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SODIUM/IODIDE SYMPORTER – NIS

Abundant and Important in Gastric Mucosa

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Akademisk avhandling i ämnet klinisk medicin med inriktning öron-, näs- och halssjukdomar som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds Universitet offentligen försvaras fredagen 6 februari 2009, kl 9.15 i GK-salen, BMC, Sölvegatan 19, Lund

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SODIUM/IODIDE SYMPORTER-NIS Abundant and Important in Gastric Mucosa

Abstract

Background:
Iodine is essential for development and growth as a constituent in thyroid hormones. Biological mechanisms for iodide uptake and concentration are very important, especially as iodine is a relatively scarce element. Active iodide transport into thyroid follicular cells is mediated by the sodium/iodide symporter (NIS), powered by Na/K-ATPase. NIS is expressed also in extra thyroidal tissues, most abundantly in gastric mucosa - the focus of this thesis.
Cellular Localisation of NIS:
NIS expression was found basolaterally in gastric mucosal surface cells of several mammals including man, basolaterally in salivary gland ducts and apicolaterally in rat bile ducts. Weak NIS immunoreactivity was found basolaterally also in duodenal mucosal surface cells. Findings in gastric mucosa and salivary glands were confirmed by in situ hybridisation.
Gastric Iodide Transport:
Considerable secretion of iodide from the bloodstream into the stomach, but negligible uptake from the stomach, was shown in vivo and in vitro in rat using ^{125}I as marker. In the Ussing-chamber in vitro system both the competitive NIS-inhibitor sodium perchlorate ($NaClO_4$) and the Na/K-ATPase-inhibitor ouabain attenuated gastric iodide transport from serosal to mucosal side, proving that gastric iodide secretion is, to a large extent, mediated by NIS.
NIS in MNNG-induced tumours:
Gastric cancer was chemically induced in rats by the administration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in the drinking water. Multiple microadenomas as well as large adenomas were found in the stomachs of all MNNG-treated rats. The surface epithelium of gastric adenomas was NIS immunoreactive, but staining was less intense than in normal mucosa. In addition, almost all of the MNNG-treated animals displayed either large papillary cystadenomas in the liver, found to express NIS with varying staining intensity, and/or infiltrating carcinomas of Brunner's glands, which did not express NIS.
Conclusions:
• NIS is abundantly expressed in gastric mucosa and also expressed in salivary gland and bile duct epithelium.
• Iodide secretion into the gastric lumen is mediated by NIS. There are several theories as to the function of this transport including mediating recirculation of iodide, as well as securing presence of iodide in the stomach for antimicrobial or antioxidative purposes.
• MNNG-induced tumours in gastric mucosa and bile ducts all express NIS, which might make radioiodine an aid in treatment and diagnosis of these cancer types.

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SODIUM/IODIDE SYMPORTER – NIS

Abundant and Important in Gastric Mucosa

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Cover page: Sodium/iodide symporter (NIS) basolaterally in the surface epithelial cells of rat gastric mucosa; immunofluorescence.

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“Jag vill. Jag kan. Jag gör det!”

Birgitta Bohman

To ”Life, the Universe and Everything!”

Douglas Adams

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ABSTRACT

Background

Iodine is essential for development and growth as a constituent in thyroid hormones. Biological mechanisms for iodide uptake and concentration are very important, especially as iodine is a relatively scarce element. Active iodide transport into thyroid follicular cells is mediated by the sodium/iodide symporter (NIS), powered by Na^+/K^+-ATPase. NIS is expressed also in extra thyroidal tissues, most abundantly in gastric mucosa - the focus of this thesis.

Cellular localisation of NIS

NIS expression was found basolaterally in gastric mucosal surface cells of several mammals including man, basolaterally in salivary gland ducts and apicolaterally in rat bile ducts. Weak NIS immunoreactivity was found basolaterally also in duodenal mucosal surface cells. Findings in gastric mucosa and salivary glands were confirmed by *in situ* hybridisation.

Gastric iodide transport

Considerable secretion of iodide from the bloodstream into the stomach, but negligible uptake from the stomach, was shown *in vivo* and *in vitro* in rat using ^{125}I as marker. In the Ussing-chamber *in vitro* system both the competitive NIS-inhibitor sodium perchlorate ($NaClO_4$) and the Na^+/K^+-ATPase-inhibitor ouabain attenuated gastric iodide transport from serosal to mucosal side, proving that gastric iodide secretion is, to a large extent, mediated by NIS.

NIS in MNNG-induced tumours

Gastric cancer was chemically induced in rats by the administration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in the drinking water. Multiple microadenomas as well as large adenomas were found in the stomachs of all MNNG-treated rats. The surface epithelium of gastric adenomas was NIS immunoreactive, but staining was less intense than in normal mucosa. In addition, almost all of the MNNG-treated animals displayed either large papillary cystadenomas in the liver, found to express NIS with varying staining intensity, and/or infiltrating carcinomas of Brunner's glands, which did not express NIS.

Conclusions

- NIS is abundantly expressed in gastric mucosa and also expressed in salivary gland and bile duct epithelium.
- Iodide secretion into the gastric lumen is mediated by NIS. There are several theories as to the function of this transport including mediating recirculation of iodide, as well as securing presence of iodide in the stomach for antimicrobial or antioxidative purposes.
- MNNG-induced tumours in gastric mucosa and bile ducts all express NIS, which might make radioiodine an aid in treatment and diagnosis of these cancer types.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Josefsson M**, Grunditz T, Ohlsson T, and Ekblad E; Sodium/iodide-symporter: distribution in different mammals and role in entero-thyroid circulation of iodide. *Acta Phys Scand* 175(2): 129-137; 2002

II. **Josefsson M**, Evilevitch L, Weström B, Grunditz T, and Ekblad E; Sodium-iodide symporter mediates iodide secretion in rat gastric mucosa *in vitro. Exp Biol Med* 231: 277-281; 2006

III. **Josefsson M** and Ekblad E; Sodium/iodide symporter in N-methyl-N'-nitro-N-nitrosoguanidine-induced tumours in rat upper digestive tract. *Manuscript*

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ABBREVIATIONS

BSA	bovine serum albumin
E17	embryonic day 17
FITC	fluorescein isothiocyanate
H&E	hematoxylin and eosin
^{131}I, ^{125}I	radioactive isotopes of iodine
IV	intravenous(ly)
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
mRNA	messenger ribonucleic acid
Na^+/K^+-ATPase	sodium/potassium adenosine triphosphatase
NIS	sodium/iodide symporter
NO_3^-	nitrate
NO	nitric oxide
NO_2^-	nitrite
rt-PCR	reverse transcriptase-polymerase chain reaction
^{35}S-dATP	radioactive sulphur isotope linked to deoxyadenosine triphosphate
SNAP	S-nitroso-N-acetyl-D, L-penicillamine (an NO-donor)
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal peptide

BACKGROUND

Iodine

Iodine is essential for development and health in humans as well as in animals and the best-known function for iodine is as a constituent of thyroid hormones. The element iodine is relatively scarce in the earth crust, which may cause endemic hypothyroidism with goitre and even cretinism (impairment of physical as well as mental growth due to congenital deficiency of thyroid hormone) in certain geographical areas. Thus, the absorption, distribution, and elimination of iodine, physiologically in its ionised form iodide, have interested researchers for many years. When radioactive isotopes of iodide became readily available in the middle of the last century, iodide research was greatly facilitated as measurements of iodide in physiological concentrations became possible. The physiological role of iodide, as well as its therapeutic potential, became the focus of much interest for several decades. In addition, it became possible to study extra-thyroidal iodide metabolism, which had been impossible before owing to the very low physiological extra-thyroidal iodide concentrations (see review by (Brown-Grant 1961). During this period of intensive research it was established that iodide is absorbed from the small intestine, and that gastric mucosa, salivary glands and mammary glands concentrate iodide actively. However, the extra-thyroidal organs were found to concentrate only inorganic iodide, whereas iodide in the thyroid is incorporated into large organic molecules (thyroglobuline) and stored in this form to meet future needs. The molecular basis for iodide concentration was still unknown.

Substantial iodide concentration, independent of acid secretion, was found in the gastric juice, and some accumulation of iodide could be detected in the gastric wall. A practical consequence that came out of this knowledge on iodide accumulation in gastric contents was the assuming of safety measures when handling vomit from patients receiving radioiodine therapy. The accumulation of iodide in gastric wall and juice is also suspected to be responsible for the elevated incidence of, and mortality in, gastric cancer after ^{131}I-therapy (Hall et al. 1992; Holm et al. 1991). However, the functional role of iodide secretion into the gastric lumen has remained elusive. Studies on bovines showed a recirculation of iodide and this was suggested to be an important iodide-conserving mechanism (Miller et al. 1975b). Other proposed functions are that iodide acts as an antioxidant in the gastric lumen (Venturi and Venturi 1999), or that the antimicrobial effects of iodide are important in the gastric lumen(Majerus and Courtois 1992). Gastric antimicrobial defences are linked to gastric carcinogenesis via the established carcinogenic effects of Helicobacter pylori. Inoculation with Helicobacter pylori has been described to alter cell cycle kinetics in the direction of carcinogenesis in mouse (Loogna et al. 2001) and eradication of Helicobacter pylori has been identified as an effective approach for prevention of gastric cancer.

Sodium/iodide symporter - NIS

The sodium/iodide symporter (NIS) is a transmembrane glycoprotein consisting of 618 amino acids (in rat), containing 13 trans-membrane regions, with the C-terminal located intracellularly and the N-terminal extracellularly (Levy et al. 1998)(figure 1). In the thyroid gland, iodide is actively transported into the follicular cells by NIS. The transport of iodide against a gradient into the cells is powered by the Na^+-gradient generated by sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) and the iodide transport is competitively inhibited by perchlorate (see review by Carrasco 1993). With the revelation of the cDNA-sequence of rat NIS (Dai et al. 1996), soon followed by the sequencing of human NIS (Smanik et al. 1996), a new era of intensive iodide research started (Dohan et al. 2003; Nilsson 1999).

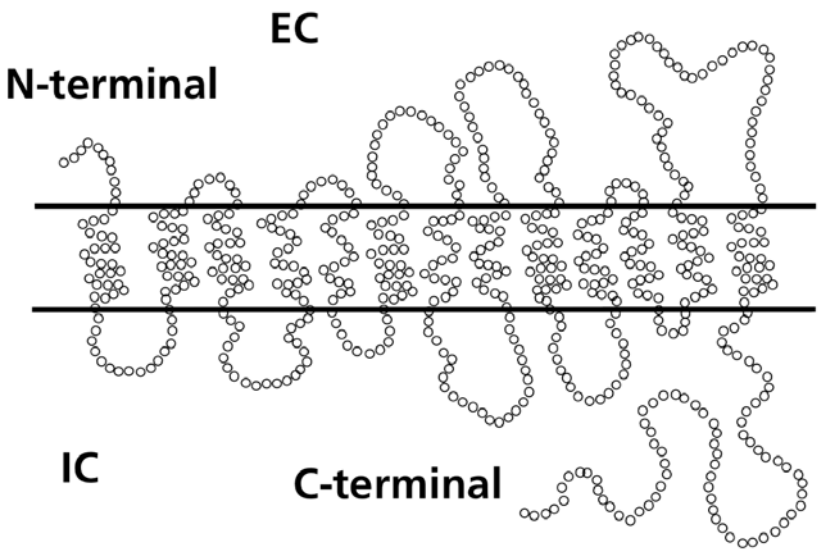

Figure 1: NIS secondary structure (freely after Levy et al.). Rings symbolise the 618 amino acids of rat NIS. The N-terminal is located extracellularly (EC) and the C-terminal intracellularly (IC).

Gastric NIS

NIS was soon identified also in the gastric wall of rat (Kotani et al. 1998) as well as man (Vayre et al. 1999). The cDNA-sequence of human gastric NIS was found to be identical with that of thyroid NIS (Spitzweg et al. 1998), while minor differences have been found between rat gastric and thyroid NIS cDNA (three nucleotide substitutions leading to two amino acid substitutions in the NIS protein) (Kotani et al. 1998). However, human as well as rat gastric NIS protein has a different molecular weight from thyroid NIS and this is mainly due to differences in glycosylation (Kotani et al. 1998; Tazebay et al. 2000).

NIS is abundantly expressed in the gastric mucosa and in rats it appears at the same gestational age (embryonic day 17(E17)) as thyroid NIS (Josefsson and Ekblad 2009). Thus, it is hard to imagine that gastric NIS is unimportant. However, the function and regulation of NIS in gastric mucosa is still poorly understood. Iodide transport across gastric mucosa and accumulation in gastric contents had been described, but no studies describing the contribution of NIS in this transport were available at the outset of work on this thesis. On the other hand there were reports suggesting that NIS function in gastric mucosa was to absorb iodide from the gastric lumen (Kotani et al. 1998). Thus, clarifying the direction of iodide transport over gastric mucosa, as well as linking this transport to NIS activity, is included in the aims of the thesis.

NIS is not an exclusive iodide transporter, but also transports other anions. Studies on NIS transport-capacity for different anions have mainly been performed on thyroid-derived systems like cells transfected with thyroid NIS or thyroid slices (see Eskandari et al. 1997 and Wolff 1998 for reviews). There are some differences in anion selectivity between the transport in NIS-transfected cell lines and physiologic transport studies (Wolff 2002) but these methods are in agreement on the selectivity of NIS for some of the anions including $I^- >> NO_3^- > Br^- > Cl^-$ (Van Sande et al. 2003; Wolff 1998). This leaves us with the possibility that an important role of gastric NIS may be transportation of anions other than iodide. Gastric NIS may, however, have somewhat different transport properties than thyroid NIS due to the differences in glycosylation.

Cancer, iodide and NIS

Gastric cancer is one of the most common neoplasms worldwide and the diagnosis also carries a bad prognosis. Interestingly there are reports of gastric cancer being more prevalent in areas with iodine deficiency. On the other hand also in populations with very large iodine intake, e.g. from seaweeds, a high incidence of gastric cancer is

reported (Venturi et al. 2000). This indicates that the iodide secretion into the gastric lumen, mediated by NIS, may be an important factor in gastric carcinogenesis. In this context it is also interesting to note that NO_3^-, often suggested to be a risk factor for gastric cancer, is also transported by NIS, and that high levels of NO_3^- certainly would reduce iodide transport competitively. The transport of NO_3^- is less efficient than that of iodide (Eskandari et al. 1997), but since plasma concentration of NO_3^- is normally much higher than that of iodide, the bulk transport of NO_3^- may still be considerable. A higher prevalence of thyroid disease (non toxic goitre and autoimmune thyroid disease) in subjects with gastric cancer compared to matched controls has been reported (Kandemir et al. 2005). A weakness in this report is that the authors do not provide any information on whether subjects with thyroid disease had received radioiodine therapy, which has previously been reported to elevate incidence of as well as mortality in gastric cancer (Hall et al. 1992; Holm et al. 1991).

Apart from the possible functional role of NIS-mediated iodide transport in gastric carcinogenesis, NIS expression may possibly serve as a diagnostic tool for gastric cancer recurrence or metastasis as indicated in a case report by Wu et al. (1984). On the other hand Altorjay et al. (2007) found NIS expression to be absent or low in gastric carcinoma, and suggest that decreased NIS expression in gastric lesions could be used as a sign of malignancy. More studies are needed to evaluate NIS expression in different types and stages of gastric cancer development. A contribution from this thesis, are the results in paper III on NIS presence in induced gastric tumours.

