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Substance P in an Argentaffin Carcinoid of the Caecum: Biochemical and Biological Characterization

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Summary. An argentaffin carcinoid tumour of the caecum which contained serotonin (167 μg/g) and consisted predominantly of EC_1-cells, was analysed for the presence of peptides using immunohistochemical, biochemical and pharmacological methods.

A very high content of 3.9 μg/g of immunoreactive substance P was found. The distribution of cells staining positively for substance P matched that of cells containing serotonin. While some immunoreactive somatostatin (3.2 ng/g) was present in the tumour, neurotensin, glucagon, gastrin, and motilin were not found.

Part of the substance P immunoreactivity measured most likely represents authentic substance P: it behaved like substance P in two chromatographic systems and in two bioassays, and its activity on the guinea pig ileum was abolished by specific tachyphylaxis towards substance P.

Key words: Carcinoid tumour – substance P

Introduction

Substance P-like immunofluorescence has been identified in intestinal nerves of intrinsic (Schultzberg et al. 1978; Costa et al. 1980) and extrinsic origin (Costa et al. 1980). In addition, endrocine cells of the intestine show substance P-like fluorescence (Nilsson et al. 1975; Heitz et al. 1976; Sundler et al. 1977 a, b). Since these cells also contain serotonin (Heitz et al. 1976; Sundler et al. 1977 a), they are identical with enterochromaffin cells of the EC_1-type. Carcinoid tumours originating from enterochromaffin cells contain serotonin (Lembeck 1954; Ratzenhofer and Lembeck 1954). Lembeck (1954) described the presence of a gut-contracting peptide in an extract of a carcinoid tumour. His assumption that it might be substance P is supported by more recent immunohistochemical (Håkanson et al. 1977; Alumets et al. 1977; Wilander et al. 1977, 1979) and

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radioimmunoassay data (Skrabanek et al. 1978) on substance P in carcinoid tumours. Elevated plasma levels of immunoreactive substance P have also been found in some patients with carcinoid tumours (Håkanson et al. 1977; Ingemansson et al. 1977; Skrabanek et al. 1978). Carcinoid tumours containing several peptides have also been described (Wilander et al. 1977, 1978, 1979; O'Brian et al. 1980).

While it has been shown that substance P in equine intestine is identical with the bovine hypothalamic undecapeptide (Studer et al. 1973), the substance P immunoreactivity present in carcinoid tumours has not been characterized by biological or biochemical methods.

We therefore examined whether the substance P, detected in a serotonin-containing carcinoid tumour by immunohistochemistry and radioimmunoassay is biologically active and biochemically indistinguishable from synthetic substance P.

Case Report

The patient (53 y., female) underwent surgery in 1954 (appendectomy), 1956 (resection of stomach, Billroth II because of a duodenal ulcer), and in 1972 (cholecystectomy). She reported that for six months meteorism with liccups had increased, as had sensations of pressure in the upper part of the abdomen. Faeces were normal. Episodes of flushing with reddening of the face, dyspnoea, sweating and tachycardia were first observed 5 months before presentation and had increased in number up to 5 times a day. During the last 6 weeks before admission observed pencil-like stools, free of blood. An endoscopy carried out because of sub-acute obstruction revealed a tumour of walnut size located at the upper ileocoecal valve. Biopsy showed an atrophic mucosa with inflammatory reaction and an alveolar tumour of carcinoid type extending into the submucosa. The values of serum-gastrin (85 pg/ml) and 5-hydroxyindoles in urine (9.6 mg/day) were normal. Hemicolectomy was performed and an obstructive tumour of $2^1/_2 \times 2^1/_2$ cm was resected from the ileocoecal region. Following surgery the patient recovered normally. Flush symptoms have not recurred. Five months after surgery the levels of immunoreactive substance P in plasma (16 pg/ml) and of 5-hydroxyindoles in urine (5,2 mg/day) were not elevated. Endoscopy revealed normal passage at the site of the anastomosis.

