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# The Use of Chromogranin, Synaptophysin and Islet Amyloid Polypeptide as Markers for Neuroendocrine Tumours

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## INTRODUCTION

### The neuroendocrine system

The diffuse neuroendocrine (NE) system consists of peptide hormone producing cells in different locations throughout the body (125). Usually, the cells in the anterior lobe of the pituitary, the thyroid C-cells, the parathyroid glands, the adrenal medulla, the islets of Langerhans and the hormone producing cells in the gastrointestinal tract are included in the NE system. A common feature for all these tissues and cells is their ability to secrete bioactive amines. Based on these observations, the amine precursor uptake and decarboxylation (APUD) concept was introduced (120). He suggested that the NE cells were derived from cells in the neural crest which probably migrated to their final position during embryogenesis. However, there has been some debate about this concept, which has led to a re-definition of the subject. All APUD cells are peptide hormone producing cells, derived from specialised ectoderm and programmed for ultimate NE function (121).

### Neuropeptides

*Synthesis and storage*

A common feature of the NE cells is their ability to produce and secrete neuropeptides. These neuropeptides are also found in neuronal tissue, co-existing with classical neurotransmitters (86). The peptides are synthesised as pro-peptides in the ribosomes. N-terminally located signal peptides direct the pro-peptides into the trans-Golgi regulated pathway. Here the peptides are processed by specific enzymatic cleavage, usually at di-basic amino acid positions. The enzymes responsible for the processing of insulin from pro-insulin have recently been characterised (145). The mature neuropeptides are C-terminally amidated, which is a result of a specific enzyme cleavage at a di-basic amino acid position with a glycin-residue located N-terminally. The peptides are then stored in granules called large dense core vesicles (LDCV), and can be released upon stimulation.

The LDCV granules are the hormone- or neuropeptide-containing organelles found in hormone producing cells, NE cells and neuronal cells. An interesting group of proteins found in the granules are the Chromogranins, which are described in more detail below. In addition to the LDCV granules, other organelles called small synaptic vesicles (SSV), are present in neuronal and NE cells (100,109). In neuronal cells, these vesicles contain the classic neurotransmitters

such as acetylcholine, noradrenalin or serotonin. There is also a protein called synaptophysin (Sy) which is located in the membrane of the SSV and will be further discussed below.

*Peptide families*
Some NE peptides share amino acid sequence homology, although the length of the peptide and the biological functions can vary. Due to the varying sequence homologies, peptides have been grouped into different families. The gene structures indicate that each family of peptides may have evolved from a common ancestral gene. Some families of peptides are shown in Table 1.

**Table 1:** Some examples of neuropeptides families.

| Family | Family members |
|---|---|
| **Glucagon - Secretin** | Glucagon<br>Secretin<br>Vasoactive Intestinal Polypeptide (VIP)<br>Gastric Inhibitory Polypeptide (GIP)<br>Peptide Histidin Isoleucinamide (PHI)<br>Growth Hormone Releasing Peptide (GRF) |
| **Gastrin - Cholecystokinin** | Gastrin<br>Cholecystokinin (CCK)<br>Caerulein |
| **Gastrin Releasing Factor** | Gastrin Releasing Factor (GRF)<br>Neuromedin B<br>Neuromedin C<br>Bombesin<br>Alytesin<br>Litorin<br>Ranatensin |
| **Pancreatic Polypeptide** | Pancreatic Polypeptide (PP)<br>Peptide YY (PYY)<br>Neuropeptide Y (NPY) |
| **Tachykinins** | Substance P<br>Neurokinin A<br>Neurokinin B<br>Eledoisin<br>Physalaemin<br>Kassinin |

*Secretion of neuropeptides*
Neuropeptides are stored in LDCV granules in the NE cells and are secreted upon stimulation. This involves a fusion of the granules with the plasma membrane and exocytosis of the granule content. The neuropeptides are believed to affect the target cells in 4 different ways:

- Endocrine transmission, where NE cells release peptides into the circulation which have effects on some distant target cells.

- Paracrine transmission, where NE cells release peptides, which have regulatory effects on adjacent target cells. Often these NE cells have cytoplasmic processes, reaching the vicinity of the target cells.
- Neurocrine transmission, where neurons release peptides, which have regulatory effects on adjacent cells.
- Autocrine transmission, where the releasing NE cells themselves have receptors for the peptides released, which modulate the release.

## Neuroendocrine tumours

Tumours derived from NE tissue are often highly differentiated and retain their ability to produce biologically active peptides and amines. As a consequence, these tumours may exert pronounced biological effects due to hormone secretion, sometimes long before the tumour itself can be visualised by radiological methods. Another consequence is that these neuropeptides can be measured in the circulation of the patient, and hence serve as specific tumour markers.

*Carcinoid tumours*

In 1907, Oberndorfer described a group of intestinal tumours which grew more slowly than adenocarcinomas, and introduced the name "Karzinoide" for these tumours (110). Later it was recognised that these tumours were associated with a clinical syndrome that included flushing, wheezing, watery diarrhoea, and valvular heart disease (158).

The tumours originate from NE cells and can be found almost anywhere in the body (143), although the most common place is the gastrointestinal tract. Due to different features of the carcinoid tumours originating in different parts of the body, the terms foregut, midgut and hindgut have been introduced (178). Foregut carcinoids, which account for about 10% of the carcinoids, are mostly found in the lungs, the thymus, duodenum and in the stomach. These tumours often secrete different kinds of neuropeptides like gastrin, somatostatin, tachykinins, pancreatic polypeptide (PP), and bioactive amines. The midgut carcinoids, which account for about 70% are located mostly in the small intestine and the appendix. They often secrete serotonin, bradykinin and tachykinins. The hindgut carcinoids, about 20%, are located in the distal 2/3 of colon and in rectum. They can occasionally produce neuropeptides such as somatostatin, PP and peptide YY, but they usually do not.

Carcinoid tumours are the most frequent NE neoplasms, with an incidence of 2.1/100,000/year in Sweden (7). Midgut carcinoid tumours are more malignant and upon admission, 90% of the patients have liver metastases (103). In untreated patients the 5-year survival was about 20% (91), while in patients treated with chemotherapy (streptozotocin and 5-fluorouracil), the median survival from start of treatment was 8 months, as compared to treatment with interferons, where the median survival was more than 80 months (115).

*Endocrine pancreatic tumours*

The first case of a patient with an endocrine pancreatic tumour was described in 1902 (38), while the first clinical syndrome (hypoglycaemia) associated with a pancreatic tumour hormone was described in 1927 (176). The tumours are probably derived from cells in the pancreatic islets and share the common APUD concept.

Due to overproduction of hormones, at least 5 major clinical syndromes associated with endocrine pancreatic tumours are recognised. The insulinoma syndrome is recognised by hypoglycaemia and overproduction of insulin or pro-insulin. The Zollinger-Ellison syndrome is

associated with diarrhoea, peptic ulcers and overproduction of gastrin. The WDHA syndrome (or Verner-Morrison syndrome) is characterised by excessive watery diarrhoeas, hypokalemia and overproduction of vasointestinal polypeptide (VIP). The glucagonoma syndrome is associated with marked weight loss, decreased glucose tolerance, skin lesions and overproduction of glucagon. The somatostatinoma syndrome shows a clinical picture of diabetes mellitus, diarrhoea, weight loss and overproduction of somatostatin. In addition to these clinical syndromes, there are patients with endocrine pancreatic tumours that show no defined clinical syndrome. These tumours, although they can secrete bioactive neuropeptides in minor amounts, usually secrete neuropeptides without prominent biological effects. Hence, these tumours are called non-functioning tumours.

Endocrine pancreatic tumours are more uncommon than carcinoid tumours. In Sweden, the incidence is about 0.4/100,000/year (34). Endocrine pancreatic tumours usually respond to treatments with interferon, somatostatin analogues or chemotherapy (streptozotocin and 5-fluorouracil). The median survival from start of interferon treatment is about 50 months (37).

*Multiple endocrine neoplasia syndrome 1 (MEN1)*
MEN1 is an inherited disease affecting the hormone producing cells in the anterior pituitary, the parathyroid gland, the pancreatic islets and in some cases the adrenal cortex. The gene, which is supposed to be a suppresser gene, is located on the long arm of chromosome 11 (76). To acquire the disease, a defective gene must be inherited and furthermore a somatic mutation in the intact allele must occur. The patients, which have inherited the disease, usually present their first tumours at 20-40 years of age. The tumours are often multiple in location and peptide production, and can affect one or more organs. In Sweden, members of the identified families are routinely checked at fixed intervals in order to discover new tumours as early as possible (139). Except for the presence of multiple lesions, the tumours grossly display the same characteristics as sporadic NE tumours from the same organ.