In the future it may be possible to use radioiodine accumulation by NIS for treatment of different types of cancer. To achieve this, ways to induce or enhance NIS expression in cancer cells must be explored. Gene transfer has been suggested as one method of inducing NIS expression (for a review see Dohan et al. 2003) and chemical induction or enhancement of NIS expression by retinoic acid has been reported in cell lines (Kogai et al. 2000).

AIMS OF THE THESIS

- To examine the localisation of NIS protein and the expression of NIS mRNA in the digestive tract from different mammals

- To evaluate the direction of iodide transport across the gastric mucosa *in vivo* and *in vitro*

- To establish a connection between the finding of NIS protein as well as NIS mRNA in gastric mucosa and the accumulation of iodide in the gastric lumen after administration of radioactive iodide

- To study possible regulation of gastric NIS activity by biologically active substances

- To evaluate spontaneous NIS expression in MNNG-induced tumours of the rat gastrointestinal tract

METHODS

Study material

Human specimens

Normal thyroid and gastric tissues were obtained from patients undergoing thyroid surgery (n=4) or gastrectomy (n=4). The procedures were approved by the ethical committee at the University of Lund.

Animals

In all three original papers, the main work was made in Sprague-Dawley rats, female or male as specified. In paper I, female NMRI mice and Dunkin-Hartley guinea pigs were also used. The animals had free access to standard food pellets and tap water. Animal care was in accordance with the European Council Convention of 1986 and the study was approved by the research animal ethics committee of Malmö/Lund. Specimens were also collected immediately post mortem from pigs (n=3) used for educational purposes at the University of Lund.

Induction of tumours

To induce cancer in the upper gastrointestinal tract of rats, the established carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used (Schoental 1966; Sugimura and Fujimura 1967). Control animals had free access to tap water throughout the study period. MNNG-treated animals had free access to tap water containing 100µg/ml MNNG (Fischer Scientific GTF AB, Västra Frölunda, Sweden) during the first 25 weeks of the study period, and then ordinary tap water. MNNG was dissolved in distilled water to 2.5 mg/l, frozen in aliquots, and after thawing further diluted in tap water to 100µg/ml. The solution was protected against light at all times. All animals were given 1 ml 10% NaCl solution by gastric catheter once a week for the first 6 weeks in order to enhance the carcinogenic effect of MNNG (Hu et al. 2004; Tatematsu et al. 1975).

Microscopy

Results in papers I and III are based on microscopic evaluation of fixed tissue specimens with emphasis on immunohistochemical visualisation of NIS. In addition, *in situ* hybridisation was used to visualise NIS messenger ribonucleic acid (mRNA) in paper I and sections stained with hematoxylin and eosin (H&E) for evaluation of morphological changes in paper III.

Tissue processing

Frozen specimens (paper I)

Specimens were fixed in Stefanini's fixative (2% formaldehyde and 0.15% picric acid in phosphate buffer, pH 7.2) for 12-24 h and then repeatedly rinsed in Tyrode buffer containing 10% sucrose, all at 4° C. Specimens were frozen on dry ice, sectioned in a cryostat at -20° C to a thickness of 10 µm, and thaw-mounted onto Superfrost plus slides.

Paraffin embedded specimens (paper III)

Specimens were fixed in 4% neutral buffered formalin for 24 h at 4°C and rinsed twice in 70% ethanol at room temperature before dehydration in ascending concentrations of ethanol and xylene. Paraffin embedded specimens were sectioned in a microtome to 5 µm thickness and mounted onto Superfrost plus slides. Sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol.

Antigen retrieval (paper III)

To enhance antigen retrieval in sections from paraffin embedded specimens, microwave heating in citrate buffer was performed prior to immunohistochemistry. Initially several buffers at different pH were tested and citrate buffer was found to be most suitable for our purposes (Kan et al. 2005; Kim et al. 2004). Slides were transferred to plastic containers suitable for microwave boiling, immersed in 0.01M citrate buffer (pH 6,0) and boiled at 650W for two cycles of 7 min. Between cycles, buffer level was checked and fresh buffer added when needed. After boiling, the container was immersed in cold tap water for 10 min and the sections then rinsed in cold tap water for 20 min, followed by 5 min in PBS buffer before immunostaining.

Immunohistochemistry

Antisera

A peptide containing the intracellularly situated eight C-terminal amino acids of rat-NIS was synthesised and conjugated to bovine serum albumin (BSA). Antiserum was raised in rabbit using the peptide-BSA conjugate together with Freund's complete adjuvant. Three rabbits were immunised with the synthetic peptide and the resulting antisera were tested in our lab for their ability to visualise NIS-containing cells. The antiserum with the lowest background staining and yielding the strongest immunostaining at low concentrations, which staining was quenched by the addition of synthetic peptide, was used in the studies (Euro-Diagnostica AB, Malmö, Sweden, code number 9806). In paper I, an affinity purified polyclonal rabbit antiserum (TIT11-A, Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) raised against the 16 C-terminal amino acids of rat NIS was also tested. As the immunostaining obtained

by TIT-11A was very weak compared to that obtained with antiserum 9806, only antiserum 9806 was used.

Immunofluorescence

Immunofluorescence was used as it is a sensitive and reliable method (Coons et al. 1955). Additionally, there is profound knowledge and experience of this method in the lab. In paper I, only frozen specimens were used as the available antisera hardly recognised NIS in paraffin embedded specimens. The main work in paper I was done in the years 1998-99 and since then microwave boiling of paraffin sections in different buffer solutions has become standard procedure to enhance antigen retrieval (Boon and Kok 1994; Shi et al. 2001a; Shi et al. 2001b). With microwave treatment antiserum 9806 recognises NIS at least as well in paraffin embedded specimens as in frozen ones, with the added advantage of a better-preserved morphology. Thus, all immunohistochemical studies in paper III were made on paraffin embedded specimens.

The protocol for immunohistochemical staining is described in detail in paper I. In brief, sections were incubated with primary antiserum over night in a moist chamber at 4° C and the site of the antigen-antibody reaction was revealed by fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit IgG or Texas red-labelled donkey anti-rabbit IgG, both diluted 1:100. Incubation time with labelled antibody was 45-60 min in room temperature. For control purposes, the primary antiserum was incubated with 100 µg/ml of the synthetic rat-NIS peptide over night at 4° C before use.

In situ hybridisation

The detailed protocol for *in situ* hybridisation is described in paper I. Briefly an oligonucleotide probe complementary to rat thyroid NIS mRNA 570-602 was constructed (Biomolecular Resource Facility, University of Lund, Sweden). The probe was then coupled to a radioactive sulphur isotope linked to deoxyadenosine triphosphate (^{35}S-dATP). Cryostat sections were cleared in chloroform, acetylated and hybridised with the probe over night at 37° C, washed in saline sodium citrate, dehydrated, and dipped in photographic emulsion. Exposure time was 5 weeks, after which the slides were developed. For control purposes, hybridisation was also performed after incubation with the RNA digesting enzyme RNase A or in the presence of a 100-fold excess of unlabelled probe in the hybridisation buffer.

Iodide transport studies

In vivo studies

In order to study the direction of iodide transport across gastric mucosa, rats were given radioactive iodide (^{125}I) either by gastric catheter (intragastric administration) or intravenously (IV). To selectively measure iodide uptake from the stomach and also to accurately measure secretion into the stomach, it was necessary to prevent iodide passage into the intestines by ligating the pylorus in one group of rats. Pyloric ligation necessitated general anaesthesia and opening of the abdomen. Therefore, all rats underwent this procedure, regardless of pyloric ligation or not. The surgical procedures are described in detail in paper I. Sixty minutes after iodide administration by gastric catheter or IV, the rats were killed. Radioactivity was analysed primarily in blood-samples, thyroid, stomach and washing fluid from stomach. Washing fluid from stomach was used to evaluate luminal iodide content. Iodide transport was reflected in radioactivity and expressed as % of total administered ^{125}I-dose per organ or sample.

In vitro studies in Ussing-chamber

Specimens

The glandular part of the stomach was dissected out, emptied of contents, and divided along the major and minor curvatures to yield two separate specimens. The muscular layer was stripped off, using fine forceps and scissors, before mounting the mucosal layer in the Ussing-chamber. Pars proventricularis of the stomach, known to lack NIS expression (see paper I), was used as reference specimen.

Ussing-chamber model

To study transport over biological membranes without the confounding factors occurring in *in vivo* experiments, the Danish professor Hans Ussing and co-workers in the 1950's developed an experimental set up consisting of two separate chambers, connected via an aperture where the biological membrane to be studied (e.g. skin or gastrointestinal specimens) was mounted (Ussing and Zerahn 1951)(figure 2). This device, named Ussing-chamber, was used extensively and further developed during the next decades. With the adaptations of Grass and Sweetana (1988) it became possible to commercially manufacture Ussing-chamber systems with different sizes of the aperture and chambers, thus making the method suitable for a wide range of specimens, differing in size and permeability. In paper II, Ussing-chambers (Navicyte, San Diego, CA, USA) with round, relatively small apertures (0,64 cm^2) were used. Mounting of the gastric mucosal specimen yielded a polarised system with one chamber representing the mucosal (intraluminal) side and the other the serosal side.

The procedure is described in detail in paper II. Briefly, ^{125}I was added to one of the chambers (donor side) and samples then drawn from the other chamber (receiver side) at 15, 30, 45 and 60 min. Samples were replaced by an equal volume of fresh buffer solution. By analysing the samples, iodide transport from donor to receiver side could be calculated. Transport was expressed as nmol iodide cumulated at each time point with correction for the removed sample volumes.

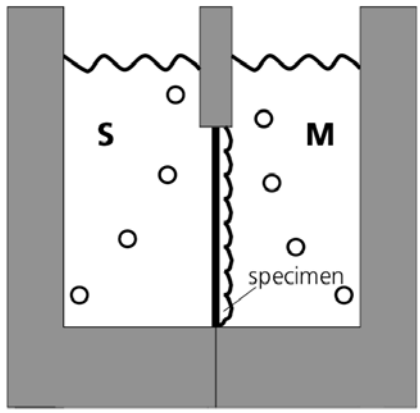

Figure 2: Cross-section of Ussing-chamber. The device consists of two half-chambers connected via a window, which is covered by a gastric mucosal specimen. This arrangement yields a polarised system with one mucosal (M) and one serosal (S) side. Circles symbolise continuous bubbling with 95% O_2 and 5% CO_2, which keeps pH stable and ensures sufficient oxygen for the specimen.

The tracer ^{125}I was added either to the serosal or to the mucosal side chamber and in this way transport could be measured in both directions, although not in the same specimen. As each animal yielded two specimens transport could be measured in both directions in the same animal. In a first set of experiments, different concentrations of iodide and the direction of iodide transport were studied. These experiments revealed that the direction of iodide transport was from the serosal to the mucosal side. Separate series of experiments were designed in order to test possible inhibitory effects on the iodide transport using perchlorate (competitive inhibitor of NIS-mediated iodide transport) and ouabain (inhibitor of Na^+/K^+-ATPase, the driving

force of NIS). In another series of experiments the possible regulation of the iodide transport was investigated by the addition of thyroid stimulating hormone (TSH), thyrotropin releasing hormone (TRH), vasoactive intestinal peptide (VIP), histamine, or the NO-donor S-nitroso-N-acetyl-D, L-penicillamine (SNAP).

Statistics

As groups were relatively small, all values were presented as medians or ranges and non-parametric statistical methods were employed. Statistical methods are further specified in each paper.

RESULTS AND DISCUSSION

NIS localisation in normal tissue

In paper I, the cellular localisation of NIS protein was studied using immunohistochemistry and NIS mRNA expression by *in situ* hybridisation. The aim was to study NIS expression in different organs with focus on the digestive tract. Another aim was to compare NIS expression in different species. Man, pig, guinea pig, rat and mouse were studied. The antiserum used for immunohistochemistry (see Methods) recognised NIS in all species studied. Expression of NIS was confirmed by demonstrating NIS mRNA by *in situ* hybridisation in mouse, rat and guinea pig, but the *in situ* probe used unfortunately did not recognise NIS mRNA in porcine or human tissue. As the cellular localisation of NIS in the thyroid of man and rat was previously known, thyroid was included as a reference organ. In paper III, our focus was on NIS localisation in pre-malignant and malignant tissue in rat, but as the immunohistochemical methods (i.e. antigen retrieval) had improved since the work on paper I was performed, new data on NIS expression in liver and duodenum were added.

Thyroid (paper I)

NIS immunoreactivity was intense and distinctly localised to the basolateral cell membrane of the thyroid follicular cells in guinea pig, rat and mouse. In man and pig some follicular cells displayed a basolateral localisation of NIS, but a large number of the NIS immunoreactive cells were diffusely stained throughout the cytoplasm. Others have reported on a strict basolateral localisation of NIS in normal human follicular cells (Jhiang et al. 1998; Vayre et al. 1999). This seems, however, to be a more prominent feature in Graves disease (Castro et al. 1999b; Spitzweg et al. 1999), in which the expression of NIS is higher than normal. For comparison it is noteworthy that the immunostaining of NIS in papillary and follicular carcinoma of the thyroid has been reported to be diffuse (Castro et al. 1999b). A strictly basolateral localisation of NIS may be a feature only in active follicular cells.

In rat and mouse virtually all follicular cells were stained, whereas in man, pig, and guinea pig the NIS immunoreactive material was unevenly distributed within follicles and the number of stained cells varied between follicles. This patchy immunoreactivity had been described previously in man (Caillou et al. 1998; Castro et al. 1999a). *In situ* hybridisation in rat and mouse showed intense autoradiographic labelling of the thyroid gland representing presence of NIS mRNA, which corresponded to the immunohistochemical findings, showing that the bulk of follicular cells expressed NIS. In guinea pig the distribution of NIS mRNA was patchy as was the immunostaining. The patchy distribution of NIS may reflect differences in metabolic activ-

ity of the thyroid follicular cells in these species. In this context it is worth mentioning that organic iodide, as studied by analytical ion microscopy, has a similarly patchy distribution in normal human thyroid (Fragu et al. 1989). However, it has not been studied whether the cells with a high content of organic iodide are the same as those rich in NIS.

Gastric mucosa (paper I)

In the oxyntic and pyloric mucosa of man, rat, and mouse, intense NIS immunoreactivity was found basolaterally in the epithelial surface cells including the epithelial cells lining the gastric pits. NIS immunoreactivity of moderate intensity was also noted in the mucosal surface epithelium (including gastric pits) in pig and guinea pig. These findings are in accordance with the findings of Vayre et al. (1999) who found the same pattern of immunoreactivity in man (with a polyclonal antiserum raised against human NIS) and with Ajjan et al. (1998) who utilised southern blot and rt-PCR and found high levels (>80% of thyroid level) of NIS mRNA in rat gastric mucosa, but in contrast to Kotani et al. (1998) who reported that NIS was preferentially located at the apical border of the gastric epithelium in rat. Gastric NIS in pig, guinea pig, and mouse had not been studied previously. Localisation of NIS mRNA corresponded well to the pattern of immunoreactivity seen in guinea pig, rat, and mouse in that a strong autoradiographic labelling of the epithelium was detected.

