GFAAS	I
Wheat Flower GBW 8503[c]	31 ± 2 µg/kg	29.3 ± 4 µg/kg					4	GFAAS	III
Wheat Flower GBW 8503[c]	31 ± 4 µg/kg	30.8 ± 1 µg/kg					4	GFAAS	IV
Skim Milk Powder BCR No. 151[d]	101 ± 8 µg/kg	95 ± 4 µg/kg					6	GFAAS	I
Lyophilised Bovine Liver BCR 185[d]	298 ± 25 µg/kg	299 ± 12 µg/kg					5	GFAAS	III
Lyophilised Bovine Liver BCR 185[d]			142 ± 3 mg/kg	141 ± 2 mg/kg	214 ± 5 mg/kg	214 ± 3 mg/kg	14	FAAS	III
Lyophilised Bovine Liver BCR 185[d]	298 ± 25 µg/kg	293 ± 97 µg/kg					2	GFAAS	IV
Lyophilised Pig Kidney BCR 186[d]	2.71 ± 0.15 mg/kg	2.59 ± 0.13 mg/kg					6	GFAAS	III
Lyophilised Pig Kidney BCR 186[d]			128 ± 3 mg/kg	127 ± 1 mg/kg	299 ± 10 mg/kg	307 ± 9 mg/kg	14	FAAS	III

[a]mean ± 95% confidence interval
[b]mean ± SD
[c]Cereal and Oil Chemistry Institute, Ministry of Commerce, Beijing, China.
[d]Community Bureau of Reference, Brussels, Belgium

Experimental design of paper II

Sprague-Dawley rats, 15 dams with litters, were standardised to 10 pups per dam and the pups were randomly assigned to one of five diet groups and killed at intervals between 2 h and 12 days after a single oral dose of $^{109}CdCl_2$ dissolved in one of the test diets on postnatal day (PND) 11. The formulas included were soy formula, cow's milk formula, wheat/oat/milk follow-up formula and wholemeal/milk follow-up formula recommended from 0, 4, 6 and 12 months, respectively. Water was used as control diet. The infant formulas were prepared

according to the manufacturers' instructions and incubated with $^{109}CdCl_2$ during gentle shaking for 24 h in + 4ºC. The radioactivity of the incubated diets was measured in a gamma counter (Searl Nuclear Chicago Analytic, Des Plaines, Ill.; model 1185). The pups were anaesthetised and killed by heart puncture and blood was collected in heparinised syringes. The whole-body retention of ^{109}Cd was measured in a whole-body gamma counter (NaI well crystal, diameter 80 mm, depth 120 mm) before the pups were killed. Kidneys, liver, brain, and gastrointestinal tract, including stomach and colon, were removed and transferred into counting vials. The small intestines, were separated from the stomach and caecum and cut into segments. The radioactivity in all tissues and in the small intestinal rinsing solutions was determined by gamma counting. The fractional retention and uptake of ^{109}Cd were calculated by dividing the radioactivity in the whole bodies and tissues with the measured radioactivity in the oral dose of the respective diets. ^{109}Cd in blood, was expressed as fractional uptake of oral dose in the estimated total blood volume of the rat pup. The animal experiment was approved by the Uppsala Ethics Committee of Animal Experiments (permit number C 253/97).

Experimental design of paper III

Eleven piglets of Yorkshire breed were held indoors together with the sow. The newborn piglets received $CdCl_2$, dissolved in deionized water or wheat/oat/milk follow-up formula recommended from 6 months of age, twice daily from PND 0 to 10 with a syringe connected to a 40 cm gastric tube. On the basis of the daily body weights, a calculated volume of a Cd stock solution was added to the respective test diet to reach a Cd dose of 2 or 20 µg/kg body weight/day. Two control piglets suckled the sow without Cd exposure or gastric intubation. On PND 11, the piglets were killed and blood, liver, kidney, duodenum, ileum, gastric content and faeces were collected for trace element analyses. The small intestinal samples were cut open and rinsed. Also, liver, kidney, duodenum and distal jejunum from one control and one Cd exposed piglet were sampled for MT analyses. The animal experiment was approved by the Uppsala Ethics Committee of Animal Experiments (permit number C 198/0).

Determination of MT in piglet tissues

Proteins were electrophoresed on 18 % polyacrylamid gels containing 0.1% SDS under reducing conditions according to Laemmli (1970). Rabbit kidney MT (Sigma) was electrophoresed as a positive control. Two gels were run in parallel; one for Coomassie staining to assess the separation of the proteins and one for blotting. Kaleidoscope protein standards assessed the molecule weights of the bands. Proteins were electroblotted onto nitro-cellulose membranes. The membranes were blocked with 0.5 % Tween-20 before incubated at room temperature for 2 h with the primary monoclonal MT-antibodies. After washing, the membranes were incubated with horse-radish-peroxidase (HRP)-conjugated secondary rabbit-anti-mouse antibodies for semiquantitative detection of HRP by Enhanced Chemiluminescence Western Blotting detection reagents.

Experimental design of paper IV

Simulated gastrointestinal digestion of five different infant foods, wholemeal porridge, fruit porridge, wheat/oat/milk follow-up formula, liver casserole and pasta Bolognese (Table 1), was combined with exposure of digest supernatants to the human intestinal cell line, Caco-2. A previous method by Crews and co-workers (1983), for simulated digestion, was modified to fit the digestion conditions of an infant. Briefly, the pH of the simulated infant gastric juice was maintained between 5.5 and 6, and the concentrations of the digestive enzymes and the bile salts were half of the concentrations of the previous method. Solubility of Cd in the infant foods was compared after simulated adult and infant digestion, respectively. Gastric juices were prepared by adding pepsin to saline solution (0.5 and 1 % w/v for infant and adult juice, respectively). The pH of the gastric juices was adjusted to 5.5 and 1.8 to simulate infant and adult gastric conditions, respectively. Intestinal juices were prepared by adding equal volumes of pancreatin (1.5 and 3 % w/v for infant and adult juice, respectively) and bile salts (0.075 and 0.15 % w/v for infant and adult juice, respectively) to saline solution.

In vitro digestion procedure of infant foods

Infant and adult gastric juices were added to the infant food samples in polypropylene tubes and incubated at 37° C for 4 h during gentle shaking. The pH was checked and adjusted after 2 h to stay between 1.8 and 3.5 in the adult gastric juice, and between 5.5 and 6.0 in the infant gastric juice. After 4 h of gastric digestion, half of the samples were centrifuged at 2000 x g for 60 min. The supernatants containing the soluble fractions of Cd were subjected to Cd analyses. In the remaining samples, the pH was adjusted to 7.4 and infant and adult intestinal juices, respectively were added. The samples were again incubated at 37° C for another 4-hour-digestion and centrifuged as above. Blanks without infant food, only containing gastric and intestinal juices, were run in parallel. The solubility of Cd, determined as Cd in the 2000 x g supernatants, was thus measured both after the gastric stage and after the intestinal stage. An *in vitro* digestion control experiment, described in detail in paper IV, was performed to examine whether Cd from the digestive enzymes stayed in solution or was bound to the pellets of the infant food samples, after the digestion and centrifugation procedures.

