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Nickel Permeation Pathways in the Small Intestine and the Olfactory System

Jonas Tallkvist

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

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The present studies have aimed at characterizing the intestinal nickel absorption, and at determining whether nickel is taken up from the olfactory mucosa into the brain via the olfactory neurons and the mechanisms for such a potential uptake.

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Oral administration resulted in higher nickel levels in several tissues of iron-deficient rats as compared to controls. These data support the assumption that nickel is taken up from the intestines by the iron transport mechanism. Studies in which rats were given nickel intraperitoneally indicate that the iron-status also affects the uptake of nickel from the blood into the tissues.

Studies on the transport of nickel across Caco-2 cells showed a passage of the metal over the epithelium in both directions. The results indicated that the transport occurred actively, with no paracellular passage in intact monolayers, and that also in the Caco-2 cells the nickel may participate in an absorptive process for iron.

Intranasal instillation of nickel in rats resulted in an uptake of the metal in the olfactory epithelium and a migration along primary olfactory axons to the glomeruli of the olfactory bulbs, and further to the rostral parts of the cerebral hemispheres.

Studies in pikes, in which nickel was applied in the olfactory chambers, showed that the metal was transported by slow axonal transport in the primary olfactory neurons. Cell fractionations and gel filtrations of the olfactory tissues of pikes and rats showed that nickel was bound both to particulate and cytosolic cellular constituents.

Keywords: nickel, mechanisms, intestinal absorption, *in vitro* perfusion, Caco-2 cell culture, iron-status, olfactory system, slow axonal transport, brain, rat, pike.

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To my family

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Papers discussed

Papers I-VI

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

I. Tallkvist, J. and Tjälve, H. (1994) Nickel absorption from perfused rat jejunal and ileal segments. *Pharmacol. Toxicol.*, 75, 233-243.

II. Tallkvist, J., Moberg Wing, A. and Tjälve, H. (1994) Enhanced intestinal nickel absorption in iron-deficient rats. *Pharmacol. Toxicol.*, 75, 244-249.

III. Tallkvist, J. and Tjälve, H. (1997) Effect of dietary iron-deficiency on the disposition of nickel in rats. *Toxicol. Lett.*, 92, 131-138.

IV. Tallkvist, J. and Tjälve, H. (1997) Transport of nickel across monolayers of human intestinal Caco-2 cells. *Manuscript.*

V. Henriksson, J., Tallkvist, J. and Tjälve H. (1997) Uptake of nickel into the brain via olfactory neurons in rats. *Toxicol. Lett.*, 91, 153-162.

VI. Tallkvist, J., Henriksson, J., d'Argy, R. and Tjälve, H. (1997) Transport and subcellular distribution of nickel in the olfactory system of pikes and rats. *Manuscript.*

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Contents

Introduction

Nickel is a metal which is widely spread in the environment, where it is present in the form of insoluble species, such as metallic, sulfidic and oxidic nickel, or soluble species, such as nickel chloride and nickel sulfate (NAS, 1975; Sunderman, 1977; WHO, 1991). Nickel ranks as the 24th metal in order of abundance, and natural pollution of the metal in the environment may occur by erosion of surface deposits, by emission of nickel-containing gases due to volcanic activity, by air-borne particles spread by windblown dusts and by atmospheric input of nickel via sea salt sprays (WHO, 1991). Combustion of fossile fuels, waste incineration and discharges during various steps in the production of nickel, such as mining, smelting and refining, constitute important sources of man-made input of the metal into the environment (Schmidt and Andren, 1980).

Due to its physical and chemical properties there has been a rapid growth in industrial demand for nickel. Nickel is currently the fourth most produced metal, and only iron, chromium and lead are produced in larger amounts (Grandjean *et al.*, 1989). The metal plays a significant role in the production of stainless-steel, in various metal alloys, in the manufacturing of nickel-cadmium batteries and in nickel electroplating factories.

Non-occupational nickel exposure occurs mainly via ingestion of the metal in food and drinking water. The average daily intake of nickel has been estimated to be about 2-10 µg/kg body weight depending on diet (Schroeder *et al.*, 1962; Sunderman, 1975; Myron *et al.*, 1978; Clemente, 1980; Smart and Sherlock, 1987). The highest nickel contents are found in cacao and dark chocolate, soya beans and other soya products, dried legumes, hazel nuts, oatmeal and buckwheat (Ellen *et al.*, 1978; Nielsen and Flyvholm, 1984). Nickel may, in addition, leach from nickel containing kitchen utensils during food preparation, contributing to the daily dietary intake of the metal (Boudène, 1979). The levels of nickel in the drinking water are usually low (< 10 µg/l), but considerably higher levels of the metal (200-2500 µg/l) have been shown in municipal tap water in the vicinity of nickel producing industries (Punsar *et al.*, 1975; Dominok *et al.*, 1980; Shuhmann, 1980; Méranger *et al.*, 1981).

Additional sources of non-occupational human nickel exposure are attributed to dermal contact with nickel containing jewellery and coins, inhalation of nickel containing tobacco smoke, leakage from nickel containing tissue-implants and nickel contamination of dialysate fluids (Menden *et al.*, 1972; Gutenmann *et al.*, 1982; Moffa, 1982; Sunderman, 1983, 1989; Hopfer *et al.*, 1989; Gollhausen and Ring, 1991; Vilaplana *et al.*, 1991).

Occupational nickel exposure occurs mainly by inhalation of the metal in various workplaces (Norseth, 1975; NIOSH, 1977; Tandon *et al.*, 1977; Bernacki *et al.*, 1978; Tola *et al.*, 1979; Adamsson *et al.*, 1980; Zober, 1982; IARC, 1990; WHO, 1991; Lidén, 1994). Additionally, in occupationally exposed individuals a significant amount of inhaled nickel may be swallowed after clearance of the nasal and mouth deposits, contributing to the dietary exposure (Grandjean *et al.*, 1989).

The uptake of nickel in the body is dependent on the route of exposure and on the water-solubility of the nickel compounds. In general, only soluble nickel species are absorbed from the gastrointestinal tract and the airways (Sunderman, 1977; Grandjean *et al.*, 1989; WHO, 1991), whereas insoluble nickel species are phagocytized (Coogan *et al.*, 1989). This phagocytosis may play an important role in the toxicity associated with these nickel compounds (Coogan *et al.*, 1989).

In the plasma nickel is bound to albumin, an α-2 macroglobulin (termed nickeloplasmin) and ultrafiltrable ligands (e. g. histidine) and is mainly distributed to the kidneys and the lungs. The-half life of absorbed nickel in man has been estimated to be about 1-2 days, and elimination of the metal occurs primarily via urinary excretion (Sunderman, 1977; Lucassen and Sarkar, 1979; Sarkar, 1984; Grandjean *et al.*, 1989; WHO, 1991).

Human health hazards from exposure to nickel compounds particularly concern cancer and contact allergy. Metallic nickel and some nickel compounds have been classified as human carcinogens by IARC (1990). The carcinogenic hazards have primarily been associated with inhalation exposure to nickel compounds with low water solubility following a considerable latency time after the initial exposure. Primary targets for the carcinogenesis are the nasal passages and the lungs among workers exposed in nickel refinery and processing industries.

Occupational inhalation of nickel has also been reported to result in impaired olfactory function. Thus, hypo-osmia and anosmia have been observed among nickel electrolysis workers, who were exposed to soluble nickel compounds (Tatarskaya, 1960; Kucharin, 1970; Sunderman, 1977; WHO, 1991).

It is well known that nickel exposure from jewellery and coins frequently causes dermatitis, and about 10-15% of females and 1-2% of males have been estimated to be hypersensitive to the metal. Although dermal contact is of major significance in nickel allergy, results from provocation experiments suggest that oral intake of the metal may cause exacerbation or development of eczema in nickel sensitive individuals (Menné and Thorboe, 1976; Cronin *et al.*, 1980; Fischer, 1989).