In accordance with Spitzweg et al. (1999), strong NIS immunoreactivity was also noted in a large number of the parietal cells in man and to a lesser extent in pig, guinea pig, and mouse, whereas such staining was absent in rat. However, the presence of authentic NIS in these cells is questioned since no NIS mRNA labelling could be detected in guinea pig or mouse (the species in which the probe recognised NIS mRNA) by *in situ* hybridisation. In addition it may be argued that the selectivity of the antiserum is highest toward rat NIS since it was raised against synthetic rat NIS. Thus, the possibility that the NIS immunoreactive material in parietal cells represents cross reactivity with some other protein/transporter must be considered.

In the rumen (*pars proventricularis*) of rat and mouse (the only two species studied possessing this gastric portion) no NIS was found, which is not surprising considering that this part of the rodent stomach is lined by squamous epithelium and not gastric glandular mucosa as in the rest of the stomach.

The presence and distribution of NIS and NIS mRNA expression have been explored in the rat gastric mucosa and thyroid during embryonic development and throughout the neonatal period (postnatal day 0-13) (Josefsson and Ekblad 2009). Gastric NIS was detected by immunohistochemistry and NIS mRNA by *in situ* hybridisation. Expression of NIS in gastric mucosa occurs already at E17, which coin-

cides with the appearance of NIS protein and NIS mRNA within the thyroid (Josefsson and Ekblad 2009). At this time-point gastric NIS immunoreactivity is intense and located in the basolateral cell membranes of the epithelial surface cells. The topographic distribution and staining intensity noted in gastric mucosa at E17 persist during the later part of embryonic development and also throughout the neonatal period. Thus the presence and expression of gastric NIS in pre- and postnatal rats are identical to those of adult rats. This is in contrast to the neonatal versus adult expression of thyroidal NIS, which although localised in the basolateral cell membranes of the follicular cells, shows a patchy distribution throughout the gland indicating differences in metabolic activity or in maturation of the thyroid follicular cells (Josefsson and Ekblad 2009). In adult rats, NIS is evenly distributed throughout the thyroid.

Salivary glands (paper I)

In rat, weak NIS immunoreactivity could be detected in the ductal cells of the parotid gland, whereas in guinea pig and mouse parotid glands NIS immunoreactivity was intense basolaterally in the ductal cells. In mouse submandibular gland intense NIS immunoreactivity was found in both intra- and interlobular ducts, while in the sublingual gland only a weak immunostaining of NIS was detected in ductal cells. No NIS immunoreactive material could be detected either in guinea pig or in rat submandibular gland. NIS mRNA labelling by *in situ* hybridisation was barely detectable in rat parotid ductal cells, but strong in guinea pig and mouse parotid ductal cells, which is in accordance with the immunohistochemical findings.
During the work on paper III, paraffin embedded and microwave treated sections of rat submandibular glands were checked for NIS immunoreactivity for comparison with the results of paper I. The antigen retrieval procedure revealed a consistent NIS immunoreactivity basolaterally in the ductal cells just as in mouse. Theses results indicate that differences in NIS expression between species may be more a question of expression level than of expression or not. This is in line with the finding of NIS in a very wide range of tissues using rt-PCR (Kotani et al. 1998; Spitzweg et al. 1998). Very low NIS expression in a certain tissue probably reflects low iodide transport capacity.

Duodenum (papers I and III)

In paper I, an extended investigation on the presence of NIS and NIS mRNA in oesophagus, duodenum, jejunum, colon and rectum was undertaken in rat. Only a few scattered NIS immunoreactive endocrine cells were found in the small intestine, without any detectable labelling of NIS mRNA by *in situ* hybridisation. No NIS immunoreactive material or NIS mRNA could be detected either in the oesophagus

or in the large intestine. In paper III, however, NIS immunoreactivity was found basolaterally in the surface epithelium of duodenal villi. Duodenal NIS immunoreactivity was much lower than gastric. The strongest NIS staining was noted at the tips of villi and staining became gradually lower basally, close to the crypts, which did not express any NIS. The difference between papers I and III is explained by the use of microwave antigen retrieval in paper III, which unmasks a NIS expression below the detection limits of the method in paper I. This is in accordance with the finding of NIS expression in duodenum by rt-PCR (Kotani et al. 1998). Brunner's glands, situated in the submucosa, did not display any NIS immunoreactivity.

Bile ducts and liver (paper III)

Bile ducts and liver were not studied in paper I. In paper III, focused on induction of gastric tumours, the unexpected development of frequent, cystic tumours in the liver drew the attention to NIS expression in this organ. In normal rats, large intrahepatic bile ducts displayed NIS immunoreactivity in the apical and lateral cell membranes of the ductal epithelium, but no NIS immunoreactivity was found in hepatocytes. This is in contrast to the basolateral NIS expression in human normal bile duct cell membranes described by Liu et al. (2007). The apical localisation of NIS in bile ducts is also in contrast to the localisation of NIS in gastric epithelium and thyroid in the rat. The apical location of NIS suggests uptake of iodide from the bile rather than secretion of iodide into it.

Iodide transport and the role of NIS

In vivo studies (paper I)

Gastric iodide transport has earlier been studied *in vivo* in animals, mostly dog and rat (Brown-Grant 1961), but also in other animals e.g. bovines (Miller et al. 1975a). The conclusions reached were that iodide is transported primarily from the bloodstream into the gastric lumen, and not in the opposite direction. However, these conclusions had been all but forgotten for many years, as the clinical use of radioiodine became routine. When the iodide transport research was revitalised by the sequencing of NIS (see "Background") towards the end of the last century, NIS was identified in the surface epithelium of gastric mucosa. Now the suggestion that the function of NIS was to absorb iodide from the gastric lumen was put forward (Kotani et al. 1998). With these different ideas in mind, the studies of NIS localisation in various organs and species (paper I) were extended with an *in vivo* study to elucidate the extent of iodide uptake from the gastric lumen and iodide transport from the bloodstream into the gastric lumen.

In vivo thyroid accumulation of ^{125}I tended to be higher after intragastric than after intravenous administration. Pyloric ligation virtually abolished thyroid ^{125}I accumulation after intragastric administration, but did not affect thyroid ^{125}I accumulation after intravenous administration (figure 3). The utterly low uptake of ^{125}I in the thyroid after intragastric administration with ligated pylorus was accompanied by a low content of ^{125}I in blood compared to the other groups. This indicates that iodide uptake is intestinal and not gastric. In the group receiving ^{125}I intravenously, a substantial part of the total administered dose was found in the gastric contents at the end of the experiment (60 min after injection), with the higher values in the group with pyloric ligation (figure 3). This finding directly proves gastric secretion of iodide.

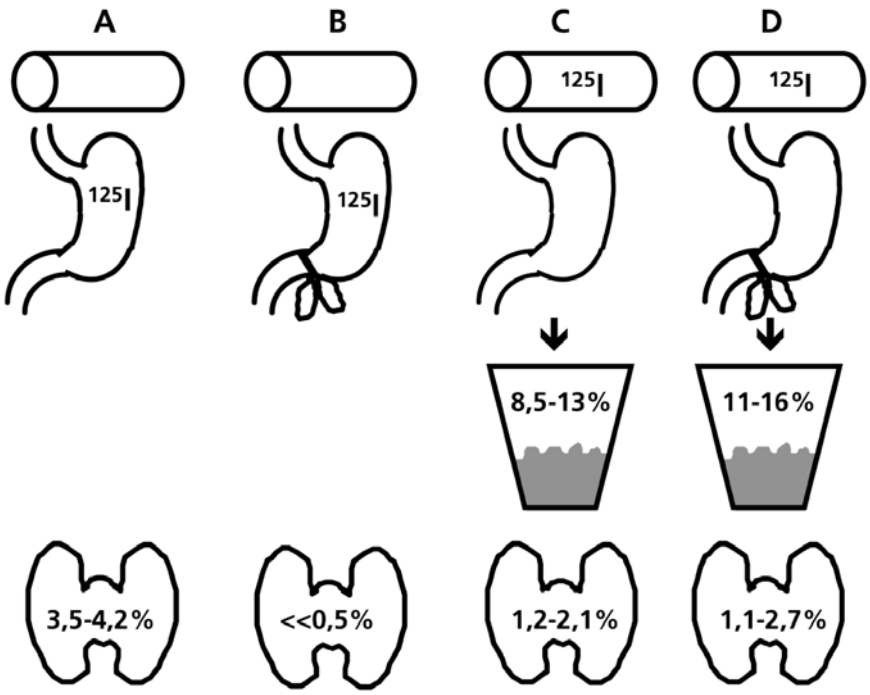

Figure 3 Accumulation of ^{125}I 60 minutes after administration *in vivo*. Upper row: Blood vessels. Second row: Stomachs with (B and D) or without (A and C) pyloric ligation. Location of the symbol ^{125}I within each group (column) signifies route of administration; by gastric catheter in A and B, intravenously in C and D. Radioactivity of gastric contents (third row) and thyroid glands (bottom row) expressed as % of total administered ^{125}I-dose (ranges).

In vitro studies (paper II)

The Ussing-chamber is a well-established *in vitro* model for studies on transport of, and permeability for, various types of substances through epithelia (Grass and Sweetana 1988; Nejdfors et al. 2000). In paper II, the Ussing-chamber model was adapted for studying iodide transport across rat gastric mucosa. The ability to transport iodide across gastric mucosa was tested by the addition of a wide range of different iodide concentrations to either the serosal or the mucosal side chamber. These experiments revealed a concentration dependent transport from the serosal to the mucosal side, which was linear over time. Negligible amounts of iodide were transported in the opposite direction, i.e. from the mucosal to the serosal side. Pars proventricularis of rat stomach was used as a reference specimen and in this specimen no transport was detected in either direction.

Linking NIS to gastric iodide secretion

There are several established competitive inhibitors of the NIS-mediated iodide transport in the thyroid including anions as bromide, chlorate, perchlorate, and thiocyanate. In earlier studies gastric iodide transport was reported to be attenuated by thiocyanate, and even more effectively by perchlorate (Brown-Grant 1961). In paper II, perchlorate, the most widely used and well-characterized inhibitor of NIS activity, acting highly competitively in iodide transport, was used (De La Vieja et al. 2000; Van Sande et al. 2003). Perchlorate inhibited the iodide transport from serosal to mucosal side by 47%, which indicates that NIS is a putative candidate for mediating this transport. However, it is notable that in spite of using high concentrations of perchlorate, a total inhibition of iodide transport, as reported to occur in cultured NIS expressing cells (De La Vieja et al. 2000; Eskandari et al. 1997), could not be achieved. This probably reflects that the availability of the NIS molecule to perchlorate is different in cultured cells as compared to full-thickness mucosa *in vitro*. In order to facilitate diffusion, the muscular layer of the stomach wall was removed before mounting the mucosa in the Ussing-chamber. However, a substantial amount of connective tissue still remains in the lamina propria and in the submucosa, which hampers the possibility for both iodide and perchlorate to reach NIS, located as it is in the apical mucosa. Perchlorate, which is a larger molecule than iodide, is probably most affected by such impediments. In an attempt to overcome this we therefore added perchlorate to both the serosal and the mucosal chambers.

Iodide transport mediated by NIS is dependent on Na^+/K^+-ATPase (De La Vieja et al. 2000). To further test the hypothesis that NIS mediates gastric iodide transport, ouabain, which is an inhibitor of Na^+/K^+-ATPase, was used. Presence of ouabain attenuated the iodide transport from serosal to mucosal side by 23%. Besides low availability caused by diffusion impediments, the lack of total inhibition by both perchlorate and ouabain may indicate that NIS mediated transport is not the only

route by which iodide can pass the gastric mucosa. However, NIS mediates a substantial part of the iodide transport across the gastric mucosa into the gastric lumen.

Regulation of gastric NIS activity

Thyroid NIS is primarily regulated by thyroid stimulating hormone (TSH) but also by other factors e.g. iodide and cytokines as described in a review by Dohan et al. (2003). However, TSH is unable to change the rate by which iodide is transported over gastric mucosa *in vivo* (see review by Brown-Grant 1961) and also *in vitro* (paper II). In order to identify a possible neuroendocrine regulation of gastric NIS activity, several substances besides TSH were tested, but neither of TRH, VIP, histamine nor the NO-donor SNAP influenced gastric iodide transport. TSH is known to regulate thyroid iodide uptake activity mainly by increasing NIS expression and synthesis, but regulation of NIS activity has also been suggested (Paire et al. 1997). It was therefore of interest to test the effects of TSH in the Ussing-chamber, measuring gastric iodide transport *in vitro*. TRH, VIP, histamine and NO are all important messengers in the gastric mucosa mediating or modulating a number of physiological activities like vasodilatation, acid secretion and bicarbonate secretion (for a review see Ekblad et al. 2000). None of these putative regulatory substances were found to affect gastric iodide transport in the Ussing-chamber. It must, however, be emphasized that the experimental conditions only allow studies on acute regulatory effects and that changes in e.g. NIS mRNA synthesis are beyond detection. The lack of response to the tested neuroendocrine signalling substances may, as previously suggested for perchlorate and ouabain, also be due to diffusion impediments. So far, no known regulators either of gastric NIS expression or gastric NIS activity have been identified.

Gastric NIS and gastric iodide functions

Why is the essential and often scarce iodide first absorbed into the body and then secreted into the gastric lumen? One suggestion is that it is part of an iodide conserving mechanism, increasing the apparent iodide space and preventing excessive excretion, as iodide is readily reabsorbed in the small intestine (for a review see (Brown-Grant 1961)). This suggestion is supported by the finding of a gastrointestinal recirculation of iodide in the dairy cow, in which iodide re-entry from the circulation to the gastrointestinal tract was suggested to be mediated via secretion of iodide by the gastric mucosa of the abomasum (Miller et al. 1975b). Iodide is a component not only of gastric juice but also of saliva, bile and duodenal secretions (Scott et al. 1966). NIS immunoreactive material has been detected in ductal cells of human salivary glands (Jhiang et al. 1998; Spitzweg et al. 1999) and this was found, in paper I, to apply also to guinea pig, rat, and mouse. This provides a morphological basis for the suggestion that also salivary glands contribute to the recirculation of iodide. In paper III, NIS immunoreactivity was found to be present also in biliary

epithelium and in duodenal mucosa, further strengthening the theory that NIS plays an important role in iodide conservation by mediating part of an entero-thyroid recirculation. As NIS is predominantly localised basolaterally in the surface epithelium of the gastric mucosa, it is reasonable to assume that NIS mediates transport of iodide into the epithelial cells in analogy with the transport into thyroid follicular cells. The subsequent efflux of iodide into the gastric lumen may be passive or mediated by some other as yet unknown system. Pendrin, which mediates iodide transport from the thyroid follicular cell into the colloid (Royaux et al. 2000), has been suggested as a candidate, but no pendrin has been found within the gastric mucosa (Lacroix et al. 2001). It is easy to leap to the conclusion that iodide transport to the gastric lumen from the circulation is mediated by NIS, but studies linking NIS directly to secretion of iodide into the gastro-intestinal tract were still missing at this time. The next step was to adapt an *in vitro* model to study iodide transport across gastric mucosa in greater detail.