Caco-2 cell experiments

The infant diets were digested under infant digestion conditions as described above. Radioactive $^{109}CdCl_2$ was added to each sample immediately after the addition of the gastric juice, allowing native and radioactive Cd to equilibrate during the 2 x 4-hour-digestion period. After digestion, the samples were centrifuged as described above and ^{109}Cd was determined in the supernatants. Caco-2 cells were grown in Dulbecco's Modified Eagle medium (DMEM) containing 10 % v/v foetal calf serum, 10 mM HEPES and 50 µg/ml Gentamicin. The cells were maintained at 37° C in an incubator with 5 % CO_2/95 % air atmosphere at 95 % relative humidity. Cells were harvested at 80 % confluence and seeded onto semipermeable filters at a density of 500 000 cells/cm^2 and allowed to differentiate on the filters for 21 days before the bioavailability

experiments. To investigate possible adverse effects on the Caco-2 cells, of infant food components or digestive enzymes present in the supernatants, the integrity of the cells was carefully assessed in a control experiment, described in detail in paper IV, prior to the bioavailability experiments. On the basis of the results of the control experiment, the bioavailability experiment was carried out for 180 minutes at 37° C with 1.5 ml DMEM on the basolateral side and 0.5 ml of non-heat-treated supernatant of the infant food digests containing soluble ^{109}Cd on the apical side. At the end of the experiment, the filter-supports with the Caco-2 cell monolayers were taken from the basolateral chamber and lyzed in 0.5 M NaOH overnight. The basolateral solution was collected and the radioactivity in the cells and in the basolateral solution was counted by gamma counting (Cobra 5003, Packard).

The fractional retention (% of administered dose) of a compound in blood and various tissues is often used as an indicator of bioavailability and in the present thesis, this has been applied for assessing the bioavailability of Cd from infant foods in the animal experiments in papers II and III. In paper IV, intracellular retention and transcellular transport of Cd in Caco-2 cells were used to assess the Cd bioavailability.

Statistics

The Statview 5.0 software was used for the statistical analyses of all data. The data in paper I met the required assumptions for using parametric statistics. Non-parametric statistics were applied on the data from papers II and IV, due to the limited number of samples in the treatment groups. In paper III, individual data of the piglets were presented without making statistical comparisons due to treatment groups of only two to three animals.

The distribution of data in paper I, was checked for normality by the Kolmogorov-Smirnov test. In paper II, the homogeneity of variance in the rat pup groups was checked with Bartlett's test. Un-paired t-test was used for comparisons of Cd concentrations in the different infant formulas in paper I. The Kruskall-Wallis analysis of variance in combination with Scheffé's post hoc test tested for differences in bioavailability of Cd between the rat pup groups in paper II, between Caco-2 cell monolayers in paper IV, and for solubility differences of Cd between infant diets in paper IV. Wilcoxon signed rank test checked for changes in TEER and LDH release in the Caco-2 cell monolayers between times 0 and 270 minutes after incubation with heat-treated or non-treated supernatants, respectively in the integrity control experiment in paper IV. The Mann-Whitney U test analysed differences in release of LDH from Caco-2 cell monolayers incubated with non-treated and heat-treated supernatants in the control experiment, and differences in Cd solubility of an infant diet after infant and adult digestion conditions, respectively and between gastric and intestinal digestion of a diet. Differences were considered to be significant at $p \leq 0.05$.

Results and discussion

Cd exposure from infant formulas and porridges (paper I)

The Cd levels of the infant diets in paper I ranged between 0.74 and 27 µg/kg concentrated formula (Table 1), which is in line with levels reported in corresponding products from Canada (Dabeka and McKenzie, 1987), Austria (Tiran *et al*., 1994) and France (Biego *et al*., 1998). Higher Cd levels were found in Swedish cow's milk formula in a previous study from the 1970s (Jorhem *et al.,* 1984), with a five times higher mean value (0.005 mg/kg) and a 30 times higher maximum value (0.061 mg/kg) compared to the mean and maximum levels of 1.10 and 1.89 µg Cd/kg, respectively in the cow's milk formulas in the present thesis. It can not be excluded that the higher levels in the former study could be due to sample contamination, or improved analytical techniques in the present study. However, quality control was included in the former study and cereal and milk-based follow-up formulas had similar Cd levels in the two studies, which indicate that the concentration differences in cow's milk formula from the two studies are true. Different compositions of cow's milk formulas from the 70s and the 90s could also explain the differences, or purer quality of the fortifying minerals used in the formulas in the latter compared to the former study. For example, fortifiers such as Zn in animal feed have previously been shown to be highly Cd contaminated (Sapunar-Postruznik *et al*., 2001) and vitamin-mineral premixes have been shown to be major contributors to Cd in Swedish pig feed (Lindén *et al*., 1999).

The formula ingredients seemed to have a strong impact on the Cd levels reported in paper I (Table 1). Cow's milk formula had the lowest (0.74-1.89 µg/kg) and wholemeal-containing formulas generally the highest Cd levels (8.28-27.0 µg/kg). Low lactational transfer of Cd in cattle (Smith *et al*., 1991) is probably the reason for low Cd levels in cow's milk formulas. Cd levels in wheat bran are approximately five times higher than in wheat flour because Cd accumulates in the outer part of the grain (Jorhem and Sundström, 1993), which explain the higher levels in the wholemeal formulas reported in paper I. The cereal containing formulas had approximately 1.5 to 21 times higher mean Cd levels than cow's milk formulas, which is a result of Cd being taken up by the root systems of the growing cereals. Furthermore, the uptake of Cd differs among plant species and cultivars (Sillanpää and Jansson, 1991; Wenzel *et al*., 1996; Gray *et al*., 1999; Kurz *et al*., 1999) and the Cd levels were higher ($p<0.0001$) in formulas containing oatmeal, wheat flour or rye flour compared to formulas based on corn starch, rice flour and cow's milk. Corn-, and rice-based formulas generally had higher Cd levels (0.86-4.18 µg/kg) than cow's milk formula but lower levels than soy formula (4.94-6.56 µg/kg, after correction for the 75 % water content).

The Cd levels analysed in the Swedish infant foods are low in comparison with most foodstuffs. However, the higher energy intake per kg body weight and the uniform food habits in infants compared to adults make formulas and porridges significant sources of dietary Cd to infants and children. Shortly after the recommendation for introduction of cereals had been lowered from six to four

months in Sweden (Lindberg, 1996), oatmeal porridges from four months became available on the Swedish market. Oats contain less gluten and generally less Cd than wheat (Sillanpää and Jansson, 1991) and Cd levels could therefore be expected to be lower in the four-months-porridges than in wheat-based porridges for older infants. However, the average Cd level in oatmeal porridge from manufacturer B (Findus) ranged between 17.3 and 19.6 µg/kg, which is similar to the levels in the wholemeal products from manufacturer B (Findus), recommended from 8 to 12 months that contained 14.8 to 27.0 µg Cd/kg. Higher Cd levels in the oats used by manufacturer B (Findus) than in the oats used by manufacturer A (Semper) could be the reason for this, but the most probable explanation is the addition of wheat flour in the four-month-porridge from manufacturer B (Findus) (Table 1).