Methods

Histology. Unfixed tumour tissue was cut with a cryostat and the following peptides were studied by immunofluorescence: substance P (antibody Rd 2, Leeman), motilin (antimotilin ucb Bruxelles), neurotensin (antineurotensin INC 28 Hz), glucagon (antiglucagon ucb Bruxelles I 601, cross-reacting with human enteroglucagon), and gastrin (antigastrin ucb Bruxelles I 600). In addition formalin-induced fluorescence was examined in samples exposed to formaldehyde vapor at 60° C for 90 min.

Part of the tumour was fixed in 10% formaldehyde and stained with haematoxylin-eosin, van Gieson stain, Alician blue, and PAS, and with the silver techniques of Masson-Fontana, Grimelius, Sevier-Munger, and Bodian (Romeis 1968). Substance P was stained in formaldehyde-fixed sections with the peroxidase-antiperoxidase technique (antibody Rd 2, diluted 1:350).

For electronmicroscopy, 4 different pieces of the tumour were fixed in 3% glutaraldehyde and embedded in Epon. Semithin sections were examined, some of them after staining with silver.

Biochemistry and Pharmacology. A cross-section of the tumour was extracted for 5-hydroxyindoles and peptides within 2 h of surgery. 5-hydroxyindoles were determined according to Weissbach et al. (1958). Peptides were extracted with acetone-HCl (Chang and Leeman 1970) and measured with specific radioimmunoassays using the assay procedure described by Mroz and Leeman (1979). Antibodies used were: Rd 2 and f 2 for substance P (Leeman), M-6 for somatostatin (Arnold and Fernstrom 1980), and HC-8 for neurotensin (Carraway 1979).

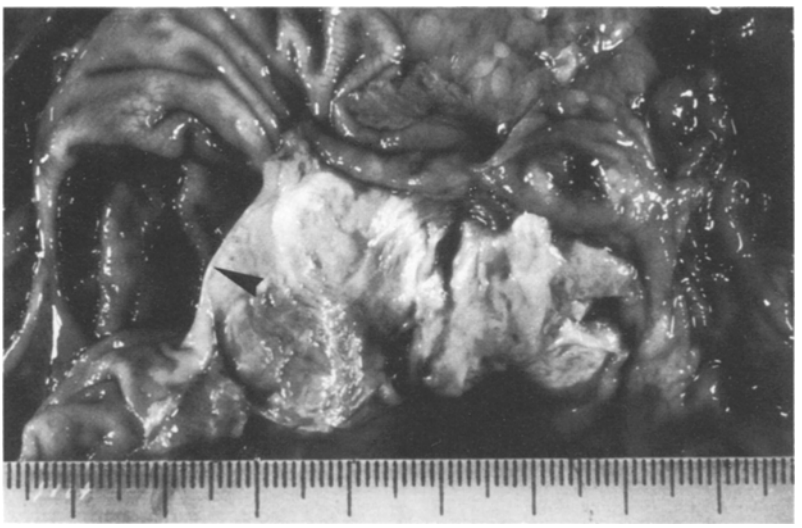

Fig. 1. Resected intestine with cross-sectioned tumour. The arrow points to the very atrophic mucosa covering the tumour

For determination of the molecular weight of the substance P immunoreactivity, an aliquot of the extract was chromatographed on Sephadex G-25 (column 1.2 × 95 cm; eluent: assay buffer; fraction volume 2 ml).

For further characterization of the immunoreactivity in the crude extract and for determination of its bioactivity, chromatography on Sephadex G-10 was performed (column 1.5 × 6 cm; eluent: 0.1 M acetic acid). Two fractions of 5 ml each were collected and lyophilized: fraction A, comprising compounds eluting in the void volume (e.g. substance P), and fraction B, comprising low molecular weight substances (e.g. serotonin and salts).

An aliquot of fraction A was chromatographed on reverse phase high pressure liquid chromatography (column: μ Bondapak C_{18}, 3.9 × 300 mm; eluent: 0.01 M acetic acid containing 0.1% pentane sulfonic acid, linear gradient of 20 to 60% acetonitrile; flow rate 2 ml/min; fraction size 2 ml).