Apart from the previously suggested iodide-conserving function of NIS, there are several other theories about the functional reason for the abundant NIS expression in gastric mucosa. A challenging aspect of iodide function worth exploring is its suggested antimicrobial effect (Majerus and Courtois 1992). In this context we must also consider that NIS is not an exclusive iodide transporter. NIS also transports other anions among which are nitrate (NO_3^-) as described in the "Cancer, Iodide and NIS". NO_3^- is reduced to nitrite (NO_2^-) by bacterial enzymes and, in the acidic environment of gastric lumen, then nonenzymatically reduced to nitric oxide (NO) (McKnight et al. 1997; Weitzberg and Lundberg 1998) - a powerful antimicrobial agent. Thus both iodide and NO_3^- probably play important roles in our defence against ingested microbes (Fite et al. 2004). In addition, Fite et al. (2004) also indicates synergism between the two in that presence of iodide enhances the antimicrobial effect of NO. Interestingly an entero-salivary recirculation of NO_3^- has been suggested by several groups (for an overview see Duncan et al. 1997) and the salivary glands, as previously described, also express NIS. Salivary accumulation and secretion of dietary NO_3^- has been suggested as the source for the nonenzymatic NO production, which abundantly occurs in the stomach (McKnight et al. 1997; Weitzberg and Lundberg 1998). NO_3^- secretion via gastric NIS offers an additional possibility to increase local NO_3^- concentration in the gastric juice and one step further also NO content in the stomach.

A further theory as to the function of iodide in the gastric lumen is that it acts as an antioxidant (Venturi and Venturi 1999).

Presence of NIS in MNNG-induced tumours (paper III)

NIS-mediated iodide transport may influence gastric cancer development through antioxidative and antimicrobial effects of gastric iodide. Furthermore, the findings of abundant NIS expression in gastric mucosa (paper I) and NIS-mediated gastric iodide secretion (paper II) fostered the idea that this transport mechanism might be manipulated and utilised to accumulate radioiodine within gastric tumours. To study the potentials of NIS and iodide as diagnostic or therapeutical aids, as well as their potential role in carcinogenesis, an animal model of gastrointestinal cancer is needed. In paper III, the established carcinogen MNNG (Schoental 1966; Sugimura and Fujimura 1967) was used to induce cancer in the upper gastrointestinal tract of rats. We aimed to evaluate spontaneous NIS expression in MNNG-induced tumours of the gastrointestinal tract.

Gastric mucosa

Gastric mucosa, both oxyntic and pyloric, in control animals displayed three distinct layers; The surface layer consisting of surface mucous cells including gastric pits, the glandular neck layer containing mucous neck cells and in the oxyntic portion parietal cells, and the basal glandular layer containing chief cells, mucous cells, and parietal cells (oxyntic portion). In MNNG-treated animals the layer structure was altered with signs of proliferation reflected in an increased glandular neck region, which is the proliferation zone. Further, the normally distinctly delineated transition between glands and gastric pits as well as the delineation between neck region and basal glands were irregular. In contrast to control animals, which harboured parietal cells within the pyloric mucosa only close to the oxyntic mucosa transition, MNNG-exposed animals often harboured parietal cells in the pyloric mucosa also in areas distant from the transition to oxyntic mucosa.

MNNG-treated animals displayed frequent microadenomas (epithelial bulging and hypertrophy) and multiple adenomas, but unexpectedly no macroscopically visible or infiltrating gastric carcinomas were found. Intestinal metaplasia (glands with goblet cells) was noted in some pyloric microadenomas. Neovascularisation was often seen to accompany microadenomas, and sloughing was frequently seen in the surface epithelium. In the control animals no adenomas or microadenomas were found.

NIS immunoreactivity was intense basolaterally in the gastric surface epithelium of both controls and MNNG-treated animals. In areas adjacent to epithelial sloughing NIS staining was less intense. In microadenomas, NIS was expressed basolaterally in the surface epithelium as in normal mucosa, but staining was weaker than normal. NIS was also expressed in surface epithelium of the large adenomas. However, here NIS immunoreactivity was patchy and in some areas clearly reduced.

Duodenum with Brunner's glands

Control animals had normal duodenal mucosa, with long and slender villi constituting the main part of total mucosal thickness, whereas animals receiving MNNG had both shorter and broader villi. MNNG-treated animals frequently displayed areas of flattened mucosa with loss of villi and varying degrees of inflammation and fibrosis - probably representing ulcers in varying stages of healing. No tumours were found in the duodenal mucosa.

In the duodenal submucosa of control animals, the mucus-secreting Brunner's glands, with regular acinar arrangement and occasional extensions of ducts into the base of the crypts, occurred in approximately the first 4-5 mm. Brunner's glands were more prominent in the MNNG-treated animals with areas of proliferation and distorted glandular structure. Infiltration into the mucosa and/or the muscle layer was noted in half of the MNNG-treated animals.

Duodenal NIS immunoreactivity displayed the same pattern in MNNG-treated animals as in controls, but due to ulcers and frequent loss of villi over-all NIS expression was lower in MNNG-treated animals. Brunner's glands displayed no NIS expression either in control or in MNNG-treated animals.

Bile ducts and liver

In control animals, large intrahepatic bile ducts were readily visible, but no widening of ducts or adenomas was seen. Five out of eight animals receiving MNNG displayed large papillary cystadenomas lined with cuboidal to low cylindric epithelium. In animals with large cysts also multiple smaller cystadenomas were seen separate from the larger ones.

Cystadenomas displayed NIS immunoreactivity in the apical and lateral cell membranes of the ductal epithelium similar to that in large intrahepatic bile ducts. However, in the cystadenomas the intensity of NIS staining varied, with areas of distinct expression adjacent to areas with almost absent expression, especially in the large papillary cystadenomas.

Comments on induced tumours and NIS

No infiltrating gastric adenocarcinomas were seen in our study, which is surprising in comparison with previous studies on MNNG-induced cancer (Chakroborty et al. 2004; Sugimura et al. 1970). Possible explanations for this might be differences in rat strains as well as differences in virulence and prevalence of Helicobacter strains in

different parts of the world. NIS was consistently expressed in the surface epithelium of gastric adenomas, but the intensity of NIS immunoreactivity was more irregular than in normal epithelium, and in some areas substantially reduced. This is in accordance with Altorjay et al. (2007), who found NIS expression to be absent or low in gastric carcinoma in man and suggested that low NIS expression might be used as an indicator of malignancy in gastric lesions. A weaker than normal NIS staining apically in the epithelial proliferations may on the other hand be interpreted as a sign of decreased epithelial viability, eventually resulting in epithelial sloughing.

Unexpectedly, almost all of the MNNG-treated animals displayed either large papillary cystadenomas in the liver, found to express NIS, and/or infiltrating carcinomas of Brunner's glands, which did not express NIS. In some animals both tumour types were present in addition to the gastric adenomas.

The epithelium lining the liver cysts was identical to bile duct epithelium, and the hypothesis that the tumours were bile duct derived is further supported by the finding of weak NIS immunoreactivity, both in normal intrahepatic bile ducts and in the cyst epithelium. Surprisingly, we found NIS staining in the apical and lateral cell membranes of larger bile ducts, which is in contrast to the basolateral localisation of NIS in human bile ducts (Liu et al. 2007). The patchy, and in some areas undetectable NIS expression in the larger cystadenomas may indicate a progression from a high NIS content in early stages of tumour development to a loss of NIS expression in more advanced stages.

Infiltrating carcinoma of Brunner's glands is reported to be very rare in man (Ohta et al. 2008) and has not previously been described to be MNNG-induced. Brunner's glands cells express a mucin, class III mucin encoded by MUC6 gene (Krause 2000), different from that of gastric foveolar cells. However, gastric mucous neck cells also express this type of mucin and Brunner's gland cells can develop into gastric foveolar cells expressing a different mucin type in reparative situations such as in close proximity to duodenal ulcers (Kushima et al. 1999). Further knowledge on NIS expression in different situations, might make NIS a future aid in differentiating between gastric and duodenal adenocarcinoma subtypes.

In advanced, but resectable stages of gastric cancer radical surgical resection is the primary treatment at present, but long-term survival is low, and adjuvant therapies as chemotherapy and radiation are currently tested (Moehler et al. 2007). New palliative or adjuvant therapies would be most welcome in the treatment of gastric as well as hepato/biliary carcinoma, and in the future radioiodine treatment may become one of them. This is, however dependent on NIS expression in the tumours. Spontaneous NIS expression or lack of it might thus be a useful marker, not only for diagnosis, but also when judging the feasibility to treat tumours and metastasis with radioio-

dine. In addition, methods to induce or enhance NIS expression in cancer cells need to be explored since NIS expression, based on present knowledge, seems to be reduced or absent in most cases of gastric carcinoma (Altorjay et al. 2007). In cell types, like gastric surface epithelium, with an inherent potential of NIS synthesis, chemical induction of NIS expression may be an appealing alternative or a complement to the more widely suggested method of gene transfer (see review by Dohan et al. 2003). Chemical induction or enhancement of NIS expression by retinoic acid in breast cancer cell lines has been reported (Kogai et al. 2000). There are so far no reports on chemical induction of NIS in gastric cells, but trials on MNNG-treated rats could be a way of testing potential candidates for NIS induction *in vivo*.

CONCLUSIONS

Based on papers I - III the following conclusions were reached:

NIS is abundantly expressed in the basolateral cell membranes of the gastric mucosal surface epithelium of all studied mammals, including man.

Salivary glands, bile ducts and duodenal mucosa also express NIS, but to a lesser extent than gastric mucosa (and thyroid).

MNNG-induced tumours in gastric mucosa and bile ducts both express NIS, but expression is of varying intensity often lower than in the corresponding normal tissue. NIS expression might, in the future, be used in the diagnosis and characterisation of gastric and biliary cancers. The absence of NIS expression might also be used as a sign of malignancy in these cancer types. Spontaneous or induced NIS expression in these tumours might make radioiodine an aid in treatment and diagnosis.

Brunner's glands are prominent in MNNG-treated animals with infiltration into the mucosa and/or the muscle layer in 50% of the animals, a cancer form not previously described as MNNG-induced. Neither normal nor infiltrating Brunner's glands express NIS.

Iodide is secreted across the gastric mucosa into the gastric lumen, but not absorbed from gastric contents.

Iodide secretion into the gastric lumen is, to a large extent, mediated by NIS.

The regulation of gastric NIS expression and activity are still unknown. Tests with neuroendocrine mediators (TSH, TRH, VIP, histamine and SNAP) *in vitro* did not influence the rate of iodide secretion.

The physiological reason for abundant gastric NIS expression and gastric iodide secretion are still not fully understood. One important function could be recirculation of iodide to prevent the rare trace element from being lost. Other suggested functions are antimicrobial and antioxidative effects of iodide in the gastric lumen, consistent with a role for NIS in gastric carcinogenesis. Transport of other anions, such as NO_3^-, could be yet another function fulfilled by gastric NIS. However, none of the suggested functions exclude the others, which further supports the concept that NIS is not only abundant but also important in gastric mucosa.

SAMMANFATTNING (SUMMARY IN SWEDISH)

Jod är nödvändigt för utvecklingen och hälsan hos alla högre djur inklusive människa, men det är ett relativt sällsynt grundämne. Man får i sig jod via kosten och det högsta jodinnehållet har fisk och skaldjur eftersom havssalt innehåller jod. Jod finns även i mejeriprodukter och i mindre mängder i grönsaker, men jodinnehållet i dessa livsmedel är starkt beroende av jodhalten i jordskorpan. I många regioner är intaget av havssalt via fisk och skaldjur otillräckligt och jodnivåerna i jordskorpan så låga att allmän berikning, vanligen i bordssalt, krävs för att inte en betydande andel av befolkningen skall drabbas av jodbristrelaterade sjukdomar, av vilka de mest kända är struma (förstoring av sköldkörteln) och brist på sköldkörtelhormon. I Sverige berikas såväl bordssaltet som mjölkkornas foder, vilket gör att jodbrist knappast förekommer här.

Mot bakgrund av ovanstående är det begripligt att mekanismerna för människans förmåga att ta upp och lagra jod i kroppen tilldragit sig betydande intresse genom åren. Mycket forskning på detta område gjordes decennierna efter mitten av 1900-talet då man fick tillgång till radioaktiva former av jod. Detta gjorde det möjligt att följa jodfördelning mellan olika organ efter tillförsel och sedan även mäta jodutsöndring i exempelvis urin och avföring. Redan under denna forskningsperiod noterades att jod ackumuleras i magsäcken. Det har länge varit känt att jodtransporten in i sköldkörtelcellerna sker tillsammans med natrium via natrium/jod-symporten (NIS), som transporterar jod mot en koncentrationsgradient. I slutet av 1990-talet fastslogs uppbyggnaden (aminosyrasekvensen) av NIS och nu väcktes på nytt intresset i forskningsvärlden för jodtransport, med speciellt fokus på NIS. Vid denna tid påbörjades avhandlingens första delarbete (arbete I), vilket var ett av de första som studerade NIS lokalisation i magsäcken.

I arbete I beskrivs förekomst och cellulär lokalisation av NIS i sköldkörtel, magtarmkanal, spottkörtlar och bukspottkörtel. Här visades att NIS-protein (undersökt med immunhistokemi), och även NIS mRNA (mallen för proteinets sammansättning, undersökt med *in situ* hybridisering), rikligt syntetiseras i magsäckens ytliga cellager samt även i stora öronspottkörtelns utförsgångar hos flera däggdjur. I detta arbete kunde även jodsekretion från blodet, över magslemhinnan och ut i magsäckslumen påvisas hos levande råtta.

Det andra delarbetet (arbete II) fokuserar på att reda ut mekanismerna bakom magslemhinnans förmåga till jodsekretion. För att mera detaljerat studera betydelsen av NIS användes en uppställning, s.k. Ussing-kammare, för studie av vävnadsbitar, i detta fall från råttmagsäck. Dessa försök visar en betydande transport av jod från blodsidan ut i magsäckslumen (sekretion), men inte i omvänd riktning. Det sker alltså inget jodupptag över magslemhinnan, vilket hävdats i en del tidigare arbeten.

Magsäckens jodsekretion i dessa försök reduceras kraftigt vid tillsats av två olika farmakologiska substanser (perklorat och ouabain), vilka på olika sätt hämmar NIS. Detta indikerar starkt att NIS aktivitet medierar denna jodsekretion. I arbete II testades även huruvida NIS aktivitet och jodsekretion i magen regleras neurohormonellt. Jodtransporten förblev dock oförändrad i närvaro av thyroideastimulerande hormon (TSH), thyrotropinfrisättande hormon (TRH), vasoaktiv intestinal peptid (VIP), histamin eller NO-givare (SNAP).