There was an over all tendency of higher Cd levels in products from manufacturer B (Findus), compared to the corresponding products from manufacturer A (Semper). The Cd levels were significantly higher in the following products from manufacturer B (Findus); in wheat/oat/milk follow-up formula from six months ($p=0.0007$), in the wholemeal/milk follow-up formula from 8-12 months ($p=0.0001$), in the oatmeal porridge from four months ($p=0.0002$) and in porridge recommended from eight months ($p=0.0002$). This may be explained by differences in the geographical origin of the cereals used by the two manufacturers. By the time the infant diets were analysed, manufacturer B (Findus) obtained cereals from the province of Skåne, in the south of Sweden, where the levels and the plant availability of soil Cd is higher compared to most regions in Sweden (Eriksson and Söderström, 1996). Manufacturer A (Semper) obtained cereals from the province of Västergötland, in the south-west of Sweden, an area with lower Cd levels in soil compared to most regions in Sweden, including Skåne (Andersson and Pettersson, 1981). Another explanation could be different compositions of corresponding formulas. For example, at the time the food samples were analysed, manufacturer B (Findus) often gave cereals as main ingredient on the package labelling, whereas manufacturer A (Semper) gave skimmed milk or whey powder as main ingredient in a corresponding formula. Skimmed milk powder has a diluting effect on the Cd levels in a formula, as Cd levels in cow's milk are low.

The average daily intake of Cd from infant foods has been estimated to approximately 0.1 µg/kg body weight for 0-6 month-old infants, and 0.16 µg/kg body weight for 6-12 month-old infants (Ministry of Agriculture, Fisheries and Food, 1999). The average daily intakes of Cd from Swedish formulas and porridges were calculated in paper I, by using the recommendations given by the manufacturers at the time for data analyses regarding consumption amounts, and varied between the different formulas. Corn starch formula for example, contributed to 0.30 µg Cd/day, whereas wheat/oat/milk-based follow-up formula from manufacturer B (Findus) contributed to 3.27 µg Cd/day when the maximum amount of 1 200 ml was consumed. The daily Cd intake ranged between 0.01 and 0.44 µg/kg, provided that the recommendations for age and consumption amounts were followed for each formula, assuming average body weight of the child. The highest Cd exposure (0.44 µg/kg/day), was estimated in 6-month-old children, with an average weight of 7.5 kg, consuming the recommended maximum amount (1

200 ml/day) of wheat/oat/milk follow-up formula from manufacturer B (Findus) containing 18.2 µg Cd/kg powder (Figure 1). The corresponding exposure from the same type of formula but with a different composition, bought and analysed in 2000, would be 0.27 µg Cd/kg/day. Despite the low levels of Cd in the cow's milk formula, it was estimated from our results that the Cd intake was twice as high in an infant consuming 1000 ml cow's milk formula compared to the same amount of breast milk, and from soy formula, 12 times higher than from breast milk (Figure 1).

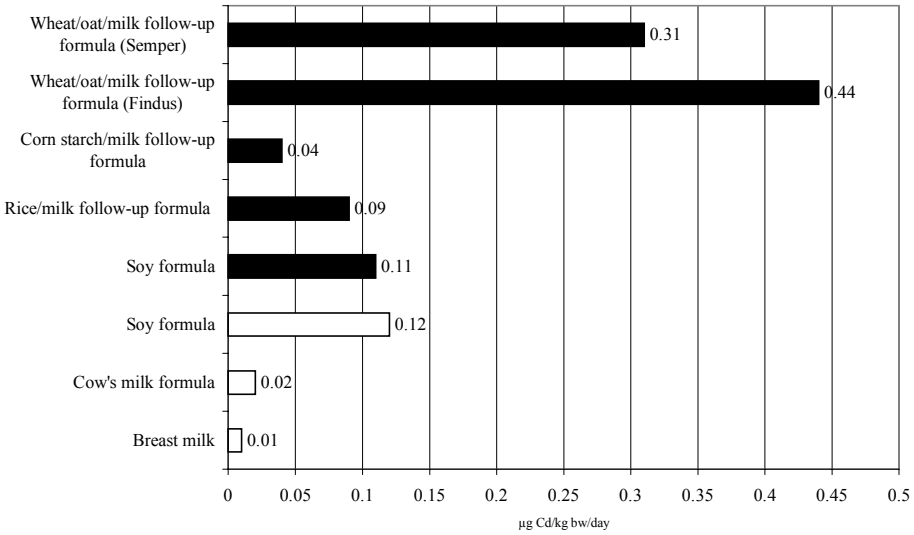

Figure 1. Daily exposure of Cd in a 4-month-old infant weighing 6.5 kg (open bars) fed 1000 ml breast milk or infant formula and in a 6-month-old infant weighing 7.5 kg (filled bars), fed 1200 ml of different formulas. The Cd contribution is calculated from the analysed mean levels of the respective formula, not including the Cd contribution from water. The breast milk data are from Palminger Hallén *et al*., (1995b).

It should be noted that the calculated Cd intakes from the formulas in paper I, do not include the contribution of Cd from drinking water, which is added when the formulas are prepared for consumption. Cd levels in drinking water are generally below 1 µg/l (WHO, 1989) and in Sweden levels have been reported to range between < 0.001 and 0.21 µg/l (Bensryd *et al*., 1994; Olsson *et al*., 2002). Thus, the contribution of Cd from Swedish drinking water is most likely negligible in most cases. However, preparation of a corn starch formula, as in the example above, with water containing 0.21 µg Cd/l, results in a significant addition of Cd as it nearly doubles the Cd contribution from the ready-to-serve formula, 0.55 compared to 0.30 µg/day. Furthermore, a guideline value of 3 µg/l for Cd in drinking water has been recommended by the WHO (1993) and preparation of a follow-up formula containing 18.2 µg Cd/kg powder, like manufacturer B's (Findus) wheat/oat/milk follow-up formula from six months, with water containing 3 µg

Cd/l and serving it as in the example above, results in an intake of 0.86 µg Cd/kg body weight per day in an infant, which is nearly 90 % of the PTWI.

Circumstances at the infant food manufacturers' have changed since the analyses of the infant foods in paper I. The compositions of some formula types have changed, and the manufacturers do not give recommendations regarding consumption amounts for cereal-based follow-up formulas any longer. However, recommended consumption amounts up to 1200 ml are given for infants up to five months of age on the cow's milk formula packages. Furthermore, both manufacturers are concerned about choosing cereals with a low Cd content and manufacturer B (Nestlé) now obtains most cereals from the same regions as manufacturer A (Semper), in Västergötland.

Uptake of a single oral dose of Cd from infant diets in newborn rat pups (paper II)

The whole-body retention of ^{109}Cd tended to be higher in rat pups that had received Cd in water or in cow's milk formula compared to soy formula or cereal-based formula at all survival times between 2 hours and 12 days (Table 3). Four days after the oral dose, whole-body retention ranged from 67 and 65 % in the water and cow's milk formula groups, respectively to 52 % in the wholemeal/milk formula group. This is higher than the previously reported whole-body retentions in adult rodents of 6 (Engström and Nordberg, 1978) and 5 % (Lind and Wicklund Glynn, 1997), three to five days after single oral doses of $^{109}CdCl_2$ in water. Sørensen and co-workers (1993) reported even lower whole-body retention, 1 % in adult mice, three days after a single oral dose of $^{109}CdCl_2$.

