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Substance P and 5-HT in Granules Isolated from an Intestinal Argentaffin Carcinoid

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Summary. Intestinal argentaffin carcinoids, thought to originate from enterochromaffin cells, occasionally contain large amounts of substance P-like immunoreactivity in addition to 5-HT. The cytoplasmic granules of one such tumour were isolated. The granules, which in the electron microscope were shown to be argentaffin, contained both substance P-like immunoreactivity and 5-HT. The results support the view that substance P is localized in a population of enterochromaffin cells where it is stored in the cytoplasmic granules together with 5-HT.

Introduction

The gut is a rich source of substance P which occurs in intrinsic nerves as well as in endocrine-like cells (Nilsson et al., 1975a; Pearse and Polak, 1975). Several observations (recently reviewed by Heitz et al., 1976; Sundler et al., 1977) seem to favour the view that the endocrine-like cells harbouring substance P belong to the enterochromaffin cell system. Particularly pertinent is the presence of substance P-like immunoreactivity in certain intestinal argentaffin carcinoid tumours (Håkanson et al., 1977). These tumours are thought to be derived from the enterochromaffin cell system which is the main site of production and storage of 5-HT. The amine, which is thought to be responsible for the argentaffin and chromaffin reactions (see Vialli, 1966; Vialli and Prenna, 1969; Håkanson et al., 1971), is stored in cytoplasmic granules similar to granules in peptide hormone-secreting cells (cf. Owman et al., 1973). It is a common finding that the granules in such cells store the peptide hormone together with an amine and it seems unlikely that the enterochromaffin cell should represent an exception. In the present study we have isolated the cytoplasmic granules of a carcinoid tumour and shown them to contain both substance P and 5-HT.

Materials and Methods

Case History. Male, 40 years old, with epigastric pains since 1962 was admitted to the Department of Surgery, University Hospital, Lund, in 1976 because of flushing, diarrhea, tachycardia, anemia and greatly elevated urinary 5-HIAA. High peripheral plasma levels of substance P were found

by radioimmunoassay (see Håkanson et al., 1977). Laparotomy revealed multiple ileal tumours and large liver metastases. The tumour was tentatively diagnosed as a carcinoid upon routine histological examination. Ileocecal resection and resection of the left liver lobe were performed leaving a non-resectable large metastasis in the right liver lobe. Four months later hepatic dearterialization was performed. During the latter operation several liver metastases were enucleated; they were subjected to subcellular fractionation or processed for histochemical and ultrastructural analysis as described below.

Material. Antiserum, raised in rabbits against synthetic substance P, was kindly put to our disposal by Dr. G. Nilsson, Department of Pharmacology, Karolinska Institute, Stockholm, Sweden. The antiserum, which has been used in previous immunohistochemical studies (Nilsson et al., 1975a; Håkanson et al., 1977; Sundler et al., 1977) has been characterized by Nilsson et al. (1975b). For control experiments it was inactivated by the addition of excess antigen (10 μg synthetic substance P per ml diluted serum). Fluorescein isothiocyanate-labeled anti-rabbit IgG was purchased from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. It was used in dilution 1:20. Peroxidase-antiperoxidase complex (PAP) was obtained from Cappel Laboratories, Downington, Pa., USA and used in dilution 1:80.

Preparation of Tissue. Fresh tumour tissue (9.6 g) was minced carefully and hand-homogenized for a few min with a Potter-Elvehjem type homogenizer (Teflon pestle) in 20 volumes of 0.33 M sucrose. These and subsequent manipulations were carried out at 0° C. The homogenate was centrifuged at $1000 \times g$ for 20 min in a refrigerated centrifuge (Sorvall Superspeed RC 2-B) and the supernatant was then passed through a series of MF Millipore® filters (47 mm in diameter) with diminishing pore size: 8000, 5000, 3000, 1200 and 800 nm. The filtrate was divided into three equal portions, which were centrifuged at $40{,}000 \times g$ for 30 min. One pellet was used for determination of 5-HT (see below). The second pellet and small pieces of the tumour were fixed in a few ml of 2.5% glutaraldehyde in 0.075 M phosphate buffer (Sörensen), pH 7.2 (for electron microscopy). Most of the specimens were post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol solutions, contrasted in a mixture of 1% phosphotungstic acid and 0.5% uranyl acetate and embedded in Epon. Other pellet fragments were processed in the same way but without osmium tetroxide. Ultrathin sections were cut on an LKB Ultratome, contrasted with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope. Other tumour specimens and the third pellet were frozen to the temperature of liquid nitrogen in a mixture of propane and propylene and freeze-dried (for fluorescence and light microscopy). The freeze-dried material was fragmented and the various fragments were exposed either to formaldehyde gas (Björklund et al., 1972) or diethylpyrocarbonate vapour (Pearse et al., 1974) and embedded in paraffin.