Det tredje delarbetet (arbete III) planerades för att studera om NIS uttrycks i tumörer utgående från magslemhinnan. Om så är fallet skulle man på sikt kunna tänka sig att utnyttja detta kliniskt, då anrikning av radiojod i tumören ger bestrålning motsvarande den behandling som ges vid sköldkörtelcancercancer och även vissa godartade sköldkörtelsjukdomar. För denna studie användes nitrosaminexponerade råttor då det sedan länge är känt att råttor som får N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) i dricksvattnet utvecklar magsäckscancer. Studien gav det oväntade resultatet att inga fullt utvecklade magsäckscancrar uppstod. I stället förändrades slemhinnan i precancerös riktning med en kraftigt ökad proliferationszon i slemhinnans körtelrör och med multipla områden i slemhinnan med adenom (mindre tumörer utan inväxt i omgivande vävnad). De MNNG-inducerade förändringarna får betraktas om förstadier till cancer och hade ett påtagligt uttryck av NIS, vilket är intressant för framtida utveckling inom diagnostik och terapi av magsäckscancer. Utöver magsäcksförändringarna uppvisade nästan samtliga de MNNG-exponerade djuren stora cystiska tumörer i levern och/eller cancer i Brunners körtlar som ligger strax under slemhinnan i tolvfingertarmen. Dessa tumörtyper har inte tidigare rapporterats vid MNNG-exposition vilket föranledde att lever och tolvfingertarm studerades speciellt, även med avseende på NIS förekomst i normal vävnad. Såväl i normala gallgångar som i de cystiska levertumörerna fanns NIS, medan Brunners körtlar, med eller utan tumörer, saknade NIS. Tack vare förbättringar i sättet att preparera vävnadsprover kunde även en mindre mängd NIS påvisas i det ytliga lagret i tolvfingertarmens slemhinna, vilket inte kunde påvisas i arbete I.

Slutsatser baserade på de ingående delarbetena

- NIS finns i stor mängd i magsäcksslemhinnan hos ett flertal däggdjur, däribland människa.
- NIS finns i mindre mängd även i spottkörtlar, gallgångar och tolvfingertarm hos råtta.
- NIS finns även i inducerade precancerösa magsäcksförändringar hos råtta, om än i något lägre grad än i normal magslemhinna.
- NIS finns även i inducerade tumörer utgångna från gallvägarna hos råtta.
- NIS-uttryck och jodtransportförmåga skulle på sikt kunna användas för såväl diagnostik av magsäcks och gallvägscancer som för terapi med radiojod.

- Jod transporteras aktivt från blodbanan ut i magsäckslumen och denna transport medieras av NIS.
- Den fysiologiska funktionen såväl som regleringen av den stora mängden NIS i magsäcken är ännu otillräckligt kända. Några möjliga funktioner för jodtransport till magsäckslumen är antibmikrobiella och/eller antioxidativa effekter av jod i magsäcken samt recirkulation av jod i kroppen för att spara på detta viktiga spårämne.
- Ingen av de föreslagna funktionerna för NIS utesluter de övriga, vilket stärker övertygelsen att NIS inte bara är rikligt förekommande, utan också har stor betydelse i magsäcken.

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Paper I

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Sodium/iodide-symporter: distribution in different mammals and role in entero-thyroid circulation of iodide

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ABSTRACT

The sodium (Na^+)/iodide (I^-)-symporter (NIS) is abundantly expressed and accumulates iodide in thyroid follicular cells. The NIS is also found in extrathyroidal tissues, particularly gastric mucosa. Controversies exist on the localization of extrathyroidal NIS. We have studied the presence of both NIS peptide and NIS messenger RNA (mRNA) in the digestive tract and thyroid from different mammals. The role of gastric NIS is enigmatic and we aimed to unravel its possible involvement in iodide transport. Methods: Distribution and expression of NIS were studied using immunocytochemistry and *in situ* hybridization. Iodide transport in the gastrointestinal tract was measured after oral or intravenous (i.v.) administration of ^{125}I to rats with or without ligation of the pylorus. Results: All thyroid follicular cells in rat and mouse expressed NIS, whereas a patchy staining was noted in man, pig and guinea-pig. Gastric mucosa surface epithelium in all species and ductal cells of parotid gland in guinea-pig, rat and mouse expressed NIS. In parietal cells and in endocrine cells of intestines and pancreas NIS immunoreactivity but no NIS mRNA was found. Studies of ^{125}I uptake showed marked iodide transport from the circulation into the gastric lumen. Conclusions: The localization of NIS varies slightly among mammals. To establish expression of NIS in a particular cell type the need to correlate the presence of both NIS protein by immunocytochemistry and NIS mRNA by *in situ* hybridization is emphasized. An entero-thyroidal circulation of iodide mediated principally by gastric NIS, but possibly also by NIS in salivary glands is suggested.

Keywords entero-thyroid circulation, gastric mucosa, iodide transport, NIS, salivary glands, thyroid gland.

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Iodide is an essential component of thyroid hormones. It is absorbed from the gastrointestinal tract and highly concentrated in the follicular cells of the thyroid. The mechanisms governing iodide transport over cell membranes in the thyroid and in the gastrointestinal tract are not known in detail.

The sodium (Na^+)/iodide (I^-)-symporter (NIS) is a transmembrane protein mediating iodide transport into the follicular cells of the thyroid gland. As the complementary DNA (cDNA) sequences of rat NIS in a thyroid derived cell line (FRTL) (Dai *et al.* 1996) and human NIS in papillary thyroid carcinoma (Smanik *et al.* 1996) were revealed, studies on iodide transport and NIS protein structure as well as function have been vitalized (Nilsson 1999, de la Vieja *et al.* 2000).

The NIS has been detected by immunocytochemistry in rat thyroid (Paire *et al.* 1997) and gastric mucosa (Kotani *et al.* 1998). In addition, reverse transcriptase–polymerase chain reaction (RT–PCR) technique has demonstrated the presence of NIS mRNA in rat brain, stomach, duodenum, skin, mammary gland, placenta, uterus and ovary but not in salivary glands (Kotani *et al.* 1998). In man, NIS-immunoreactive material has been found in thyroid follicular cells, gastric and large intestinal mucosa, ductal cells of salivary glands, lachrymal gland and some epithelial cells of mammary gland (Jhiang *et al.* 1998, Spitzweg *et al.* 1999, Vayre *et al.* 1999). Spitzweg *et al.* (1999) also found NIS-immunoreactive material in pancreatic endocrine and exocrine cells while in the study by Vayre *et al.* (1999) no NIS immunoreactivity could be detected in pancreas. Presence of NIS messenger RNA (mRNA), as demonstrated by RT–PCR technique, has been reported in human pituitary, thyroid, salivary glands, gastric

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mucosa, pancreas, adrenal gland, heart, lung, thymus, ovary, mammary gland, testis and prostate (Spitzweg *et al.* 1998).

In man thyroid and gastric NIS cDNA has been found to be identical (Vayre *et al.* 1999), while rat gastric NIS cDNA has been found to differ from rat thyroid NIS cDNA by three nucleotide substitutions leading to two amino acid substitutions in the NIS protein (Kotani *et al.* 1998). Gastric NIS has been suggested to be of an immature form, easily degraded and functioning to trap iodide from the gastric lumen although not very efficiently (Kotani *et al.* 1998).

The aims of this study were to examine the localization and the expression of NIS and NIS mRNA in the thyroid and digestive tract from different mammals and the role of gastric NIS for iodide transport in the gastrointestinal tract.

MATERIALS AND METHODS

Animals

A total of five female NMRI mice (20–30 g, Alab, Stockholm, Sweden), 24 female Sprague–Dawley rats (170–200 g, Alab, Stockholm, Sweden) and three female Dunkin–Hartley guinea-pigs (250 g, Sahlins, Malmö, Sweden) were used. The animals had free access to standard food pellets and tap water. Animals were killed by bleeding from a cardiac incision during deep diethyl ether or chloral hydrate anaesthesia. Specimens were also collected immediately postmortem from three pigs used for educational purposes at the University of Lund. Animal care was in accordance with the European Council Convention of 1986 and the study was approved by the research animal ethics committee at the University of Lund.

Human material

Normal thyroid and gastric tissues were obtained from patients undergoing thyroid surgery ($n = 4$) or gastrectomy ($n = 4$). The procedures were approved by the ethical committee at the University of Lund.

Specimens and tissue processing for microscopic examination

Thyroid and stomach (oxyntic and pyloric region; from rat and mouse also pars proventricularis) from all species as well as parotid and submandibular glands from guinea-pig, rat and mouse were examined. In addition specimens from oesophagus, duodenum, jejunum, colon descendens, rectum and pancreas from rat were included. Specimens were fixed in Stefanini's fixative (2% formaldehyde and 0.15% picric acid in phosphate buffer, pH 7.2) for 12–24 h and then repeatedly rinsed in Tyrode buffer containing 10% sucrose, all at 4 °C. Specimens were frozen on dry ice, sectioned in a cryostat at –20 °C to a thickness of 10 μm, and thaw-mounted onto Superfrost plus slides (Tamro lab AB, Mölndal, Sweden). The sections were then processed either by immunocytochemistry or by *in situ* hybridization.

Immunocytochemistry

A peptide containing the eight C-terminal amino acids of rat-NIS (GHDVETNL) was synthesized in a SYRO multisynthesizer (Multisyn Tech, GmbH). Purity as analysed by high-performance liquid chromatography (HPLC) was >60%. The rational for choosing the C-terminal as antigen was that it is located intracellularly, thus well preserved during tissue processing and that it has no significant homologies with other known proteins, including the major thyroid antigens and transporters (Benvenga *et al.* 1999, GeneBank, NCBI, sequence similarity search, www.ncbi.nlm.nih.gov/BLAST). The N-terminus of the peptide was conjugated to bovine serum albumin (BSA) and an antiserum was raised in rabbit using the peptide-BSA conjugate together with Freunds complete adjuvans. Sections were incubated with the primary antiserum (code number 9806, Euro-Diagnostica AB, Malmö, Sweden) diluted 1 : 1600 in BSA-containing phosphate buffered saline (PBS) over night in a moist chamber at 4 °C. In addition an affinity purified, polyclonal rabbit antiserum (TIT11-A, Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) raised against the 16 C-terminal amino acids of rat NIS was used in dilution 1 : 160. The site of the antigen–antibody reaction was revealed by fluorescein isothiocyanate (FITC)-labelled pig antirabbit immunoglobulin G (IgG) (Dako, Copenhagen, Denmark) diluted 1 : 80. Incubation time with FITC-labelled antibody was 45–60 min in room temperature. For control purposes, the primary antisera were incubated with 100 μg mL^{-1} of the respective synthetic peptide overnight at 4 °C before use.

In situ hybridization

For the detection of NIS mRNA a 33-mer oligonucleotide probe complementary to rat thyroid NIS mRNA 570–602 (TCATCCTGAACCAAGTGACCG GGTTGGACATCT) (Dai *et al.* 1996) was constructed (Biomolecular Resource Facility, University of Lund, Sweden). The probe was 3′-endtailed with ^{35}S-deoxyadenosine triphosphate (dATP) by use of terminal transferase (both supplied by NEN, DuPont, Stockholm, Sweden), yielding a specific activity of approximately 2×10^9 CPM μg^{-1}. Cryostat sections were prepared as described previously. The hybridization protocol has previously been described in detail (Mulder

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et al. 1994). Briefly, after clearing in chloroform, the sections were acetylated and hybridized with the probe (1 pmol mL^{-1}) overnight at 37 °C. The slides were washed in saline sodium citrate (4 × 15 min, 55 °C), dehydrated and dipped in emulsion (Ilford K5, Mettssons Foto AB, Sweden). Exposure time was 5 weeks, after which the slides were developed in Kodak D-19 (Gerh. Ludwigsen, Partille, Sweden). For control purposes, hybridization was also performed after incubation in ribonuclease (RNase) A (45 μg mL^{-1}, Sigma, St Louis, MO, USA; 30 min at 37 °C) or in the presence of a 100-fold excess of unlabelled probe in the hybridization buffer.

Radioiodide uptake in rat

The ^{125}I (25 μCi in 0.5–1 mL of saline) was administered to rats either intravenously (i.v.) or orally. All rats were fasted overnight before experimentation. Rats appointed to receive ^{125}I orally ($n = 7$) were anaesthetized with diethyl ether and the abdomen was opened. In four of these rats pylorus was ligated and in the other three left unligated. The abdomen was closed and the rats were allowed to wake up before placing the gastric catheter for iodide administration. Rats appointed to receive ^{125}I i.v. ($n = 6$) were anaesthetized with chloral hydrate (300 mg kg^{-1} intraperitoneally), the abdomen was opened, pylorus was ligated in three of the rats and left unligated in the other three. The abdomen was then closed and ^{125}I was injected into the femoral vein. Sixty minutes after iodide administration orally or i.v., 0.3 mL blood was collected by cardiac puncture under chloral hydrate anaesthesia, after which the rats were killed by bleeding. The blood samples were put directly in test tubes with 0.7 mL of saline. Thyroid–larynx complex, stomach, jejunum (approximately 2 cm), colon descendens (approximately 2 cm), and kidney were removed and weighed. All stomachs were ligated at both ends before removal and the stomachs as well as the intestines were cut open and washed in 8 mL of physiological saline for 1 min before measurements. The washing fluids obtained by this procedure from stomach and colon of i.v. treated rats (with and without pyloric ligation) were used for analysis of luminal ^{125}I content. Blood samples, washing fluids, and excised organs were measured for radioactivity in an automatic NaI(Tl) scintillation well counter (1282 Compugamma; LKB, Finland) and radioactivity was expressed as percentage of total administered ^{125}I per organ or sample.

Statistics

Values are single observations or ranges. For statistical analysis, values of radioactivity for washing fluids were expressed as ratio of washing fluid radioactivity to corresponding organ sample radioactivity. This was to compensate for sample size differences and a possible leakage of ^{125}I from the tissue. Comparisons were made with Wilcoxon's signed rank test.

RESULTS

Localization of NIS

Two different antisera were tested. The NIS antiserum 9806 resulted in strong and intense immunolabelling of the thyroid glands in all species studied, whereas the immunolabelling obtained by TIT-11A was weak and restricted to rat and mouse thyroid glands. The staining pattern obtained using the TIT-11 A antibodies was identical to that of antiserum 9806 in that the thyroid follicles possessed a basolateral immunolabelling. The immunolabelling was abolished by pre-incubating the antisera (both 9806 and TIT-11 A) with excess peptide. The TIT-11A failed to demonstrate NIS immunoreactivity in human, pig and guinea-pig thyroid glands. Therefore, the immunocytochemical results are mainly based on the NIS antiserum 9806.

The oligonucleotide probe generated for *in situ* hybridization showed consistent autoradiographic labelling in guinea-pig, rat and mouse thyroid gland, gastric mucosa and salivary glands demonstrating presence of NIS mRNA. In these locations no autoradiographic labelling was obtained in the control experiments. In man and pig no autoradiographic labelling was detected in any of the specimens including thyroid, indicating that the probe does not recognize the NIS mRNA sequences of these species.

Thyroid gland. In general NIS immunoreactivity predominated basolaterally in the follicular cells (Fig. 1a). This staining pattern was less obvious in man (Fig. 1b) and pig. In these two species some follicular cells displayed a basolateral localization of NIS, but a large number of the NIS-immunoreactive cells were diffusely stained throughout the cytoplasm. In rat (Fig. 1a) and mouse virtually all follicular cells were stained, whereas in man (Fig. 1b), pig and guinea-pig the NIS-immunoreactive material was unevenly distributed within follicles and the number of stained cells varied between follicles. *In situ* hybridization on rat (Fig. 2a) and mouse thyroid gland showed intense autoradiographic labelling representing the presence of NIS mRNA, which corresponded to the immunocytochemical findings in that the bulk of follicular cells expressed NIS. In guinea-pig the distribution of NIS mRNA was patchy (Fig. 2b) as was the immunocytochemical staining.