Structure and physiology of the small intestine and the olfactory system

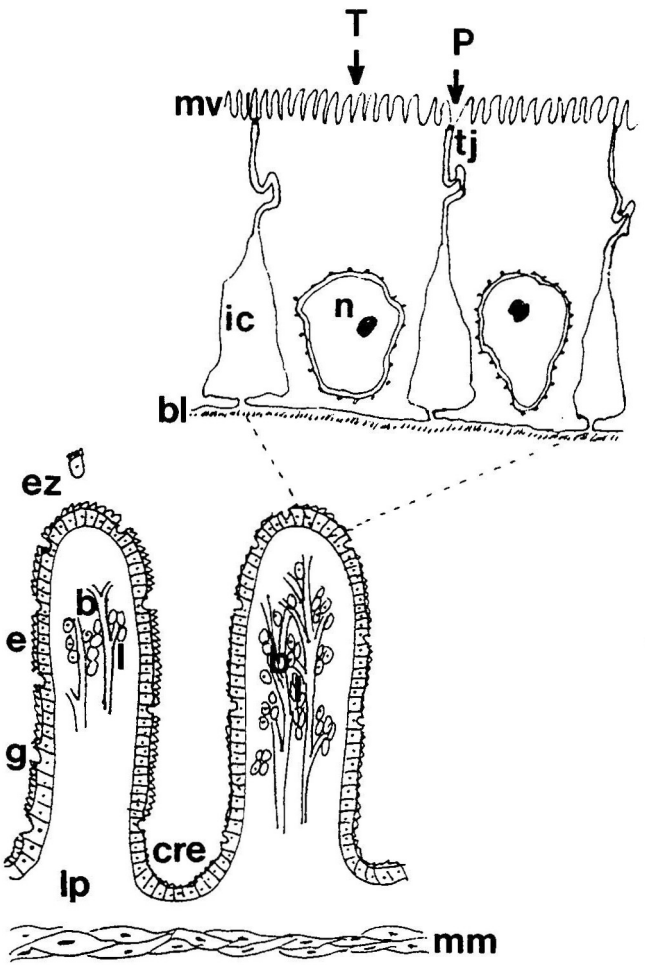
The present thesis focuses on the nickel permeation processes in the small intestine and the olfactory system. Therefore, as a background, a brief survey of the structure and physiology of these tissues will be given below.

The small intestine

The small intestine consists of the duodenum, jejunum and ileum. The mucosal interface is composed of an epithelial cell layer, a *lamina propria* and a *muscularis mucosa*. (Carr and Toner, 1984). The luminal surface of the small intestine is so arranged that the area available for contact with its contents is greatly amplified (Madara and Trier, 1994). *Plicae circulares* constitute infoldings of the intestinal mucosa, which increase the luminal surface about 3 times. These folds are covered with villi which increase the surface an additional 10 times. The villi are lined with columnar epithelial cells from which small fingerlike extensions project into the lumen. These extensions of the cell surface, called microvilli, increase the membrane area of the epithelium about 20 times and enhance the rate of absorption into the cells. Taking the *plicae circulares*, villi and microvilli into consideration, the luminal surface of the small intestine in man is estimated to be about 200 m^2 (Junqueira *et al.*, 1986, Mackay *et al.*, 1991).

The epithelial cell layer consists of a covering of a continuous monolayer of polarized columnar cells which are attached to each other by various junctional complexes. The apical region of these cells faces the intestinal lumen and the basolateral region the *lamina propria*. (Madara and Trier, 1994). The principal cells of the upper intestinal villi are the differentiated absorptive cells (enterocytes) and some interspersed mucous producing goblet cells (Mackay *et al.*, 1991). The crypt epithelium between the villi (crypts of Lieberkühn) is composed of intestinal precursor cells (enteroblasts), goblet cells, enteroendocrine cells and secretory Paneth cells (Madara and Trier, 1994). The enteroblasts migrate from the crypts towards the tips of the villi during differentiation. At the tip of the villus mature enterocytes are extruded into the intestinal lumen and excreted along with the feces. The epithelium of the small intestine is thus constantly renewed with a turnover rate of about 3-5 days (Johnson and McCormack, 1994).

Beneath the epithelial cell layer is a basal lamina present which separates the epithelial monolayer from the *lamina propria*. The *lamina propria* is composed of loose connective tissue containing blood and lymph vessels, nerve fibers and smooth muscle cells penetrating the core of the intestinal villi (Junquieira *et al.*, 1986; Madara and Trier, 1994). The deepest layer in the mucosa is the muscularis mucosa which consists of a continuous thin sheet of smooth muscle.



Schematic diagram illustrating the histologic organization of the mucosa of the small intestine. The transcellular (T) and paracellular (P) pathways are indicated with arrows. b, blood vessels; bl, basal lamina; cr, crypt epithelium; e, enterocytes; ez, extrusion zone; g, globular cells; ic, intercellular space; l, lymph vessels; lp, lamina propria; mm, muscularis mucosa; mv, microvilli; n, nucleus; tj, tight junction.

In order to maintain the physiological function of the intestinal epithelium the extracellular fluid surrounding the apical and basolateral membranes of the enterocytes must be kept separate. This is accomplished by tight junctions and adherens junctions which connect and mediate adhesion between the epithelial cells (Citi, 1993; Madara and Trier, 1994). The tight junctions, which are more narrow in the distal parts of the small intestine as compared to the proximal,

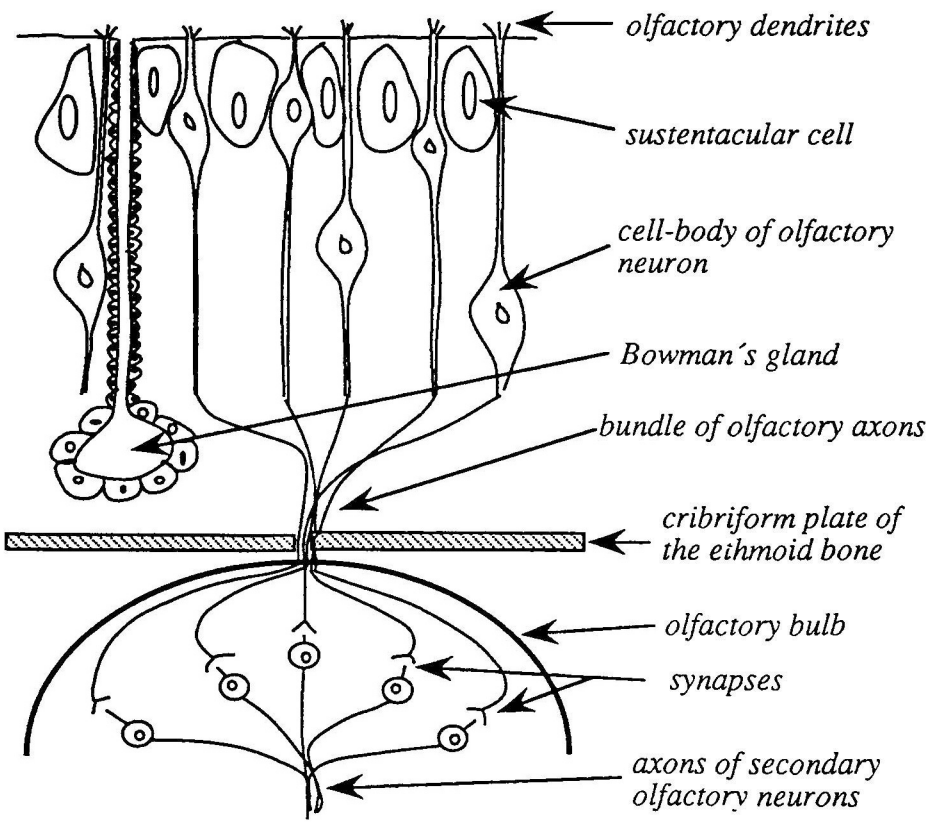
are considered to be connected to the actin cytoskeleton of the intestinal epithelial cells (Powell, 1981, 1987; Artursson *et al.*, 1993; Madara and Trier, 1994). Other junctions include desmosomes, which contribute to the tensibility, and gap junctions which mediate communication between the epithelial cells (Alberts *et al.*, 1994).

