Bioactivation and Transport of Foreign Materials in the Olfactory System

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Abstract

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The brain is protected from circulating toxicants by the blood-brain barrier. In the olfactory mucosa the primary olfactory neurons are in contact with the external environment in the nasal cavity and these neurons also reach the olfactory bulbs of the brain. Uptake in the olfactory epithelium and transport along the olfactory neurons may therefore pose an alternative route of entry for foreign materials to the brain. The olfactory mucosa also contains many metabolising enzymes, which may participate in the bioactivation of xenobiotics in this tissue.

The aim of the present thesis was to increase the knowledge of the olfactory system as a site of bioactivation and a route of entry to the brain of foreign materials.

Studies in horses showed that aflatoxin B_1 (AFB_1) is bioactivated in the olfactory mucosa to DNA-binding metabolites. The localization of cells with bioactivating capacity correlated with the localization of cells containing the cytochrome P450 (CYP)-enzymes CYP 3A4 and CYP 2A6, which are known to be involved in the bioactivation of AFB_1.

Studies in rats showed that intranasal administration of benzo(a)pyrene (BP) leads to a bioactivation of the compound in some cells in the olfactory mucosa and a transfer of BP and/or BP-metabolites to the olfactory bulbs of the brain.

Studies in rats and pike showed that cobalt and zinc are taken up in the olfactory mucosa and transported along the primary olfactory neurons to their terminations in the olfactory bulbs. These metals also continue to the interior of the bulb and to the anterior parts of the olfactory cortex.

Cadmium was shown to be transported bound to metallothionein in the olfactory system of rats and pikes.

It is concluded that the olfactory system is an important site of bioactivation of xenobiotics and a route by which foreign materials may gain access to the brain.

Keywords: olfactory pathway, inhalation, metal exposure,
mycotoxin, intranasally, autoradiography, CNS

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Contents

Papers discussed

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-V):

I. Larsson, P., Persson, E., Tydén, E. & Tjälve, H. Cell-specific activation of aflatoxin B_1 correlates with presence of some cytochrome P450 enzymes in the olfactory and respiratory tissues in horse. Accepted for publication in *Research in Veterinary Science*

II. Persson, E., Larsson, P. & Tjälve, H. 2002. Cellular activation and neuronal transport of intranasally instilled benzo(a)pyrene in the olfactory system of rats. *Toxicology letters* 133, 211-219.

III. Persson, E., Henriksson, J. & Tjälve, H. Uptake of cobalt from the nasal mucosa into the brain via olfactory pathways in rats. Manuscript

IV. Persson, E., Henriksson, J., Tallkvist, J., Rouleau, C. & Tjälve, H. Transport and subcellular distribution of intranasally administered zinc in the olfactory system of rats and pikes. Manuscript

V. Tallkvist, J., Persson, E., Henriksson, J. & Tjälve, H. 2002. Cadmium-metallothionein interactions in the olfactory pathways of rats and pikes. *Toxicological sciences* 67, 108-113.

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Introduction

The brain is protected from circulating toxicants by the blood-brain barrier. This barrier consists of specialised endothelial cells without fenestrae and joined together with tight junctions. These cells also have high enzyme-activities. Since the primary olfactory neurons are in contact with both the external environment in the nasal cavity and the central nervous system (CNS), the olfactory mucosa may pose an alternative route of entry for foreign materials to the brain.

It has been shown that metals such as nickel, cadmium, mercury and manganese can use this pathway (Tjälve & Henriksson, 1999). Among these metals, manganese has a unique ability to be transported via the olfactory pathway into most parts of the brain. It has also been demonstrated that other materials, e.g. amino acids and viruses, can be taken up and transported from the olfactory mucosa to the brain (Margolis & Grillo, 1977; Lee *et al*., 1999).

The olfactory mucosa is a highly metabolically active tissue. Thus, this tissue contains high levels of some cytochrome P450 (CYP)-enzymes and some other enzymes, which have the capacity to bioactivate xenobiotics (Dahl & Hadley, 1991). Bioactivation of compounds to toxic metabolites in this tissue may lead to tissue damage.

Inhalation of foreign materials may result in a high local exposure of the nasal tissues, thus making the olfactory mucosa a possible site of bioactivation and of entry to the brain.

In some neurodegenerative disorders loss of smell or lesions in the olfactory mucosa and olfactory related regions of the brain are seen in an early stage of the disease. (Ferreyra-Moyano & Barragan, 1989; Hawkes, Shepard & Daniel, 1999).

The olfactory system

The olfactory mucosa

The olfactory mucosa (Fig. 1) is situated in the upper posterior part of the nasal cavity. There are large differences between species in the proportion of the surface area of the nasal cavity that is covered by olfactory mucosa. In humans this area is approximately 10 cm^2 (Proctor, 1977). In animals that are highly dependent on the sense of smell, like the rat, this area covers a much greater proportion.

The organization and morphology of the olfactory mucosa is similar in most vertebrate species (Harkema, 1991). The olfactory epithelium has a pseudostratified columnar structure and consists of three main cell types. The olfactory receptor cells are bipolar neurons that have a short peripheral process (dendrite) towards the surface of the epithelium and a long central process (axon) towards the brain. The dendrite expands into a small knob at the surface of the mucosa. From the knob several cilia arise, which contain the olfactory receptors that interact with odorous molecules. These neurons are unique in that they have a capacity to be regenerated. Adjacent to the neurons are the supporting or sustentacular cells. The third major cell-type in the epithelium is the basal cell.

These cells are believed to be the progenitors of regenerating olfactory neurons (Hurtt et *al*., 1988). The olfactory mucosa is covered by a mucus layer. This mucus is produced by cells of the Bowman's glands that are located in the lamina propria. The axons of the olfactory neurons form bundles, which become surrounded by Schwann cell processes. The bundles join to form the olfactory nerve that passes through the cribriform plate of the ethmoid bone to reach the olfactory bulb.

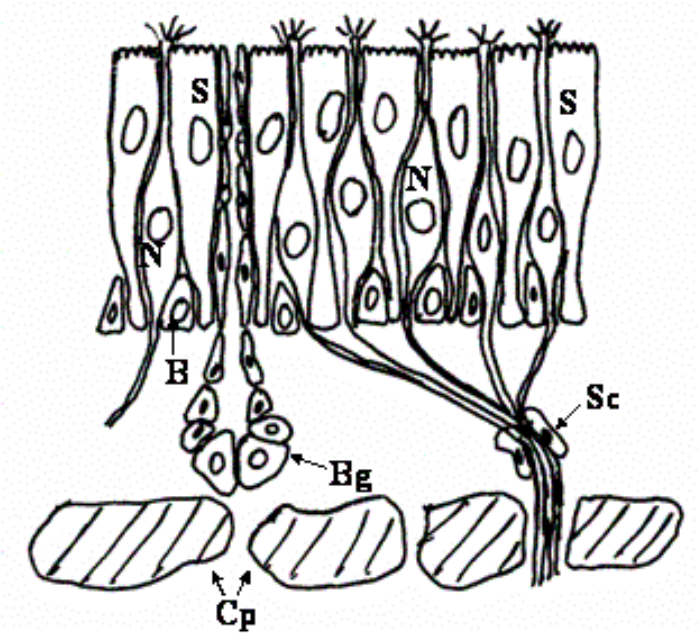

Fig. 1. Morphological organization of the olfactory mucosa. Legends: B, basal cells; Bg, Bowman's glands; Cp, cribriform plate; N, olfactory neuron (receptor cell); S, sustentacular cell; Sc, Schwann cell.