The whole-body retention in the rat pups was mainly a reflection of the small intestinal retention, which was remarkably high still nine days after the oral dose, indicating a slow regeneration of the epithelial cells in the intestinal tract and a long absorption period in newborns (Table 3). Higher values in the small intestines compared to the whole-bodies of the pups is probably explained by a lower counting efficiency in the whole-body gamma counter, 6.5 % than in the tissue gamma counter, which had 50 % counting efficiency and determined the radioactivity in the rinsed small intestines. Four days after the Cd dose, significant differences in the small intestinal retention were detected between the two cereal-based formula groups, which retained 54 to 55 % of the dose, and the water control, the cow's milk formula and the soy formula groups, which all retained approximately 70 % of the dose. Nine days after ^{109}Cd dosage, on PND 20, retention in the small intestine varied greatly within the diet groups, for example between 0.7 and 49 % within the soy formula group. This is probably due to individual variations among the rat pups, in the onset of enterocyte re-generation, which occurs between 15 and 21 days of age (O'Connor, 1966). The slow regeneration of enterocytes in the pups is associated with a prolonged small intestinal retention creating possibilities for a continuous absorption site of Cd. The intestinal mucosa appeared to be a continuous site for Cd absorption, even 12 days after the oral Cd dose.

Table 3. Cd retention (% of dose) in rat pups given a single oral dose of $^{109}CdCl_2$ in infant formulas or water on PND 11. Data are means ± SD of 5-6 animals. Values not sharing a letter are significantly different ($p<0.05$) within the same column and survival time.

Survival after dose	Diet	Whole body	Rinsed small intestine	Blood	Liver	Kidney
4 days	Water	67 ± 7[a]	71 ± 8[a]	0.036 ± 0.016[a]	1.63 ± 0.43[a]	0.37 ± 0.05[a]
	Cow's milk formula	65 ± 6[ab]	70 ± 5[a]	0.020 ± 0.004[ab]	0.74 ± 0.13[b]	0.27 ± 0.04[b]
	Soy formula		70 ± 4[a]	0.016 ± 0.008[b]	0.52 ± 0.11[b]	0.26 ± 0.02[b]
	Wheat/oat/ milk formula	56 ± 3[bc]	54 ± 8[b]	0.011 ± 0.005[b]	0.53 ± 0.13[b]	0.22 ± 0.04[b]
	Wholemeal /milk formula	52 ± 3[c]	55 ± 3[b]	0.010 ± 0.003[b]	0.31 ± 0.15[b]	0.22 ± 0.03[b]
9 days	Water	41 ± 22[a]	24 ± 10	0.028 ± 0.008[a]	1.28 ± 0.36[a]	0.72 ± 0.31
	Cow's milk formula	34 ± 15[ab]	22 ± 16	0.021 ± 0.006[ab]	0.82 ± 0.25[bc]	0.92 ± 0.43
	soy formula		18 ± 21	0.024 ± 0.005[a]	1.07 ± 0.17[ab]	1.06 ± 0.44
	Wheat/oat/ milk formula	21 ± 7[ab]	26 ± 15	0.013 ± 0.003[b]	0.69 ± 0.13[bc]	0.74 ± 0.32
	Wholemeal /milk formula	15 ± 7[b]	11 ± 9	0.013 ± 0.002[b]	0.62 ± 0.06[c]	0.78 ± 0.29

By mistake the whole-body retention in the soy formula group was not measured on survival days 4 and 9, and could consequently not be presented.

The duodenal retention of Cd in the rat pups, 24 hours after dosage, was lower, 5 to 17 % of the dose, than from studies with adult rodents. For example, Lind and Wicklund Glynn (1997) reported 40 to 60 % duodenal retention of a single oral dose in adult mice. The duodenal retention in the adult animals is most likely explained by the low pH of the gastric contents emptying into the duodenum. Distal to the pancreatic duct the pH increases and Cd will rapidly be chelated by various dietary components and is then thought to be less available for intestinal uptake. The lower retention in the duodenum of the rat pups, which may be explained by newborns having a higher pH in the stomach, could be expected to

lead to a lower Cd absorption in the rat pups than in adults. However, by reducing the binding of Cd in the duodenum, more Cd is passed further down into the jejunum. Thus, the distribution of retained Cd in the small intestine differed from that reported in adult animals. The significance of this is that in newborns, the duodenum might not be the major site for Cd absorption. One pathway could be paracellular transport facilitated by looser tight junctions between the enterocytes, compared to adult rats, or Cd absorption in the rat pups might occur through pinocytosis (Jones, 1978; Keller and Doherty, 1980; Weaver, 1992), as previously discussed in the introduction section.

Significant differences between the diet groups in blood and tissue levels of ^{109}Cd were detected at day four after dosage (Table 3). Levels of ^{109}Cd in blood were higher in the water group (0.04 % of the dose) compared with the soy formula group (0.02 %) and the two cereal-based formula groups (0.01 %, respectively). Likewise, on day four, the retention of ^{109}Cd in the liver and kidneys was significantly higher in the water group compared to all four formula groups. Unexpectedly, the ^{109}Cd levels in blood and liver of the rat pups were higher two than 24 h after dosage, which indicates a high initial absorption. Normally, MT in the enterocytes is proposed to bind Cd and delay (or prevent) the metal from being absorbed into the circulating blood (Foulkes and McMullen, 1986, Min *et al*., 1991). It could be that the basal rate of the MT production in the enterocytes of an individual receiving a single dose of Cd is too slow to delay the Cd absorption, resulting in elevated Cd absorption shortly after the oral dose. Despite the immature renal functions in newborns, the rat pups were able to accumulate approximately 1.5 % of a single dose in the kidneys, 12 days after the oral administration (on PND 23), and at this time point the levels were still increasing in all treatment groups. Thus, in order to detect endpoints, such as Cd accumulation in kidney cortex, it is important to extend studies on Cd absorption in newborns over a long time period. Moreover, on survival day 12, significant differences in kidney Cd could no longer be detected between the diet groups, and it may be that differences in Cd retention from different diets is erased with time, due to prolonged small intestinal retention and absorption of Cd in neonates.