The small intestinal epithelium is "leaky", which means that an extracellular or paracellular route for the transepithelial passage of materials exists in addition to a transcellular one (Csáky, 1984; Armstrong, 1987). Thus, when examining the movement of materials across the intestinal epithelium one has to consider the transport across the apical membranes of the intestinal epithelial cells, the transport across the basolateral membranes of these cells and also the leakage between the cells across the junctional complexes. The apical and basolateral membranes can be considered, as far as their permeability properties are concerned, as lipid barriers. Thus, lipophilic substances can permeate by transcellular diffusion, whereas hydrophilic substances pass only by an interaction with active or carrier-mediated transport mechanisms. The paracellular route is permeable to water and some cations, anions and uncharged molecules. This is a purely passive route for permeation of materials across the intestinal epithelium. Ionic permeation via this route appears to take place by way of aqueous pores or channels (Jackson, 1987). After the passage through the epithelial barrier, either by the transcellular or the paracellular route, the substances or ions will reach the *lamina propria* richly endowed with blood- and lymph-vessels for further transport to various tissues via the systemic circulation (Armstrong, 1987).

Water and glucose absorption are two important physiological functions of the intestinal epithelium, which in the present thesis were used as viability criteria of the intestinal preparations. Water absorption occurs by a mechanism known as standing gradient osmosis (Guyton, 1986), which implies that there is no osmotic gradient between the luminal contents and the blood in the intestinal capillaries. Briefly, active extrusion of intracellular sodium into the intercellular spaces by basolaterally localized Na/K-ATPases and facilitated diffusion of chloride via the junctional complexes or adjacent cells into the intercellular spaces between the intestinal epithelial cells result in a local hypertonicity in these areas and, as a consequence, water enters by osmosis from the intestinal lumen via the junctional complexes (Powell, 1987). Glucose enters the enterocyte by means of a sodium dependent co-transport at the brush border membrane and is then transferred into the intercellular spaces, probably by facilitated diffusion, for further movement towards the *lamina propria* (Guyton, 1986). Glucose may also be transferred directly into the intercellular spaces via the junctional complexes by "solvent drag". The latter pathway is probably predominating at higher concentrations of glucose in the intestinal lumen (Guyton, 1986).

The olfactory system

The olfactory mucosa occupies the superior-posterior aspects of the nasal septum and the turbinates, and is composed of an epithelium and a *lamina propria* (Farbman, 1994). The olfactory epithelium consists of neuronal cells, supporting cells and basal cells. The neuronal cells constitute the receptor cells for the sensation of smell. The dendrites of these cells extend to the epithelial surface, whereas the unmyelinated axons pass through the basement membrane into the *lamina propria*, which in addition contains tubulo-alveolar glands (Bowman's glands) and blood-vessels. The axons group into nerve bundles which penetrate the cribriform plate of the ethmoid bone to synapse with secondary neurons (mitralis and tufted cells) in the glomeruli of the olfactory bulbs of the brain. The secondary neurons, in turn, project to other regions of the central nervous system (Shepherd and Greer, 1990; Farbman, 1992, 1994).



The olfactory mucosa and its neuronal connections with the brain.

Since the olfactory neurons are in contact with both the environment in the nasal cavity and the brain, they provide a pathway by which various exogenous compounds may gain direct access to the brain without the interference of the blood-brain barrier. The uptake takes place via the axons of the primary olfactory neurons and occurs in an anterograde direction (from the soma to the periphery of the neuron). The process of axonal transport has been divided into several classes depending on the speed of the movement of materials (Sabri, 1986). The major classes are termed slow, intermediate and fast. Membrane bound vesicles undergoing fast axonal transport may move at a velocity of about 250 mm/day, whereas polymerized cytoskeletal proteins and various soluble enzymes, undergoing slow axonal transport, may move at a rate < 4 mm/day (Vallee and Bloom, 1991; Nixon, 1992). Exogenous materials which have been shown to be transported in the olfactory neurons include metals, such as cadmium, and manganese (Tjälve *et al.*, 1986; Gottofrey and Tjälve, 1991; Evans and Hastings, 1992; Tjälve *et al.*, 1995, 1996), organic substances, such as wheat germ agglutinin-horseradish peroxidase (WGA-HRP) (Shipley, 1985; Itaya, 1987) and solvents and their metabolites (Ghantous *et al.*, 1990), and viruses, such as neurotropic arbovirus (Monath *et al.*, 1983) and herpes simplex virus (Esiri and Tomkinson, 1984).

Aims of the thesis

For most people nickel exposure occurs mainly via trace levels of the metal in food and drinking water. Occupational exposure to nickel may, furthermore, occur by inhalation. One objective of the present thesis has been to characterize the processes involved in the transfer of nickel across the small intestinal epithelium. Another has been to investigate whether nickel is taken up in the brain from the olfactory epithelium via the olfactory neurons and to study the mechanisms for such a potential uptake.

Materials and Methods

Chemicals

$^{63}NiCl_2$ (specific radioactivity 284.5-484 MBq [7.69-13.08 mCi]/mg Ni^{2+}) and ^{14}C-mannitol (specific radioactivity 11.1 MBq [0.3 mCi]/mg) were obtained from DuPont Scandinavia AB (Stockholm, Sweden). Iodoacetate, phloridzin, verapamil, antimycin A, 2-deoxy-D-glucose, nitrilotriacetic acid, sodium azide, dextran blue, bovine serum albumin, carbonic anhydrase, cytochrome C, aprotinin, neurotensin, cytidin, L-histidine and L-carnosine were obtained from Sigma Chemical Company (St. Louis, MO, USA). BAY K8644 was purchased from Research Biochemicals International (Natick, MA, USA) and tetramethrin from Promochem AB (Ulricehamn, Sweden). The luciferin/luciferase reagent was purchased from Biothema AB (Dalarö, Sweden). Cell culture media were obtained from Gibco, through Laboratorie Design AB (Lidingö, Sweden), and cell culture plastic wear from Costar Europe Ltd (Badhoevedorp, the Netherlands). Sephadex G-75 Superfine and Superdex 30 Prep grade were delivered from Pharmacia Biotech AB (Uppsala, Sweden). Merckotest kits, used for glucose- and Hb-determinations, were obtained from E. Merck (Darmstadt, Germany). All other chemicals used in the studies were delivered from Kebo AB (Stockholm, Sweden).

Animals

Rats

In Papers I, V and VI male Sprague Dawley rats, weighing 150-350 g, were used. They were obtained from Bantin and Kingman AB (Sollentuna, Sweden). The rats were fed a standard pellet diet, R 36, purchased from Lactamin AB (Vadstena, Sweden), and were given tap water *ad libitum*.

In Papers II and III male Sprague Dawley rats, also obtained from Bantin and Kingman, weighing about 50 g, were used. These rats were divided into two experimental groups in which one was fed an iron-sufficient and the other an iron-deficient diet. The basic composition of both diets was identical, consisting of 60% potato starch, 20% milk protein, 10% corn oil, 5% mineral salts, 4% α-cellulose and 1% vitamins. The iron concentration in the iron-supplemented diet was 150-220 ppm and in the diet not supplemented with iron 4-5 ppm. The rats in Papers II and III were given deionized water *ad libitum*. All rats were housed in macrolon cages in a 12/12 h light/dark cycle (2-6 animals/cage depending on size). The temperature was about 22°C and the humidity above 55%.