The biological activity of the fractions A and B was tested on the rabbit blood pressure (urethane anaesthaesia, 1 mg/kg atropine) and the isolated guinea pig ileum (Tyrode solution, no antagonists). Since many compounds are known to cause contraction of the ileum and since no substance P antagonist is yet available, the specificity of the contraction induced by fraction A was examined on ileum made tachyphylactic with high doses of substance P (100 ng/ml), serotonin (1 μg/ml) or substance P plus serotonin.

Results

Morphology

The tumour situated at the ileo-caecal valve was found to be clearly separated from the surrounding tissue and to be growing towards the atrophic mucosa (Fig. 1). In one of seven lymphatic nodes in the vicinity of the tumour metastatic tissue was seen macroscopically. Histologically the tumour was categorized as a solid-trabecular carcinoid extending throughout the intestinal wall and growing towards the adjacent fatty tissue. Islets of tumour cells were seen in the muscle coats, the muscularis mucosae and the mucosa.

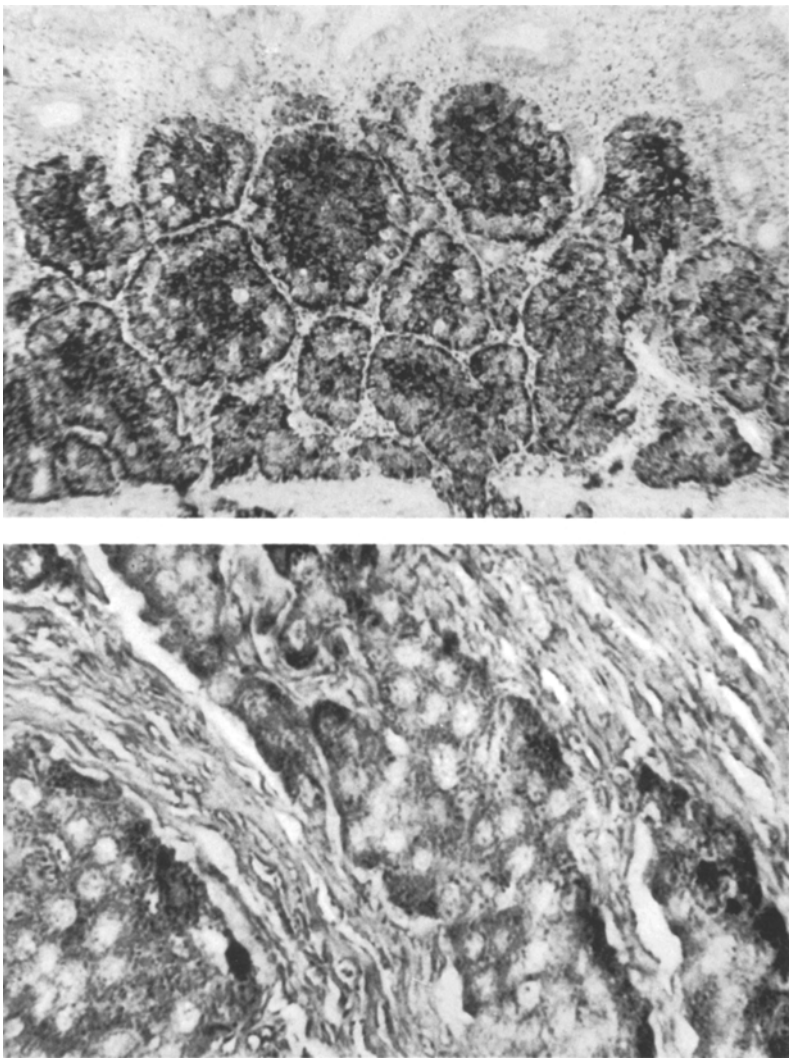

Fig. 2. Masson-Alzian blue staining of the carcinoid. Note extensive infiltration of the mucosa (upper part) by strongly argentaffin tumour cells. In the lower part the remainder of the lamina muscularis mucosae can be seen. Magn. ×63

Fig. 3. Immunohistochemical demonstration of substance P in the carcinoid. Cells containing reaction product are preferentially localized in the marginal zone of the tumour cell complexes. PAP technique, dilution of the SP-antiserum: 1:350. Magn. ×400