The olfactory bulbs

The axons from the primary olfactory neurons terminate in the olfactory bulbs, which are the first relay stations in the olfactory system. The olfactory bulbs are two small, ovoid bodies that develop from the telecephalon and rest on the cribriform plate of the ethmoid bone. Within the olfactory bulbs the axons synapse with mitral cells and tufted cells, which constitute the secondary olfactory neurons (Shepard & Greer, 1998). The synapses are localized in the outer part of the bulb in specialized spherical structures called glomeruli. Periglomerular cells, short axon cells and granule cells, are additional neuronal populations in the olfactory bulb (Greer, 1991).

Olfactory pathways in the brain

The axons from the mitral and tufted cells pass via the olfactory tract to terminate in the olfactory cortex. This area includes the piriform cortex, the anterior olfactory nucleus, the olfactory tubercle, the entorhinal cortex, the agranular insula and the amygdaloid cortex (Haberly, 1998). From the olfactory cortex, tertiary olfactory neurons project to multiple cortical and subcortical regions of the brain. Such areas that receive olfactory inputs from the olfactory cortex include the thalamus, the neocortex, the hypothalamus, the hippocampus and the deep nuclei of the amygdala (Price, 1985).

Xenobiotic-metabolizing enzymes in the olfactory mucosa

High levels of some CYP-enzymes are present in the olfactory mucosa (Dahl & Hadley, 1991). This is an important group of isoenzymes involved in the metabolism of endogenous compounds as well as xenobiotics. In rats and some other species the olfactory mucosa contains levels of CYP-enzymes that are second only to the liver (Thornton-Manning & Dahl, 1997). In some instances there are large variations in the P450 isoenzymes profile in the olfactory mucosa between species. Generally it is difficult to induce the activity of CYP-enzymes in the olfactory mucosa. In the olfactory mucosa the CYP-enzymes are present mainly the sustentacular cells and the cells of Bowman's glands (Voigt, Guengerich & Baron, 1985; Brittebo, 1997; Thornton-Manning & Dahl, 1997). Besides CYP-enzymes the olfactory mucosa has also been shown to contain e.g. epoxide hydrolase and the glutathione (GSH)-conjugating enzyme glutathione-S-transferase (GST) (Baron et al., 1988).

Axonal transport

The normal function of axonal transport is to transport proteins and other cell constituents to and from the cell body. The transport can take place both in anterograde (from the cell body) and retrograde (to the cell body) direction and occurs with various velocities. The slow anterograde axonal transport mainly occurs for materials, such as cytoskeletal proteins and various cytoplasmic enzymes (Vallee & Bloom, 1991). The fast anterograde transport mainly occurs for particulate materials, including a variety of membranous cellular structures, like organelles. Calcium is also transported at this rate (Knull & Wells, 1975). Retrograde transport occurs at a fast rate. Organelles such as mitochondria, lysosomes and pinocytotic vesicles have been shown to undergo retrograde transport. Axonal transport can be depressed by colchicine, which binds to tubulin, thus inhibiting the formation of microtubules (Crothers & McCluer, 1975).

Compounds examined

Aflatoxin B_1

Aflatoxin B_1 (AFB_1) is a mycotoxin that is produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. It can be found e.g. in nuts, grains and mouldy

hay. AFB_1 is bioactivated by CYP-enzymes to the reactive intermediate AFB_1-8,9-epoxide, which binds to tissue-macromolecules, such as proteins and nucleic acids, and is considered to excert the negative effects of the mycotoxin (Essigman *et al*., 1982). Several forms of CYP enzymes, such as CYP 2A6 and CYP 3A4 can catalyse the formation of the AFB_1-8,9-epoxide. Exposure to AFB_1 may lead to acute toxicity (aflatoxicosis) and also to tumourigenesis. The liver is the main target for the negative effects of the mycotoxin, but several extrahepatic tissues may be affected. Inactivation of the AFB_1-8,9-epoxide occurs mainly by conjugation with GSH. In horses, aflatoxicosis gives rather unspecific clinical signs, including fever, depression, nasal discharges and cough (Angsubhakorn *et al*., 1981).

Benzo(a)pyrene

Benzo(a)pyrene (BP) is a polycyclic aromatic hydrocarbon (PAH) and is often used as a prototypic compound in studies of PAHs. BP is a widely distributed PAH, which is metabolized to active metabolites that are tumourigenic. BP is formed during incomplete combustion of organic materials and can be found e.g. in diesel exhaust, cigarette smoke and in some work-place atmospheres (Saunders, Ramesh & Shockley, 2002). BP is bioactivated by some CYP-enzymes, particularly CYP 1A1, and by epoxide hydrolase to form metabolites such as BP-7,8-diol-9,10-epoxide, 9-OH-BP-4,5-epoxide and BP-7,8-dione, which bind to DNA and other tissue-macromolecules (Sims *et al*., 1974; Burczynski & Penning, 2000).

Cobalt

Cobalt is an important metal, which is used in various industrial applications such as the production of alloys and as a component in the hard metal industry (Jensen & Tüchsen, 1990) Occupational exposure to cobalt occurs mainly via inhalation of cobalt-containing dusts or fumes. Exposure of rats and mice to cobalt sulphate by inhalation results in atrophy and metaplasia of the olfactory epithelium (Bucher *et al*., 1999). Long-term occupational exposure via inhalation has been shown to cause memory deficits related to short term verbal memory and the allocation of attentional resources (Jordan, Whitman & Harbut, 1997). Cobalt has been used as an intracellular dye to stain neurons and in experimental models to produce epileptic seizures (Hartman, Colasanti & Craig, 1974; Tsuda *et al*., 1997; Quast *et al*., 2001).

Zinc

Zinc is an essential metal important for many biological functions, e.g. as a co-factor for many enzymes. Considerable levels of zinc are present in the brain, particularly in the hippocampus. Zinc is present in glutamatergic neurons, in which it is considered to serve as a modulator in synaptic neurotransmissions (Frederickson & Bush, 2001). Zinc is used in the industry e.g. in the galvanizing of iron and the manufacture of brass (Barceloux, 1999). Inhalation of zinc can lead to metal fume fever. The potential neurotoxicity of zinc has not been determined in detail. However, it has been proposed that zinc may contribute to the plaque

formation in Alzheimer's disease by inducing precipitation of amyloid beta (Frederickson & Bush, 2001).

Cadmium

Cadmium is used e.g. in batteries, for galvanization and as a colour pigment. For the general population the most important sources of cadmium exposure are food and cigarettes. The major target organ for the toxicity of cadmium is the kidney. An important binding ligand for cadmium is metallothionein (MT), which is a cystein-rich protein that is present in several tissues and is highly inducible by cadmium (Nordberg & Nordberg, 2000). Occupational exposure to cadmium by inhalation has been reported to result in impairment in olfactory function (Rose, Heywood & Costanzo, 1992; Sulkowski *et al*., 2000).

Aims of the thesis

The general aim of the present thesis was to increase the knowledge of the olfactory system as a site of bioactivation and a route of entry to the brain for foreign materials.

The specific aims of this thesis were:

- to study the bioactivation of AFB_1 in the olfactory and respiratory tissues in horse, and to determine whether the distribution in the tissues of cells having capacity to bioactivate the mycotoxin correlates with the distribution of cells containing some CYP-enzymes

- to examine the bioactivation of BP in the nasal olfactory mucosa of rats and to determine if BP and/or its metabolites can be transported from the olfactory mucosa to the brain via olfactory pathways

- to study the uptake and transport of cobalt and zinc to the brain via olfactory pathways in rats

- to determine if cadmium is bound to MT when transported from the olfactory mucosa to the brain via the olfactory neurons in rats and pikes

Materials and methods

Chemicals

$^{65}ZnCl_2$ (specific activity 0.15 µCi/µg), $^{57}CoCl_2$ (specific activity 6.3 µCi/µg) and ^{3}H-BP (specific activity 65 mCi/µmol) were purchased from Amersham, Sweden. $^{109}CdCl_2$ (specific activity 2.07 µCi/µg) was obtained from NEN Life Science Products (Belgium). ^{3}H-AFB_1 (specific activity 14.8 mCi/µmol) was purchased from Moravek Biochemicals (Brea, Ca, USA). Polyclonal CYP-antibodies were obtained from Chemicon International Inc., USA. One of these antibodies showed immunoreactivity against human CYP 3A4; the other against human CYP 2A6 and 2B6. The monoclonal antibody against MT and the secondary antibodies were purchased from Dako. The PicoGreen ds DNA quantitation reagent was purchased from Molecular Probes, USA. All other chemicals were of analytical grade and purchased from regular commercial sources.