Uptake of low-dose Cd in newborn piglets after repeated oral administrations, effects on Zn and Fe (paper III)

The uptake of oral Cd in newborn piglets was diet- and dose dependent (Table 4). Piglets that had received Cd in water retained twice as much of the total Cd dose in the liver as piglets exposed to Cd in follow-up formula, 2 versus 1 %. These results are comparable to those of the rat pups in paper II, which also retained approximately 1 to 1.5 % of the dose in the liver, 12 days after exposure to a single oral dose. Previously, total (Sullivan *et al*., 1984b) and liver (Sasser and Jarboe, 1980) retention of radioactive Cd have been reported to be about 2 % in newborn piglets at similar survival times. However, the administered Cd doses in the former studies, are either unclear (Sullivan *et al*., 1984b) or much higher, about 2 mg/kg body weight (Sasser and Jarboe, 1980) and were administered as single oral doses in water. In the present study, the major part of the orally administered Cd passed

the gastrointestinal tract of the piglets without being absorbed. The gastrointestinal Cd uptake in our study was highest in the duodenum, which is in contrast to observations from Sullivan and co-workers (1984b), who found most of the small intestinal Cd further down jejunum and ileum. In our study, duodenal retention was approximately twice as high in piglets exposed to 20 µg Cd/kg/day in water, compared to piglets that had received the same dose in infant follow-up formula, 3.1 versus 1.4 mg/kg. The mean Cd levels in blood and tissues of piglets exposed to 20 µg Cd/kg in water were approximately ten times higher than the corresponding levels in piglets exposed to 2 µg/kg.

The follow-up formula reduced the Cd uptake in comparison with water, but the distribution of absorbed Cd to the kidneys was 19 % higher in the formula group compared to the water group exposed to the same Cd dose (20 µg/kg/day). One explanation could be that Cd administered in formula binds to phytochelatins in the cereals and form complexes that resembles Cd-MT, which are distributed directly to the kidneys without being trapped in the enterocytes. In rats, oral administration of Cd-phytochelatin complexes leads to increased distribution of Cd to the kidneys compared to $CdCl_2$ (Fujita *et al*., 1993). Another explanation could be that the maturation of the renal function in the newborn individual is triggered by some compound(s) in the formula, making the re-absorption of Cd in the proximal tubular cells more efficient

The piglet model enabled detection of MT in Cd exposed, as well as in unexposed animals. The MT levels in liver, kidney and duodenum of the piglet exposed to 20 µg Cd/kg/day in water, were two to six times higher compared to the levels in the unexposed control piglet. Thus, even low-dose exposure to dietary Cd induces MT levels at this age. Although MT sequesters Cd and acts like a detoxifying agent inside cells (Klaassen and Liu, 1997), it also functions as a carrier of Cd between tissues, for example from the liver to the kidneys, and absorbed Cd-MT complexes exert toxic effects in the kidneys at much lower doses than inorganic Cd does (Min *et al*., 1986). Foetal and neonatal tissues of humans contain relatively high concentration of MT in comparison with adults (Riordan and Richards, 1980; Bakka and Webb, 1981; Yoshida et al., 1998), and previous studies have shown that the half-life of MT is 12 times higher in animal neonates, compared to adults (Kershaw and Klaassen, 1992). Thus, it may be that binding of Cd to MT in the enterocytes of newborns leads to a higher retention of Cd and that the MT sequestering of Cd in the intestinal mucosa is further prolonged due to the late onset of enterocyte re-generation in neonates in comparison to adults resulting in a prolonged absorption time.

Table 4. Mean Cd levels in blood (µg/l) and tissues (µg/kg) of newborn piglets orally exposed to $CdCl_2$ in deionized water or follow-up formula for the first 10 days of life, n= 2-3. For comparison, human data are also given.

Species	Exposure	Duo-denum	Ileum	Blood	Liver	Kidney	Reference
Suckling piglets	unexposed control	8.2	5.7	0.12[a]	0.46	0.42	Paper III
Suckling piglets	2 µg Cd/kg/d in deionized water	427	37	0.27	6.0	6.0	Paper III
Suckling piglets	20 µg Cd/kg/d in deionized water	3054	275	2.0	77	54	Paper III
Suckling piglets	20 µg Cd/kg/d in follow-up formula	1391	560	0.63	43	64	Paper III
Human foetuses and infants[b]						1-8	Lutz *et al*., 1996
Human Infants[c]					50	610	Yoshida *et al*., 1998
Human Infants[d]				0.4			Kraschler *et al*., 1999
Children[e]				0.5-6.0			Moon *et al*., 2003
Human adults				0.2-0.4			Kraschler *et al*., 1999; Baecklund *et al*., 1999; Björkman *et al*., 2000; Olsson *et al*., 2002

[a]The Cd uptake in blood from unexposed control is from piglet No. 1. The blood sample from piglet No.2 was accidentally spilled and therefore not analysed.

[b]Data are reported range in second trimester abortions and deceased infants before 3 months of age.

[c]Data are reported in autopsy samples from infants, 0-12 months of age.

[d]Data are reported in infant sera.

[e]Data are reported range in children, 4-10 years old.

The Zn level in the livers of piglets exposed to 20 µg Cd/kg/day was only about half of the corresponding level in the unexposed control piglets. Similarly, the mean duodenal Zn level in piglets exposed to 20 µg Cd/kg/day in water, was less than half of the level in the controls. This could reflect a competitive binding of Cd instead of Zn at the absorption sites in the small intestine, which would reduce the Zn absorption. Furthermore, Cd has a higher affinity to MT than Zn (Waalkes *et al*., 1984), and Cd might thus replace Zn on hepatocyte MT, resulting in lower Zn

levels in the liver. It is also possible that Cd interferes with the expression and/or the function of Zn transporting proteins in the enterocytes and/or the hepatocytes. Divalent cations, such as Cd^{2+} and Fe^{2+}, have previously been shown, *in vitro*, to inhibit Zn uptake mediated by a human Zn transporting protein belonging to the ZIP (Zrt-, Irt-like Protein) family, hZip2 located in plasma membranes (Gaither and Eide, 2000), and Cd^{2+} could possibly affect the Zn transporters in the intestines of the piglets in the same fashion. In contrast to our results, Zn levels in kidneys and livers of Cd exposed pigs increased significantly compared to controls in a previous study (Cousins *et al*., 1973). However, the latter study was conducted with adult pigs exposed to much higher doses of Cd (450-1350 ppm in pig feed) than in our study. In the present study, the levels of Fe were similar among Cd exposed and control piglets, ranging from 0.01-0.02 mg/kg dry weight, in the gastric content, to 0.14-0.30 mg/kg in faeces. Only in the livers of piglets in the high-dose water group could a tendency of lower Fe levels (0.31-0.40 mg/kg) be detected, compared to unexposed controls (0.54-0.61 mg/kg).

Bioavailability of Cd from infant food in Caco-2 cells after simulated infant digestion (paper IV)

The pepsin and pancreatic enzymes used in the *in vitro* digestive experiments, contained endogenous Cd, 10.6 and 32.7 µg/kg powder, respectively, which contributed to 12 to 16 % of the total Cd content of the infant food samples. Enzyme derived Cd could overestimate the amount of soluble Cd from digested food samples, and it is surprising that this has not been considered in previous *in vitro* digestion reports (Crews *et al*., 1983; Hocquellet *et al*., 1997). Pancreatic fluid is a well-known, although minor, excretion route for Cd (Friberg *et al.,* 1985), and it is likely that Cd in the pancreatic enzymes originates from digestive metallo-enzymes, for example from carboxypeptidase A, which normally binds Zn (Stryer, 1988). However, the control experiment (described in detail in paper IV) revealed that the enzyme derived Cd was soluble and stayed in the supernatants, rather than bind to the pellets, after the digestion and centrifugation of the infant food samples. Thus, the Cd concentration in supernatants of blank samples containing only the digestive enzymes, without infant food sample, was subtracted as background from the Cd concentration in the supernatants of the digested infant food samples in the *in vitro* digestion experiment.