*Pheochromocytoma and neuroblastoma*
Pheochromocytoma and neuroblastoma tumours originate from the adrenal medulla or the sympatic neural crest. These tumours often produce the bioactive amines adrenalin and noradrenalin and neuropeptides such as neuropeptide Y or VIP. The clinical symptoms are sudden onset of high blood pressure and headache. In most cases, these tumours are benign and the treatment is surgical removal of the tumour.

## Circulating markers for tumour growth

*Tumour markers in general*
The possibility to discover tumour growth by analysis of a single blood sample would be a major step towards better management of tumour diseases. To assure a confident discrimination between tumour and non-tumour growth, the tumour marker must either be produced only by tumour cells or the tumour cells must secrete the molecule in much higher concentrations than non-neoplastic cells. For tumour growth in general, no such compound has been identified. However, a variety of tumour markers with association to different kinds of tumour diseases have been introduced. Some of these are shown in Table 2.

Tumour markers can be used for different purposes:
- Screening for tumour growth in general.
- Screening for a certain kind of tumour disease.
- Verifying the tumour diagnosis.

- Follow up of treatment.

In general, the tumour markers used today do not have predictive values sufficient for screening purposes. However, for verification of the tumour diagnosis and in particular, follow up of treatments the tumour markers can be very useful. NE tumours are often highly differentiated and retain their ability to secrete neuropeptides and amines. This offers an advantage, compared to other neoplasias.

**Table 2:** Examples of non-neuroendocrine tumour markers and tissue types applicable.

| Tumour marker | Tissue and cell types applicable |
|---|---|
| **Cancer embryonic antigen (CEA)** | Colon<br>Rectum<br>Pancreas<br>Stomach |
| **Alpha Feto Protein (AFP)** | Liver<br>Embryonal Cancer<br>Teratocarcinoma |
| **Tissue Polypeptide Antigen (TPA)** | Breast<br>Colon<br>Rectum<br>Lung |
| **Prostate Specific Antigen (PSA)** | Prostate |
| **Tymidin Kinase (TK)** | Lymphatic cells<br>Leukaemia cells |
| **Cancer Antigen 19-9 (CA 19-9)** | Pancreas<br>Colon<br>Rectum<br>Stomach |
| **Cancer Antigen 125 (CA 125)** | Ovary |
| **Cancer Antigen 72-4 (CA 72-4)** | Stomach |
| **Cancer Antigen 15-3 (CA 15-3)** | Breast |
| **Cancer Antigen 50 (CA 50)** | Colon<br>Stomach<br>Pancreas |

*Bioactive amines as markers for neuroendocrine tumours*
As mentioned above, NE tumours produce metabolic active amines, such as serotonin, histamine, adrenalin or noradrenalin. These amines are rapidly metabolised or secreted into the urine. This makes the amines difficult to measure in the blood, but urine measurements of the amines and their degradation products can be used as markers for tumour growth. For midgut carcinoid tumours, urine measurement of the degradation product of serotonin, 5-hydroxyindolacetic acid (5-HIAA), is used and for pheochromocytoma, urine measurements of adrenalin, noradrenalin and their metabolites 4-hydroxy-3-methoxymandelic acid (HMMA) and vanillylmandelic acid (VMA) are used.

*Neuropeptides as specific markers for neuroendocrine tumours*
Reliable biochemical markers are crucial for efficient management of patients with NE tumours. Conventional radiological investigations may sometimes be of limited value for monitoring therapeutical effects and diagnostic attempts. NE tumours can be too small to be visualised and medical intervention can be successful without detectable regression of tumour size. In such situations, knowledge of correlation between circulating peptide levels and tumour status is of great importance. From that point of view, radioimmunoassay measurements of different neuropeptides in plasma samples, introduced a new way to monitor NE tumour growth. These measurements allowed biochemical characterisation of the tumour secretion products, i.e. the specific neuropeptides released from a certain tumour. Furthermore, a direct correlation between the secreted peptide and specific symptoms and syndromes could be established.

Neuropeptides are almost ideal as tumour markers for NE tumours since they are specific for a certain type of cells and their circulating levels during tumour growth are usually clearly elevated. In the management of carcinoid tumours, gastrin, substance P, neuropeptide K, growth hormone releasing hormone and corticotrophin releasing hormone are used as tumour markers (36). In the management of endocrine pancreatic tumours, insulin, C-peptide, pro-insulin, gastrin, VIP, calcitonin, glucagon, somatostatin and other neuropeptides can be used as specific tumour markers (36). However, as mentioned above, some NE tumours are clinically non-functioning and thus no measurable amounts of peptides are secreted. Furthermore, some tumours produce unknown peptides and others change the neuropeptide production during the course of the disease. Consequently, as some NE tumours can not be detected by measurements of neuropeptides and a large number of neuropeptides must be measured to ensure a safe diagnosis, some more general markers for NE tumour growth would be useful.

*General markers for neuroendocrine tumours*
To be attributed as a general marker for NE tumours, the marker must be able to detect a majority of the tumours. Although PP must be considered as a neuropeptide specific for PP cells, it can also be used as a general marker for both carcinoid and endocrine pancreatic tumours. It has been shown that PP can be elevated in about 75% of patients with endocrine pancreatic tumours (36), although the PP was secreted from the tumours only in some of the cases (2,116).

Measurements of proteins exclusively associated with NE tissue have been used as general markers for NE tumours. Plasma measurements of CgA can be used as a general marker for NE tumour growth (30). Most of the patients with NE tumours could be detected by this method, including the non-functioning NE tumours (142). CgA will be further discussed below. Other proteins of NE origin, such as Neuron Specific Enolase (NSE) and S-100, have been used as circulating markers, but with less success than CgA. Human Chorionic Gonadotropin (HCG) and the subunits HCG-α and HCG-β have been used as markers for NE tumours (67,119,130). About

12-70% of the patients displayed elevated levels of HCG or the subunits. Furthermore, an association with malignancy was also found (67,119).

*Tumour markers of non-neuroendocrine origin*
Since NE tumours usually offer reliable markers for tumour growth, measurements of conventional tumours markers may be of limited value. However in a study, CEA, Ca 19-9 and Ca 50 were measured in serum from patients with endocrine pancreatic tumours. Ca 50 was found elevated in 82% of the cases and there was an association to survival, while CEA and Ca 19-9 were less useful (36).

## Islet Amyloid Polypeptide

Islet Amyloid Polypeptide (IAPP) or Amylin was first purified from the amyloid deposits in an insulin producing endocrine pancreatic tumours (169,170). Shortly after, IAPP was also isolated from amyloid deposits in pancreas from patients with type 2 diabetes mellitus (26). The isolated peptide was a C-terminally amidated 37 amino acid polypeptide with about 50% homology to Calcitonin Gene Related Peptide (CGRP).

The gene encoding for IAPP is located on Chromosome 12 (12p12.3) and consists of 3 exons and 2 introns (96,102). The cDNA encodes for a 89 amino acid propeptide, which is further processed to the mature IAPP peptide. Chemically, human IAPP forms amyloid spontaneously. This tendency has been assigned to the amino acid sequence 20-29 in the mature molecule, where the amino acids 25-26 are the most important for amyloid formation (168). In this part of the molecule, there are interspecies variations and hence amyloid formation has been found only in human, cat and racoon, while rodent IAPP does not form amyloid. In this context it is notable that only the species which form amyloid develop non-insulin dependent diabetes mellitus.

It has been shown, by immunocytochemistry, that IAPP is located in the pancreatic islet β-cells and ultrastructurally, IAPP co-exists with insulin within the β-cell granules (22,85). Immunoreactive IAPP has also been found in NE cells in the lung and in NE cells of the gastric and intestinal mucosa (159). These results have been verified by radioimmunoassay (RIA) measurements of tissue extracts (99) and presence of mRNA in these tissues has also been shown (40).