Animals

Sprague-Dawley rats with a body weight of about 150 g were obtained from Bantin and Kingman (Sollentuna, Sweden). Males were used in paper III-V, and females in paper II. The rats were kept in macrolon cages, in a 12h light/dark cycle. They were fed a standard pellet diet and had free access to tap water. Pikes (*Esox lucius*) weighing about 3 kg, were caught by net fishing in Lake Mälaren. They were housed in a 200 l aquarium with a water temperature of 10ºC. Tissues from healthy horses (north Swedish trotters) of both sexes were obtained from a local slaughterhouse.

All studies were approved by the Local Ethics Committee for Animal Research.

Intranasal administration

For intranasal administration the rats were first anaesthetized with pentobarbital sodium (30-40 mg/kg body-weight). A plastic tube connected to a Hamilton syringe was then inserted into the right nostril. When the movement stopped, the tubing was retracted a few millimetres and 10 µl of the solution containing the material to be examined was instilled. In the pikes the administration was performed by a micropipette applied to the olfactory chambers as described previously (Gottofrey & Tjälve, 1991).

Autoradiography

In papers II-IV, whole body autoradiography with tape sections of the heads of rats or pikes was performed according to Ullberg, Larsson & Tjälve, (1982). *In vivo* and *in vitro* microautoradiography (dipping technique) were used in papers I and II to determine the cellular localization of the firmly bound materials in the tissues (Ullberg, Larsson & Tjälve, 1982).

Immunohistochemistry

Immunohistochemistry was used to trace the presence of CYP 2A6/2B6 and CYP 3A4 in the olfactory and respiratory tissues of horses (paper I). In paper IV and V the same technique was used to study the expression of MT in the olfactory system of rats. All reactions were performed according to the avidin-biotin technique according to the supplier's recommendation.

Cell fractionation and gel filtration

For cell fractionation (paper IV and V) the tissues were homogenized and then centrifuged in several steps according to Webb & Weinzierl (1972). In paper V gel filtration was performed with a Superdex 30 column with Tris-HCl as running buffer. For the experiments in paper IV a Superdex 75 column was used. The column was modified according to Lönnerdal (1980) and connected to an automated liquid chromathography system (ÄKTA prime). In both systems the absorbance at 280 nm was recorded. Fractions were collected and the radioactivity was determined by gamma-spectrometry.

In vitro activation

In paper I, microsomes were prepared by homogenization of the tissues followed by centrifugation at 10000 x g for 25 min. The supernatants were then centrifuged at 105000 x g for 1 hour. This was repeated twice. The microsomes (0.2 mg protein) were then used for incubations with ^{3}H-AFB$_1$ and calf thymus DNA. The incubations were performed for 20 min in 37ºC. In some experiments the incubations were performed in the presence of naringenin (0.3 mM), metyrapone (0.5 mM) or coumarin (0.2 mM). Incubations were also performed with GSH (0.5 mM) in the buffer. The reactions were stopped by adding 4.5 M NaCl and 3 % SDS. The amount of DNA-bound ^{3}H-AFB$_1$-metabolites was determined as described earlier (Tjälve *et al*., 1992), with the exception that the DNA-quantitation that was performed with PicoGreen Reagent according to Singer *et al*. (1997).

Other assays

Beta-spectrometry was used to quantify the ^{3}H-AFB$_1$ (paper I) and ^{3}H-BP (paper II). Gamma-spectrometry was used to quantify ^{57}Co (paper III), ^{65}Zn (paper IV) and ^{109}Cd (paper V). The protein determinations were assayed by the method of Lowry et al. (1951) in paper II and by the method of Smith *et al*. (1985), adapted for microplate readers, in paper I. Histopathology of the nasal cavity was performed in papers III and IV as previously described (Henriksson, Tallkvist & Tjälve, 1997).

Statistics

Statistical significance was judged with the two-tailed Student's t-test for differences between mean values. A value of $P<0.05$ was considered statistically significant. Statistical analysis of ratios was conducted on the logarithms of the data, using the theory of normal distribution. The obtained ratio and their 95% confidence interval were then anti-logarithmated. A difference was considered significant if 1.0 was not included in the interval.

Results and Discussion

Tracing of AFB_1-bioactivating cells and cells containing CYP-enzymes in the olfactory and respiratory tissues in horses (paper I)

All examined olfactory and respiratory tissues in the horse had a capacity to form DNA-bound AFB_1-metabolites in vitro. The highest bioactivation was found in the olfactory mucosa, with at least ten times higher capacity than the other tissues (Fig. 2). Similar results have previously been reported for other farm animals, such as cattle and swine (Tjälve *et al*., 1992; Larsson & Tjälve, 1996).

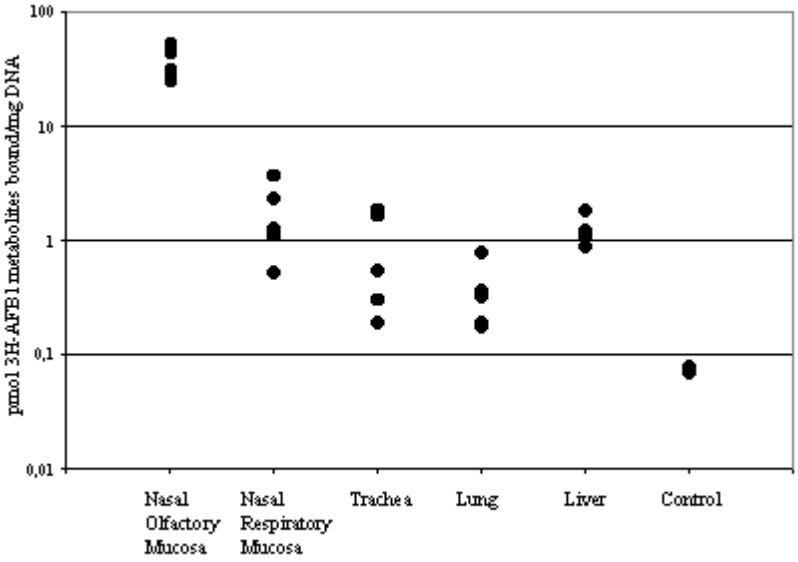

Fig 2. Amount of DNA-bound AFB_1-metaboliotes formed in incubations with ^3H-AFB_1 and microsomes from various tissues of horse. Incubations were carried out for 20 minutes with 0.5 mg DNA, 0.5 µCi ^3H-AFB_1 (0.16 µM AFB_1) and 0.2 mg microsomal protein.

Incubations with metyrapone, which is a non-specific CYP-inhibitor (Testa, 1990), depressed the levels of AFB_1-DNA-binding in the incubations with microsomes from the olfactory mucosa. Narigenin, which is an inhibitor of CYP 3A4 (Halpert *et al*., 1994), and coumarin, which is an inhibitor of CYP 2A6 (Béréziat *et al*., 1995) also lowered the levels of AFB_1-DNA-binding (Table 1). The results also showed an inhibition of AFB_1-activation in the liver by metyrapone and naringenin, whereas coumarin instead increased the levels of AFB_1-DNA-binding. It is known that the same compound may exert different effects on different isoenzymes (Rendic & Di Carlo, 1997). It is possible that CYP 2A6 plays a minor role in the bioactivation of AFB_1 in the liver of the horse

and that coumarin may act as an activator on some other CYP isoenzyme in this tissue.

