

Multiple Endocrine Cell Types in Thyroid Medullary Carcinoma

Evidence for Calcitonin, Somatostatin, ACTH, 5HT and Small Granule Cells*

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Summary. 10 cases of thyroid medullary carcinoma (TMC) have been studied ultrastructurally and histochemically. Well differentiated calcitonin-producing C cells were present in all tumours, being prevalent in 9 cases. 5-Hydroxytryptamine (5HT) storing cells were found in two cases, somatostatin immunoreactive cells in at least 5 cases and ACTH-immunoreactive cells in 4 cases. Ultrastructurally, at least 3 types of apparently non-C cells were observed. Type 1 cells with large, poorly osmiophilic granules resembling those of gastroenteropancreatic D cells, were present in 6 cases; they appeared to correlate well with somatostatin immunoreactive cells. Type 2 cells with large osmiophilic granules were found in 5 cases; they resembled ACTH-MSH cells of the human pituitary and may correspond to the ACTH-immunoreactive cells of light microscopy. Type 3 cells with small granules and an unknown function were found in 6 cases, always in scarce number. It is concluded that TMC, although mainly made up of C cells, usually contains large proportions of other endocrine cell types.

Key words: Thyroid medullary carcinoma – Calcitonin cells – Somatostatin cells – ACTH cells – 5HT cells.

Introduction

The discovery that thyroid medullary carcinoma (TMC) produces calcitonin (Cunliffe et al., 1968; Melvin and Tashjian, 1968) and reproduces histochemical and ultrastructural findings of thyroid C cells (Williams, 1966; Gonzales-Licea et al., 1968; Meyer and Abdel-Bari, 1968; Bussolati et al., 1969; Solcia et al.,

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1970) lead many Authors to conclude that this tumour is to be interpreted as a C cell neoplasm. However, besides calcitonin, other humoral agents are known to be produced by the tumour, including ACTH (Donahower et al., 1968; Williams et al., 1968b), corticotropin-releasing factor (CRF) (Birkenhäger et al., 1976), prolactin production-stimulating material (Birkenhäger et al., 1976), 5-hydroxytryptamine (Moertel et al., 1965), prostaglandins (Williams et al., 1968a), kallikrein (Williams et al., 1968a) and histaminase (Baylin et al., 1970). Moreover, histochemical and ultrastructural findings reported in occasional tumors (Ljungberg, 1970; Bordi et al., 1972; Bussolati et al., 1973) suggest that more than one cell type might be present in such tumours.

In this paper we have systematically investigated the histochemical and ultrastructural patterns of 10 TMCs. Ultrastructural and/or histochemical evidence for the presence of other endocrine cell types, besides C cells, has been gained in 9 cases.

Material and Methods

Specimens of 10 thyroid tumours showing histological patterns diagnostic for TMC were studied. The main clinico-pathological findings of the 10 cases are reported in Table 1.

For *light microscopy* investigations tumour specimens were fixed with 10% formol, 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3, Bouin's fluid or Zamboni's buffered picric acid-formaldehyde mixture. Paraffin sections were stained with haematoxylin-eosin, Congo red, Grime-lius' silver (1968), lead haematoxylin with and without mild acid hydrolysis (Solcia et al., 1969a), HCl-toluidine blue (masked metachromasia) technique (Solcia et al., 1968), the Masson-Fontana argentaffin reaction and diazonium, xanthydrol and yellow fluorescence reactions for 5-hydroxytryptamine (Solcia et al., 1969b). Samples from tumour 8 were frozen in liquid nitrogen, then freeze-dried with a thermoelectric apparatus (Edwards-Pearse), fixed with formaldehyde vapours according to the technique of Falk and Owman (1965) and embedded in paraffin. Sections were examined with a Leitz Orthoplan fluorescence microscope equipped with an XBO75 lamp and Ploem illuminator; fluorescent cells were analyzed with a microspectrofluorimeter (by Dr. G. Prenna, Histochemistry Center of the National Research Council, Pavia, Italy) and the emission curve recorded.

An indirect immunofluorescence procedure (Coons et al., 1955) using rabbit anti-human calcitonin serum (from Dr. Mary Clark, Royal Postgraduate Medical School, London), rabbit anti-somatostatin serum (from Drs. L. Orci and M.P. Dubois; see Rufener et al., 1975) or rabbit anti-ACTH serum (Wellcome) followed by goat antirabbit γ -globulin serum (Hyland) or pig antirabbit γ -globulin serum (Dakopatts) has been applied to paraformaldehyde- Bouin- or Zamboni-fixed sections. Controls were performed a) by absorbing the specific antihormone sera with excess purified hormone, b) by substituting non-immune serum for the specific serum, and c) by omitting the first step of the indirect test.

Other sections, after incubation with the immune serum were treated first with swine anti-rabbit serum IgG, then with peroxidase-antiperoxidase (P.A.P.) immune complex (Dakopatts, Denmark) and finally with 3,3'-diaminobenzidine- H_2O_2 mixture (Sternberger, 1974). Controls were done as for immunofluorescence.