Determination of 5-HT. The pellet was blotted dry, weighed and transferred to 5 ml of 80% aqueous acetone. After 24 h at +4° C the acetone extract was evaporated to dryness *in vacuo*, the dry residue was dissolved in 1 ml of 0.1 N HCl, made alkaline with 0.5 ml of 10% Na_2CO_3 and extracted with 15 ml of n-butanol. 5-HT was then returned to an aqueous phase by shaking with 2 ml 0.1 N HCl and determined fluorometrically after condensation with OPT according to Maickel et al. (1968).

Histochemistry. Both tumour tissue and bellets were analyzed. Paraffin sections were cut at 5 μ. The formaldehyde-fixed specimens were mounted in Entellan and examined for formaldehyde-induced fluorescence. The diethylpyrocarbonate-fixed specimens were subjected to the indirect immunofluorescence procedure (Coons, Leduc and Conolly, 1955) or the PAP procedure (Sternberger, 1974) for the demonstration of substance P-like immunoreactivity. The substance P antiserum was used in dilution 1:20 (immunofluorescence) or 1:80 (PAP staining).

In one series of experiments, sections from the diethylpyrocarbonate-fixed granule pellet were stained with the PAP procedure using active or inactivated anti-substance P serum. The staining intensity was evaluated semi-quantitatively in the following manner: Arbitrarily selected visual fields were photographed at magnification 125X (12.5X eye-piece, 10X objective) with a photo-cell-connected automatic camera. The time of exposure, which is proportional to the staining intensity, was recorded.

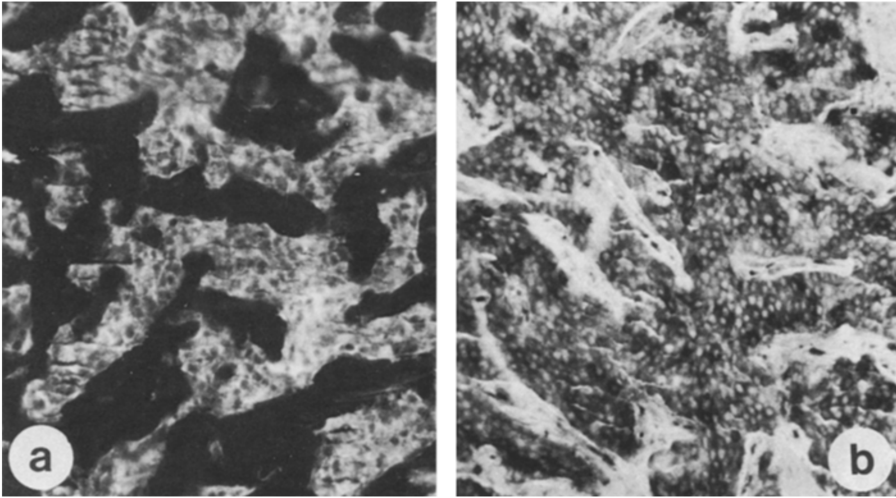

Fig. 1a and b. Carcinoid tumour. **a** Formaldehyde vapour fixation. The bulk of tumour cells exhibit intense 5-HT fluorescence. **b** Diethylpyrocarbonate fixation. Immunohistochemical staining (*PAP*) of substance P. ×150