The solubility of Cd was significantly lower after infant than adult digestion. Only a pasta Bolognese ready-to-use dish had comparable amounts of soluble Cd in the supernatants after complete digestion (Table 5). Furthermore, infant digestion generally resulted in less soluble Cd in the gastric juice compared to the intestinal juice, which was in contrast to the solubility after adult digestion conditions. The higher pH in the infant gastric juice, compared to the adult gastric juice, is probably the main reason for that, as it means less dissociation of Cd from the food components. Moreover, the lower enzyme concentrations in the infant digestive juices, compared to the adult juices, probably lead to less proteolytic degradation of the food with consequent changes in binding sites for Cd. Pepsins for example, have a pH optimum between 1.6 and 3.2 (Ganong, 2003). The pH of the infant

gastric juice was thus too high (5.5-6) for the pepsins to be fully active. The pH of the infant intestinal juice however, was presumably optimal for the pancreatic enzymes to digest the infant food and this probably explains the higher solubility of Cd in infant intestinal juice compared to infant gastric juice. After complete infant digestion, the solubility of Cd in a liver casserole ready-to-use dish was higher (52 %) compared to the other diets (range 30-31 %). The Cd solubility from the wholemeal porridge was only 1 % in the infant gastric juice, and 30 % in the infant intestinal juice. The low solubility in the gastric juice could be explained by the fact that the minimum solubility of Cd-phytic acid complexes is found at pH 6 (Nolan *et al*., 1987), which corresponds to the pH of the infant gastric juice. Thus, Cd bound to phytic acid or insoluble dietary fibre would be removed by the centrifugation after the infant gastric stage. The corresponding solubility after adult digestion was 69 % for the liver casserole and 73 and 52 %, respectively for the wholemeal porridge in gastric and intestinal juice. Thus, different compositions of foods resulted in a wide variation in Cd solubility after digestion of the foods under the same digestion conditions.

Table 5. Fractional solubility of Cd after *in vitro* digestion of infant food. Data are medians (interquartile ranges, q3-q1) of n=6. Gastrointestinal conditions were adjusted to simulate infants and adults for comparison.

		Soluble Cd in digestion juices (%)			
	Endogenous Cd in food sample	Infant		Adult	
Infant food	(µg/kg)	Gastric	Intestinal	Gastric	Intestinal
Wholemeal porridge	12.8	1 (1)^{d}	30 (3)^{d}	73 (5)^{ab}	52 (9)^{a}
Fruit porridge	10.3	17 (3)^{eg}	31 (2)^{d}	74 (3)^{a}	57 (4)^{ab}
Follow-up formula	11.4	8 (1)^{de}	30 (2)^{d}	66 (9)^{ac}	50 (6)^{a}
Liver casserole	11.9	63 (16)^{f}	52 (9)^{e}	85 (7)^{b}	69 (10)^{b}
Pasta Bolognese	9.52	19 (4)^{g}	31 (5)^{d}	47 (10)^{c}	26 (2)^{c}

Data not sharing a letter in a column, indicate significantly different solubility between diets digested under the same gastrointestinal condition.

The Cd uptake in the Caco-2 cells incubated with supernatants of the infant foods varied among the diets between 4 and 6 % of the dose, and the transport of Cd over the monolayers varied between 1 and 2 % of the dose (Figure 2). Digests

containing food compounds increased the intracellular uptake and transport of soluble Cd up to 66 and 133 %, respectively compared to the enzyme control solution. Monolayers of Caco-2 cells incubated with supernatants of liver casserole had significantly higher intracellular uptake of soluble Cd compared to wholemeal porridge and a control solution containing the digestive enzymes. The reason for this could be higher availability of Cd in beef liver than in whole-wheat meal. Cd in liver is mainly bound to MT and the concentration of H^+ is the major factor known to influence the dissociation of metals from MT. However, metals begin to dissociate from MT already when the pH drops from seven to five and MT with no metals bound to it is susceptible to degradation by proteolytic enzymes (McKim *et al*., 1992). Hence, the pH of our infant gastric juice would be low enough for sufficient dissociation of Cd from MT. It could be that Cd in the liver casserole has dissociated from MT in the infant gastric juice and that the proteolytic enzymes have degraded most of the MT, rendering free Cd ions in the supernatant of the liver casserole digests. Free Cd ions in the supernatant of the intestinal liver casserole digest could then bind electrostatically to the cell membranes of the Caco-2 cells and be internalised in the enterocytes of duodenum (Foulkes, 1985), for example via metal transporting proteins like DMT1 (Picard *et al*., 2000; Tallkvist *et al*., 2001) or ZIPs (Gaither and Eide, 2001). In the enterocytes, the free Cd ions may bind to intracellular components such as MT, glutathione or other molecules containing SH-groups and this may delay the transepithelial transfer of Cd to the basolateral side.

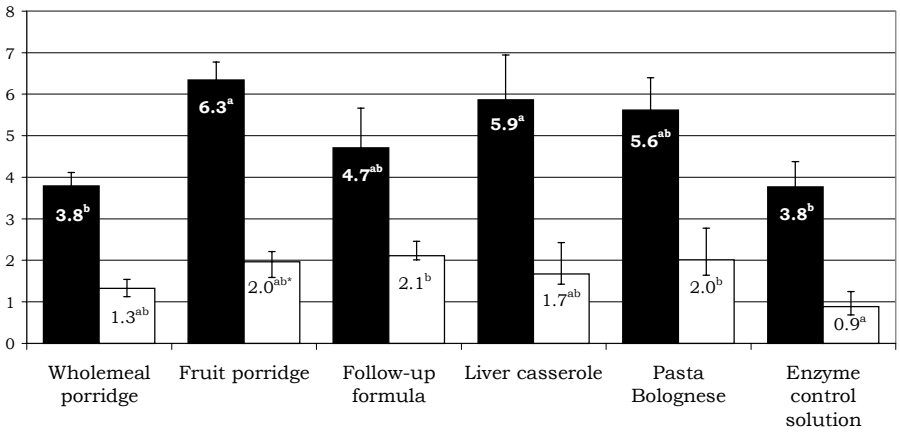

Figure 2. Uptake (filled bars) and transport (open bars) of soluble Cd in monolayers of Caco-2 cells after three hours of incubation with supernatants of *in vitro* digested infant foods. Results are expressed as % of total Cd dose, medians and interquartile ranges (q3-q1), n=6-8. Different letters indicate significant differences in Cd uptake and transport, respectively between diets. *p=0.05 as compared to enzyme control.