Pikes

In Paper VI pikes (*Esox lucius*) of both sexes, weighing about 2.5-3.5 kg, were caught by netfishing in Lake Mälaren (Sigtuna, Sweden). The pikes were kept

at our laboratory in 200 l all-glass aquaria in aerated tap water at 10 ± 0.5°C and were not fed during the experimental periods.

Experimental procedures

Intestinal perfusion (Papers I and II)

Rats were fasted with free access to drinking water 24 h prior to the experiments. They were anaesthetized with 40 mg pentobarbital sodium/kg b.wt. i.p. The abdomen was opened with a midline incision and intestinal segments of either the jejunum or ileum were isolated and perfused luminally in a perfusator which allows continuous sampling of the absorbate, as originally described by Fisher and Parsons (1949) and modified by Rummel and Stupp (1960).

A peristaltic pump, connected to the perfusator by tygon and silicon tubings, was used to recycle the perfusion medium. The jejunal segment used constituted the 10 cm portion distal to *ligamentum duodeno-jejunalis* (ligament of Treitz´) and the ileal segment the 10 cm portion proximal to *valvula ileo-caecalis*. The segments were perfused at either 37°C or 22°C with a bicarbonate buffer, pH 7.2-7.4, containing 15 mM glucose, saturated with carbogen gas (93.5% O_2 and 6.5% CO_2). In the experiments linear water and glucose absorption were used as viability criteria of the intestinal preparations. After a 30 min preincubation period, during which the isolated intestinal segments were allowed to adjust to the experimental situation, $^{63}Ni^{2+}$ was added to the perfusion medium. In some cases Zn^{2+}, Co^{2+}, Cd^{2+} or Hg^{2+} was added together with the $^{63}Ni^{2+}$ at various molar ratios. In other cases iodoacetate or phloridzin was added to the perfusion medium after 30 min of perfusion.

Absorption samples were collected at the serosal side of the intestinal preparations at successive 15-min-intervals for 1 h and 45 min. The level of $^{63}Ni^{2+}$ which accumulated in the mucosa of the perfused intestinal segment was registered and expressed on the basis of the protein contents of the tissue. The amount of water absorbed per 15-min-interval was also determined and related to the protein contents of the tissue.

The perfusion method used in the present study has previously been used to examine intestinal transfer of electrolytes, iron, cadmium and organic xenobiotics (Rummel and Stupp, 1960; Richter and Strugala, 1985; Schümann *et al.*, 1986, 1990 a, b; Schümann and Elsenhans, 1988).

In vivo experiments (Papers II, III, V and VI)

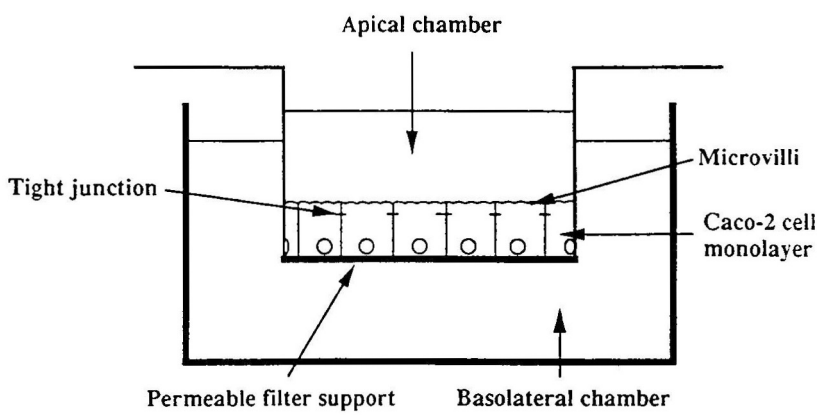
In Papers II and III the effect of iron-deficiency on the intestinal absorption and on the distribution of $^{63}Ni^{2+}$ was studied *in vivo* in rats. The animals were given deionized water *ad libitum* and were deprived of their respective diets 5-16 h prior to oral or intraperitoneal administration of 4-12 µg $^{63}Ni^{2+}$/kg b. wt.

The iron-deficient and control rats were deprived of food for the same period of time. After various survival intervals (ranging from 3-168 h) the animals were killed by CO_2-asphyxiation and exsanguination. Samples of internal organs were taken and used for ß-spectrometry.

In Papers V and VI the uptake of nickel into the brain via olfactory neurons was examined. Rats were anaesthetized (see above) prior to the intranasal instillations of the $^{63}Ni^{2+}$, which were performed according to Tjälve *et al.* (1996). Application of $^{63}Ni^{2+}$ into the olfactory chambers and determination of the transport rate of nickel in the olfactory nerves of pikes were accomplished as described by Gottofrey and Tjälve (1991).

Cell culture (Paper IV)

The Caco-2 cells, originally obtained from American Cell Culture Collection (Rockville, MD, USA), were maintained and expanded in normal tissue culture flasks at 37°C in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated calf serum and 1% non-essential amino acids, in an atmosphere of 90% air and 10% CO_2 in 95% relative humidity as previously described (Artursson, 1990; Artursson *et al.*, 1996). Cells between passages 90-105 were harvested at 80% confluence and seeded onto permeable Transwell polycarbonate filter supports (Ø 12 mm, pore radius 0.4 µm) at a density of 4.2 x 10^5 cells/cm^2. The cells were then allowed to differentiate on the filters for 21-31 days prior to the nickel transport experiments. In some cases the cells were allowed to differentiate on the filter supports in iron-supplemented DMEM. Hydroxylation and precipitation of the iron were prevented by dissolving the metal in a four-fold molar excess of nitrilotriacetic acid before the addition to the DMEM. The levels of iron in the ordinary and iron-supplemented media were 5 and 65 µM, respectively. After about 3 weeks of differentiation in their respective DMEM the level of iron in the control cells was 20 ppm and in the iron-loaded ones 103 ppm.



Schematic diagram showing a Caco-2 cell monolayer and its surrounding compartments.

The nickel transport experiments were performed at 37°C or 4°C in sterile filtered Hank´s balanced salt solution (HBSS), pH 7.4, containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). In the experiments performed at 37°C the monolayers were preincubated for 30 min in prewarmed HBSS, whereas in the experiments carried out at 4°C this procedure was carried out in refrigerated HBSS. After preincubation the buffer was removed and the filter supports were moved to new wells where fresh and prewarmed or refrigerated HBSS was added to the apical and basolateral chambers. Radiolabelled nickel was then added to one of the two chambers. When the transport of $^{63}Ni^{2+}$ was studied in the apical to basal -direction the filter supports were transferred to new wells containing HBSS at successive 30 or 45 min-intervals for 3 h. During the transfer of the filter supports the filter plates were kept on a heat plate placed in an LAF-bench. At the end of the experiments samples were withdrawn from the basolateral chambers and the radioactivity was measured by ß-spectrometry. When the transport of $^{63}Ni^{2+}$ was examined from the basolateral to the apical chamber samples were taken at regular time intervals from the apical side, as described above. In order to compensate the volume of the withdrawn sample new HBSS was added to the apical chamber. A plate shaker was used to agitate the cell monolayers at a rate of 100 rpm during the transport experiments. To determine the uptake of nickel in the cells at the end of the transport experiments the monolayers were rinsed with ice-cold EDTA and then dissolved in NaOH followed by ß-spectrometry.

Radioluminography (Paper VI)

In Paper VI, one pike was given $^{63}Ni^{2+}$ in the right olfactory chamber and killed after 8 days. The head of the pike was embedded in carboxymethyl cellulose (CMC) and put in a hexane-bath cooled to -78°C with CO_2-ice. Sections were taken on tape (20 µm thick) at -20°C according to Ullberg *et al.* (1982), whereafter imaging plates were exposed to the tape fastened sections. The imaging plates were then analyzed using a bioimaging analyser system (BAS 2000, Fuji, Japan). In this system the radiation energy stored on the imaging plate is emitted as photostimulated luminescence (PSL), having an intensity that is proportional to the radiation energy stored (Ahr and Steinke, 1994). The PSL-values are transformed into concentrations by means of radioactive standards. The obtained image is displayed on a monitor, and areas of interest can be selected for calculation of average concentration within the selected areas. A calibration curve was obtained by applying known concentrations of $^{63}Ni^{2+}$ in gelatine capsules, which were embedded in CMC and sectioned on tape along with the head of the pike. The concentrations of $^{63}Ni^{2+}$ in the capsules vs. the obtained PSL-values were plotted in order to obtain the calibration curve.