Incubation together with GSH inhibited the formation of DNA-bound AFB_1-metabolites in all tissues examined. In the olfactory mucosa the binding was depressed to about 25-30 % of control (Table 1). In cattle and rodents, the GSH-conjugation of AFB_1-epoxide has been shown to be catalysed mainly by cytosolic GST (Hayes *et al*., 1991; Tjälve *et al*., 1992), whereas in sheep and swine microsome-associated GST has been reported to catalyze this reaction (Larsson, Busk & Tjälve, 1994; Larsson & Tjälve, 1996). In this study, the inhibition of the AFB_1-DNA-binding by GSH was seen in incubations performed with microsomes, thus indicating that horses have microsomal associated GST.

Table 1. Effects of various compounds on the formation of DNA-bound metabolites by microsomal preparations of nasal olfactory mucosa and liver from horses. Incubations were performed for 20 minutes with 0.5 mg DNA, 0.2 mg microsomal protein and 0.16 μM ^3H-AFB_1

		AFB_1-metabolites bound (% of control)	
Compound	Concentration	Nasal olfactory mucosa	Liver
Naringenin	0.3 mM	4-12	21-83
Coumarin	0.2 mM	40-77	113-177
Metyrapone	0.5 mM	3-12	27-80
GSH	5 mM	25-31	30-62

The results from the microautoradiography of the olfactory mucosa showed that the activation of AFB_1 was mainly located to the sustentacular cells and the cells of Bowman's glands (Fig. 3). The bound labelling was preferentially localised over cell nuclei.

The immunohistochemistry of the olfactory mucosa showed staining for CYP 2A6/B6 in the sustentacular cells, mainly confined to the outer parts of the cytoplasm, and in addition in some cells of Bowman's glands (Fig. 4). The staining of the cells of Bowman's glands varied between different glands. For CYP 3A4 a similar staining pattern was seen in the olfactory mucosa (Fig. 4). Results from experiments with nasal respiratory, tracheal, bronchial and bronchiolar mucosa showed presence of some cells in the surface epithelium and in some glands with a capacity to accumulate bound AFB_1-radioactivity. Immunohistochemistry revealed that cells in these tissues which were stained for

CYP 3A4 and CYP 2A6/2B6 had a similar distribution as the cells having capacity to bioactivate AFB_1.

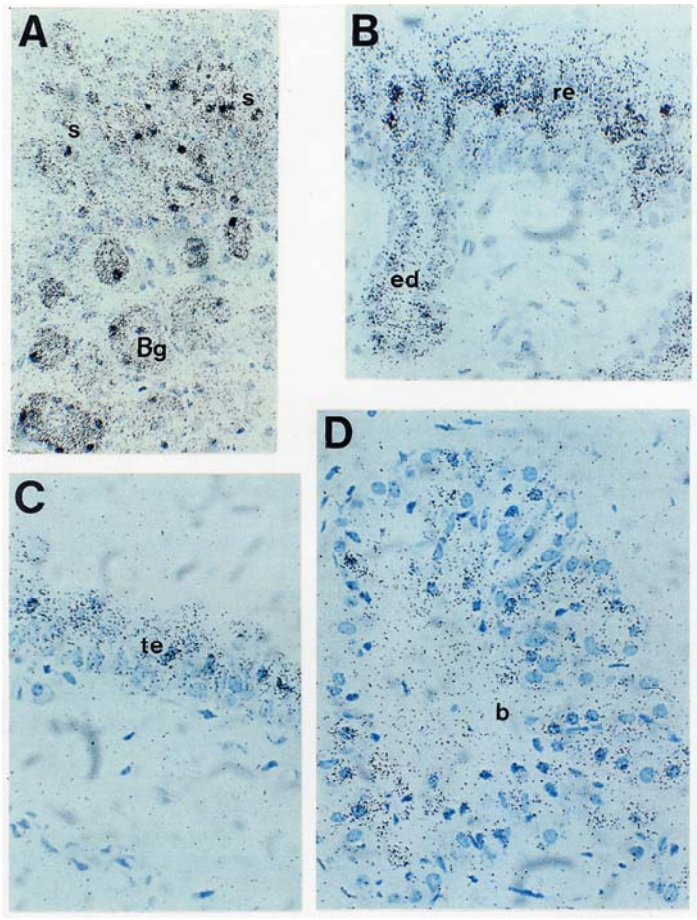

Fig. 3. Microautoradiography of the nasal olfactory mucosa (A), nasal respiratory mucosa (B), tracheal mucosa (C) bronchus (D) in horse. Tissues were incubated with ^3H-AFB_1 (0.16 µM) for 1 h in a buffer in O_2 atmosphere. b = lumen of bronchus; Bg = Bowman's glands; ed = excretory duct; re = respiratory epithelium; s = sustentacular cells; te = tracheal mucosa.

Thus, both in the olfactory mucosa and respiratory tissues the localisation of the cells containing covalently bound AFB_1-metabolites was well correlated with the localisation of the cells containing P450-enzymes capable of bioactivating AFB_1.

Inhalation of AFB_1 from contaminated feed may lead to a bioactivation in the olfactory mucosa and formation of DNA-bound metabolites. This in turn may lead to tumour formation. Nasal tumours have been observed in farm animals exposed to dust particles with high AFB_1-contamination (Sreekumaran & Rajan, 1983). Nasal tumours are sometimes seen in horses, but the potential role of AFB_1 in the pathogenesis has not been explored.

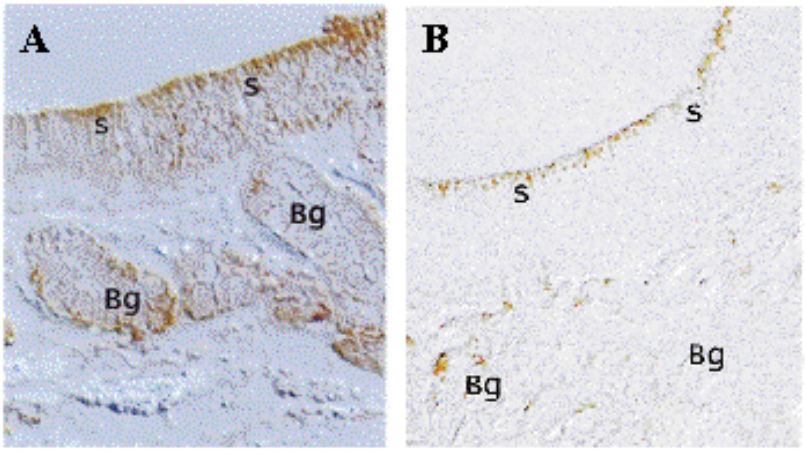

Fig. 4. Immunohistochemical staining of CYP2A6/2B6 (A) and CYP 3A4 (B) in the nasal olfactory mucosa in horse. Bg = Bowman's glands; s = sustentacular cells.

Uptake and bioactivation of BP in the olfactory pathways of rats (paper II)

Microautoradiography of ^{3}H-BP instilled into the nasal cavity in rats showed that the compound is activated in the olfactory mucosa to form metabolites which bind covalently to tissue macromolecules (Fig. 5). Most of the covalently bound material was found in cells of Bowman's glands in the lamina propria and in the sustentacular cells of the surface epithelium. BP is bioactivated by CYP 1A1, which has been reported to be expressed in the sustentacular cells and the cells of Bowman's glands (Voigt, Guengerich & Baron, 1993). In addition, BP-hydroxylation has been shown in these cells in vitro (Voigt, Guengerich & Baron, 1993). The labelling of cells of Bowman's glands varied in intensity between glands. This observation may be explained by varying levels of bioactivating enzymes between different glands.