It could be that intracellular uptake of Cd is inhibited or delayed by soluble food components in the digest of the wholemeal porridge, reducing the intracellular retention of Cd. However, it could be speculated that food-bound Cd internalised

by the enterocytes, interacts with intracellular components to a lesser degree than free Cd ions do, since we found lower intracellular uptake, but higher transport of Cd in digests of food samples than Tallkvist and co-workers (2001) did in a similar experiment, where Caco-2 cells were exposed to $^{109}CdCl_2$ of similar concentration in saline solution. Thus, food-bound Cd may facilitate the transport over the epithelial cells by shortening the intracellular time period. Significantly higher transport of Cd was seen over monolayers incubated with supernatants of digested follow-up formula and pasta Bolognese, compared to monolayers incubated with the digestive enzyme control solution. The reason for this is not known, but it could be speculated that the transport of Cd over the monolayer is facilitated by certain dietary constituents which are formed during the digestion of the food. For example, casein phosphopeptides (CPP's) are formed when proteolytic enzymes in the digestive tract degrade casein in milk. CPP's enhance the absorption of Ca in chickens (Mykkänen and Wasserman, 1980) and in rats (Kitts *et al*., 1992) and improve Zn and Ca bioavailability from high phytate infant meals in rat pups (Hansen *et al*., 1996). It could be that CPP's have a similar effect on the absorption of Cd^{2+} associated with casein in the follow-up formula, which had skimmed cow's milk powder as a main ingredient.

The results of the experiments in paper IV, show that the solubility of an element does not always correlate with its bioavailability. What may be of more importance, the uptake of Cd in the Caco-2 cells or in the gastrointestinal tract is up to an order of magnitude lower than the solubility. The fractional solubility of Cd after the infant digestion ranged from 1 to 63 %, but the fractional uptake and transport of Cd was only between 1 and 6 %. Furthermore, it is not given that the food type with the highest solubility of Cd is the food type with the highest bioavailability of Cd. In our study, liver casserole clearly had the highest fractional Cd solubility (52-63 %) in infant digestion and a higher intracellular uptake (5.9 %) than wholemeal porridge and enzyme control solution, which may indicate a higher risk of exposure due both to a higher Cd dose accessible to the intestinal cells, and a higher cellular uptake. However, the solubility of Cd from the follow-up formula was low, 8 and 30 % respectively, in the infant gastric and intestinal juice. Still, the transepithelial transport of the soluble Cd from this formula was 133 % higher compared to the enzyme control solution.

Relevance of the bioavailability models

Data for risk assessment of Cd exposure in human infants are practically lacking and to improve the risk assessment for infant Cd exposure, knowledge of the bioavailability of dietary Cd and health effects of early exposure is of paramount importance. However, ethical and practical considerations make it difficult to conduct controlled studies in the human infant. Thus, relevant models for bioavailability studies of Cd in food must be developed. The experimental conditions in the bioavailability models used in this thesis were carefully elaborated to resemble infant conditions as much as possible with regard to Cd doses, diet types and physiological conditions.

In paper II, we used oral administration of radioactive Cd equilibrated in infant diets to newborn rat pups. This model has been used in previous bioavailability studies with essential trace elements from cow's milk formula in newborns (Lönnerdal *et al*., 1993; Hansen *et al*., 1996). Studies with radioactive elements with a high specific activity have the advantage of using low concentrations of the element in question, and the amount of the radioactive element that is retained is not affected by contamination, and it allows easy quantification of the uptake of the label. However, it involves the assumption that the extrinsic label has fully equilibrated with the non-labelled intrinsic mineral of the tested food. However, the distribution of ^{65}Zn (Sandström *et al*., 1983), ^{54}Mn (Lönnerdal *et al*., 1985a), ^{64}Cu (Lönnerdal *et al*., 1985b), ^{28}Mg (Lönnerdal *et al*., 1993) and ^{203}Pb (Palminger Hallén and Oskarsson, 1995a) have previously been shown to be virtually identical to those of the endogenous elements in milk and in infant formulas. Furthermore, in a recent study, Cd solubility was similar after simulated digestion of extrinsically and intrinsically Cd contaminated lettuce (Waisberg *et al*., 2003). Similarly, in paper IV of the present thesis, the distribution of ^{109}Cd was similar to that of the endogenous Cd after the simulated digestion and centrifugation procedures.

However, the relevance of existing bioavailability models for Cd with rodents is limited by the fact that the physiological conditions differ in many ways from those of humans. Rats and mice have a rapid metabolism and intestinal transit time for food. Rodents are coprophagous which enables them to assimilate microbial amino acids and vitamins. Pigs, on the other hand, are omnivorous with anatomical and physiological conditions of the gastrointestinal tract that resemble those of man. Particularly the upper part of the small intestines, where the absorption of trace elements takes place, is comparable with humans. Composition of the diet, the pH and transit time for food in the gastrointestinal tract are also similar to that of humans and microbial degradation in the large intestines of man and pig is of less importance compared to rodents (Moughan *et al*., 1992; Miller and Ullray, 1987). Moreover, the kidneys of the pig and man are similar in that they are both multipyramidal, meaning that the medulla is arranged as many pyramidal masses, with a portion of the cortex capping each pyramid at its base, whereas the apex of the pyramid points toward the renal sinus (Dyce *et al*., 1987).

In paper III, repeated administrations of oral Cd in low doses to newborn piglets by gastric intubation was manageable by one person alone and also allowed the administration of Cd to be strictly controlled in each animal. The lowest dose in the piglet model (2 µg Cd/kg/day) is in line with the daily intake of dietary Cd in German children, which recently was reported to be up to 2.06 µg/kg/day (Wilhelm *et al*.. 2000), and it was sufficient to result in Cd levels above the detection limit in blood and tissues after 11 days of exposure.

The advantage with animal models is that the accumulation of Cd is measured in a biological system rather than estimated from an *in vitro* model or limited human clinical data. However, a rather large number of animals is required to meet the assumptions behind statistical evaluations, and there is the inter-species variations to consider when extrapolating the results to human infants. Simulated infant digestion combined with uptake and transport of soluble Cd from the digests, in

fully differentiated human enterocytes (Caco-2 cells) is a practical and manageable complement for estimations of the Cd availability in newborns, and obviously does not require experimental animals. Furthermore, the Caco-2 cell model enables mechanistic studies, for example to investigate induction and/or inhibition of transport proteins for Cd or other trace elements. Caco-2 cells show many of the functional and morphological properties of human enterocytes (Pinto *et al*., 1983; Hidalgo *et al*., 1989). They differentiate into polarised enterocyte-like monolayers with microvilli and act similarly to small intestinal cells. However, the Caco-2 cell model in combination with simulated infant digestion needs to be validated against an *in vivo* model, preferably in humans but the ethical and practical difficulties with controlled infant studies as discussed before, make the described piglet model a relevant alternative. For example, Cd bioavailability from the same diets (soy formula, cow's milk formula, cereal and milk-based formula) could be tested in the Caco-2 cell-, and in the piglet model. The gastrointestinal absorption of Cd in infants might be higher than the results from the Caco-2 cell experiments in paper IV indicate, as the intestinal epithelium of infants is not fully developed with looser tight junctions than adults, which could not be simulated in the Caco-2 cell model. Furthermore, the enterocyte turnover is lower in infants than in adults, which might lead to longer retention periods of Cd in the gut mucosa with increased risk for Cd to be absorbed.