Other methods (Papers I-VI)

ß-spectrometry (Papers I-VI) was used to quantify the $^{63}Ni^{2+}$. The level of glucose in the absorbates obtained from the perfused intestines (Papers I and II) as well as the Hb-levels (Papers II and III) were determined spectrophotometrically by using Merckotest kits. All protein determinations were performed according to Lowry *et al.* (1951). Atomic-absorption spectrometry according to Moberg Wing *et al.* (1992) was applied to measure the levels of iron in tissues, diets, cells and cell culture media (Papers II-IV). The integrity of the monolayers (Paper IV) was assessed by measuring either the transepithelial electrical resistance in a diffusion chamber according to Karlsson (1995), or by determining the permeability of the paracellular marker ^{14}C-mannitol, as described by Artursson *et al.* (1996). Cellular ATP-depletion was accomplished according to Bacallao *et al.* (1994), and the luciferin/ luciferase reaction (Lundin, 1990) was used to determine the cellular levels of ATP (Paper IV). In addition to radioluminography, autoradiography according to Ullberg *et al.* (1982) was used to visualize $^{63}Ni^{2+}$ in whole-body tissue sections (Paper V). Quantification of the autoradiograms (Paper V) was performed by densitometric measurements with the aid of computer-assisted image analysis (d'Argy *et al.*, 1990). Cell fractionations (Paper VI) were performed by homogenization and centrifugation according to Webb and Weinzierl (1972). Gel filtrations on Sephadex G-75 and Superdex 30 were performed to examine the cytosolic distribution of the nickel in various tissues of the olfactory system of rats and pikes (Paper VI).

Statistical analysis

The two-tailed Student's t-test for unpaired comparisons was applied to determine the significance of the difference between mean values.

Results and Discussion

Nickel absorption in perfused intestinal segments (Paper I)

Since very little is known about the mechanism of nickel uptake over the intestinal epithelium, the objective of the study in Paper I was to examine the absorption of the metal in perfused intestinal segments of rats by varying the experimental conditions. Jejunal and ileal segments were perfused luminally with a nickel containing medium, and absorbates were collected via blood- and lymph-vessels emerging on the serosal side of the intestinal preparations.

The isolated segments were shown to maintain physiological characteristics, such as glucose and water absorption, which were used as viability criteria. The results showed that addition of iodoacetate, which blocks glycolysis, or phloridzin, which is an inhibitor of cellular glucose uptake, led to a rapid decrease of the water and glucose transfer through the intestinal mucosa. It is apparent that the perfused intestinal preparations are dependent on intact cellular metabolism.

In the experiments with nickel there was a slow increase in the transfer rate of the metal. In most instances an equilibrium was obtained after 45-60 min, but in some cases the transport rate did not equilibrate within the experimental period. The reason for the slow increase in the transfer of the metal is unknown. However, one can presume that a certain time period is required until the metal has passed the intestinal epithelium and the *lamina propria* and will leave the intestinal preparation via blood- and lymph-vessels. The absorptive process may also *per se* occur at a slow rate. Additionally, various negatively charged ligands, such as sulfhydryl-, carboxyl-, phosphate-, and imidazole-groups which are present in the tissues, may bind the metal and retard its transfer across the intestinal mucosa. It is possible that a reversible binding of nickel to such ligands, which may be present both in the surface epithelium and in the *lamina propria*, requires a long equilibration period and that a saturation of the transfer process will not be reached until such an equilibrium is obtained.

Several observations indicate that nickel is actively absorbed via the transcellular pathway in the jejunal segments of the small intestine. Thus, in the perfusions there was a saturation of the rate by which the metal was transported: When the concentration of nickel in the perfusion medium was increased by 100 times (from 4 μM to 400 μM) the cumulative amount of nickel in the absorbate increased only about 30 times. The results also showed that the absorption of nickel was much higher in the jejunum than in the ileum. The nickel absorption in jejunum was significantly decreased when the experimental temperature was decreased from 37°C to 22°C, but still higher than the absorption from the ileum at 37°C. In the perfusions no correlation

22°C was higher than in the ileum at 37°C, whereas less water was absorbed in the former than in the latter perfusions. These data indicate that solvent drag is not the mechanism by which nickel is absorbed.

Addition of zinc, cobalt, cadmium or inorganic mercury to the jejunal perfusates affected the nickel absorption to varying extents. Thus, zinc had minor effects on the nickel absorption. However, it has been shown in a study by Foulkes and McMullen (1986) that high levels of zinc may suppress the uptake of nickel in the jejunal mucosa.

Cadmium and inorganic mercury strongly depressed the cumulative nickel absorption. This effect is probably to a considerable extent related to a decrease in the water absorption induced by these metals. Besides affecting the water absorption inorganic mercury depressed the glucose absorption. However, at a low cadmium-concentration (4 μM) in the perfusate the nickel absorption was decreased in spite of the fact that the metal did not affect the water absorption and thus not the viability of the intestinal preparation. It has been shown that iron-deficiency may lead to increased intestinal cadmium absorption (Valberg *et al.*, 1976; Flanagan *et al.*, 1978, 1980; Moberg Wing *et al.*, 1992; Berglund *et al.*, 1994).

The addition of cobalt to the perfusates resulted in a depressed cumulative nickel absorption at all intervals as compared to perfusions with the nickel alone. There are indications that cobalt competes for iron uptake and/or transfer in the intestinal mucosa (Valberg and Flanagan, 1983). Since nickel also may use the iron transfer mechanism in the small intestine cobalt may interfere in this process.

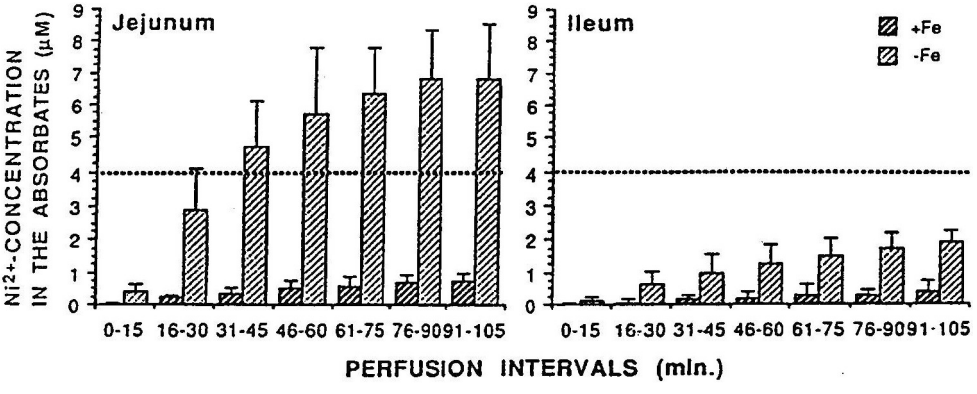
Conceivably, nickel, like cobalt and cadmium, may interact in the absorptive mechanism for iron in the mucosa of the small intestine.

Nickel absorption and iron-deficiency (Papers II and III)

The objective of these studies was to assess whether the transport mechanism for iron in the small intestinal mucosa is operative for nickel. This matter has only been examined by using isolated everted intestinal sacs of rats. Thus, Forth and Rummel (1971) and Becker *et al.* (1980) used everted sacs of small intestine of rats to study the transfer of some metals, including nickel, through the intestinal wall and found a higher mural transfer of nickel in segments obtained from iron-deficient rats as compared to normal ones.