The data showed a preferential localization of silver grains over the nuclei in the cells of Bowman's glands, with an increasing ratio over time (Table 2). This result may be related to induction via the aryl hydrocarbon receptor (AhR). BP induces CYP 1A1 via interaction with the AhR. The liganded AhR is translocated into the nucleus where it binds to the AhR-nuclear translocator. This complex then binds to the CYP 1A1 gene, thereby inducing its expression (Fujii-Kuriyama *et al*., 1994; Whitlock *et al*., 1996). It has been proposed that the reactive BP-7-8-dione is translocated into the nucleus via the AhR (Burczynski & Penning, 2000). Activated BP may in this way reach the nucleus. In the sustentacular cells the silver grains were preferentially localized over the cytoplasm, but there was an increase of the labelling over the nuclei over time (Table 2). This observation may

be explained by a lower enzyme induction in these cells compared to the cells of Bowman's glands.

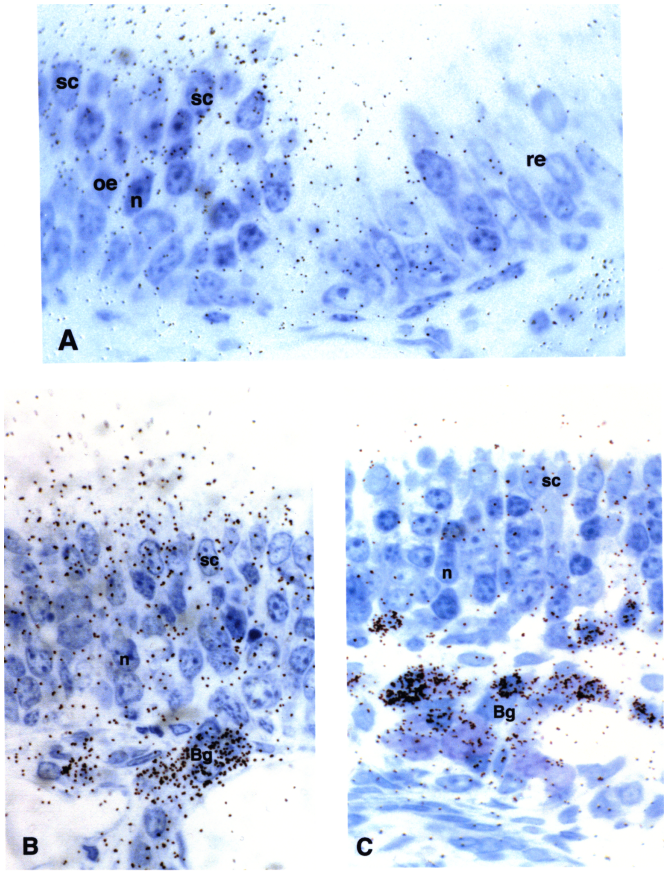

Fig. 5. Microautoradiograms of the right nasal mucosa of rats killed 4 h (A), 1 day (B) and 4 days (C) after instillation of ^{3}H-BP (10 µCi; 40 ng) in the right nostril. Legends: Bg, cells of Bowman's glands; n, neuronal cells; sc, sustentacular cells; oe, olfactory epithelium; re, respiratory epithelium.

The tape-section autoradiography showed a labelling of the right olfactory mucosa after instillation of ^{3}H-BP in the right nostril. The underlying nerve fascicles, and the olfactory nerve layer, the glomerular layer and adjacent layers of the right olfactory bulb were also labelled (Fig. 6). These results suggest that BP and/or its metabolites are taken up into the primary olfactory neurons in the olfactory mucosa and transported along the axons of these neurons to the olfactory bulbs. There was no distinct demarcation of the labelling at the glomerular layer, but instead the labelling faded out into the centre of the bulb. This suggests that the labelled material leaves the terminals of the primary olfactory neurons in the glomeruli.

The mechanism for the axonal transport of BP in the primary olfactory neurons is not known. Since there was no covalently bound BP-metabolites in the nerve fascicles in the olfactory mucosa or in the olfactory bulb it is suggested that the transported material is BP and/or some of its non-reactive metabolites.

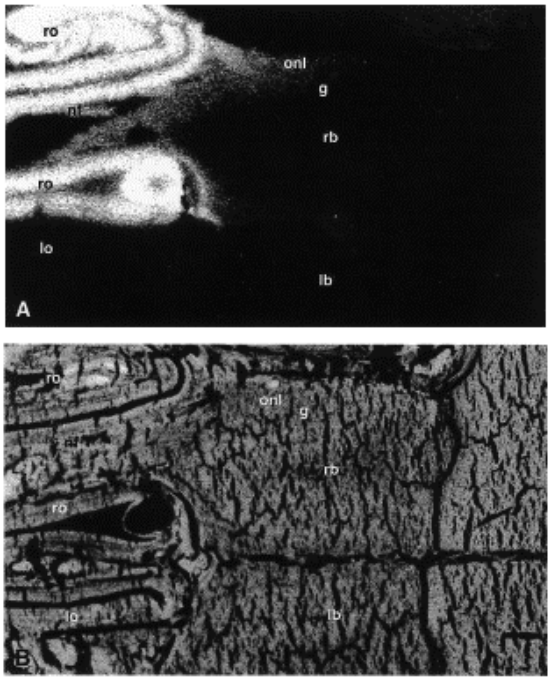

Fig. 6. (A) Autoradiogram of the head of a rat killed 1 day after instillation of ^{3}H-BP (10 μCi; 40 ng) in the right nostril. White areas denote radioactivity. (B) Corresponding tissue section. Legends: g, glomerular layer; lb, left olfactory bulb; lo, left olfactory mucosa; nf, nerve fascicle; onl, olfactory nerve layer; rb, right olfactory bulb; ro, right olfactory mucosa.

Table 2. Ratios of the density of silver grains (number of grains per μm^2) over nuclei versus cytoplasms in cells of the olfactory mucosa in microautoradiograms of rats after intranasal administration of ^{3}H-BP

	Ratios of density of silver grains over nuclei versus cytoplasms[a]	
Survival interval	Sustentacular cells	Cells of Bowman's glands
4 hours	0.33 (0.20-0.55)[b]	2.62 (1.53-4.50)[b]
1 day	0.71 (0.36-1.38)	4.70 (3.57-6.25)[b]
4 days	0.74 (0.42-1.29)	3.90 (1.82-8.35)[b]

[a] Values shown are the ratio of the mean with the 95% confidence interval within parenthesis. [b] Significantly different,1 is not included in the 95% confidence interval

It has been reported that the olfactory bulbs lack CYP 1A1-activity (Morse *et al*., 1998). However, it has been shown that increased levels of CYP 1A1 mRNA can be induced in the olfactory bulb by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Huang *et al*., 2000). Implantation of BP-pellets into the brain of rats has been shown to induce brain tumours (Hopewell & Wright, 1969). It is possible that exposure of the brain parenchyma to BP for prolonged periods leads to bioactivation and tumour formation, even if the levels of bioactivating enzymes are very low.

Uptake of zinc and cobalt to the brain via olfactory pathways in rats and pikes (paper III and IV)

Studies in rats and pikes showed that both zinc and cobalt were taken up in the olfactory mucosa and were transported along the primary olfactory neurons to the olfactory bulbs. Both metals accumulated in the terminal parts of the primary olfactory neurons in the glomeruli of the bulb (Fig. 7-10). In addition, the metals were seen to continue to the interior of the bulbs and to the anterior parts of the olfactory cortex. These results indicate that the metals can leave the terminations of the primary olfactory neurons.