When comparing the results from the rat pup and the piglet model, it was found that fractional Cd retention in the livers of newborn rat pups and piglets was similar, 1-2 % of the dose, at survival days 12 and 11, respectively, after exposure to a single Cd dose of approximately 8 µg Cd (300 µg Cd/kg bw) to the rat pups and continuous exposure of 20 µg/kg bw/day (500 µg Cd in total) to the piglets. However, a quantitative comparison is difficult to make between the different models. Furthermore, qualitative differences were also found between the rat pup and the Caco-2 cell model. In the rat pup model, the wheat/oat/milk follow-up formula reduced the Cd retention and uptake in comparison to water and cow's milk formula. However, in the Caco-2 cell model, the transport of Cd over monolayers incubated with digests of wheat/oat/milk follow-up formula was significantly higher, as compared to a enzyme control solution.

The biological half-life of Cd in human kidneys is up to 30 years. Thus, if the Cd absorption by the human infant resembles the high rate of absorption of the neonatal rat and swine, Cd represents a much greater hazard than it would if it was rapidly excreted. According to our calculations, the exposure of Cd from infant formulas is higher than from breast milk and efforts are being made to design infant and follow-up formulas to optimise the bioavailability of minerals and trace elements, such as Fe. There is a risk in doing so, that the bioavailability of toxic metals, for example Cd, also will be increased. On the other hand, the ingredients of commercial infant foods in Sweden are thoroughly analysed for contaminants, such as Cd, and by choosing cereal species and cultivars grown in soil types with low Cd release, Cd levels in infant foods may be reduced.

Concluding remarks

The most important findings in the present work were as follows:

- Infants that are not breast-fed are exposed to up to 12 times more Cd from infant formulas compared to breast milk. An infant might be exposed to 50 % of the PTWI by consuming follow-up formula in amounts that cover the infant's daily energy and nutritional need.

- The Cd levels in infant formulas are affected by the formula composition so that cow's milk formulas generally have the lowest and wholemeal formulas the highest Cd levels.

- Cereal based follow-up formulas reduce the bioavailability of Cd in comparison to water and the composition of a formula affects the bioavailability of Cd so that it is generally lowest from wholemeal formulas and highest from cow's milk formulas.

- The whole-body retention of a single oral Cd dose in water or infant formula is higher in newborn rats compared to previously reported in adult animals.

- Prolonged small intestinal retention of dietary Cd, probably due to late onset of enterocyte re-generation and a high half-life of Cd-sequestering MT in the enterocytes, prolongs the absorption period and increases Cd uptake in neonates compared to adults.

- Despite the immature renal function, newborn rat pups and piglets are capable of accumulating Cd in the kidney cortex.

- The distribution of absorbed Cd to the kidneys is higher in piglets when Cd is given in cereal-based follow-up formula than in water.

- The solubility of Cd from infant food is lower at infant compared to adult digestion conditions, but food compounds in the infant food digests might enhance the intracellular uptake and transport of soluble Cd compared to a control solution.

- The uptake and transport of Cd in human intestinal Caco-2 cell was approximately one order of magnitude lower than the solubility of Cd after simulated digestion of infant food.

Bioavailability of dietary Cd in neonates needs to be further investigated to improve the risk assessment of Cd exposure in infants and children. The proposed Caco-2 cell line model combined with simulated infant digestion could be suitable for screening studies of Cd bioavailability in larger numbers of infant foods, whereas the piglet model is more appropriate for validation studies of Cd bioavailability with a limited number of infant diets.

It is concluded that age-specific digestion conditions as well as diet composition affect both the solubility and the bioavailability of dietary Cd. The calculated Cd intake from the previously recommended intake of infant formulas is below the PTWI. However, the PTWI does not include a safety factor and is based on renal effects in adults. Neonatal, compared to adult Cd exposure, could pose a risk of elevated absorption and a greater life-time body burden of Cd with possible adverse health effects. This must be taken into consideration when setting standards for Cd exposure and making risk assessment for infants and children.

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Acknowledgements

This work was carried out at the Department of Pharmacology and Toxicology and at the Department of Food Hygiene, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

I wish to express my sincere gratitude to all of those who have supported me in different ways in the making of this thesis, and especially to:

Agneta Oskarsson, my supervisor, for giving me the opportunity to take on this project. For invaluable scientific guidance and inspiring support and for showing trust in me to take initiatives of my own. For always turning scientific dead-ends into something positive, which so many times has pushed me into the right direction.

my co-supervisors, *Gösta Samuelson* and *Anders Glynn* for very much appreciated discussions and valuable advice during the planning of the projects, the writing of the papers and completion of this thesis.

Anna (Lindén) Widell, co-author and loyal friend, for so generously sharing your vast knowledge on the mysteries of food chemistry and statistics with me, and for the many good laughs at the lab.

Jonas Tallkvist, my co-author, for friendliness and for never loosing patience with me when guiding me through the molecular techniques at the lab. I sure wasn't splendid all of the times - but you were. You are a cool person to work with.

my co-author, *Kierstin Petersson Grawé*, for making me feel welcome at the Division of Toxicology at the National Food Administration during the work with paper II and for being a supportive friend to turn to from then on.

Agneta Boström, at the Department of Pharmacology and Toxicology for excellent technical assistance at the lab and for calmly dealing with panic matters that should have been completed yesterday. To *Inga Lill Gadhasson, Elvy Netzel* and *Kerstin Pödra* for the warm welcome and expert technical support at your lab at the National Food Administration. It has been a pleasure to work with you all.

Birgitta Berthas, secretary at the Department of Pharmacology and Toxicology, for helping me out with all sorts of administrative paper work and for being the amiable person you are.

all present and former colleagues at the Department of Pharmacology and Toxicology, SLU and at the Department of Food Hygiene, SLU.

Börje Danell, my Mentor. A lucky star crossed my path on the day I met you. You are truly a rare specimen of humankind. Thank you for the good talks we have had and for the ones to come.

Elin Johansson, for spending endless hours on the highway between work and home with me over the years, analysing life and veterinary matters.

Marie, Lena, Helen, Maja, and *Suppan*, childhood best friends. Thank you ladies for always showing me where the party is.

my parents, *Birgitta* and *Hans* for never-ending encouragement and for showing such a great interest in my work, for being best-ever grand-parents to my children. This thesis would never have been completed without your help and support. Words can not express how much it means to me. It was never taken for granted.

Annika, dearest sister, for caring and for always being around when I need you. I'll see you at the gym on Saturday. To *Wille* and *Daniel*, sweet cousins of my children, who make me a proud auntie and godmother. To *Peter*, brother in-law, friendly bloke, local rock star and easy-rider V-max driver, all in one person.

my family, for giving me a place of rest and recreation to return to at the end of the day - our home where love is all around. To *Mats*, who has my heart and of course is my true challenge in life, and to our beautiful boys, *Love Isak* and *Theo Ossian* - you make all the difference.

I also wish to thank the Ivar and Elsa Sandberg Foundation, the Swedish Council for Forestry and Agricultural Research (SJFR), the Foundation for Strategic Environmental Research (MISTRA, Food 21), The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), the Swedish Fund for Research without Animal Experiments and the Scholarship Fund donated by Municipal Veterinarian Dr. Billström for financial support. The Royal Swedish Academy of Forestry and Agriculture (KSLA) and Knut and Alice Wallenberg's Foundation for giving me the opportunity to attend conferences abroad.