Most of the studies concerning the biological functions of IAPP have focused on the putative diabetogenic effects of IAPP (25,65,146). The effects of IAPP on glucose homeostasis can be summarised as follows:

- IAPP is co-released with insulin from the β-cells upon stimulation (3,17,39,49,54,62,68,93,111,147), although in some instances, the molar ratio of IAPP and insulin can vary.
- IAPP can inhibit stimulated insulin (and IAPP) release (4,50,72,98,112,138), although negative results have also been recorded (15,105,122,124,156).
- IAPP can inhibit insulin-stimulated glucose uptake and glycogen synthesis in liver and skeletal muscle (21,28,51,56,74,77,186). The effects of IAPP are mediated via binding to CGRP-receptors (8,19,47,94,148,164,185).

In addition to the effects on glucose homeostasis, IAPP also displays other biological functions:

- IAPP can inhibit osteoclast function and lower the blood calcium levels, similar to the effects of calcitonin (27). These effects are probably mediated via binding to calcitonin receptors.
- IAPP can induce vasodilation and can decrease the blood pressure in rats and rabbits (11).

- Intrahypothalamic administration of IAPP can decrease the eating in rats and mice (18,95).

Although IAPP was first isolated from an EPT, surprisingly few reports have investigated the potential use of IAPP as a marker for NE tumour growth. This topic will be discussed below.

## Synaptophysin

Synaptophysin was first isolated from the brains of calf (172) and rat (63). By immunocytochemical studies of nervous tissue, it has been shown that Sy is located in the membranes of the SSV (100). In NE tissue, Sy is found in similar kinds of vesicles (100,109).

The Sy gene which covers about 20 kb of DNA and contains 7 exons and 6 introns, is located on the X-chromosome, Xp11.22-p11.23 (114). In humans, the translated 2.5 kb mRNA encodes for a 296 amino acid chain with 4 hydrophobic regions and 5 hydrophilic regions (78,149). Based on the amino acid sequence, the molecular weight is 33 kDa (16,78), but SDS-gel electrophoresis experiments have revealed molecular weights of about 38-42 kDa (100,126). This is probably due to different degree of N-glycosylation of Sy in different tissues. It has also been shown that Sy can bind calcium (126) and can be phosphorylated at serine positions in a calcium-dependent manner (131). The repeating hydrophobic and hydrophilic regions indicate that the Sy molecule is membrane bound. Thus, it has been shown that Sy forms a membrane bound hexameric homo-oligomer structure, which resembles the structure of a channel-protein complex (149,157). Transfection experiments (where mRNA encoding for Sy was introduced into non-NE cells by micro-injection) stimulated the cells to produce small vesicles, similar to SSV (66,79). Summing up, these results indicate that Sy may be involved in the process of formation and intracellular transport of synaptic vesicles and/or the release and uptake of neuronal transmitters and NE bioactive amines.

Immunocytochemical stainings of Sy have often been performed with the monoclonal antibody SY-38 (Boehringer-Mannheim, Mannheim, Germany) developed by Wiedenmann (172), although other antibodies have also been used. Antibodies against Sy immunostain almost all normal NE tissue and NE tumours (for an extensive review see (173)). However, with the exception of some immunoblotting methods (133,174), no methods for quantitative measurements (e.g. radioimmunoassay) of Sy have been presented so far.

## The Chromogranin - Secretogranin family of proteins

The Chromogranin family of proteins consists of Chromogranin A, Chromogranin B (also called Secretogranin I), Chromogranin C (often called Secretogranin II) and peptides derived from the Chromogranins.

CgA, originally called secretory protein I, was first isolated from chromaffin cells in the bovine adrenal medulla (10). CgB too was isolated from chromaffin cells in the adrenal medulla (42) while CgC was isolated from anterior pituitary (41,129). Adrenal chromaffin granules contain about 40 soluble proteins (42) and about 40% of the soluble protein material consists of CgA (179). CgA and CgB are present in about equal amounts, while CgC is less abundant (53,136). Granules isolated from other tissues show different relative amounts of the Chromogranins. The main source of circulating CgA is the adrenal medulla, although other NE tissues also contribute (151).

The human CgA gene is located on chromosome 14 (14q32) (97), while location of the genes for human CgB and CgC is not known. In animals, the biosynthesis of Chromogranins can be regulated by different stimuli. For example, in male rats oestrogen treatment decreased the CgA mRNA levels, but increased the CgB and CgC mRNA levels (43). However, the physiological significance of the regulation is not fully understood though some possible biological functions of CgA have been postulated (30):

- Regulation of secretory granule function.
- Regulation of secretion of co-resident hormones.
- Precursor of biologically active peptides.

Common chemical characteristics of the Chromogranins are:

- High glutamic acid content (about 25%) and consequently an acidic pI (141),
- Ability to be phosphorylated, sulphated or glycosylated (129),
- Calcium binding properties, which induce conformational changes (183,184),
- Presence of several dibasic amino acid positions, which are potential cleavage sites for production of polypeptides (59).

Biologically active polypeptides with amino acid sequence homology with CgA, CgB and CgC have been isolated. Some of these are:

- Pancreastatin (PS) = CgA 249-301 (155),
- β-granin = CgA 1-114 (32,60),
- Chromostatin = CgA 124-143 (48)
- Vasostatin = CgA 1-76 (1),
- GAWK = CgB 420-493 (6,182),
- BAM-1745 = CgB 580-593 (44),
- CCB = CgB 597-565 (6),
- Secretoneurin = CgC 154-186 (71).

The localisation of Chromogranins to normal tissues has been studied using immunocytochemistry, radioimmunoassay, Northen blot and *in situ* hybridisation techniques. Chromogranins have been found in almost all endocrine and NE tissues and have been used as reliable markers for endocrine and NE tissues (166,173,175,180). By immunocytochemical techniques, antibodies against all Chromogranins have been used for detection and characterisation of tumours derived from endocrine and NE tissues (9,30,80,173,175). In addition, some assays for the measurement of circulating CgA and some for PS have been described (5,31,35,89,106,107,154). Due to its abundance in the NE tissues and its hydrophilic properties, CgA seems to be an outstanding circulating marker for NE tumour growth. No assays for the measurement of circulating CgB or CgC have been presented so far, although some assays for tissue extracts have been reported (24,61,71).

## MATERIALS AND METHODS

### Patient profile

Blood and urine samples were obtained from patients with NE tumours, treated at the Endocrine Unit, Department of Internal Medicine. Different sets of patients participated in the different studies. In the IAPP study 44 patients with carcinoid tumours and 58 patients with endocrine pancreatic tumours were studied and in the chromogranin studies 44 patients with carcinoid tumours and 28 patients with endocrine pancreatic tumours were evaluated. For comparision

blood samples and urine samples from healthy individuals were collected for setting up reference ranges.

### Collection of blood and urine samples

If not stated otherwise, the blood samples were collected after an overnight fast. The blood samples for measurements of NE peptides, were collected in chilled Vacutainer tubes, with addition of sodium heparin and 400 KIU/ml Aprotinin (Bayer, Leverkusen, Germany). The tubes were centrifuged at 3000g for 10 minutes at +4°C. The plasma was collected and frozen within 2 hours, usually in small aliquots. Urine samples were collected either as a single morning sample or as a 24-hour collection.

### Metabolic stimulation tests

Blood samples were also collected during mixed meal stimulation tests (117,140), oral glucose tolerance tests (75 g glucose), intravenous glucose tolerance test (0.35 g/kg body weight) and euglycaemic insulin clamp test (29).

### Synthesis of peptides

Peptides were synthesised by an automated solid-phase system (Applied Biosystems model 430A, Foster City, Ca., USA). The selected amino acid sequences of the peptides had homologies less than 50% to any other known protein in the Protein Resource Bank, version 28 (Riverside Scientific Enterprises, Bainbridge Island, Wa., USA)

### Antibody production

The peptides were coupled to albumin with glutaraldehyde (108). The antigenic complex was mixed with Freunds adjuvants and injected into rabbits using the multiple intradermal technique (161). The rabbits were boostered every 3 to 6 months. Blood was collected two weeks after booster injections. Almost all rabbits responded with useful antibodies after the second booster injection.

### Immunocytochemistry

Immunocytochemical stainings were performed as follows. Tissue specimens were obtained from biopsies or from surgically removed tissue. The specimens were fixed in buffered formalin, dehydrated, paraffin-embedded and cut in 4 μm thick sections. The sections were immunostained with the antibodies produced in this study and commercially available antibodies. To compare the immunostaining of the Sy antibodies, the commercially available monoclonal antibody SY-38 (Boehringer-Mannheim, Mannheim, Germany (172)) was used. Accordingly, to compare the immunostaining of the CgA antibodies, the monoclonal antibody LK2H10 (Boehringer-Mannheim (82)) was used. The immunostainings were visualised by the ABC technique (Vector Laboratories, Burlingame, Ca., USA).