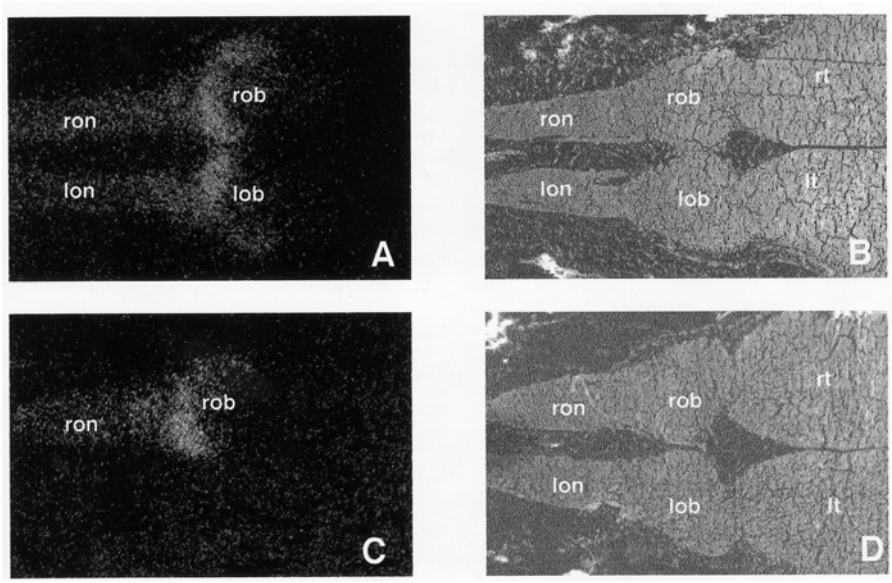

Fig. 7. Autoradiogram of the head of a pike killed 2 weeks after instillation of $^{65}Zn^{2+}$ (0.12 µg; 20 µCi) in both olfactory chambers. (B) The corresponding tissue section. (C) Autoradiogram of the head of a pike killed 2 weeks after instillation of $^{65}Zn^{2+}$ (0.12 µg; 20 µCi) in the right olfactory chamber. (D) The corresponding tissue section. Legends: lob, left olfactory bulb; lon, left olfactory nerve; lt, left telencephalic hemisphere; rob, right olfactory bulb; ron, right olfactory nerve; rt, right telencephalic hemisphere.

Rats have a "septal window" in the nasal septum (Kelemen & Sargent, 1946). Some of the metal solution applied in the right nostril may therefore have passed over to the left nasal cavity. This may explain the large individual differences in

the levels of metals on the right and left side of the nasal cavity which were sometimes were observed. However, there was a correlation between the levels of metal in the epithelium on either side of the septum and the levels in the corresponding olfactory bulb.

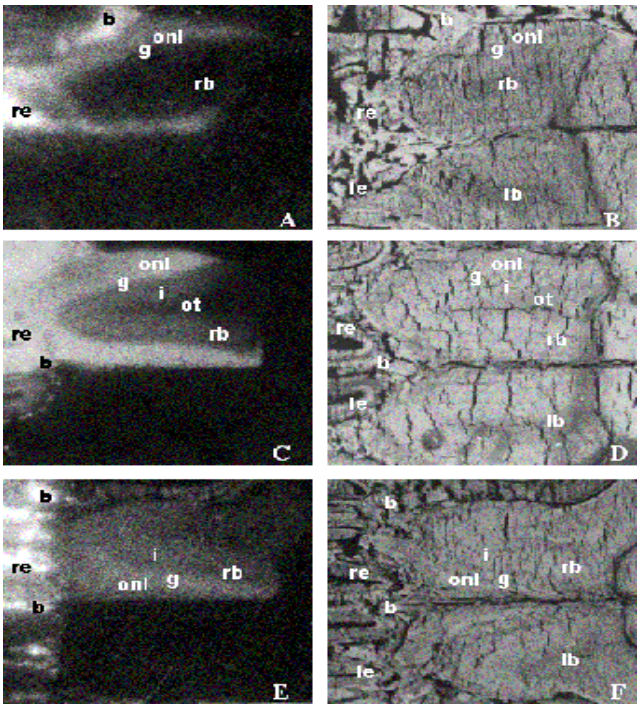

Fig. 8. Autoradiograms of the heads of rats killed at (A) 1 day, (C) 1 week and (E) 6 weeks after instillation of $^{65}Zn^{2+}$ (0.03 µg; 5 µ Ci) in the right nostril; (B), (D) and (F) are the corresponding tissue sections. Legends: b, bone; g, glomerular layer; i, interior of the bulb (external plexiform layer, mitral cell layer, internal plexiform layer and granular cell layer); lb, left olfactory bulb; le, left olfactory epithelium; onl, olfactory nerve layer; ot, olfactory tract layer; rb, right olfactory bulb; re; right olfactory epithelium.

The mechanisms by which cobalt and zinc are taken up in the olfactory neurons are not known. The divalent metal transporter (DMT1) is expressed in several parts of the brain and has a broad range of substrates (Gunshin *et al*., 1997). DMT1 may be involved in the uptake of cobalt and zinc in the olfactory epithelium. Another possibility is that the metals may use mechanisms for the uptake and transport of calcium. Cobalt as well as zinc has been proposed to substitute for Ca^{2+} in different reactions and exchange mechanisms (Jennette, 1981; Colvin *et al*., 2000).

The results of the present studies indicate that cobalt and zinc are transfered from the olfactory mucosa to the brain via the olfactory nerve pathway (Jackson, Tigges & Arnold, 1979; Mathison, Nagilla & Kompella, 1998). Another possibility for intranasally instilled materials to enter the brain is via the so-called olfactory epithelial pathway (Mathison, Nagilla & Kompella, 1998). Thus, agents may be taken up in sustentacular cells or Bowman's glands or enter paracellularly

to reach the lamina propria and after entering the perineural space reach the cerebrospinal fluid (CSF) that surrounds the brain. As concern metals uptake from the CSF into the brain parenchyma occurs to a very limited extent. This makes the olfactory epithelial pathway an unlikely route of uptake of metals in the brain.

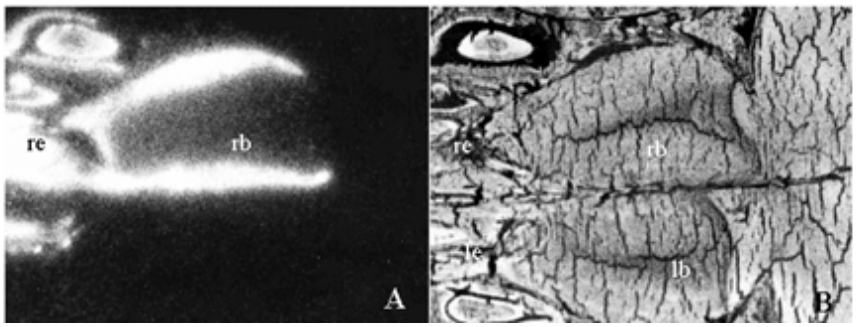

Fig. 9. (A) Autoradiogram of the head of a rat killed 1 day after intranasal administration of $^{57}Co^{2+}$ (0.8 ng; 15 μCi) in the right nostril. White areas denote high levels of radioactivity. (B) The corresponding tissue section. Legends: lb, left olfactory bulb; le, left olfactory epithelium; rb, right olfactory bulb, re, right olfactory epithelium.

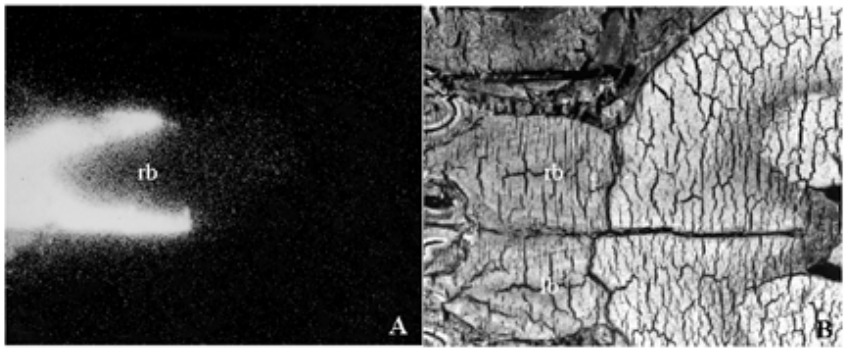

Fig. 10. (A) Autoradiogram of the head of a rat killed 1 week after intranasal administration of $^{57}Co^{2+}$ (0.8 ng; 15 μCi) in the right nostril. White areas denote high levels of radioactivity. (B) The corresponding tissue section. Legends: lb, left olfactory bulb; rb, right olfactory bulb.