Stomach. In the oxyntic and pyloric mucosa of man, rat and mouse, intense NIS immunoreactivity was

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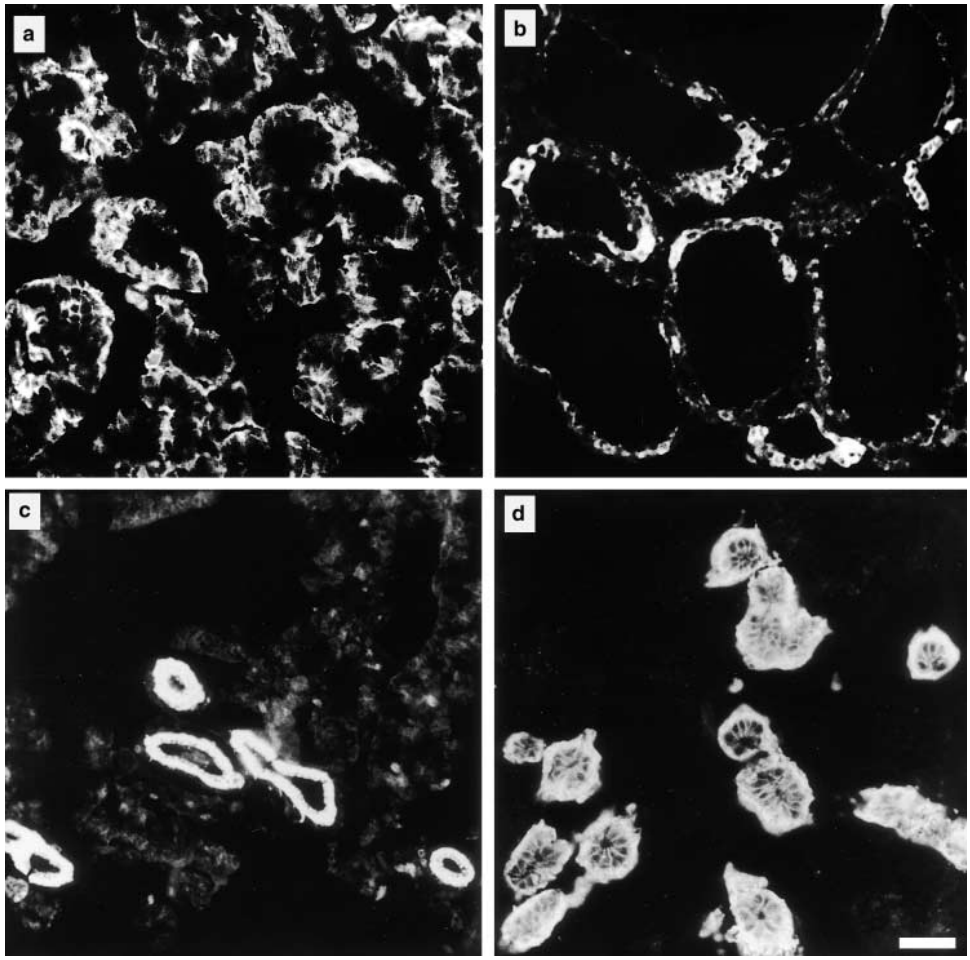

Figure 1 Cryostat sections of rat thyroid (a), human thyroid (b), mouse parotid gland (c) and guinea-pig parotid gland (d) immunostained for NIS. The NIS immunoreactivity is preferentially localized basolaterally in thyroid follicular cells. The NIS is present in virtually all follicular cells in rat, but has a more patchy distribution in man. In parotid gland NIS immunoreactivity is contained within the ductal cells and mainly found basolaterally. The scale bar in (d) represents 80 μm (a, b and d) or 125 μm (c).

found basolaterally in the epithelial surface cells including the epithelial cells lining the gastric pits (Fig. 3). NIS immunoreactivity of moderate intensity was also noted in the mucosal surface epithelium (including gastric pits) in pig and guinea-pig. In addition strong NIS immunoreactivity was noted in a large number of the parietal cells in man (Fig. 3c) and to a lesser extent in pig, guinea-pig and mouse (Fig. 3a), whereas such staining was absent in rat (Fig. 3b). In the pars proventricularis no NIS immunoreactivity was found in rat or mouse (the only two species studied possessing this gastric portion). Autoradiographic labelling of NIS mRNA corresponded well to the pattern of immunoreactivity seen in guinea-pig, rat (Fig. 2c) and mouse in that a strong autoradiographic labelling of the epithelium was detected. The parietal cells in guinea-pig, rat and mouse expressed no NIS mRNA as studied by *in situ* hybridization.

Oesophagus and intestines. An extended investigation on the presence of NIS and NIS mRNA in oesophagus, duodenum, jejunum, colon and rectum was undertaken in rat. In the small intestine a few scattered NIS-immunoreactive endocrine cells were found, but these

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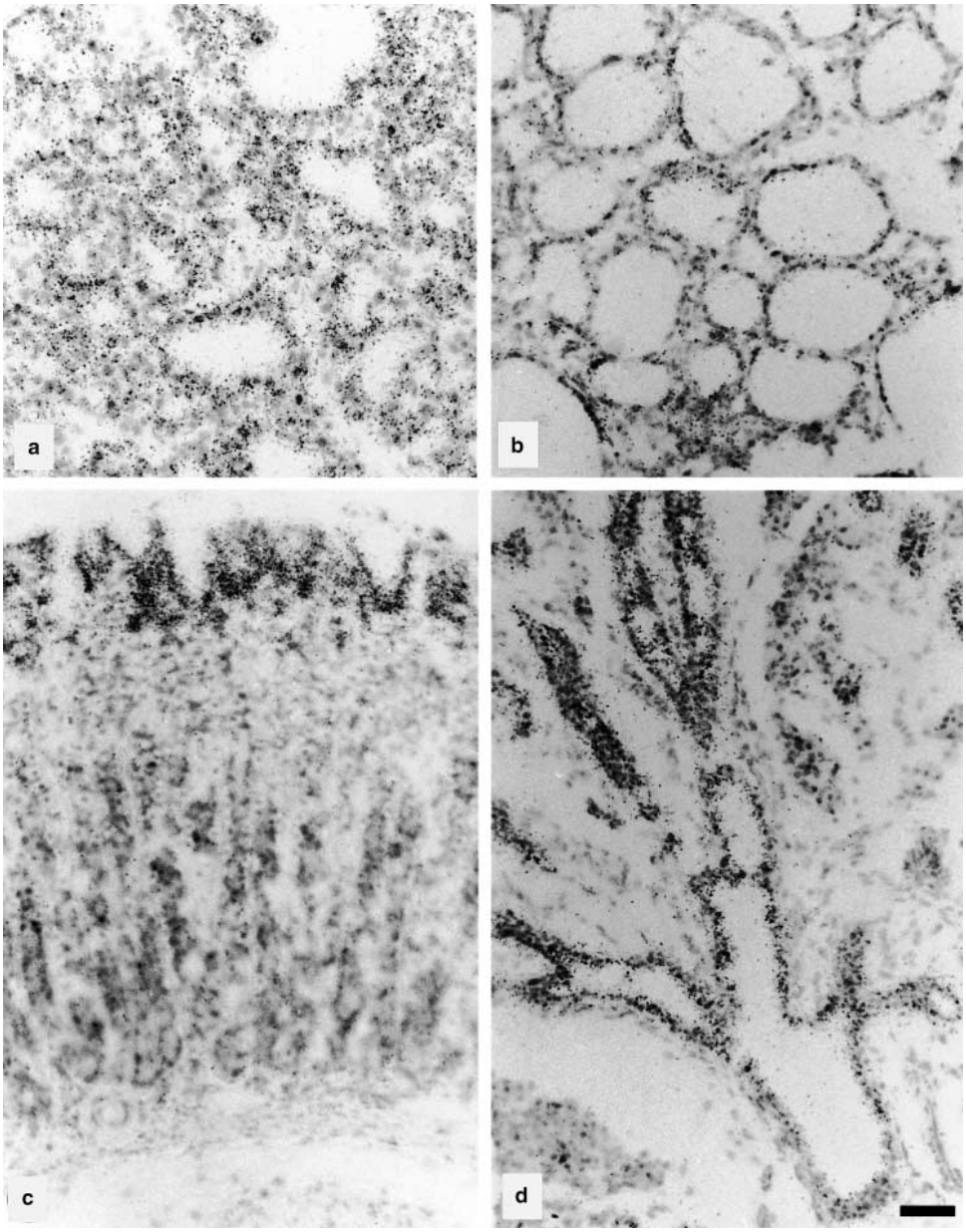

Figure 2 Cryostat sections of rat thyroid (a), guinea-pig thyroid (b), rat stomach oxyntic mucosa (c) and guinea-pig parotid gland (d) autoradiographically labelled for NIS mRNA. The labelling is homogeneously distributed in rat thyroid follicular cells (a) but patchy in guinea-pig thyroid (b). Intense labelling is seen in the gastric surface epithelium (c) and in ductal cells of the parotid gland (d). The scale bar in (d) represents 80 μm (a–c) or 60 μm (d).

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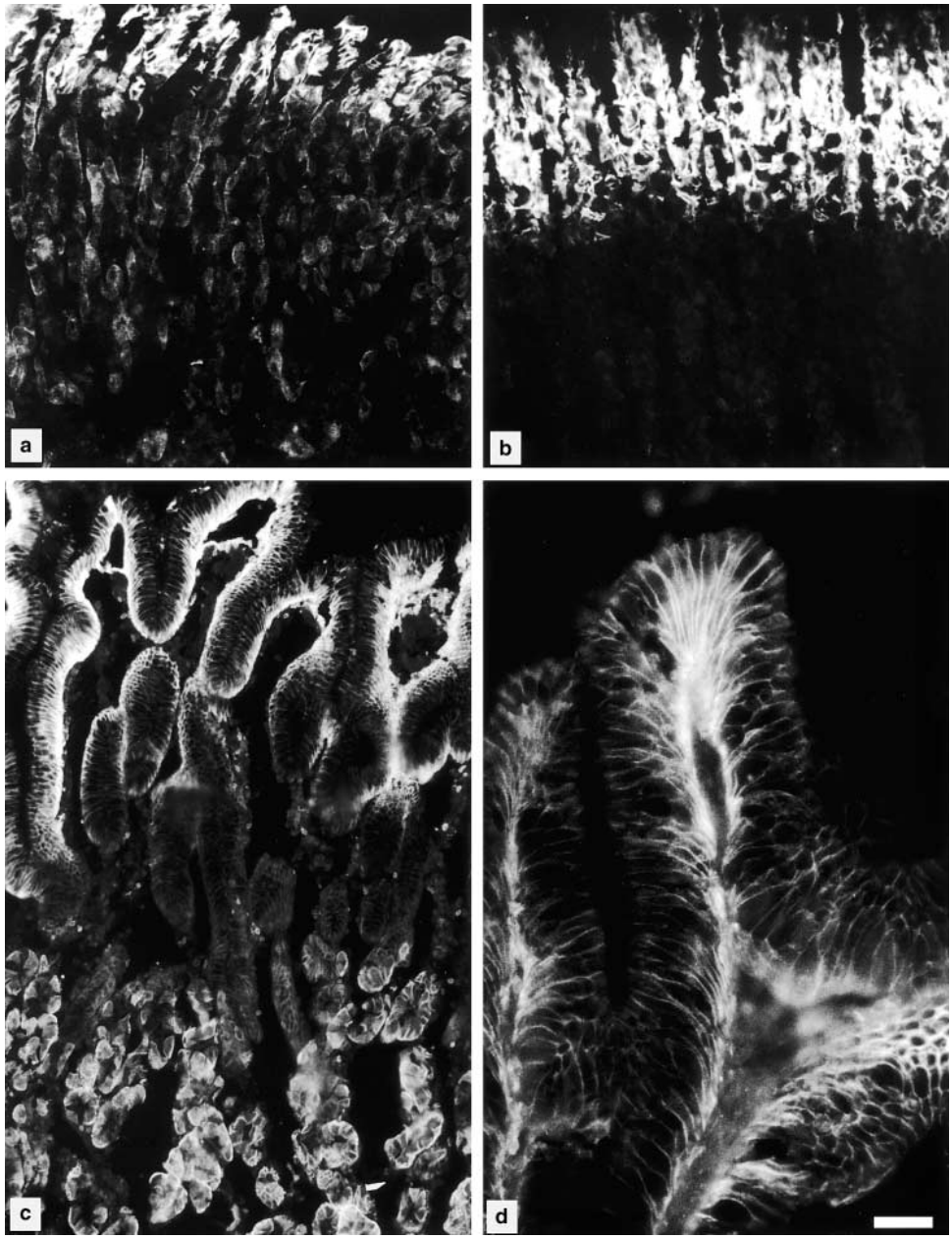

Figure 3 Cryostat sections of gastric oxyntic mucosa from mouse (a), rat (b) and man (c and d) immunostained for NIS. The NIS-immunoreactive material is found basolaterally in epithelial surface cells (a–d) and localized to the cell membrane in parietal cells (a and c). The scale bar in (d) represents 80 μm (a and b), 125 μm (c) or 50 μm (d).

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cells showed no detectable labelling of NIS mRNA by *in situ* hybridization. No NIS-immunoreactive material or NIS mRNA could be detected in the oesophagus or large intestine.

Salivary glands. In rat, weak NIS immunoreactivity could be detected in the ductal cells of the parotid gland, whereas in guinea-pig and mouse parotid glands NIS immunoreactivity was intense basolaterally in the ductal cells (Fig. 1c, d). In mouse submandibular gland intense NIS immunoreactivity was found in both intra- and interlobular ducts, while in the sublingual gland only a weak immunostaining of NIS could be detected in ductal cells. No NIS-immunoreactive material could be detected either in guinea-pig or in rat submandibular gland. The NIS mRNA labelling by *in situ* hybridization was barely detectable in rat parotid ductal cells but strong in guinea-pig (Fig. 2d) and mouse parotid ductal cells, which are in accordance with the immunocytochemical findings.

Pancreas. In rat pancreas a moderate number of NIS-immunoreactive cells was found within the islets. Such cells displayed, however, no NIS mRNA labelling. No NIS immunoreactivity or NIS mRNA was detected in the exocrine parenchyma.

Radioiodide uptake in rat

With open (unligated) pylorus iodide uptake in the thyroid was high after both oral and i.v. administration of ^{125}I (Table 1). With closed (ligated) pylorus the thyroid uptake of ^{125}I after oral administration of iodide was almost negligible, whereas in rats receiving iodide i.v. pyloric ligation did not hamper uptake of ^{125}I (Table 1). The utterly low uptake of ^{125}I in the thyroid after oral administration with closed pylorus was accompanied by a low content of ^{125}I in blood compared with the other groups (Table 1) ($P = 0.006$). Presence of iodide within the gastric wall was of the same magnitude irrespective of iodide administration route or pyloric ligation (Table 1). Jejunum had no obvious accumulation of iodide and kidneys consistently showed activity slightly above that of the blood samples in all four groups (Table 1).