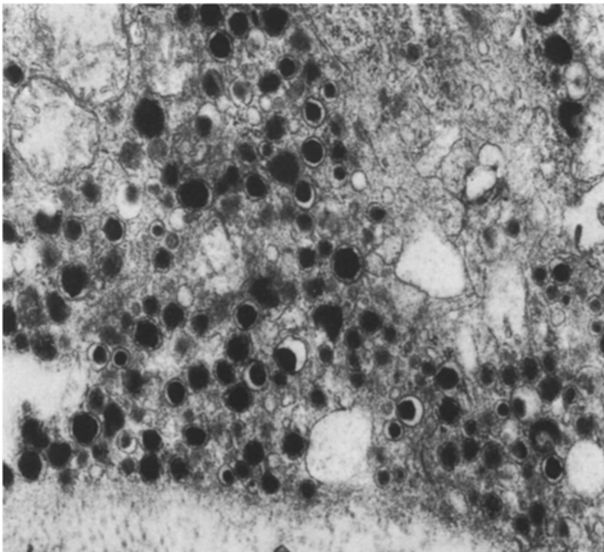

Fig. 2. Electron micrograph of carcinoid tumour cell. Formaldehyde-glutaraldehyde-osmium tetroxide fixation. Numerous pleiomorphic, highly electron-dense cytoplasmic granules. ×23,500

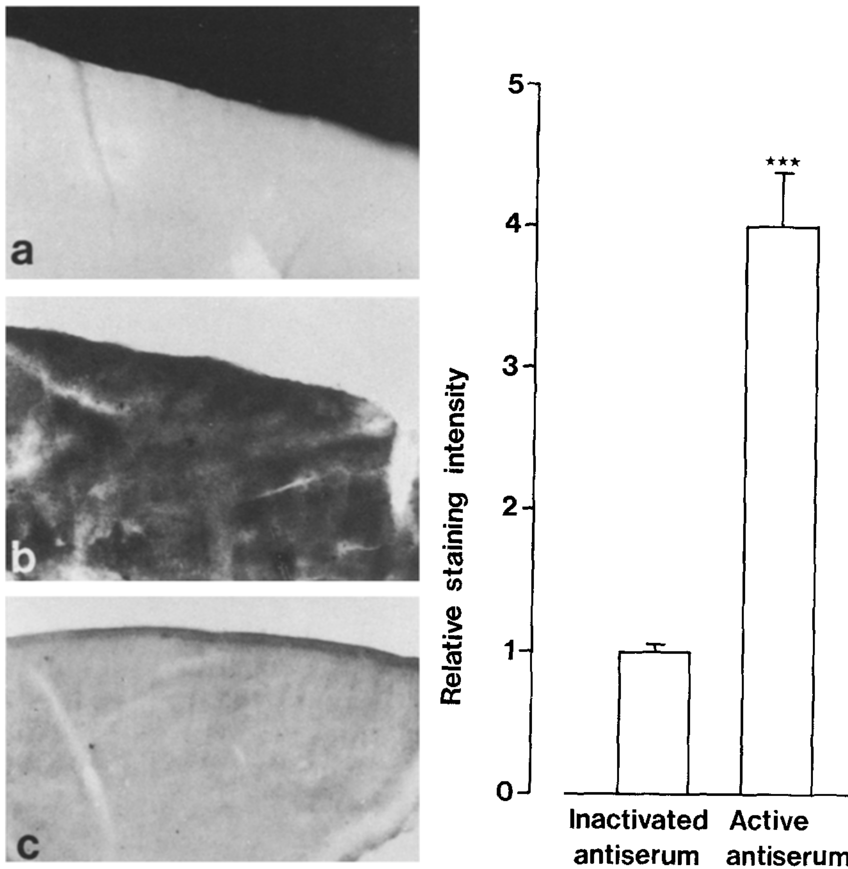

Fig. 3a–c. Granular pellet. **a** Formaldehyde vapour fixation. Intense 5-HT fluorescence. **b** and **c** Diethylpyrocarbonate fixation. PAP-staining for substance P using active anti-substance P serum (b) and inactivated antiserum (c) ($\times 150$). Panel showing intensity of PAP stain (arbitrary units) in sections from the granular pellet using active and inactivated antiserum, respectively. Each value is the mean of 10 separate recordings. Vertical bars give S.E.M. The difference is significant by $p < 0.005$ (Student's t-test). For details see text

Paraffin sections from tumour material and ultrathin sections from non-osmicated pellet fragments (placed on nickel grids) were stained with ammoniacal silver nitrate in the argentaffin procedure of Singh (1964) (cf. Håkanson et al., 1971).