Intraperitoneal injections of the cobalt and zinc in rats resulted in low levels in the brain for both metals, indicating that these metals are transported to the brain from the systemic circulation to a low extent.

The uptake of materials in the olfactory mucosa may be affected by damage to the epithelium (Lewis, Hahn & Dahl, 1994). Thus, epithelial damage may lead to loss of receptor cells, which in turn may result in a decreased uptake and transport along the olfactory axons. However, such damage may also disrupt the tight junctions between the cells in the the olfactory epithelium, facilitating entrance to the perineural space. It can be noted that high concentrations of zinc (about 0.17 M) have been used experimentally to destroy the olfactory epithelium (Margolis *et al*., 1974). However, in our study a very low concentration of zinc was used (46

μM) and the histological evaluation of the nasal cavity that showed no signs of damage.

Published data have shown large variations in the ability of different metals to be taken up into the brain via olfactory pathways (Tjälve & Henriksson, 1999). Among the metals so far examined manganese has been found to have a unique ability to reach all parts of the brain (Tjälve *et al*., 1996). The results for zinc and cobalt show patterns that are similar to the pattern for nickel (Henriksson, Tallkvist & tjälve, 1997).

Occupational exposure to cobalt and zinc occurs in many workplaces (Jensen & Tüchsen, 1990; Walsh *et al*., 1994). Inhalation of metal-containing dusts or fumes may result in an uptake of the metals in the brain via the olfactory pathway. It is not known whether this may result in neurotoxicity. However, it has been reported that long-term exposure to cobalt-containing dust may cause memory deficits (Jordan, Whitman & Harbut, 1997). Dysregulation of zinc, resulting in excessive extracellular levels in the brain, has been implied to play a role in Ahlzheimer's disease (Frederickson & Bush, 2001). In addition, it has been observed that impairment of the sense of smell, and lesions in the olfactory regions are present in early stages of neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (Ferreyra-Moyano & Barragan, 1989; Hawkes, Shepard & Daniel, 1999)

Subcellular localization of zinc and cadmium in the olfactory pathways of rats and pikes (paper IV and V)

Subcellular fractionations of the olfactory mucosa and bulb of rats given zinc intranasally showed that the metal was present in these tissues in association with both particulate cellular constituents and cytosolic components. (Table 3). The results from gel filtrations of the olfactory mucosa and bulb from rats given zinc indicated that the metal in the cytosol in part is present in association with MT. Thus, the elution profiles for the olfactory epithelium and the olfactory bulb showed a peak corresponding to a molecular weight of about 6-7 kDa (Fig. 11) The same profile was found when ^{65}Zn and MT were mixed in vitro. For the olfactory bulb however, an additional peak was found, corresponding to a molecular weight of about 30-35 kDa. The identity of this ligand is not known.

Results from subcellular fractionations in rats and pikes, given cadmium in the nasal cavity or olfactory chamber respectively, showed that this metal was found mainly in the cytosol (70-85 %). Gel filtrations indicated that cadmium is transported as a CdMT complex along the axons of the primary olfactory neurons. Thus, the gel filtrations of cytosols from rat olfactory epithelium, rat olfactory bulb and pike olfactory nerve in animals killed 48 h after administration of cadmium showed that the metal was eluted in a peak corresponding to a molecular weight of about 6-7 kDa (Fig. 12). The same peak was found when ^{109}Cd and MT were mixed *in vitro* (Fig 13). In gel filtrations with cytosols from rats killed 2 h after the administration of cadmium an additional peak was observed, corresponding to a

molecular weight of about 1 kDa (Fig. 13). This peak may be cadmium bound to GSH. Previous investigations have demonstrated that GSH provides an initial cellular defence against cadmium toxicity prior to the induction of MT synthesis (Zaroogian & Jackim, 2000). This suggestion is supported by the fact that the same peak was found when ^{109}Cd and GSH were mixed *in vitro* (Fig. 13). In rats killed at 2 h, which had been pretreated with cadmium to induce MT, the cadmium was eluted in only one peak corresponding to CdMT (Fig. 13).

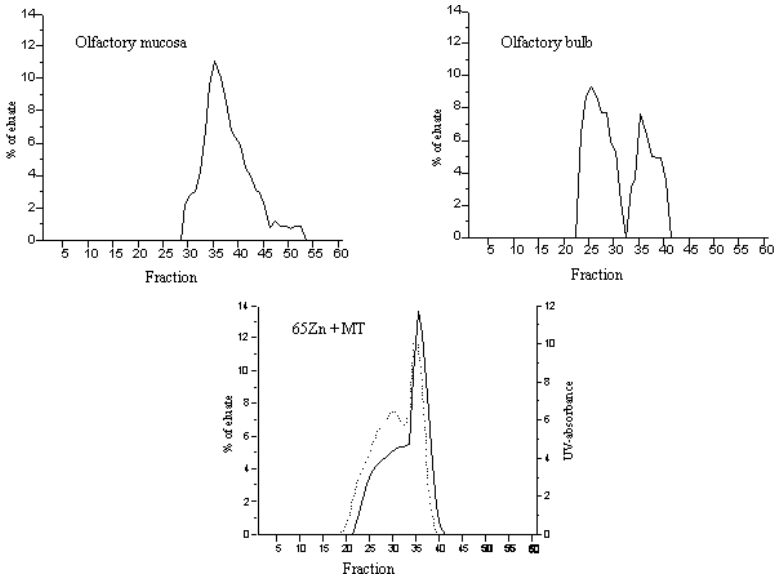

Fig. 11. Representative Superdex 75 filtration profiles of cytosol of the olfactory mucosa and olfactory bulb in rats killed 1 day after intranasal administration of $^{65}Zn^{2+}$ (0.03 µg; 5 µCi) and of $^{65}Zn^{2+}$ mixed with MT *in vitro*. Solid line, $^{65}Zn^{2+}$; dotted line, UV-absorbance (280 nm).

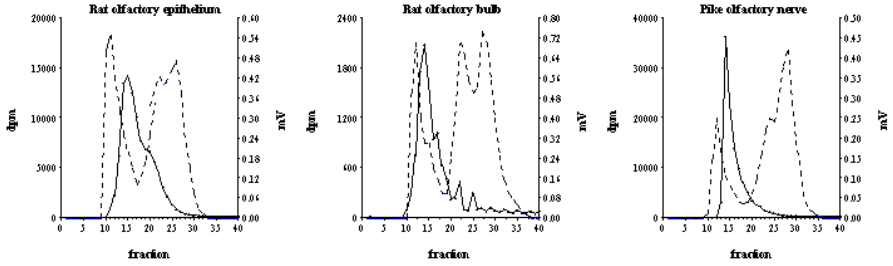

Fig. 12. Representative Superdex 30 filtration profiles of cytosol of rat olfactory epithelium, rat olfactory bulb, and pike olfactory nerve. The rat samples are from controls killed 48 h after intranasal administration of $^{109}Cd^{2+}$ (5 µg). Solid line, $^{109}Cd^{2+}$; dotted line, UV-absorbance (280 nm).

Table 3. Proportions of zinc in subcellular fractions of the olfactory mucosa and the olfactory bulb in rats killed 1 d after intranasal administration of $^{65}Zn^{2+}$ (0.03 µg, 5µCi). Data are presented as means ± SD (n=3).