### Radioimmunoassays

The original radioimmunoassays presented in this study, were competitive binding assays. Other immunoassays were performed as previously described (34,103).

*Radioactive labelling*
Radioactive labelling of peptides and proteins with $^{125}I$ was performed with the chloramine-T method (58). Usually 0.25 nmol peptide was labelled with 0.5 mCi carrier free $^{125}I$ (Amersham International, Buckinghamshire, England). The labelling reaction was initiated by addition of 7 µg chloramine-T and stopped after 60 sec with addition of 120 µg sodium metabisulphite. The whole labelling procedure was performed at 0°C with chilled reagents. After labelling, the reagents were separated on gel filtration column. The content and the size of the column was selected to achieve optimal separation of the labelled peptide or protein from the non-desired components in the reaction mixture. In most cases, a simple separation on a small Sephadex G-15 column or on a prepacked PD-10 column (Pharmacia-LKB, Uppsala, Sweden) was sufficient.

*Sample preparation*
To reduce non-specific interference during radioimmunoassay measurements of plasma samples, an extraction procedure was used. To 1 ml plasma 2 ml acidified ethanol (0.1% concentrated formic acid in 96% ethanol) was added. The samples were mixed vigorously for 30 minutes on vortex mixer and centrifuged at 3000g for 15 minutes. The supernatants were collected, evaporated and finally dissolved in 1 ml assay buffer. An alternative procedure with extraction of plasma on Sep-Pak C-18 cartridges (Waters Associates, Milford, Ma., USA) was also investigated. One ml plasma was diluted with 1 ml 0.1% trifluoroacetic acid (TFA) in water and the sample was loaded onto the cartridge. After washing with 2 ml of 0.1% TFA in water, the samples were eluted by 1 ml of 0.1% TFA in a solution of 20% water and 80% acetonitrile. The samples were evaporated and then dissolved in assay buffer. The recoveries of the extraction procedures were checked by adding tracer amounts of $^{125}I$-IAPP or standard amounts of IAPP to the plasma samples before extraction.

*Assay conditions*
All dilutions were made in an assay buffer of 40 mmol/l sodium phosphate at pH 7.4, containing 75 mmol/l sodium chloride, 0.5% Tween 20, 2 g/l human serum albumin and 3 mmol/l sodium azide. To 100 µl of pre-diluted antibody was added 100 µl of standard or unknown sample and 100 µl of tracer (20,000 cpm). The tubes were incubated for 3 days at +4°C. Separation of antibody-bound and -free tracer was accomplished by addition of a second antibody coupled to a solid phase (Decanting suspension 3, Pharmacia-LKB). The results were calculated with a logit-log transformation program (Multicalc, Pharmacia-LKB).

## Protein separation

Separation of proteins were performed by means of gel filtration, rHPLC and an affinity separation where zinc ions were used for the affinity binding. In all experiments the fractions collected were measured by radioimmunoassay to reveal the positions of the proteins and peptides. All separations were performed on a FPLC System with prepacked columns (Pharmacia-LKB). Also SDS-gel electrophoresis and IEF electrophoresis (Phast System, Pharmacia-LKB) was used. All these separations were performed on ready-made gels which were stained according to the manufacturer's instructions.

## Western Blot

A partly purified sample of Sy was subjected to SDS-gel electrophoresis. The proteins were electro-transferred to a nitro-cellulose filter. The filters were blocked with milk proteins and then immunostained with antibodies against Sy. The immunostaining was visualised with the ABC-technique (Vector Laboratories).

### Northern Blot

mRNA was isolated from a cell line (LCC-18) according to (20). The RNA was electrophoretically separated and transferred to a nitro-cellulose filter. The probe, which covered the same sequence as the synthesised Sy peptide (amino acids 246-260 in the human sequence), was labelled with $^{32}$P-ATP (Amersham International) using T4 polynucleotide kinase. The filters were exposed for 20 h, using an intensifying screen (Cornex, Du Pont, USA) and Hyperfilm MP (Amersham International).

## RESULTS AND DISCUSSION

### Islet amyloid polypeptide

*Introduction*

Although IAPP was first isolated from an endocrine pancreatic tumour, surprisingly few reports have studied the presence of IAPP in NE tumours. IAPP and amyloid have not only been identified in insulin producing endocrine pancreatic tumours (101,160), but also in other endocrine pancreatic tumour syndromes (128) and in carcinoid tumours (33). Radioimmunoassay measurements of tissue extracts and plasma from patients with different endocrine tumours disclosed the presence of IAPP in both tumour tissue and in the circulation (14). Finally, plasma measurements of IAPP in patients with non-NE pancreatic carcinomas revealed slightly elevated levels in some patients (123).

*Plasma levels of IAPP*

Production of IAPP has been described in normal pancreatic β-cells, in endocrine cells of the lung and in the gastrointestinal tract (22,40,99,159). Consequently, tumours derived from NE cells in these organs, i.e. carcinoid tumours and endocrine pancreatic tumours, would be possible candidates for IAPP production. Data from plasma measurements of 102 patients with carcinoid or endocrine pancreatic tumours are presented. In total, 12 of the patients showed elevated plasma levels of IAPP. Five of these 12 patients suffered from sporadic EPT, 3 from MEN1 syndrome with endocrine pancreatic tumours and 4 from midgut carcinoid tumours. None of the 9 foregut or hindgut carcinoids showed any elevated levels of IAPP. Within the different endocrine pancreatic tumour syndromes, 1/11 patients with insulinoma, 2/16 with gastrinoma, 0/2 with glucagonoma, 0/3 with VIPoma and 5/26 patients with non-functioning tumours showed elevated plasma levels of IAPP. In 10 of the 12 patients with elevated plasma levels of IAPP, the levels were moderately increased, about 2-5 times the upper reference limit. However, in the remaining 2 patients the plasma levels of IAPP were very high and both these patients presented abnormal glucose homeostasis, which will be further discussed below.

In response to ingestion of a standardised mixed meal (117,140), plasma IAPP increased significantly. The increase was parallel to the insulin, PP and gastrin responses. Parallel responses of IAPP and insulin to a mixed meal have previously been shown for normal subjects and patients with non-insulin dependent diabetes mellitus (17). In 5 patients with MEN1 syndrome and elevated blood calcium levels due to parathyroid adenoma, the plasma levels of IAPP were significantly higher before than after surgical removal of the tumours. This indicates that the response of IAPP to elevated levels of calcium may be physiological. However, there was no correlation between plasma IAPP levels and calcium levels when calcium was within the normal range.

*Immunocytochemistry*
Positive immunostaining of IAPP was found in 34% of the patients examined. In a study of 87 patients with gastroenteropancreatic endocrine tumours (33), 22% of the tumours were IAPP immunoreactive. This discrepancy can be due to a larger number of insulinomas in our study, since these tumours have a higher abundance of IAPP immunoreactivity than other endocrine tumours (33,101,128,160). In the study where the concentration of IAPP was measured in extracts from tumour tissue by radioimmunoassay technique, the number of IAPP imunoreactive endocrine pancreatic tumours and carcinoid tumours was 57% (14). These results may indicate that radioimmunoassay measurements of tissue extracts are more sensitive than immunocytochemical stainings.