The mucosa was extremely atrophic but not ulcerated. Some occasional small PAS-positive areas were found. A positive substance P-immunofluorescence was seen in unfixed tissue, while no cells reacted with antibodies against motilin, gastrin, glucagon, and neurotensin. The tumour exhibited strong formalin-induced fluorescence, particularly near the mucosa. This fluorescence, corre-

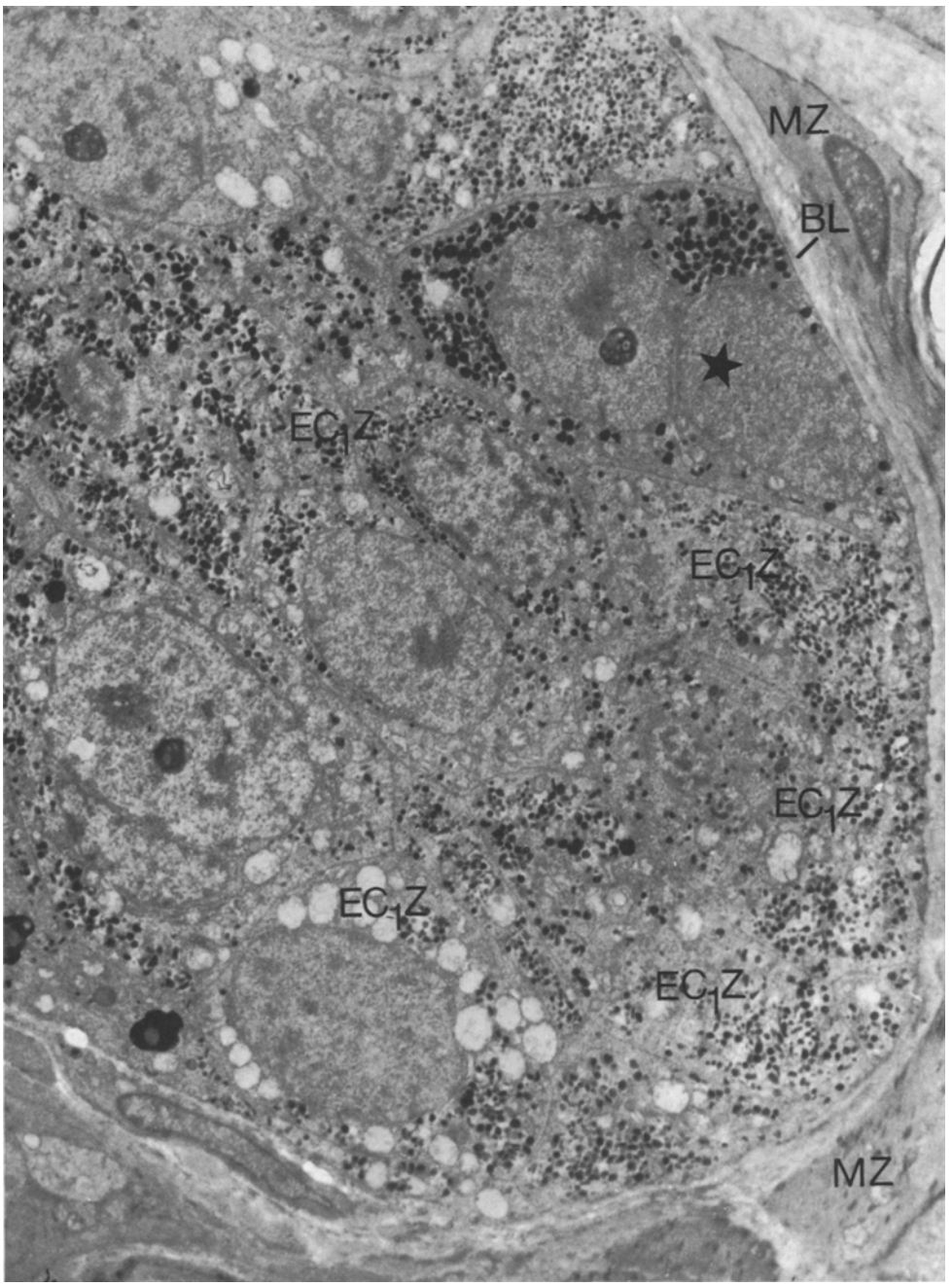

Fig. 4. Electron micrograph of a tumour area consisting of at least 13 cells. The representative section consists almost exclusively of cells of the EC_1-type (EC_1Z);* shows a tumour cell containing large, electron-dense granules, which thus possibly belongs to the EG- or EC_2-type. *BL*, basal lamina; *MZ*, smooth muscle cell. Magn. ×8,400