	Percent of Zn in the respective subcellular fraction			
Tissue	600 g pellet	12,000 g pellet	105,000 g pellet	Supernatant
Olfactory mucosa	52 ± 7	10 ± 2	13 ± 1	25 ± 11
Olfactory bulb	68 ± 5	7 ± 1	7 ± 1	18 ± 5

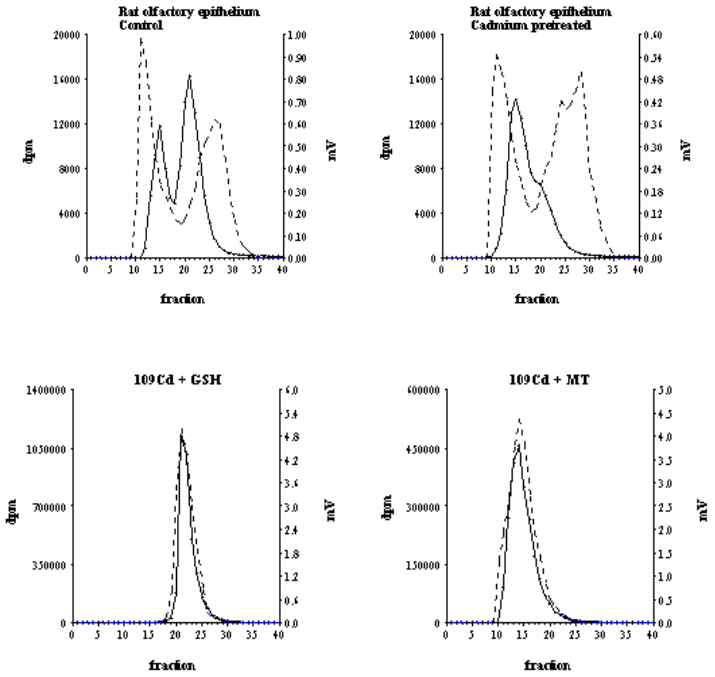

Fig. 13. Representative Superdex 30 filtration profiles of cytosol of control rat olfactory epithelium (top left), cadmium pretreated rat olfactory epithelium (top right), $^{109}Cd^{2+}$ mixed with GSH *in vitro* (bottom left), $^{109}Cd^{2+}$ mixed with MT *in vitro* (bottom right). The cytosolic samples are from animals killed 2 h after intranasal administration of $^{109}Cd^{2+}$ (5 µg). Solid line, $^{109}Cd^{2+}$; dotted line, UV-absorbance (280 nm).

Immunohistochemistry has shown that MT is constitutively expressed in some cells of the olfactory system. Thus, MT-immunoreactivity has been shown in a few sustentacular cells of the olfactory epithelium, in some Bowman's glands and in a few scattered olfactory nerve fibers in the olfactory mucosa (Shimada *et al*., 1996; Skabo *et al*., 1997).

When cadmium was instilled intranasally in the right nasal cavity in rats a strong induction of MT was seen in the olfactory tissues at the ipsilateral side. (Fig. 14). Thus, a marked staining was found in the right olfactory surface epithelium and in the underlying nerve fascicles. The olfactory nerve layer of the right olfactory bulb also showed a marked immunoreactivity. MT immunoreactivity was observed in the glomeruli of both the right and left olfactory bulbs. However, the staining was homogenously distributed whithin the entire glomeruli of the right bulb, whereas it showed a mesh-like pattern, corresponding to the localization of astrocytes, in the glomeruli of the left bulb. It is concluded that the exposure of the olfactory epithelium for cadmium results in induction of MT in the primary olfactory neurons and a transport of the metal in these neurons as a CdMT-complex. MT may play a physiological role in the olfactory mucosa in being a part of the protection of the olfactory epithelial cells, since they are exposed to materials via the inspired air.

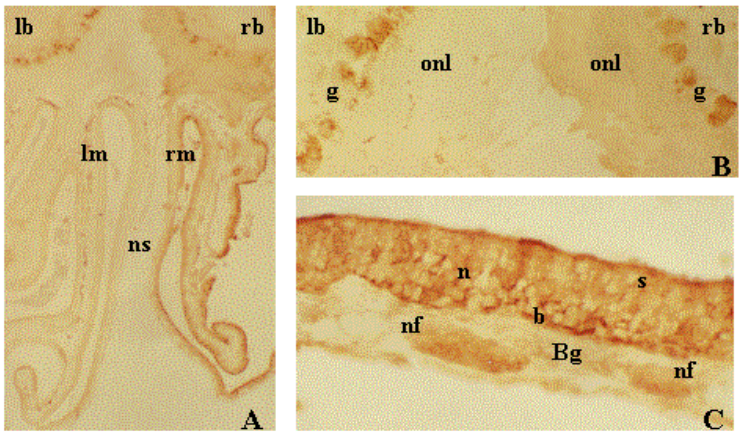

Fig. 14. Immunohistochemical staining for MT in sections of the olfactory system of rats killed 48 h after application of cadmium on the right olfactory mucosa. (A) Section showing the right and left olfactory mucosae, turbinates and bulbs. (B) Section showing the right and left olfactory bulb. (C) Detail of the right olfactory mucosa. Legends: b, basal cells; Bg, Bowman's glands; g, glomeruli; lb, left olfactory bulb; lm, left olfactory mucosa; n, olfactory nerve cell; nf, nerve fascicles; ns, nasal septum; onl, olfactory nerve layer; rb, right olfactory bulb; rm, right olfactory mucosa; s, sustentacular cell.

Conclusions

- AFB_1 can be activated and form DNA bound metabolites in the olfactory mucosa of horse. The bioactivation occurs mainly in the sustentacular cells and the cells of Bowman's glands. These cells also contain some CYP-enzymes, known to have a capacity to bioactivate AFB_1. Cells with the ability to bioactivate AFB_1 and containing CYP-enzymes are also present in respiratory tissues of the horse.

- Following application of BP on the olfactory epithelium of rats BP and/or its metabolites are translocated to the olfactory bulbs of the brain. BP is also bioactivated in the olfactory mucosa to form metabolites which bind to tissue-macromolecules. The bioactivation occurs in the sustentacular cells and in the cells of Bowman's glands.

- Intranasal administration of cobalt in rats results in a transport of the metal along the primary olfactory neurons to the olfactory bulb. Cobalt will leave the terminals of the primary olfactory neurons in the glomeruli of the bulb and continue to the interior of the bulb and to the anterior parts of the olfactory cortex.

- Zinc is transported along the primary olfactory neurons to the olfactory bulb in rats and pikes. Zinc also has the ability to pass the terminations of the primary olfactory neurons and reach the interior of the bulb and the olfactory cortex. The data indicate that during the transport in the olfactory system zinc is bound to both particulate cellular constituents and to cytosolic components. The results indicate that some of the zinc in the cytosol of the olfactory mucosa and bulb is bound to MT.

- Cadmium is transported along the primary olfactory neurons of rats and pike bound to MT. Immunohistochemistry show that intranasal administration of cadmium in rats results in a strong induction of MT in the olfactory epithelium, in nerve-fascicles in the lamina propria of the olfactory mucosa and in the olfactory nerve layer of the olfactory bulb.

The results of the studies in this thesis show that metals as well as organic compounds can be taken up in the brain via olfactory pathways. The toxicological implications of such an uptake may vary. However, this is a route by which foreign materials may circumvent the blood-brain barrier and gain access to the brain. This may potentially result in neurotoxicity.

In addition, the results of the studies in the present thesis confirme that the olfactory mucosa contains some enzymes with a high capacity to metabolize xenobiotics. Normally these enzymes may play a role in detoxification of foreign substances, including odorants, which reach the olfactory mucosa. In some instances the metabolism may instead result in bioactivation, which in turn may cause tissue damage.

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