After i.v. administration of ^{125}I, 9–16% of the administered dose was found in the stomach washing fluid irrespective of the pylorus being closed or open, whereas only 0.017–0.039% was found in the colon washing. For comparison of stomach and colon washing fluids all i.v. treated rats were considered as one group. The ratio of washing fluid radioactivity to corresponding organ sample radioactivity was clearly higher for stomach (1.9–3.2) than for colon (0.26–0.63) ($P = 0.03$).

DISCUSSION

Expression and distribution of NIS

The localization and expression of NIS in thyroid follicular cells is of great interest as NIS is a crucial protein for iodide trapping. The significance of NIS expression in other organs, such as the gastric mucosa, is less well understood. The present study verifies previous immunocytochemical observations on the presence of NIS in the gastric mucosa in man and rat, but also extends these studies to other species (pig, guinea-pig and mouse) as well as to other regions of the digestive tract. In addition we present a probe able to detect the expression of NIS mRNA by *in situ* hybridization in guinea-pig, rat and mouse, whereas it did not label NIS mRNA in pig or man.

The NIS immunoreactivity was predominantly localized basolaterally in thyroid follicular cells. This was, however, less obvious in pig and man, where many of the follicular cells displayed NIS immunoreactivity throughout the cytoplasm. Others have reported on a basolateral localization of NIS in normal human follicular cells (Jhiang *et al.* 1998, Castro *et al.* 1999, Vayre *et al.* 1999). This seems, however, to be a more prominent feature in Graves disease (Castro *et al.* 1999, Spitzweg *et al.* 1999), in which the expression of NIS is higher than normal. A strictly basolateral localization of

Table 1 Iodide uptake as percentage per organ of administered dose

Groups*	*n*	Thyroid	Stomach‡	Jejunum	Colon	Kidney	Blood (0.3 mL)
Oral	3	3.5–4.2	3.0–3.1	0.05–0.11	0.04–0.06	0.39–0.45	0.35–0.38
Oral + liga†	4	0.03–0.42	1.9–2.5	0.001–0.07	0.001–0.08	0.004–0.5	0.004–0.2
i.v.	3	1.2–2.1	3.9–4.7	0.05–0.07	0.04–0.11	0.47–0.62	0.27–0.47
i.v. + liga‡	3	1.1–2.7	4.3–5.6	0.06–0.24	0.05–0.08	0.49–0.66	0.31–0.54

*Groups are based on iodide administration route.
†Liga = pyloric ligation.
‡Glandular portion.
Values are ranges for each group.

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NIS may be a feature only in active follicular cells. The NIS and NIS mRNA were expressed in virtually all follicular cells in mouse and rat, whereas in guinea-pig, pig and man NIS immunoreactivity was patchy, which, in man, is in accordance with the findings of others (Caillou *et al.* 1998, Castro *et al.* 1999). The patchy distribution of NIS may reflect differences in metabolic activity of the thyroid follicular cells in these species. In this context it is worth mentioning that organic iodide, as studied by analytical ion microscopy, has a similarly patchy distribution in normal human thyroid (Fragu *et al.* 1989). However, it is not clear whether the cells with a high content of organic iodide are the same as those rich in NIS.

Gastric NIS in rat has been cloned and the cDNA was found to be nearly identical with thyroid NIS. However, its post-translational modification (mainly glycosylation) differs from that of thyroid NIS (Kotani *et al.* 1998). In the present study on gastric mucosa, as well as in the study by Vayre *et al.* (1999), very intense NIS immunoreactivity was found in the basolateral cell membranes of the surface epithelium. This is in contrast to the findings of Kotani *et al.* (1998) who reported that NIS was preferentially located at the apical border of the gastric epithelium. In accordance with Spitzweg *et al.* (1999) we found NIS-immunoreactive material also within the parietal cells of the gastric mucosa in man. NIS-immunoreactive parietal cells were also detected in pig, guinea-pig and mouse (present study). The identity of the parietal cells was confirmed by costaining with an antiserum directed against H^+/K^+-ATPase (data not shown). However, the presence of authentic NIS in these cells is strongly questioned as no NIS mRNA labelling could be detected. Thus, the possibility that the NIS-immunoreactive material in parietal cells represents cross reactivity with some other protein/transporter must be considered.

Presence of NIS and/or NIS mRNA has been reported in a large number of tissues, mainly by the use of RT–PCR technique (Kotani *et al.* 1998, Spitzweg *et al.* 1998). In accordance with previous findings in human salivary glands (Spitzweg *et al.* 1999) we found a high expression of NIS and NIS mRNA in salivary glands of guinea-pig and mouse, particularly within the ductal epithelium. The present finding of NIS immunoreactivity and NIS mRNA in ductal cells of rat parotid gland is noteworthy as it is in contrast to the negative findings on NIS expression in this location by Kotani *et al.* (1998). On the other hand we could not confirm the report of NIS expression in the cornification layer of rat pars proventricularis (Kotani *et al.* 1998). A novel finding reported in the present study was the detection of NIS-immunoreactive material in endocrine cells in the pancreas and small intestine of the rat which is in line with the finding of NIS immunoreactivity in pancreatic endocrine cells in man (Spitzweg *et al.* 1999). However, our failure in detecting NIS mRNA expression in these cells, parallel to our findings in parietal cells, offers two possibilities, either a very low mRNA expression (below detection limit) or cross-reactivity of the antibodies with a related antigen. Conflicting reports on the localization of NIS can, at least partly, be the result of methodological sensitivity differences. The combined use of *in situ* hybridization and immunocytochemistry in order to establish the expression of both NIS mRNA and NIS protein is crucial in order to minimize false positive results.

Gastric NIS function The great abundance of NIS and NIS mRNA in gastric mucosa raises the question of its functional role in this location. In the present study, a considerable transport of iodide from the bloodstream to the gastric lumen could be established, whereas no indication of any substantial gastric luminal uptake of iodide was found. Our suggestion is that iodide transport to the gastric lumen from the circulation is the result of an active pumping mediated by NIS located within the gastric mucosa. As the absorption of iodide occurs in the small intestine (for a review see Brown-Grant 1961), gastric NIS is likely to play an important role in iodide conservation by an entero-thyroid recirculation. This is supported by the finding of a gastrointestinal recirculation of iodide in the dairy cow in which iodide re-entry from the circulation to the gastrointestinal tract was suggested to be mediated via secretion of iodide by the gastric mucosa of the abomasum (Miller *et al.* 1973 and 1975). In addition to gastric secretion a small amount of iodide is reported to enter the gastrointestinal tract via saliva particularly from parotid glands (Brown-Grant 1961). NIS-immunoreactive material has been detected in ductal cells of human salivary glands (Jhiang *et al.* 1998, Spitzweg *et al.* 1999) and this was found, in the present study, to apply also to guinea-pig, rat and mouse. In particular the parotid glands were found to express NIS, thus providing a morphological basis for the suggestion that salivary glands contribute to the recirculation of iodide. The secretion of iodide by exocrine glands and gastrointestinal mucosa may also have an antimicrobial function (Majerus & Courtois 1992) or act as an antioxidant (Venturi & Venturi 1999).

Whether the gastric mucosa, in addition to secreting iodide, is able to take up iodide as has been suggested (Kotani *et al.* 1998) is still an open question. Our findings of negligible uptake in blood and thyroid of ^{125}I given orally to rats with pylorus ligated argue against the gastric mucosa as a site for systemic iodide uptake.

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Concluding remarks We suggest that NIS is not only of high importance for iodide trapping in the thyroid follicular cells, but also plays an important role in iodide conservation. By its presence in salivary glands and gastric mucosa, NIS mediates iodide transport from the circulation to the gastrointestinal tract via an active pumping of iodide to the lumen. The secreted iodide is then again transported to and taken up by the small intestine. An effective recirculation of iodide may thus protect against iodide deficiency.

Studies linking NIS directly to the secretion of iodide into the gastrointestinal tract as well as studies on the functional role of NIS expression in other extra thyroidal organs and the possibility that NIS may participate in the secretion of anions other than I^- such as ClO_3^- or NO_3^- would be of great interest. Also, in view of the findings that the incidence of (Holm *et al.* 1991) and the mortality in (Hall *et al.* 1992) gastric carcinoma seems to be elevated after ^{131}I-therapy, a better understanding of gastric iodide transport and the possible accumulation of iodide in this organ is desirable.

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Paper II

Sodium-Iodide Symporter Mediates Iodide Secretion in Rat Gastric Mucosa *In Vitro*

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In vivo **studies on rats have demonstrated that considerable amounts of iodide are transported from the bloodstream into the gastric lumen. The mechanisms for and functional significance of this transport are poorly understood. Active (driven by Na^+/K^+-ATPase) iodide transport into thyroid follicular cells is mediated by the sodium-iodide symporter (NIS), which is also abundantly expressed in gastric mucosa. We aimed to further investigate the iodide transport in gastric mucosa and the possible role of NIS in this transport process. Iodide transport in rat gastric mucosa was studied *in vitro* in an Ussing chamber system using ^{125}I as a marker. The system allows measurements in both directions over a mucosal specimen. A considerable transport of iodide (from the serosal to the mucosal side) was established across the gastric mucosa, whereas in the opposite direction (mucosa to serosa), iodide transport was negligible. Sodium perchlorate ($NaClO_4$), a competitive inhibitor of NIS, and ouabain, an inhibitor of the Na^+/K^+-ATPase, both attenuated gastric iodide transport from the serosal to the mucosal side. To investigate a possible neuroendocrine regulation of the iodide transport identified to occur from the serosal to the mucosal side of the stomach, thyroid-stimulating hormone (TSH), thyrotropin-releasing hormone (TRH), vasoactive intestinal peptide (VIP), histamine, or nitric oxide donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) was added. None of these substances influenced the iodide transport. We conclude that iodide is actively transported into the gastric lumen and that this transport is at least partly mediated by NIS. Additional investigations are needed to understand the regulation and significance of this transport.** Exp Biol Med 231:277–281, 2006

Key words: gastric NIS; gastric iodide secretion; ouabain; sodium perchlorate; Ussing chamber

Introduction

Iodide is transported from the bloodstream into the gastric lumen in considerable amounts *in vivo* (1–3). The mechanisms for and functional significance of this transport are as yet poorly understood.

Active iodide transport into thyroid follicular cells is mediated by the sodium-iodide symporter (NIS) (4), which is also abundantly expressed in gastric mucosa (3, 5–7). Thyroidal NIS has been extensively investigated (8) since the cDNA sequence of rat NIS was revealed (9). NIS is a transmembrane glycoprotein actively pumping iodide into the thyroid follicular cells against a gradient, and it is driven by the Na^+-gradient generated by Na^+/K^+-ATPase (4).

Gastric NIS is preferentially localized basolaterally in the surface epithelial cells (3), but little is known about function and regulation of NIS in gastric mucosa. It is noteworthy that during the 1960s perchlorate- and thiocyanate-sensitive iodide transport was suggested to occur across the gastric mucosa in rats, dogs, and humans (1). The occurrence of iodide transport from the circulation to the gastric lumen was recently confirmed in an *in vivo* model in rats (3). In this report gastric iodide transport is suggested to be mediated by NIS as part of an entero-thyroid recirculation of iodide. This is in line with the observation, described decades ago, that iodide transport into the gastric lumen serves as an important iodide-conserving mechanism in bovines (2). Other possible functions suggested for gastric secretion of iodide are antioxidative (10) and antimicrobial (6).

The causal relationship between the occurrence of both NIS protein and NIS mRNA in gastric mucosa and the accumulation of iodide in the stomach after administration of radioactive iodide has not been established. Gastric NIS

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was suggested to mediate uptake of iodide across gastric mucosa (5). However, *in vivo* experiments failed to identify any gastric iodide uptake but showed a marked secretion of iodide into the gastric lumen (3). Therefore, we sought to further investigate the extent and mechanisms of iodide transport in gastric mucosa in an *in vitro* model. The competitive NIS inhibitor perchlorate and the Na^+/K^+-ATPase inhibitor ouabain were used to establish whether gastric NIS contributes in the transport process. The possible regulation of gastric iodide transport by biologically active substances was also tested by the addition of thyroid-stimulating hormone (TSH), thyrotropin-releasing hormone (TRH), vasoactive intestinal peptide (VIP), histamine, or nitric oxide (NO)-donor S-nitroso-N-acetyl-D, L-penicillamine (SNAP).

Materials and Methods

Animals. A total of 73 male Sprague-Dawley rats (200–250 g) (Taconic M&B, Denmark, or Scanbur BK, Sweden) were used. The animals had free access to standard food pellets and tap water. The animals were killed by bleeding from a cardiac incision during deep isofluran anesthesia and the stomachs were removed. Animal care was in accordance with the European Council Convention of 1986, as well as the National Institutes of Health, USPHS, Guide for the Care and Use of Laboratory Animals. The study was approved by the research animal ethics committee of Malmö and Lund, Sweden.

Specimens. The oxyntic part of the stomach was removed, emptied of contents, and divided along the major and minor curvatures to yield two separate specimens. The specimens were pinned to a Sylgaard-coated Petri dish filled with oxygenated Krebs' buffer solution at room temperature, and the muscular layer was stripped off using fine forceps and scissors before mounting the mucosal layer in the Ussing chamber. Pars proventricularis, known to lack NIS expression (3), from four rats were used as reference specimens.

***In Vitro* Studies of Iodide Transport.** Ussing chambers (Navicyte; San Diego, CA) with round apertures of 0.64 cm^2 were used. After tissue mounting, the chambers were immediately placed in a heat block maintaining 37°C. The two reservoirs were each filled with 1.5 ml of modified Krebs' buffer solution (NaCl 110.0 m*M*, $CaCl_2$ 3.0 m*M*, KCl 5.5 m*M*, KH_2PO_4 1.4 m*M*, $NaHCO_3$ 29.0 m*M*, Na pyruvate 5.7 m*M*, Na fumarate 7.0 m*M*, Na glutamate 5.7 m*M*, and glucose 13.4 m*M*; pH 7.4) and continuously aerated with a mixture of 95% O_2 and 5% CO_2. After 15 mins of equilibration, the solution in one reservoir was replaced with fresh 37°C Krebs' buffer and in the other with Krebs' buffer containing 0–200 m*M* NaI supplemented with 0.1 ml of a 0.08 μ*M* ^{125}I solution, yielding a radioactivity of 0.2 MBq, in order to identify iodide transport across the mucosa. Iodide was added either to the serosal or to the mucosal side reservoir. To calculate iodide transport, samples of 150 μl were drawn from the iodide-containing (donor) reservoir at 0 and 60 mins (end of the experimental period) and from the other (receiver) reservoir at 15, 30, 45, and 60 mins. Samples taken at 15, 30, and 45 mins were replaced by an equal volume of fresh Krebs' buffer. In a first set of experiments, different doses of iodide and the direction of iodide transport were studied. Specimens were mounted with the donor reservoir either on the serosal side or on the mucosal side. Because these experiments revealed that the direction of iodide transport was from the serosal to the mucosal reservoir, separate series of experiments were designed accordingly in order to test possible inhibitory effects of perchlorate (20 m*M*) and ouabain (500 μ*M*) on iodide transport. In this experimental set up, all specimens were mounted with the donor reservoir (0.2 m*M* NaI and 0.2 MBq ^{125}I) on the serosal side, and the inhibitor under investigation was added to both reservoirs throughout the experiment, including the equilibrating period. In order to maintain constant concentration, inhibitor was also added to the buffer used for replacement after sampling. In a separate series of experiments (0.02 m*M* NaI and 0.2 MBq ^{125}I in the donor reservoir), the possible regulation of the iodide transport was investigated by the addition of TSH (0.1 U/ml), TRH (1 or 10 μ*M*), VIP (0.1 or 1 μ*M*), histamine (0.1 m*M*), or NO donor SNAP (0.1 m*M*) to both sides of the specimen. These experiments were carried out as described earlier.