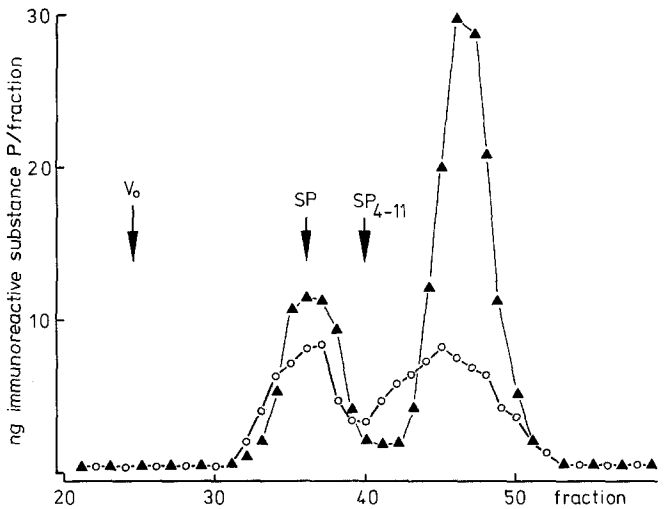

Fig. 5. Chromatography of a tumour extract on Sephadex G-25. Substance P immunoreactivity was measured by using antibodies RD 2 (o—o) and f 2 (▲—▲). The void volume (*Vo*) and the positions of synthetic substance P (*SP*) and the c-terminal substance P octapeptide (SP_{4-11}) were determined in separate runs

sponding to serotonin, was highest in the marginal cell layer of tumour islets. The same characteristic pattern was also seen with the Masson silver stain (Fig. 2). Some well defined areas of the tumour were, however, not argentaffin. Cells staining intensely with Grimelius silver technique were found, but staining according to Sevier-Munger or Bodian gave only weakly positive results. Dividing cells could be observed in semithin sections. A positive reaction was found in sections stained for substance P by the PAP-technique. The highest concentration of the reaction product was seen in cells of the outer layers of islets (Fig. 3), thus matching the distribution of serotonin.

When examined under the electronmicroscope (Fig. 4), the tumour consisted almost exclusively of endocrine cells. From the morphology of their secretory granules (a longer axis of pleomorphic rod-shaped granules up to 270 nm, diameter of round profiles up to 200 nm) could be categorized as EC_1-cells (Heitz et al. 1978). Occasionally cells were found containing larger, electrondense granules (longest extension of polymorph granules 340 nm, diameter of round profiles up to 410 nm).

Biochemical Analysis

The tumour contained 167 µg/g 5-hydroxyindoles. A content of 3.9 µg/g of immunoreactive substance P was measured with the antibody Rd 2 and a value of 5.7 µg/g with the antibody f 2. While a small amount of immunoreactive somatostatin was found (3.2 ng/g), the tumour did not contain measurable amounts of neurotensin (less than 0.1 ng/g). Serial dilutions of the extract produced parallel curves to the synthetic peptides in the substance P and somatostatin assay.