It has been reported before (33,160), and our immunocytochemical studies presented here illustrate that when amyloid occurs in endocrine pancreatic tumours it is composed of IAPP. This observation opposes any suggestion of islet amyloid as an unspecific heterogeneous protein accumulation. Since the amyloid deposits are structurally similar in islet amyloidosis and insulinoma amyloid, similar mechanisms could be assumed for its production. For that reason it may be of some interest that it has been noted by ultrastructural analysis of amyloid production in endocrine pancreatic tumours, that the amyloid fibrils occurred already at intracellular sites and in close proximity to the secretory granules (84,104). An inverse relationship between the amount of amyloid deposits in the tumours and the IAPP immunoreactivity in the tumour cytoplasma was evident, and in one tumour with strong IAPP immunoreactivity, the tumour cells lacked amyloid production. This inverse relationship is consistent with that seen in pancreatic islets from patients with type 2 diabetes mellitus, where occurrence of amyloid deposits was correlated to less intense IAPP immunostaining of the β-cells (171). Thus, IAPP immunoreactivity is more pronounced in normal β-cells than in β-cells surrounded by amyloid. The presence of amyloid in tumours initially identified as insulinomas is consistent with the observation that IAPP is a component of normal β-cells. However, after tumour transformation a disturbed concomitance seemed to appere, since some tumours were insulin immunoreactive but lacked IAPP and *vice versa.*

*Effects of high plasma levels of IAPP*
A disturbed glucose homeostasis was presented by both the patients with extremely elevated plasma levels of IAPP. One of the patients was a woman with a type 1 diabetes mellitus from childhood. At the age of 34, the insulin requirements gradually decreased and insulin treatment was withdrawn, but the patient developed severe hypoglycaemia. Eventually an endocrine pancreatic tumour with one ovarian and multiple liver metastases was diagnosed. The primary tumour and the ovarian metastasis were surgically removed with initially good results. Despite medical treatments with α-interferon, streptozotocin and doxorubicin the tumour disease progressed. The final phase was characterised by B-glucose levels oscillating between severe hypoglycaemia (0.6 mmol/l) and hyperglycaemia (60 mmol/l). Hormonal analyses demonstrated moderately increased serum levels of insulin, pro-insulin and glucagon but tremendously increased IAPP levels.

The malignant disease and the glucose homeostasis of the second patient was further evaluated. The patient, a 50-year old man with a malignant endocrine pancreatic tumour with multiple liver metastases, was admitted to the Endocrine Unit of the hospital for diagnosis and anti-tumour treatment. Three years earlier, a tumour in the pancreatic head had been found incidentally when the patient was hospitalised because of hypertension. The hypertension was easily managed by treatment with β-receptor blockade. A biopsy from the tumour revealed "carcinoid-like tumour

cells", but no anti-tumour treatment was started. Upon admission, the patient had severe jaundice and CT-scan showed a massively enlarged liver with multiple metastases. Medical treatment with recombinant α-interferon (Introna, Schering-Plough, N. J., USA) was started with initially good response.

Elevated blood glucose (6.4 mmol/l) was noted upon admission. At a check-up 1.5 years later, the fasting blood glucose levels were about 10 mmol/l. Half a year later the patient was hospitalised with acute blood glucose levels of 39 mmol/l and insulin treatment was started. The insulin requirements were about 50 IU/day which were divided into four daily doses of human insulin. From the time of his arrival at our hospital, the circulating levels of IAPP were immensely increased, varying from about 15 000 to 35 000 pmol/l. No diabetes mellitus was reported amongst first degree relatives of the patient and no islet-cell antibodies were recorded (75).

A biopsy taken from one of the liver metastases, showed relatively regular tumour cells growing in solid sheets. Some scattered tumour cells, but mainly the surrounding stroma tissue, displayed pronounced IAPP immuoreactivity. Electron microscopy showed that the hormonal granules were immunoreactive to Chromgranin and IAPP, but not to insulin (84). Amyloid was mainly found to be extracellular, but amyloid fibrils were also found in the cytoplasm. These seemed to emerge from the granules in the shape of radiating threads (84).

A mixed meal test was performed. Since the patient had an EPT, PP was expected to increase during the test, but in this case, no increase was recorded. During an oral glucose tolerance test, glucose increased from about 9 to 16 mmol/l, but no insulin response was seen. During both these tests, there were no significant changes of the IAPP levels. However, a slight reduction was seen after injection of 1 mg Atropine. No insulin responses to either intravenous or oral glucose were recorded, although blood glucose levels were again high and increased during the tests. The hormonal dynamics during an oral glucose tolerance test are shown in Fig 1. In response to an injection of glucagon, slight increases of insulin and C-peptide were seen. An euglycaemic insulin clamp test showed normal peripheral insulin sensitivity. Rat pancreatic islets cultured for 4 days with 10% serum from the patient showed decreased glucose oxidation rate and decreased insulin release in response to 16.7 mmol/l glucose, compared to islets cultured with 10% normal serum.

Summing up, these results indicate that the high circulating IAPP levels induced prominent effects on the glucose metabolism in this patient. The most obvious was the inhibited insulin response to glucose stimulation. It has been reported in experimental animals that concentrations of IAPP in the range of $10^{-8}$ to $10^{-5}$ mol/l can induce decreased glucose-stimulated insulin secretion (4,50,72,98,112,138), although some experiments have failed to show any inhibitory effects at IAPP concentrations in the range of $10^{-6}$ to $10^{-7}$ mol/l (15,105,124,156). These differensis in results may be explained to some extent by the use of different sources of IAPP since a varying biological activity of IAPP preparations has been reported (23). However, in a study where IAPP was infused intravenously into human volunteers at concentrations at about 2,200 pmol/l, decreased insulin release during an intravenous glucose tolerance test was recorded (12) while similar results could not be repeated at 1,200 pmol/l. For comparison, the patient in this study had about 10 times higher circulating concentrations of IAPP than the 2,200 pmol/l, giving decreased insulin release in healthy volunteers. Another diabetogenic effect to consider was the streptozotocin treatment. However, it is unlikely that the pancreatic β-cells should have been damaged by the streptozotocin treatment that was given as an anti-tumour treatment, since diabetes mellitus is rarely reported at doses of streptozotocin administrated to the patient (118).

Furthermore, diabetes mellitus was present before this therapy was started. Inhibited PP response to a mixed meal test also indicates inhibitory effects of IAPP on pancreatic islet cell function.

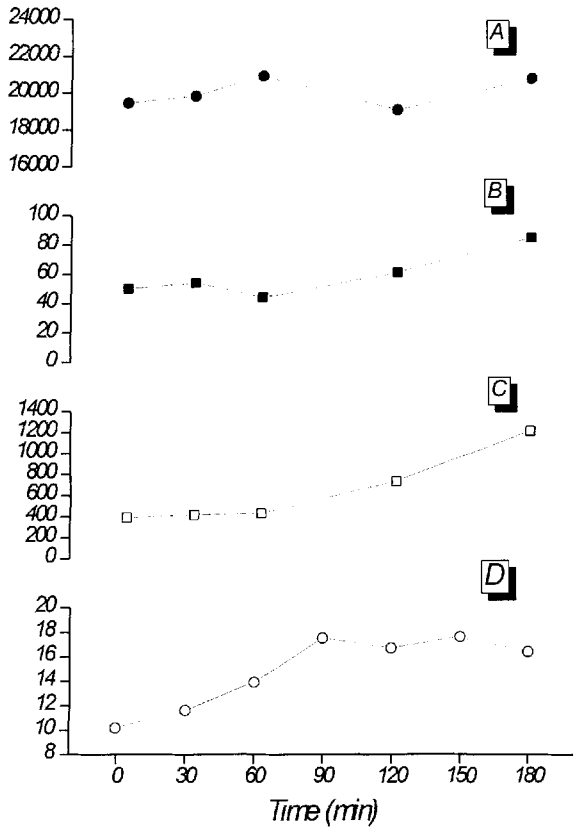


**Fig 1**: Concentrations of plasma IAPP (A), serum insulin (B), serum C-peptide (C) and blood glucose (D) during oral glucose tolerance test. Although the fasting glucose levels were clearly diabetic (10.2 mmol/l), insulin was not elevated. During the test, there was virtually no response of insulin or C-peptide, although the glucose levels increased up to about 17 mmol/l. The IAPP levels were oscillating on an extremely elevated level. The concentrations on the Y-axes are given in mmol/l for glucose and in pmol/l for IAPP, insulin and C-peptide.

Euglycaemic clamp studies and glucose tolerance tests during intravenous infusions of IAPP have been performed earlier in experimental animals. In dogs, infusion of IAPP in high doses induced increased peripheral resistance (144), while at low infusion rates (giving about 12- to 50-fold elevation of plasma IAPP), no peripheral effects were recorded (69). In rats, increased peripheral resistance was induced by IAPP infusions elevating plasma levels in the range from 220 to 9,000 pmol/l (45,73,92). However, in humans no effects on peripheral insulin sensitivity were seen in healthy volunteers at plasma concentrations of IAPP up to about 2,000 pmol/l (177).