In Paper II the absorption of nickel was studied in jejunal and ileal segments of iron-deficient and iron-sufficient rats by the same technique as described above. Our results showed that the nickel absorption was about 10 times higher in the jejunal preparations of the iron-deficient rats as compared to the controls. In fact, the nickel concentration in the jejunal absorbate of the iron-

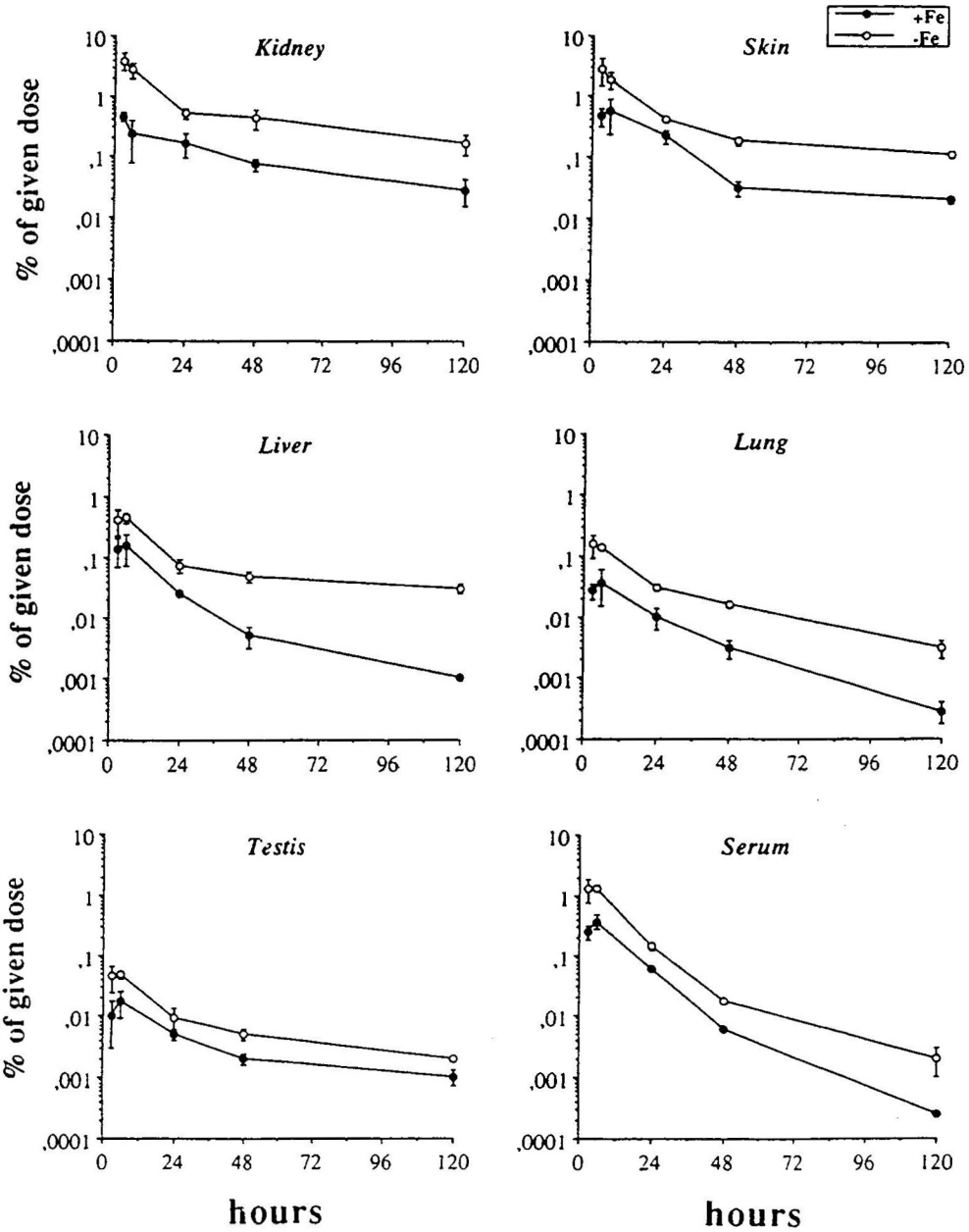
in the jejunal preparations of the iron-deficient rats as compared to the controls. In fact, the nickel concentration in the jejunal absorbate of the iron-deficient animals exceeded the concentration of the metal in the perfusion medium. This indicates that nickel is absorbed actively in the jejunum of the iron-deficient rats. Also in perfusions of ileal segments the nickel absorption was higher in preparations from iron-deficient rats as compared to iron-sufficient ones.



Concentration of nickel (µM) in absorbates collected at successive 15 min intervals (min ± S.D, n = 4-7). The nickel concentration in the perfusion medium is indicated by the dotted line (+Fe = iron sufficient rats; -Fe = iron deficient rats.

In Papers II and III the nickel was also given orally to iron-deficient and iron-sufficient rats and the levels of the metal in various tissues were examined at several time intervals. The results showed higher levels of nickel in the tissues of the iron-deficient rats at all examined intervals following oral administration of the metal as compared to the controls.

It is well known that the intestinal iron absorption is homeostatically regulated. The initial step in the uptake process seems to involve association of iron to high affinity binding sites at the brush border surface followed by a carrier-mediated transfer of the metal over the brush border membrane into the interior of the cell. The processes involved in the transport of iron through the mucosal cell and across the basolateral membrane are still poorly understood (Uchida, 1995; Qian and Tang, 1995). Our results of the present study indicate that nickel, at least in part, can utilize the absorptive mechanism for iron in the small intestine.



Percentages of the given nickel dose detected in various tissues of iron-sufficient (+Fe) and iron-deficient (-Fe) rats 3, 6, 24, 48 and 120 h following gastric intubation of 4 µg $^{63}Ni^{2+}$/kg b. wt. The values represent means ± S.D. of six rats. All values of the iron-deficient rats differ significantly from those of the iron-sufficient ones.

In addition, our results showed higher nickel-levels in the tissues of the iron-deficient rats than in the controls after intraperitoneal injection of the metal. Also, the urinary excretion of the metal was lower in the iron-deficient than in

the iron-sufficient animals. These data indicate that the iron-status affects the uptake of nickel from the systemic circulation into the tissues.

In the plasma iron is mainly bound to transferrin, and the intracellular uptake of the iron-transferrin complex is regulated by an event which has been shown to involve binding of mono- or diferric- transferrin to transferrin receptors at the surface of the cells followed by a receptor mediated endocytosis (Baker and Morgan, 1994; Qian and Tang, 1995; Morgan, 1996; Richardson and Ponka, 1997). It has been shown that nickel can bind to transferrin (Harris, 1986), and it is possible that the metal can be taken up in the tissues by the receptor-mediated endocytosis that occurs for iron. It has also been shown that, in addition to the intestinal epithelial cells (as described above), there is a non-transferrin-related uptake of iron in reticulocytes and hepatocytes (Qian and Tang, 1995; Morgan, 1996). Conceivably, nickel may also be taken up in the tissues from the circulation by the carrier-mediated transport mechanism that occurs for non-transferrin bound iron. It can be mentioned that the non-transferrin dependent uptake of iron has been shown to be competetively inhibited by nickel as well as cobalt, manganese and zinc (Morgan, 1988).

Transport of nickel across Caco-2 cells (Paper IV)

In Paper IV, the transport of nickel across monolayers of human intestinal epithelial (Caco-2) cells, originally deriving from a colonic adenocarcinoma, was examined in bicameral chambers. The purpose of this study was to further characterize the intestinal absorption of nickel and also to investigate whether the uptake and transport of the metal are dependent on the iron-status of the Caco-2 cells.

The results indicated that nickel is transported actively in both the apical to basolateral and basolateral to apical directions. In the experiments performed at 4°C no detectable transport of nickel was observed in either direction, indicating that the transport of the metal across the intestinal epithelium is a transcellular event which is dependent on temperature (Hu and Borchardt, 1990).