For *electron microscopy* small specimens of tumour tissue were fixed in 2.5% glutaraldehyde or in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3. Specimens of all cases were post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Epon or in Durcupan ACM. Sections were stained with uranyl acetate and lead citrate. Some specimens of cases 1, 2, 3 and 10 were cut with a Smith-Farquhar tissue sectioner (Sorvall); 100–150 nm sections were stained by the Masson-Fontana and Grimelius silver techniques (Vassallo et al., 1971a). They were then dehydrated and embedded in Epon. Sections of silver impregnated blocks were observed in the electron microscope with and without uranyl acetate counterstaining.

Table 1. Clinico-pathological data of 10 thyroid medullary carcinomas

Case N.	Sex, years	Famil- iarity	Thyroid tumor(s)	Metastases	Associated tumors or changes	Functional signs
1. 43595PV	♂, 22	yes	Multiple ^a	Liver ^a L.N.	Multiple mucosal neuromas Megacolon	Severe watery diarrhea Hypokaliemia Hypertension Flushing
2. 548PV74	♀, 52	no	Single ^a	—	—	—
3. 3394VA74 and 3714VA74	♀, 43	no	Single ^a	L.N. ^a	—	—
4. 60393PR and 66761PR ^b	♂, 12	no	Single	L.N. ^a Lung Liver	Right colon di- verticuli	Watery diarrhea Cushing's
5. 79664PR	♂, 28	no	Single ^a	Bones Skin	—	Mild diarrhea Alcohol indu- ced flushing
6. 94374PR	♀, 61	yes	Multiple ^a	—	—	Mild diarrhea Alcohol indu- ced flushing
7. CC2022TO	♂, 48	no	Single ^a	L.N.	—	—
8. CC2062TO	♀, 60	yes	Single ^a	—	—	Diarrhea, cured by tumour surgery
9. CC2720TO	♀, 53	no	Single ^a	—	—	—
10. 9225VA76	♀, 61	no	Single ^a	—	—	—

L.N. = Lymph nodes

^a Material on which ultrastructural studies have been done

^b See: Bordi et al. (1972) and Bussolati et al. (1973)

Homogenates of tumour 8 were examined (by Dr. L. Valzelli, Mario Negri Research Institute, Milano) with a spectrofluorimetric method for the evaluation of 5-hydroxytryptamine (5HT) and 5-hydroxyindolacetic acid (5HIAA) (Giacalone and Valzelli, 1969).

Results

Light Microscopy

All tumours investigated showed solid patterns of growth and amyloid deposits. Solid nests or trabeculae of medium-sized polygonal cells predominated in 8 tumours; a diffuse growth of spindle cells was prominent in case 5; a paraganglioma-like pattern was present in areas of case 2; occasional microacini were noted in the lymph node metastasis of case 1. Cells reacting with Grimelius'

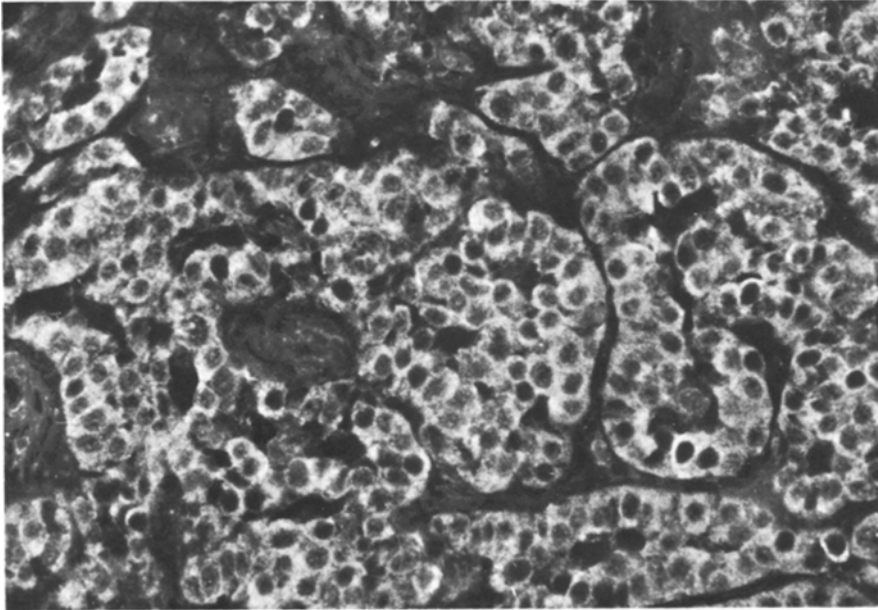


Fig. 1. Case 7: cords and nests of calcitonin cells stained with immunofluorescence. $\times 280$

Table 2. Endocrine cells identified histochemically in 10 TMCs

Case	5HT cells	Calcitonin cells	Somatostatin cells	ACTH cells
1	0	6	3	1
2	3	6	2	0
3	0	7	1	1
4	0	6	0	4
5	0	8	0	?
6	0	4	5	0
7	0	9	0	0
8	2	7	1	0
9	0	9	0	1
10	0	6	?	0

The amount of reactive cells has been graded from 10 (100% of the whole tumour cell population) to 0. 1 = less than 10%.