The insulin-mediated glucose disposal rate found in the present study was within the normal range for healthy men of the same age, although the circulating levels of IAPP were highly elevated, about 20,000 pmol/l. Thus, if IAPP contributes to the precipitation of type 2 diabetes mellitus by its effects on insulin resistance, an inhibition of the insulin-stimulated glucose disposal rate would have been anticipated by the elevated IAPP levels in this patient.

During all tests, plasma IAPP was oscillating at high levels, about 15,000 to 25,000 pmol/l. These fluctuations were probably due to the spontaneous release of the IAPP-like molecule from the tumours and apparently the tumours did not respond to the different secretory stimuli. The true nature of the peptide is not quite clear. A gel filtration experiment revealed a higher molecular weight (6.3 kDa versus 4 kDa) of the peptide than the standard IAPP. This indicates that the circulating peptide might be a pro-peptide of IAPP, maybe having other biological functions than ordinary processed IAPP molecule. Nevertheless, it is likely that the high circulating levels of IAPP in this patient may be in the range of the local concentrations reached in the intra-islet milieu, thus mimicking the autocrine/paracrine-like effects possibly exerted by IAPP (13).

Considering other putative effects of IAPP, the hypertension recorded before the tumour diagnosis spontaneously disappeared and the patient became normotonic without any anti-hypertension treatment during the period when IAPP levels were high. No effects on the blood calcium levels were recorded.

*Conclusions*
Two patients with endocrine pancreatic tumours and massive overproduction of IAPP have been identified. Both showed disturbed glucose homeostasis. Metabolic investigations on one of these patients, which developed an insulin requiring diabetes mellitus during tumour progression, were performed. The results suggest that if IAPP has a diabetogenic effect it is more likely to be exerted at the level of insulin secretion than at the level of peripheral insulin sensitivity. Consequently, if a clinical syndrome associated with overproduction of IAPP (IAPPoma) exists, disturbed glucose homeostasis would be a major symptom.

*Methodological aspects*
Reliable measurements of IAPP require an extraction procedure to minimise the effects of interfering substances in plasma. Previous reports have utilised extraction with Sep-Pak cartridges, with recoveries ranging from 60% to 95% (14,17,83,90,132,162). However, we could not achieve reliable results with Sep-Pak extraction, since the recoveries were variable and usually less than 10%. Instead, we used extraction with acidified ethanol, a reliable method previously employed for many peptide hormones. The recovery was rather low (50%) but reproducible as indicated by the similar results obtained when synthesised standard IAPP or $^{125}$I-IAPP was used. When the plasma levels were extremely elevated, interference could be minimised by dilution of plasma by 1/100 or more.

## Synaptophysin

*Introduction*
Since Sy is an established immunocytochemical marker for NE tissue it was of considerable interest to establish a sensitive radioimmunoassay for measurements in blood plasma and to evaluate a possible clinical use of monitoring circulating levels of Sy. Except for immunoblot methods (133,174) no assays for quantitative detection of Sy were available before. These

methods though, have not been used for detection of circulating Sy but have only been applied for quantification of Sy during fractionation of Sy-containing specimens.

*Antibodies against Synaptophysin*
A polypeptide, which was homologous to amino acids 246 - 260 in the human Sy molecule (150), was synthesised. The peptide was coupled to bovine albumin (108) and injected into rabbits for production of polyclonal antibodies (161). The polypeptide was selected to give antibodies against the same epitope as the commercially available monoclonal antibody SY-38, which has previously been shown to be directed to amino acids 220 - 260 in human Sy (78). Furthermore, this region had identical amino acid sequences in both human and rat which allowed use of the antibodies in tissue specimens from both species.

Three out of five rabbits responded with antibody production after the second booster injection. The immunostaining of rat pancreas with our polyclonal antisera showed identical results when compared to the monoclonal SY-38 antibody. Nerve fibres were also stained by the polyclonal antibodies. The lack of immunostaining after preabsorption of the antibodies against the synthesised polypeptide in our study indicated that the antibodies were directed against the peptide. Since this peptide had an amino acid sequence unique for Sy, it is most likely that the antibodies are directed against Sy. This was further confirmed by the Western blot analysis, where both antibodies detected the same protein band. As in the case of the SY-38 antibody, the polyclonal antibodies could only be used on rapidly frozen material or acetone or ethanol fixed material while material fixed in formalin for more than two hours could not be used (55). Long fixation time in formalin apparently destroys the antibody-binding epitopes.

*Radioimmunoassay for Synaptophysin*
A radioimmunoassay for Sy was established using the synthesised peptide for standard and tracer preparations and the polyclonal antibodies. The chemical nature of Sy with its 4 hydrophobic regions makes this molecule very "sticky" and difficult to keep in a water solution usable for reliable standard and tracer preparations. By using the polypeptide, we could overcome these difficulties. The parallel dilution behaviour obtained from the standard curve, from our partly purified Sy preparations and from the highly purified Sy preparation, further justified this procedure. Furthermore, we were able to obtain "true" concentration values since we could weigh an exact amount of peptide and express the concentrations at molar basis.

Circulating levels of Sy were measured in plasma from 10 healthy individuals and 10 patients with advanced stages of NE tumours (metastatic carcinoid and endocrine pancreatic tumours). Plasma samples were also collected from the hepatic vein during embolisation treatment (87) of liver metastases in 2 patients with carcinoid tumours. The plasma samples were measured undiluted, diluted 1/10 and 1/100 (with assay buffer) in the assay. Two ml of patient plasma was subjected to separation on a proRPC HR 5/10 column and another 2 ml was subjected to separation on a Chelating Superose HR 10/2 column (FPLC-system, Pharmacia-LKB).

However, in spite of the high sensitivity of the radioimmunoassay, no immunoreactive Sy was detected in the blood of either normal subjects or patients with NE tumours, despite the latter clearly exhibiting elevated plasma levels of chromogranin A (a reliable circulating marker of NE tumour growth (35)). As mentioned above, the Sy molecule passes through the vesicle membrane four times and is thus likely to be very closely attached to the membranes. It is therefore less likely that Sy would be released into the circulation even during stimulated hormonal release situations. However, it was more surprising that we were not able to detect any Sy in the liver vein during embolisation treatment of carcinoid metastases. By this treatment severe hypoxia and

massive cell death of the liver metastases was induced and consequently Sy was expected to be released into the circulation. But, since the cell death was somewhat delayed, a possible elevation of Sy may have been missed as we were not able to collect blood from the liver vein for more than 20 minutes after the embolisation treatment.

Cell culture media from PC-12 cells (52) cultured for 5 days contained 0.25 pmol Sy per ml (10 μg/ml). The cell homogenate contained 4 pmol SY per mg wet weight (160 ng Sy/mg). Cell culture media from LCC-18 cell line contained 0.1 pmol Sy per ml (4 μg/ml) and the cell homogenate contained 1 pmol Sy per mg wet weight (40 ng Sy/mg). The capability of the LCC-18 cell line to produce Sy was confirmed by the Northern blot analysis where clearly detectable amounts of Sy mRNA were found. Demonstration of Sy in the PC-12 rat pheochromocytoma cell line was in accordance with the previously reported investigations (163,174). The content of Sy in the cell culture media may represent secretion of Sy into the media or its release as a result of cell death. The LCC-18 cell line has also been shown to contain Sy (88). This cell line originates from a human colonic NE carcinoma. It has earlier been shown that the cells exhibit some NE properties but lack cytoplasmic secretory granules. With immunocytochemistry it was shown that the Sy immunoreactivity was confined to the plasma membrane. This observation might be explained by the fact that the cells had lost their normal peptide storage and release regulation and constantly secreted the hormones into cell culture media.

Measurements of Sy during the purification steps of Sy from rat brains were applied for optimising the separation conditions. During the centrifugation separation steps as much as 78% of the initial Sy could be recovered. During the column purifications, Sy eluted in homogeneous peaks and the combined procedure resulted in a highly purified sample of Sy.

*Conclusion*
Measurements of circulating Sy in patients with NE tumours was not successful, probably because Sy is not released into the circulation, at least not at concentrations above 40 pmol/l (1.5 mg/l). However, synaptophysin is still a reliable immunocytochemical marker for NE tumour growth.

## The chromogranin family of proteins

*Introduction*
The use of CgA as a circulating marker and Chromogranins as immunocytochemical markers for NE tumours has been thoroughly examined (see above). Development of specific antibodies for CgA, CgB, CgC and PS and establishment of sensitive radioimmunoassays for measurement of circulating levels of CgA, CgB, CgC and PS is described below.