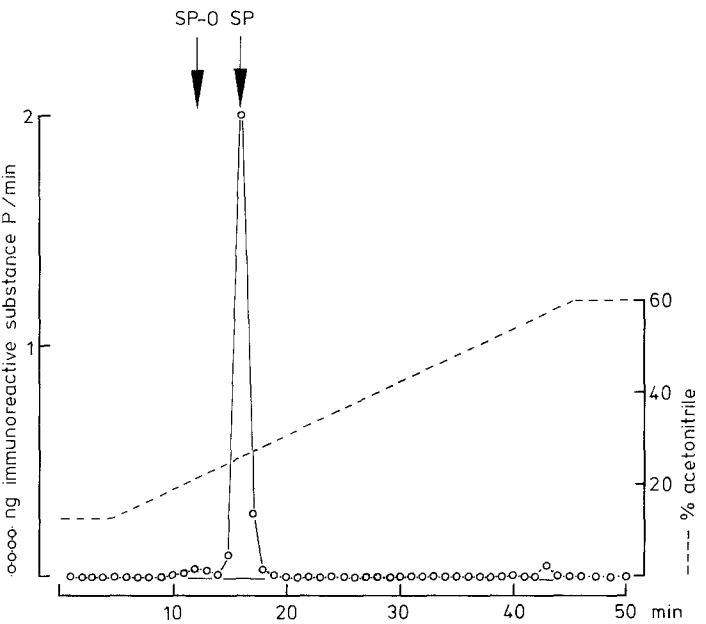

Fig. 6. HPLC chromatogram of the purified tumour extract (fraction A after Sephadex G-10). Substance P immunoreactivity was measured with antibody Rd 2. The retention time of synthetic substance P (SP) and of substance P sulfoxide (*SP–O*) were determined by UV absorption in separate runs

Chromatography of the tumour extract on Sephadex G-25 revealed two clearly separated peaks of substance P immunoreactivity (Fig. 5). The first peak eluted at the position of synthetic substance P and both antibodies recognized the immunoreactivity to a similar extent. The compound in peak 2 cross-reacted much more with antibody f 2 and accounted for about 70% of the immunoreactivity measured in the tumour extract using this antibody. When corrected for the presence of this unknown cross-reacting compound, both antisera gave a concentration of immunoreactive substance P in the carcinoid of 1.6 μg/g.

Because of the presence of this immunoreactive material and of serotonin in the tumour extract, immunoreactive substance P was separated from these contaminants by chromatography on Sephadex G-10. The purified fraction thus obtained (fraction A, see methods) was further characterized on HPLC and used for bioassay. In the chromatogram a major peak comprising 93% of the substance P immunoreactivity was found to elute at the same position as synthetic substance P (Fig. 6). Some immunoreactivity was detected at the position of substance P-sulfoxide (4.5%) and at a higher acetonitrile concentration (2.5%).

Bioassay of the Tumour Extract

Fraction A from the Sephadex G-10 column caused a fall in rabbit blood pressure and a contraction of the guinea pig ileum, that is to say, it had the

Table 1. Immunoreactivity and biological activity of the purified tumour extract (fraction A) compared to substance P

	Substance P-equivalent (ng/ml)	Relative activity
Radioimmunoassay	660 ± 54	1
Rabbit blood pressure	570	0.86
Guinea pig ileum	500	0.76

The value of the substance P-immunoreactivity (antibody Rd 2) represents mean $\pm$ S.D. of 4 serial dilutions; the values of the bioassays are the means of dose-response curves of 2 experiments each

same effects as substance P. In both test systems the dose-response curves were found to be parallel to substance P. The relative potencies of fraction A and substance P were almost identical in both bioassays and closely matched the substance P content of fraction A determined by radioimmunoassay (Table 1). A further indication of the identity of the bioactive principle in fraction A with substance P was obtained in experiments with tachyphylactic ileum. Contractions induced by fraction A were not altered by the addition of high doses of serotonin which abolished responses to serotonin. They were, however, completely inhibited when the gut was made tachyphylactic towards substance P or substance P plus serotonin. Contractions induced by acetylcholine used as controls, remained unchanged in all these experiments.

The second fraction obtained by chromatography on Sephadex G-10 (fraction B) caused hypotension and gut contraction. In contrast to fraction A, however, the dose response curve obtained on the rabbit blood pressure was considerably steeper than that of substance P. No further tests were carried out with this fraction B, due to the limited amount of material available.