Results and Comments

The bulk of tumour cells displayed substance P-like immunoreactivity of varying intensity and moderate to intense formaldehyde-induced 5-HT fluorescence (Fig. 1). As could be expected most of the tumour cells proved argentaffin. In the electron microscope, the tumour cells were characterized by the presence

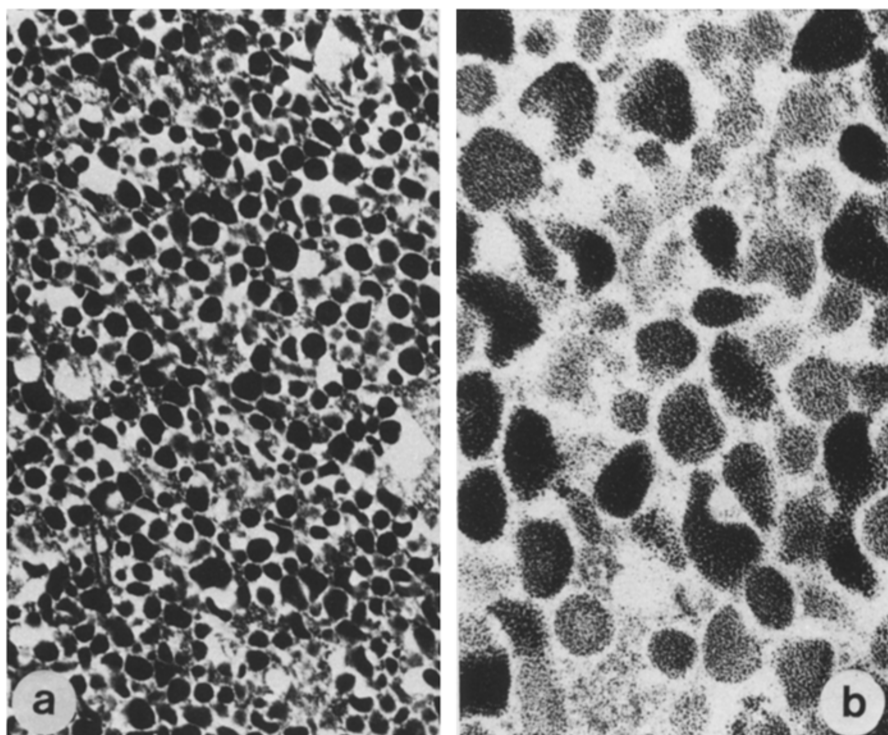

Fig. 4a and b. Granular pellet. Formaldehyde-glutaraldehyde fixation. No osmium tetroxide. **a** Conventional section-contrasting with lead citrate and uranyl acetate. Densely packed, highly electron-dense, pleiomorphic granules. ×20,000. **b** Argentaffin reaction. Silver precipitate over granules. Note varying staining intensity. No contrasting. ×63,500

of electron-dense, pleiomorphic cytoplasmic granules (Fig. 2). The granular pellet was rich in 5-HT (more than 120 µg per gram wet weight). Upon histochemical examination it was found to be homogenous, exhibiting intense formaldehyde-induced 5-HT fluorescence and moderate to strong substance P-like immunoreactivity (Fig. 3). In the electron microscope the pellet was found to consist almost exclusively of membrane-bound, pleiomorphic granules (Fig. 4a) with the appearance of enterochromaffin cell granules. All of them displayed argentaffin staining of varying intensity (Fig. 4b). We therefore conclude that both 5-HT and substance P are contained in the cytoplasmic granules of the carcinoid tumour cells. The findings of the present study support the view that substance P is produced, stored and secreted by a certain type of enterochromaffin cell and that the peptide occurs together with 5-HT in the cytoplasmic granules.

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