*Chromogranin A in urine*
The study was initiated by the observation that bands of unknown proteins were detected in the urine from patients with NE tumours by the routine agarose gel electrophoresis method used at the Department of Clinical Chemistry (64). To examine the above observation in more detail, urine samples were collected from 40 consecutive patients, with known carcinoid tumours, referred to the Endocrine Unit at the Department of Internal Medicine. The routine electrophoresis method was used for detection of the protein bands.

A total of 5 out of the 40 urine samples examined showed the presence of unknown protein bands detectable by the routine electrophoresis method. Based on the classification of the protein patterns (64) a close correlation between the existence of the unknown protein bands and a

tubular dysfunction of the kidneys was found. There was however no correlation to serum creatinine, to urinary albumin excretion nor to glomerular dysfunctions of the kidneys. The lowest concentration detectable by this method was about 1 g/l in the concentrated urine, which represents about 10 mg/l (about 400 nmol/l) in native urine.

In the normal kidney, low molecular weight proteins including many peptide hormones, are filtered through the glomerular membrane and then reabsorbed in the proximal tubules. Theoretically, increased excretion of CgA fragments in the urine depends on at least two factors. First, patients with NE tumours in advanced stages have high levels of CgA in the circulation. Thus, increased amounts of CgA fragments can be filtered and excreted into the urine due to an overload of the reabsorption capacity. Second, in patients with decreased renal function both the glomerular filtration and the reabsorption of proteins in the tubuli may be disturbed. It has been shown that CgA is filtered in the glomeruli of the kidneys and then taken up into the endocytotic lysosomal pathways of the tubular cells (167). Accordingly degradation of human pancreastatin has been shown in the presence of kidney extracts (153). A possible explanation of the presence of CgA fragments in the urine of some of our patients is that the capacity of reabsorption and removal of the filtered CgA is exceeded. This suggestion is favoured since we could show a correlation between the urine excretion of CgA fragments and a slight tubular dysfunction. The concentration of albumin in the urine, which mostly reflects the glomerular function, did not correlate to the presence of CgA fragments in the urine. It should also be pointed out that both the tubular and glomerular dysfunctions in our patients were mild and did not affect the serum creatinine levels. Furthermore, it has been shown that severely impaired renal function will result in a progressive retention of CgA and its fragments in the circulation (57).

The isolation of CgA fragments from the urine was achieved by one ion exchange chromatography separation step. The result was a mixture of CgA fragments but no other protein material was detectable. This protein mixture was immunogenic and resulted in antibodies against CgA, useful for radioimmunoassay and immunocytochemistry.

The amino acid sequence determinations of the fragments revealed two major cleavage sites in the CgA molecule, both at previously reported proteolytic degradation sites with two basic amino acids and two minor cleavage sites. The major sites were located at amino acids numbers 116 and 210 while the minor were located at amino acids numbers 273 and 395 of the intact CgA. These cleavage sites have been described earlier for tumour derived CgA-like proteins separated from NE tumours (46,60,135,137,152). Since these cleavage sites are known tryptical degradation sites it is more likely that this degradation appears in the tumours or in the circulation and not in the urine. The first 114 amino acids of CgA, which represent the putative CgA split product ß-granin (32), were not found in the present preparation. It has been shown that the degradation of CgA within the chromaffin granules starts at both C-and N-terminal cleavage sites (181). This observation is in agreement with our results and can thus explain the different fractions with different lengths with the same N-terminal cleavage sites.

The exact C-terminal degradation points could not be determined but according to SDS-electrophoretic patterns a majority of the fragments had molecular weights of about 30 kDa. The gel permeation chromatography showed that the molecular weight of the fragments was about 48 kDa. Analysis of the total amino acid composition revealed the molecular weight to be 35.2 kDa. This apparently large difference in the results from the different molecular weight determinations reflects methodological problems in molecular weight determinations of an acidic and hydrophilic protein as CgA (141). Consequently, the most reliable value of the molecular weight is the 35.2 kDa deduced from the total amino acid content. This molecular size was expected

since proteins smaller than about 40 kDa are filtered freely in the kidney and the dialysis tube used for concentration of the urine samples before electrophoresis had a cut-off value of less than 10 kDa. The IEF electrophoresis showed that the CgA fragments had isoelectric points at 4.8 and 5.0, which is in agreement with the known value of 4.9 for the intact CgA.

There was a good correlation between the amounts of CgA fragments measured in the radioimmunoassay and the existence of the unknown protein bands in the urine. Again high levels of CgA fragments in the urine correlated with tubular dysfunction in the kidneys but not with glomerular dysfunction. Patients with high plasma levels of CgA had elevated levels in the urine only if concomitant tubular dysfunction was present.

*Antibodies:*
Antibodies against CgA were raised after immunisation with the isolated CgA fragments. The use of synthesised peptides, deduced from a known protein sequence, is a reliable way to achieve specific antibodies (113). This technique was used to obtain specific antibodies against PS, CgB and CgC. The CgB and CgC peptides were selected to be unique for the respective proteins in order to avoid cross-reaction with any other substances within the assay systems. Six rabbits were used for immunisation and all 6 animals responded with useful antibodies after the second booster injection. Since the PS peptide has a sequence homology to CgA, these antibodies could be expected to cross-react with CgA. The results from the cross reactivity studies, the plasma and urine measurements and the gel permeation experiments indicate that this potential cross-reaction was negligible in the radioimmunoassay system. The background for this specificity is not fully understood. One explanation could be that in the 3-dimensional structure of the undegraded, intact CgA, the PS epitope is hidden, but when degradation of the CgA molecule occurs, the PS epitope becomes exposed on the surface of the molecule and the PS antibodies can bind. This theory is supported by our gel permeation chromatography experiments on plasma and urine samples, where patients with higher levels of PS in the plasma, compared with the CgA levels, had a lower molecular weight distribution than those with low levels of PS in the circulation compared to CgA. The theory is probably true for water solutions (e.g. in radioimmunoassay conditions) but it may be untrue under immunocytochemical conditions, as indicated by the fact that the stainings with the PS and CgA antibodies gave almost identical results. Before immunostaining, the tissue specimens are treated with denaturing processes, which may alter the 3-dimensional structure of CgA hence exposing the PS epitope for the PS antibodies to bind.

*Immunocytochemistry:*
Immunocytochemical staining of pancreatic islets, adrenal medulla and ileal mucosa, and tumours derived from these tissues, with our antisera against CgA, CgB, CgC and PS gave results similar to those reported by others (9,53,70,81,82,127,136,165,173,175) and are summarised in Table 3. However, there were minor differences in the results reported earlier. Our CgC antibodies did not detect any cells in the ileal mucosa, nor in the midgut carcinoid tumours and visualised cells in only 1 of the 4 insulinoma tumours. Furthermore, in ileal mucosa no immunostaining was seen with the CgB antibodies. These differences may be due to a number of factors. It is well known that Chromogranins exhibit considerable interspecies variation in the amino acid sequences (173), a fact which can affect the specificity of the antibodies since most of the previous immunocytochemical results are based on antibodies raised against Chromogranins from species other than human. It is also possible that different fixation methods, immunostaining conditions, and other technical procedures, as well as true differences in the tissues examined, may account for the minor differences found. As mentioned above, PS can be expected to cross-react with CgA under immunocytochemical conditions. This was clearly

shown in our study, where the PS, CgA and LK2H10 (monoclonal CgA antibodies, (82)) antibodies gave similar immunostaining results.