When we studied the transport of nickel across metabolically inhibited (ATP-depleted) monolayers an increased transfer of nickel was observed. In these experiments the epithelial passage of the extracellular marker molecule mannitol was found to increase in the same magnitude as the nickel. These data indicate that the ATP-depletion will result in a loosening of the tight junctions, reflected by the increased nickel and mannitol permeabilities of the Caco-2 cell monolayers. It has been shown that ATP-depletion increases the permeability of the tight junctions, probably by rearrangements in the actin cytoskeleton (Bacallao *et al.*, 1994).

A further indication of an active transport of nickel across the Caco-2 cells was the observation that the metal was transported against a concentration gradient. Thus, the transport of nickel in the apical to basal direction was only about 3 times lower when a 10-fold molar excess of the metal was present in the basolateral chamber. The transport rate of nickel in the basal to apical direction was even unaffected in the presence of a 10-fold higher concentration of the metal in the apical chamber.

Our results showed that the transport of nickel in the basal to apical direction occurred at a higher rate than in the apical to basal direction. The same phenomenon has been observed in the Caco-2 cell model in experiments with zinc (Rafaniello *et al.*, 1992). Additionally, *in vivo* studies with pigs have indicated that metals, such as iron, zinc, copper and manganese, are secreted from the blood into the intestinal lumen in the caecal-colon region of the gastrointestinal tract (Larsen and Sandström, 1993). It is possible, therefore, that the Caco-2 cells, which are of colonic origin, may share a property of the colonic epithelial cells to actively secrete some metals.

Results obtained from incubations with calcium-channel modulators of the L- and T-type indicate that these channels are not involved in the nickel permeation process across the intestinal epithelium. Indeed, studies on the calcium influx pathway in Caco-2 cells have indicated that these cells do not express L-type voltage dependent calcium-channels (Tien *et al.*, 1993).

Our results showed that both the transport and accumulation of nickel were depressed in the iron-loaded monolayers, indicating that the cellular iron-status is of importance for the absorption of nickel also in the Caco-2 cells. The Caco-2 cell line has previously been used to study the absorption of iron, and it has been shown that the transport and uptake of this metal respond to the iron-status of the cells (Alvarez-Hernandez *et al.*, 1991).

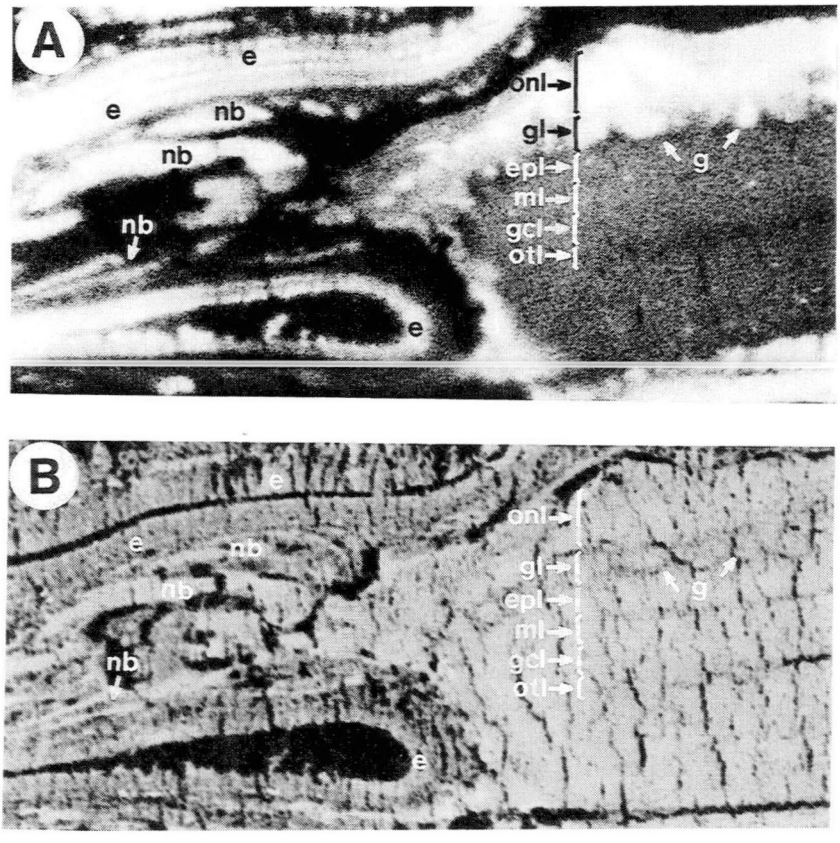
In all experiments the results showed a successive increase in the nickel transport rate at 37°C in both the apical to basal and basal to apical directions during the initial 90 min of incubation before equilibrium was reached. The reason for this may be the same as described in the experiments with perfused intestinal segments, i. e. that nickel may bind reversibly to negatively charged ligands on the luminal surface of the epithelium or within the epithelial cells and that a constant nickel-transport rate can not be obtained until the binding sites have been saturated.

The results obtained in Papers I-IV have shown that the intestinal absorption of water-soluble nickel is a transcellular process which is affected by the iron-status. As mentioned introductorily, there are indications that ingestion of nickel can cause development or exacerbation of eczema in nickel-sensitized individuals (Menné and Thorboe, 1976; Cronin *et al.*, 1980; Fischer, 1989).

Conceivably, a higher intestinal nickel absorption due to low iron-status may potentiate nickel sensitization.

Uptake of nickel into the brain via olfactory neurons (Paper V)

In Paper V rats were used to investigate whether nickel is taken up from the nasal mucosa into the brain via the olfactory pathways. Intranasal instillation of nickel resulted in an uptake of the metal from the olfactory mucosa into the olfactory bulbs via the primary olfactory neurons. Histopathology showed no signs of injury to the olfactory mucosa at the nickel-concentrations used. Autoradiography revealed that the intranasally instilled nickel accumulated in the olfactory nerve layer and in the glomeruli of the olfactory bulbs. The inner parts of the olfactory bulbs showed a lower, homogeneously distributed radioactivity, indicating that the nickel slowly passes the synapses to the secondary olfactory neurons in the bulbs, i. e. the mitral and tufted cells. Other



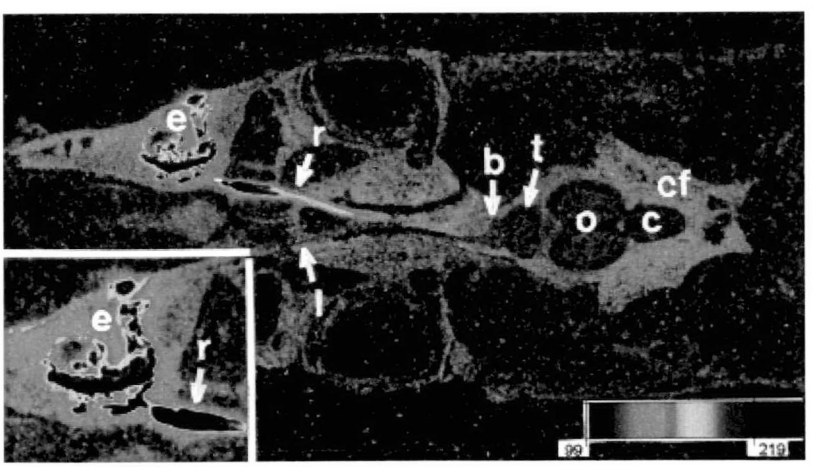
(A) Detail of a whole-body autoradiogram (horizontal section) showing the right nasal cavity and the right olfactory bulb of a rat killed one week after instillation of $^{63}Ni^{2+}$ (1.2 µg) in the right nostril. (B) Corresponding tissue-section. e, olfactory epithelium; epl, external plexiform layer; g, glomeruli; gcl, granular cell layer; gl, glomerular layer; ml, mitral cell layer and internal plexiform layer; nb, olfactory nerve bundles; onl, olfactory nerve layer; otl, olfactory tract layer.

cells in the olfactory bulbs may also take up the metal. In addition, nickel was also found to localize in the olfactory peduncle and tubercle, in the rostral parts of the olfactory cortex and in the rostral parts of the frontal and cingulate corticis. It is possible that nickel is carried to the latter areas by tertiary olfactory neurons, which connect the piriform cortex with cortical areas dorsal to the sulcus rhinalis (Price, 1985).