Discussion

Histology of the Carcinoid and Peptide Content

Carcinoid tumours derived from midgut have been shown to contain serotonin and substance P-like immunoreactivity (Håkanson et al. 1977; Wilander et al. 1979) and both compounds appear to be stored within the same granules (Alumets et al. 1977). Not all tumours reported to contain substance P immunoreactivity were argentaffin (see Table 2). Wilander et al. (1979) demonstrated enteroglucagon immunoreactivity in addition to that of substance P in 7 out of 8 carcinoids. In the carcinoid under investigation no enteroglucagon immunofluorescence in tumour cells was seen, while cells of the mucosa stained positively. In the electron microscope a second cell type was observed in addition to the EC_1-cell type. This corresponded to those cells containing serotonin and substance P immunoreactivity and contained granules of different morphology. This cell type possibly represents EG cells (enteroglucagon) or EC_2 cells. The majority of the cells were, however, EC_1 cells which is in agreement with the results of the silver staining. This is also supported by the very high content

Table 2. Substance P in Carcinoid tumours

	Stomach/Duodenum	Small intestine	Large intestine
Argentaffin	2	3	1
Non-argentaffin	4	–	6
	Wilander et al. (1979)	Håkanson et al. (1977)	Wilander et al. (1977)

of immunoreactive substance P (1.6 μg/g) which exceeded the normal content of human ileum (9.6 ng/g; Skrabanek et al. 1978) by about 150 fold. The small amount of immunoreactive somatostatin measured in the tumour extract may have originated from preexisting nerve plexuses of parts of the intestinal wall that had been overgrown by the tumour. Midgut tumours have not been shown to contain somatostatin. The absence of several other peptides in the tumour suggests that this carcinoid was not multihormonal.

Authentic Substance P in the Carcinoid

Several lines of evidence strongly suggest identitiy of part of the substance P immunoreactivity found in the carcinoid tumour with the undecapeptide substance P. On Sephadex G-25 a peak of substance P immunoreactivity eluted at the position of synthetic substance P indicating a similar molecular weight. Furthermore, similar values were obtained in this peak with both antisera used, suggesting that they recogenized the same compound. After purification on Sephadex G-10, chromatography on HPLC revealed that more than 97% of the immunoreactivity behaved like substance P and substance P – sulfoxide, which was most probably generated during the extraction procedure or in the subsequent steps of repeated lyophilization. Under the condidtions used, substance P can clearly be separated from the peptides physalaemin or eledoisin, and the c-terminal substance P nona-, hepta-, and hexapeptide fragments, which cross-react from 0.1 to 100% with the antibodies used. The presence of the substance P-octapeptide was excluded on Sephadex G-25. Additional support for the presence of substance P comes from the bioassay data. Dose-response curves of the purified extract were parallel to substance P in both bioassays, the potency was very close to the content of immunoreactive substance P and, most important, specific tachyphylaxis towards substance P abolished the activity of the extract on the guinea pig ileum. Of all substances known, only the peptides physalaemin and eledoisin shown cross-tachyphylaxis with substance P (Lembeck and Fischer 1967). Their presence in the extract could, however, be ruled out from the HPLC data. Lembeck (1954) has reported the presence of a gut-contracting substance in addition to serotonin in a carcinoid tumour. The Rf value of 0.40–0.46 which he measured in a paper chromatogram for this unknown peptide is identical with that of synthetic substance P (0.43 ± 0.01, $n=9$) chromatographed under identical conditions. Taken all these results together, there remains little doublt that carcinoid tumours produce substance P identical with the undecapeptide isolated from hypothalamus and intestine.

Coexistence of substance P and serotonin has also been observed in neurones of the central nervous system (Hökfelt et al. 1978). A possible function of substance P in these neurones could be regulation of the release of serotonin (Reubi 1980). This function may also occur in carcinoid tumours. In addition, release of substance P from carcinoids into the blood could account for some of the clinical symptoms observed.

A substance cross-reacting in the substance P assay and therefore possibly related to substance P was also found in the tumour extract. The nature of this compound is unknown. It is unlikely that it represents a substance P fragment since the highly cross-reacting c-terminal octapeptide elutes earlier in the chromatogram and shorter fragments exhibit less than 5% crossreaction. Interestingly, a compound with similar chromatographic characteristics was also found in an extract from rat ileum using the antibody Rd 2 (Holzer et al. 1980). The large amount of the cross-reacting compound, best detected with the antibody f 2, shows once more that immunohistochemical and radioimmunoassay data have to be carefully interpreted. Immunoreactive material should be characterized, particularly when dealing with tumour tissue.

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