**Table 3**: Results of immunocytochemical stainings of different NE tissues with antibodies against CgA, CgB, CgC and PS. Figures show the number of tissue specimens positively stained divided by the total number of specimens in each group.

| Tissue | CgA | CgB | CgC | PS |
|---|---|---|---|---|
| **Normal pancreas** | 4/4 | 4/4 | 3/4 | 4/4 |
| **Insulinoma** | 4/4 | 4/4 | 1/4 | 4/4 |
| **Glucagonoma** | 2/2 | 2/2 | 2/2 | 2/2 |
| **Normal adrenal medulla** | 4/4 | 4/4 | 3/4 | 4/4 |
| **Pheochromocytoma** | 3/3 | 3/3 | 3/3 | 3/3 |
| **Normal ileal mucosa** | 3/3 | 0/3 | 0/3 | 2/3 |
| **Carcinoid** | 3/3 | 3/3 | 0/3 | 3/3 |

*Radioimmunoassays*

There was no cross-reactivity between the PS, CgB and the CgC assays. Standard preparations of CgA cross-reacted in the CgB assay by less than 1% and in the CgC assay by less than 0.01%. Standard preparations of PS, CgB or CgC did not influence the binding in the CgA assay. A preparation of intact CgA cross-reacted by less than 1% in the PS assay, while the standard CgA preparation (which consists of partly degraded CgA) showed about 15% cross-reactivity in the PS assay (see above). Based on the group of healthy individuals, the reference ranges for plasma measurements of the different assays were calculated for CgA to <4.0 nmol/l, for CgB to <1.2 nmol/l, for CgC to <160 pmol/l and for PS to <1.0 nmol/l. Based on the group of patients without NE tumours, the reference ranges for urine measurements were calculated to <4.0 nmol/l, <1.5 nmol/l, <0.5 nmol/l and <0.5 nmol/l for CgA, CgB, CgC and PS, respectively. The results from plasma and urine measurements of the different Chromogranins are summarised in Table 4.

Urine samples collected with the addition of hydrochloric acid or acetic acid in the collection vessel showed significantly lower levels of all Chromogranins compared to the samples collected without any preservatives and the samples collected as a single morning sample. Since the acid preservatives seemed to decrease the measured Chromogranins, these samples were excluded from the study. A majority of the patients with increased urinary levels of CgA or PS were previously subjected to chemotherapy treatment with streptozotocin, a cytotoxic drug known to induce renal tubular dysfunctions. In some patients the urinary secretion of CgA was considerable, i.e. more than 2 µmol/l (>80 mg/l).

*Molecular forms of chromogranins*

Gel permeation chromatography showed different molecular size patterns of CgA in different patients. The elution curves from the gel permeation chromatography of plasma and urine samples confirmed that the degradation of CgA starts in the tumour or in the plasma. The intact CgA molecule is not secreted into the urine. Degraded CgA is secreted into the urine only if there is a concomitant tubular dysfunction of the kidneys. This is illustrated in Fig. 2 where a patient with normal kidney function, retains both intact and partly degraded CgA in the circulation whereas a patient with a tubular dysfunction retains only the intact CgA in the plasma and secretes the fragments of CgA.

**Table 4:** Percent values of elevated levels of Chromogranin A, Chromogranin B, Chromogranin C and Pancreastatin in plasma and urine samples of 44 patients with carcinoid tumours, 17 patients with sporadic endocrine pancreatic tumours (EPT) and 11 patients with endocrine pancreatic tumours and the multiple endocrine neoplasia syndrome 1 (MEN1).

| | Carcinoid n=44 | EPT n=17 | MEN1 n=11 |
|---|---|---|---|
| **p-CgA** | 100% | 100% | 91% |
| **p-CgB** | 86% | 100% | 73% |
| **p-CgC** | 5% | 12% | 0% |
| **p-PS** | 61% | 35% | 0% |
| **u-CgA** | 41% | 59% | 0% |
| **u-CgB** | 11% | 35% | 0% |
| **u-CgC** | 7% | 41% | 0% |
| **u-PS** | 34% | 53% | 9% |

It has been shown earlier that CgA and CgB are more abundant than CgC in human NE tissue (53,136). These observations are supported in this study by the fact that normal plasma CgA and CgB levels were in the same level of magnitude, but the CgC levels were found to be about 10-fold lower. This was even more pronounced in the patients, where only 4 out of 72 patients (6%) displayed increased plasma levels of CgC compared to 100% of the patients which showed increased levels of either CgA or CgB. The levels of the different Chromogranins in the urine samples were notable. Of all the patients in the study, only 29 of 72 (40%) had elevated levels of CgA or CgB in the urine compared to 100% in plasma. None of the MEN1 patients had increased urinary levels of any of the measured Chromogranins or PS. Majority of the patients with elevated urinary levels of CgA, CgB, CgC or PS had been subjected to treatment with streptozotocin, which is known to induce renal tubular dysfunctions. None of the patients with MEN1 syndrome had been treated with chemotherapy and hence no one displayed increased urinary levels of CgA, CgB, CgC or PS. Consequently, these results indicate that measurements of CgC and PS are less useful circulating markers for NE tumours than CgA and CgB and furthermore, that plasma measurements are more sensitive than urine measurements.

The measurements of CgB in the fractions collected during the gel permeation chromatography experiments showed that the CgB immunoreactivity eluted in a homogenous peak with a molecular weight of about 7 kDa. The complete lack of higher molecular weight CgB (and the intact CgB molecule) was unexpected. The CgB molecule has several dibasic amino acid positions, which are putative degradation points (59,180). The observed molecular weight, in the gel permeation experiments, is very similar to the deduced molecular weight of the peptide CgB306-365 that would occur from cleavage at amino acid positions 304-305 and 366-367. Since our antibodies are directed against the CgB sequence 312-331, this peptide would be the part of the CgB molecule detected in our CgB assay. Antibodies against this part of the CgB molecule have previously been used for immunocytochemical characterisation of pituitary tumours (134). In that study, all tumours were immunostained by the CgB antibodies, indicating that this part of the molecule may serve as a marker for the whole CgB molecule. In the case of CgA, the PS antibodies did not recognise the intact CgA molecule but partly degraded CgA, as discussed above. If a similar degradation of CgB would occur, some higher molecular weight fragments of CgB would have been expected. An alternative explanation is that (at least in the

patients examined) the CgB molecule is subjected to complete degradation into smaller fragments, presumably within the tumours. These patients also had a considerable excretion of the low molecular weight CgB fragment in the urine. The levels of CgC in plasma and urine were too low to be detected with sufficient accuracy in the fractions collected during the gel permeation separations and hence no molecular weight distribution results of CgC in plasma or urine can be presented.

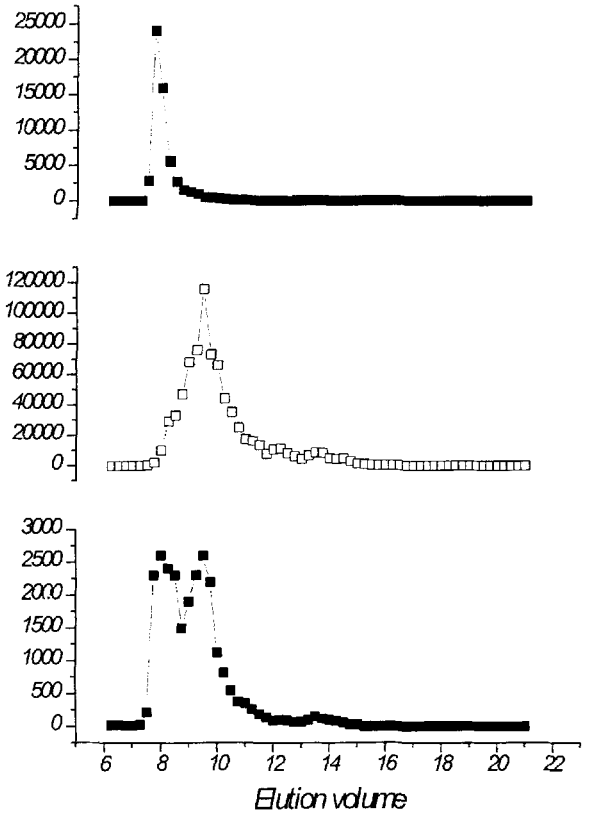


**Fig 2**: Gel filtration chromatography (on a Superdex-75 column) of plasma (top) and urine (middle) from a patient with tubular kidney dysfunction, compared to plasma (bottom) from a patient with normal kidney function. All 60 fractions collected during the separations were measured in the CgA assay. In the normal case, the circulating CgA immunoreactive material existed in two molecular forms. In case of tubular dysfunction, the higher molecular weight fraction (intact CgA) is retained and the fraction with lower molecular weight material is secreted in the urine. Concentrations of CgA (pmol/l) are given on the Y-axes.

*Conclusion*

Antibodies against CgA, CgB, CgC and PS were developed. These antibodies were useful for both radioimmunoassay measurements of circulating levels and immunocytochemical investigations of the different Chromogranins. Furthermore, plasma measurements of Chromogranins are more sensitive as markers for tumour growth than urine measurements, and CgA and CgB are better circulating markers for NE tumour growth than CgC and PS.

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