All samples were measured for radioactivity using a Packard Cobra II auto-gamma counting system. Transport was expressed as nmol iodide cumulated at each time point with correction for the removed sample volumes.

As an indicator of mucosal viability, the transepithelial potential differences were measured before and at the end of each experiment using a pair of Ag/AgCl electrodes embedded in KCl agar. Potential differences were 1–7 mV before experiments and 1–12 mV after experiments. Specimens with extremely low (<1 mV) or greatly diminishing potential differences, as well as some exhibiting obvious leakage of iodide, were excluded ($n = 20$).

Chemicals. Isofluran (Forene) was obtained from Apoteket AB (Stockholm, Sweden). ^{125}I was obtained from Amersham Biosciences (Amersham, UK) and diluted to 2 MBq/ml in saline. Sodium iodide (NaI), sodium perchlorate ($NaClO_4$), ouabain, TSH, TRH, VIP, histamine, and SNAP were purchased from Sigma-Aldrich (Stockholm, Sweden). Ouabain was solubilized in boiling water to 3 m*M*, and SNAP was solubilized in dimethyl sulfoxide (DMSO) to 10 m*M* before further dilution in buffer solution. All other chemicals were solubilized in distilled water or saline before further dilution in buffer solution.

Statistics. Values are presented as medians (interquartile range). Comparisons between groups were done with Mann-Whitney *U* test except when several doses were compared (VIP and TRH), in which case Kruskall-Wallis test was used. P values of < 0.05 were considered significant. Statistical analysis was performed using StatView 4.01 software (Abacus Concepts, Berkeley, CA). Results in the

figures are presented as boxplots showing median, quartiles, and whiskers drawn to the extreme values, except in Figure 1 where the total ranges are shown.

Results

Amount and Direction of Iodide Transport. The ability to transport iodide across gastric mucosa was tested by the addition of a wide range of different iodide concentrations (5.3 n*M*–200 m*M*) to either the serosal or the mucosal side reservoir. These experiments revealed that an increase in iodide concentration on the serosal side resulted in increased transport to the mucosal side (Fig. 1). Iodide 0.02 m*M* added to the serosal side reservoir yielded a transport to the mucosal side of 0.96 (0.86–1.1) nmol after 60 mins ($n = 11$). With an initial iodide concentration of 0.2 m*M* in the serosal side reservoir, iodide transport to the mucosal side was 9.0 (7.3–9.1) nmol after 60 min ($n = 11$). Negligible amounts of iodide were transported in the opposite direction; that is, addition of 0.02 m*M* iodide to the mucosal side reservoir resulted in the transport of 0.23 (0.16–0.26) nmol at 60 min ($n = 8$) (Fig. 2). The iodide transport across gastric mucosa was linear over time and directed from the serosal to the mucosal side (Fig. 2).

Pars proventricularis of rat stomach was used as a reference specimen, and in this specimen no transport was detected in either direction (data not shown).

Inhibition of Iodide Transport by Perchlorate. The presence of perchlorate in the Ussing chamber inhibited gastric mucosal transport of iodide (0.2 m*M* = 300 nmol/specimen) from the serosal to the mucosal side by 47% ($P = 0.0027$). Iodide transport was 5.1 (4.8–6.3) nmol to the mucosal side after 60 min with 20 m*M* perchlorate on both sides of the specimen ($n = 7$). The transport in specimens ($n = 6$) run in parallel from the same animals, but without the addition of perchlorate, was 9.6 (9.2–9.8) nmol (Fig. 3A).

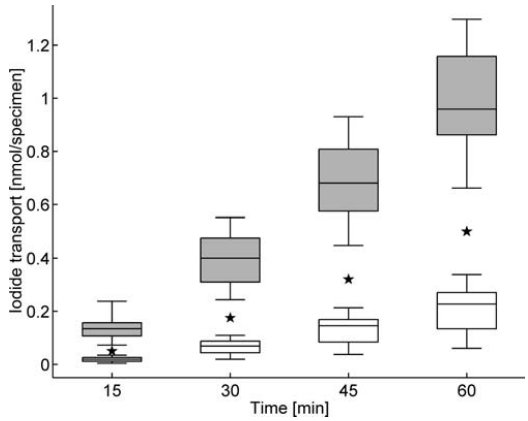

Figure 2. Boxplots showing iodide transport from serosal to mucosal side (shadowed boxes, $n = 11$) and from mucosal to serosal side (open boxes, $n = 8$). Values are nmol transported per specimen (0.64 cm^2) after 15, 30, 45, and 60 mins; medians (interquartile range); whiskers drawn to the extreme values. Initial iodide concentration was 0.02 m*M*. $^*P < 0.001$ at all time points.

Inhibition of Iodide Transport by Ouabain. Presence of ouabain in the Ussing chamber inhibited gastric transport of iodide (0.2 m*M* = 300 nmol/specimen) from the serosal to the mucosal side by 23% ($P = 0.009$). Iodide transport was 7.3 (4.8–7.9) nmol to the mucosal side after 60 mins with 500 μ*M* ouabain on both sides of the specimen ($n = 5$). The transport in specimens ($n = 5$) run in parallel from the same animals (but without the addition of ouabain) was 9.5 (9.1–9.9) nmol (Fig. 3B).

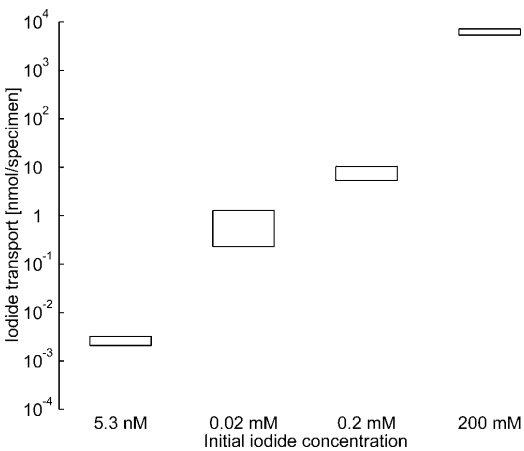

Figure 1. Boxes showing total range of transport for each tested iodide concentration. Values are nmol transported per specimen (0.64 cm^2) after 60 min. For initial iodide concentration, 5.3 n*M*, $n = 4$; 0.02 m*M*, $n = 17$; 0.2 m*M*, $n = 11$; and 200 m*M*, $n = 2$.

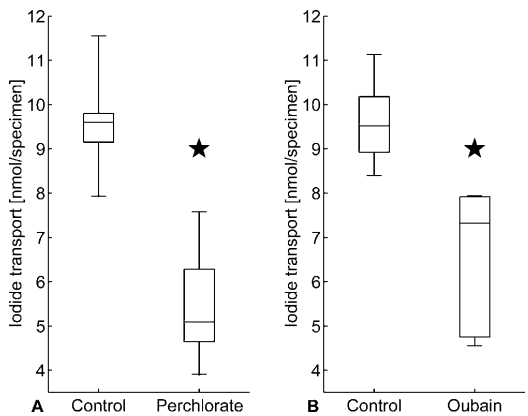

Figure 3. Boxplots showing iodide transport from the serosal to the mucosal reservoir in the presence of perchlorate 20 m*M* (A) or ouabain 500 μ*M* (B). Initial iodide concentration was 0.2 m*M*. Values are nmol transported per specimen (0.64 cm^2) after 60 min; medians (interquartile range); whiskers drawn to the extreme values, $n = 5$–7. $^*P < 0.01$ in A and B.

Table 1. Iodide Transport (nmol/Specimen in 60 Min) With and Without TSH, TRH, VIP, Histamine, and NO Donor SNAP[a]. Initial Iodide Concentration Was 0.02 m*M*.

Test substance	Transport	*n*	Transport controls	*n* controls
TSH 0.1 U/ml	0.53 (0.66–1.0)	7	0.49 (0.71–0.82)	5
TRH 1 μ*M*[b]	0.94 (0.63–1.4)	4	1.2 (1.0–1.3)	4
TRH 10 μ*M*[b]	1.3 (1.2–1.5)	4	1.2 (1.0–1.3)	4
VIP 0.1 μ*M*[c]	1.2 (1.1–1.2)	5	0.97 (0.89–1.0)	6
VIP 1 μ*M*[c]	1.1 (1.0–1.3)	7	0.97 (0.89–1.0)	6
Histamine 0.1 m*M*	0.77 (0.62–0.96)	4	0.78 (0.60–0.98)	4
SNAP 0.1 m*M*	0.75 (0.59–0.84)	6	0.66 (0.55–0.82)	7

[a] Values are median (interquartile range).
[b] Controls are the same for both doses of TRH.
[c] Controls are the same for both doses of VIP.

Effects of TSH, TRH, VIP, Histamine, and SNAP on Gastric Iodide Transport. Iodide transport from the serosal to the mucosal side was measured in the presence of TSH (0.1 U/ml, $n = 7$), TRH (1 μ*M*, $n = 4$ or 10 μ*M*, $n = 4$), VIP (0.1 μ*M*, $n = 5$ or 1 μ*M*, $n = 7$), histamine (0.1 m*M*, $n = 4$), or SNAP (0.1 m*M*, $n = 6$) (Table 1). None of these substances caused any statistically significant changes in the rate of iodide transport. For each substance, specimens that were run in parallel but without the addition of test substance served as controls ($n = 4$–7). Initial iodide concentration in this series was 0.02 m*M*.

Discussion

Iodide transport into the thyroid follicular cell is mediated by NIS, which is a transmembrane protein actively transporting iodide together with sodium (4). Iodide transport in the stomach may also be mediated by NIS since NIS is abundantly expressed in gastric mucosa (3, 7). NIS is predominantly localized basolaterally in the surface epithelium of the gastric mucosa, and thus it is reasonable to assume that NIS mediates transport of iodide into the epithelial cells as it does in thyroid follicular cells. The subsequent efflux of iodide into the gastric lumen may be passive or mediated by some other as yet unknown system. Pendrin, which mediates iodide transport from the thyroid follicular cell into the colloid (11), is one putative candidate. However, no pendrin has been found within the gastric mucosa (12).

The Ussing chamber is a well-established *in vitro* model for studies on transport of and permeability for various types of substances through epithelia (13, 14). In the present study, the Ussing chamber model was adapted for studying iodide transport across rat gastric mucosa. Iodide transport was found to be considerable from the serosal to the mucosal side but negligible in the opposite direction, suggesting that the route of iodide transport is from the circulation into the gastric lumen and that no uptake of iodide takes place in the gastric mucosa. This is in accordance with previous *in vivo* findings of Josefsson *et al.* (3). In this study radioactive iodide administered *via* an intragastric tube to rats subjected to ligation of pylorus could not be retrieved in the circulation or in the thyroid, indicating lack of uptake via the gastric mucosa. In addition, it was shown that ^{125}I given intravenously accumulated in both the stomach lavage and the gastric wall.

There are several established competitive inhibitors of the NIS-mediated iodide transport in the thyroid, including such anions as bromide (Br^-), chlorate (ClO_3^-), perchlorate (ClO_4^-), and thiocyanate (SCN^-). In the present study, perchlorate (the most widely used and well-characterized inhibitor of NIS activity and acting highly competitively in iodide transport) was used (15, 16). Perchlorate inhibited the iodide transport from serosal to mucosal side by 47%, which indicates that NIS is a putative candidate for mediating this transport. It is noteworthy, however, that in spite of using a high concentration of perchlorate, a total inhibition of iodide transport, as reported to occur in cultured NIS expressing cells (16, 17), could not be achieved. This probably reflects that the availability of the NIS molecule to perchlorate is different in cultured cells and full-thickness mucosa *in vitro*. In order to facilitate diffusion, the muscular layer of the stomach wall was removed before mounting the mucosa in the Ussing chamber. However, a substantial amount of connective tissue still remains in the lamina propria and in the submucosa, which hampers the possibility for both iodide and perchlorate to reach NIS located in the apical mucosa. Perchlorate, which is a larger molecule than iodide, is probably most affected by such impediments. In an attempt to overcome this we therefore added perchlorate, as well as ouabain, to both the donor and the receiver chambers.

Iodide transport mediated by NIS is dependent on Na^+/K^+-ATPase (16). To further test the hypothesis that NIS mediates gastric iodide transport, we used ouabain, which is an inhibitor of Na^+/K^+-ATPase. Presence of ouabain attenuated the iodide transport from the serosal to the mucosal side by 23%. Besides low availability caused by diffusion impediments, the lack of total inhibition by both perchlorate and ouabain may indicate that NIS-mediated transport is not the only route by which iodide can pass the gastric mucosa. From the present results we conclude that NIS mediates at least part of the substantial iodide transport from the bloodstream over the gastric mucosa and into the gastric lumen.

In order to identify a possible neuroendocrine regulation of gastric NIS activity, we also tested whether TSH, TRH, VIP, histamine, or NO influenced gastric iodide transport. TSH is known to regulate thyroid iodide uptake activity mainly by increasing NIS expression and synthesis, but regulation of NIS activity has also been suggested (18). It was therefore of interest to test the effects of TSH in our system, measuring gastric iodide transport *in vitro*. TRH, VIP, histamine, and NO are all important messengers in the gastric mucosa, mediating or modulating a number of physiological activities, such as vasodilatation and acid and bicarbonate secretion (for a review, see Ref. 19). In the present study, none of these putative regulatory substances was found to affect gastric iodide transport. It must, however, be emphasized that the present experimental conditions only allow studies on acute regulatory effects and that changes in, for example, NIS mRNA synthesis, are beyond detection. The lack of response to the tested neuroendocrine signaling substances may, as previously suggested for perchlorate and ouabain, also be due to diffusion impediments.

In order to understand the regulation of NIS expression and activity in the gastric mucosa as well as the functional significance of iodide transport into the gastric lumen, additional work is needed. A challenging aspect of iodide function worth exploring is its suggested antimicrobial effect. A recent study reported that the presence of iodide increases the antimicrobial activity of acidified nitrite, thereby augmenting the activity of gastric acid, thus providing a better host defense against bacteria (20). In this context we must also consider that the anion selectivity of NIS is $ClO_4^- > ReO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^-$ (15, 21). This leaves us with the possibility that the main role of gastric NIS may be transportation of anions other than iodide. An interesting putative candidate is nitrate because nitrate is a source for nonenzymatic NO production, which abundantly occurs in the stomach (22, 23). Salivary accumulation and secretion of dietary nitrate followed by reduction to nitrite by bacterial nitrate reductases and formation of NO at low pH are considered to be the mechanisms. Secretion *via* gastric NIS offers an additional possibility to increase local nitrate concentration in the gastric juice. Noteworthy is that NIS is found also in salivary glands (3), and its role in mediating nitrate accumulation and transport in this location is as yet unexplored.

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