Intraperitoneal injection of nickel resulted in an even distribution of the metal in the brain, without preferential uptake in the olfactory bulbs.

Transport and subcellular distribution in the olfactory system (Paper VI)

The objective of the study in Paper VI was to bring insight into the mechanisms involved in the transport of nickel in the olfactory system. We investigated the axonal transport and the subcellular distribution of nickel in olfactory nerves of pikes after administration of the metal in the olfactory chambers. The olfactory nerves of pike have been shown to be suitable for studying axonal transport of proteins, amino acids and metals (Gross and Kreutzberg, 1978; Gottofrey and Tjälve, 1991; Tjälve *et al.*, 1995). In addition, we examined the subcellular distribution of nickel in the olfactory system of rats following intranasal administration of the metal.



Pseudo-colouring of a radioluminographic image of a section of the head of a pike killed 8 days after adminsitration of 50 μCi (4 μg) $^{63}Ni^{2+}$ in the right olfactory chamber. The magnification in the lower left corner is a radioluminographic image of an adjacing section, showing the right olfactory chamber and the initial part of the right olfactory nerve. The intensity of the radiation energy is indicated by the inserted scale. b, olfactory bulbs; c, cerebellum; cf, cerebrospinal fluid; e, right olfactory epithelium; l, left olfactory nerve; o, optic lobes; r, right olfactory nerve; t, telencephalon.

In order to visualize the localization of nickel in the olfactory nerves of a pike radioluminography was applied. The results showed a high labelling of the right olfactory epithelium and right olfactory nerve 8 days after application of $^{63}Ni^{2+}$ in the right olfactory chamber. The labelling of the left olfactory nerve was low. The level of $^{63}Ni^{2+}$ was also low in all parts of the brain (olfactory bulbs, telencephalon, optic lobes and cerebellum).

In the experiments where we investigated the nickel transport rate in the olfactory nerves of pikes it was shown that the metal was transported slowly. Thus, the data showed that there was a wave of nickel in the olfactory nerves which moved slowly towards the olfactory bulbs. The rate of the transported nickel was determined by plotting the movement of the wave front base as a function of time. The observation that the regression line related to the movement of the wave front base was close to zero of the time axis indicates that the nickel transport is not markedly delayed due to uptake or somal process in the olfactory epithelium prior to transport of the metal in the axons. The transport of nickel in the olfactory axons occurs in an anterograde direction. This mode of transport has been shown to exhibit several distinct rate classes, the major ones being slow, intermediate and fast. Nickel was found to be transported at a rate of about 3 mm/day, corresponding to the class of slow axonal transport (0.1-4 mm/day). Materials transported at this rate involve cytoskeletal proteins and various soluble enzymes, including cytoplasmic enzymes of intermediary metabolism (Vallee and Bloom, 1991; Nixon, 1992). It is possible that nickel within the olfactory nerves will adhere to and move with components undergoing slow axonal transport.

Our results showed that the transport of nickel in the olfactory nerves of the pikes occurred at a constant rate. This finding indicates that nickel is not transported in the olfactory pathways by means of diffusion. In addition, diffusion of nickel over the relatively long distances observed in this study seems less likely.

The cellular fractionations of the olfactory neurons and the olfactory epithelium of the pikes, and also of the olfactory system of the rats, showed that the nickel was present both in the soluble cell fraction (cytosol) and in association with various particulate cell constituents. This subcellular distribution has been observed also in other tissues of nickel treated animals (Webb and Weinzierl, 1972; Oskarsson and Tjälve, 1979; Herlant-Peers *et al.*, 1983).

Gel filtrations of cytosols showed that nickel was mainly eluted at a Ve/Vo-coefficient corresponding to an MW of about 250. The same coefficient was obtained in the gel filtrations performed with nickel mixed with L-histidine *in vitro*. It is thus possible that cytosolic nickel may be bound to histidine.

However, it can not be excluded that the nickel may also bind to other amino acids or other cellular components which are similar in size to histidine.

The dipeptide carnosine (ß-alanyl-L-histidine), which is present in primary olfactory neurons (Margolis, 1974), would potentially be a binding site for nickel. However, the gel filtrations in the present study showed no elution of cytosolic nickel at the Ve/Vo-coefficient that was observed for carnosine.

The cellular fractionations of the rat tissues showed that a higher proportion of the nickel was present in the cytosols of the olfactory epithelia than of the olfactory bulbs and the basal hemisphere. It is possible that the newly absorbed nickel initially will remain in the cytosol before it is incorporated into the particulate cellular constituents. In the rat - most marked in the olfactory epithelium - nickel was present also in the cytosol, partly in association with a cytosolic component with an MW of about 25000. The identity of this component is not known, but gel filtrations of other tissues have shown similar results (Oskarsson and Tjälve, 1979).

Previous studies on the disposition of cadmium ($^{109}Cd^{2+}$) and manganese ($^{54}Mn^{2+}$) in the olfactory system of pikes have shown that these metals are transported in the olfactory nerves at a rate which is about 20 times higher as compared to nickel (Gottofrey and Tjälve, 1991; Tjälve *et al.*, 1995). When cadmium reached the terminal parts of the axon in the glomerular layer of the bulbs it appeared unable to pass the synapses to the secondary olfactory neurons. Manganese, on the other hand, easily passed the synapses and reached large areas of the brain. Similar results were obtained in rats (Tjälve *et al.*, 1996). As discussed above our study in rats (Paper V) indicated that nickel, which reaches the axonal terminations of the primary olfactory neurons, slowly passes to the interior of the bulbs and continues to the olfactory peduncle and tubercle and the anterior parts of the cerebral hemispheres. It appears that nickel, cadmium and manganese behave differently in the olfactory system. The reason for this is not known, but may be related to varying affinities of the metals for tissue constituents.

It is concluded from our results that water-soluble nickel is transported in the primary olfactory neurons by a slow axonal transport. In this process the metal is bound to both particulate and cytosolic cellular constituents, the latter mainly having a low molecular weight. This pattern of subcellular nickel distribution is found also in the other parts of the olfactory system.

Papers V and VI have shown that nickel is taken up in the brain via primary olfactory neurons by means of slow axonal transport. As mentioned introductorily, hypo-osmia and anosmia have been observed among workers who are exposed to water-soluble nickel by inhalation. It is possible that these injuries are related to an uptake of the metal in the olfactory neurons. It has

been shown that nickel can promote lipid peroxidation in the brain of rats (Hasan and Ali, 1981). Lipid peroxidation is damaging because of subsequent reactions of free radicals which are formed. Thus, by this means nickel may induce neurotoxicity.

Conclusions

The main conclusions of the present thesis can be summarized as follows:

- Nickel is actively absorbed through the intestinal epithelium via the transcellular pathway.
- The absorption of nickel proceeds at a higher rate in the jejunum than in the ileum.
- The absorption of nickel involves, at least in part, the absorptive mechanism for iron in the intestinal epithelium.
- Nickel is taken up in the olfactory epithelium from the nasal cavity and is transferred via the primary olfactory neurons to the olfactory bulbs of the brain
- The rate of the transfer of nickel in the olfactory neurons falls into the class of slow axonal transport. In this process nickel is bound to both particulate and cytosolic cellular constituents.
- At the axonal terminations in the olfactory bulbs the nickel slowly passes the synapses and continues to the interior of the bulbs and to the rostral parts of the cerebral hemispheres.

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