? Results not conclusive

silver, lead-haematoxylin or masked metachromasia techniques were found in all tumours, although various proportions of reactive cells and various degrees of reactivity were found in different tumours. Tumour cells reacting with anti-calcitonin antibodies in immunohistochemical tests were found in all cases investigated (Fig. 1). Although the proportion of reactive to non-reactive cells varied widely in different cases (Table 2), in general C cells accounted for the majority of tumour cells.

Cells reacting with antisomatostatin antibodies were found in 5 cases (Fig. 2). Sometimes they formed solid nests or cords of tumour cells; in other instances they were scattered as single elements among non-reactive tumour cells, most

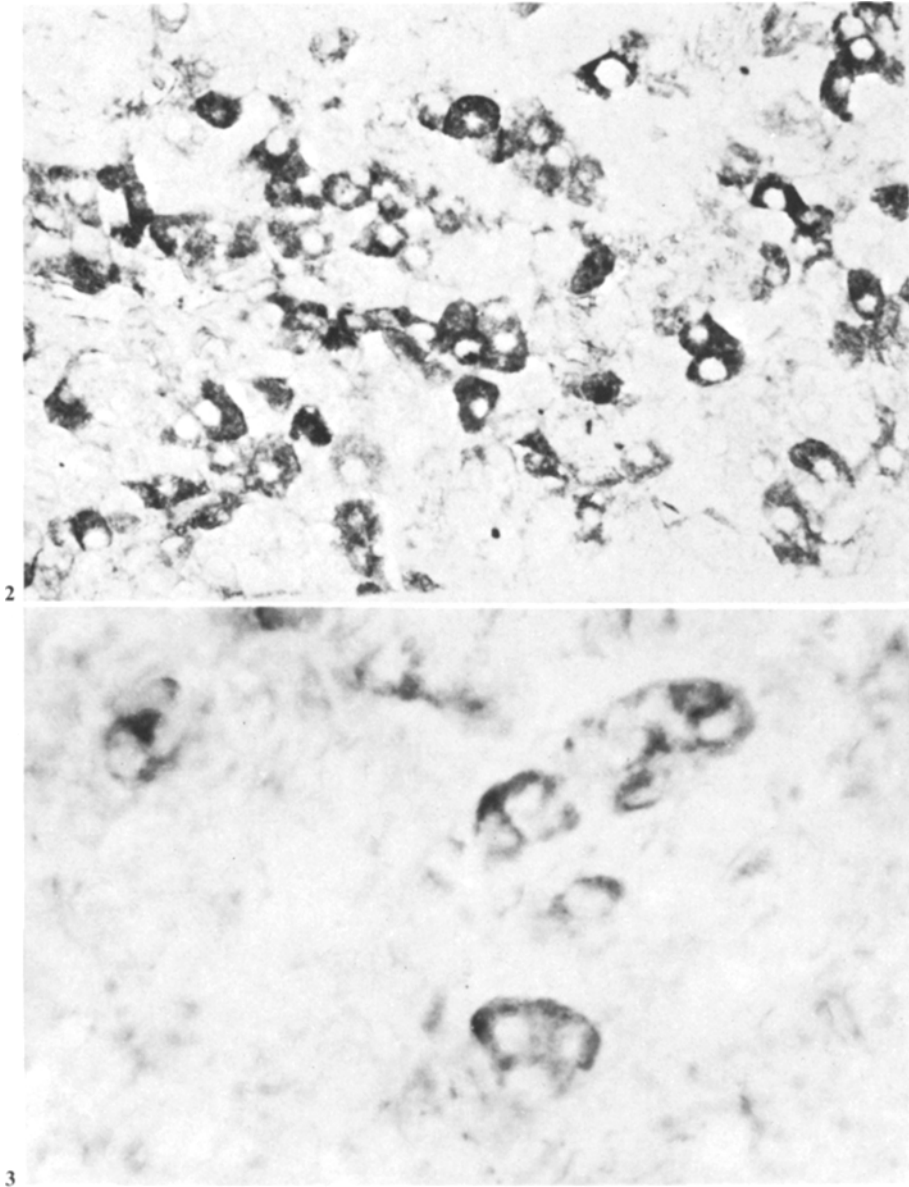


Fig. 2. Somatostatin cells scattered in tumour tissue of case 2. Immunoperoxidase. $\times 450$

Fig. 3. Case 1: groups of ACTH immunoreactive cells scattered in non reactive tumour tissue. Immunoperoxidase. $\times 700$

of which reacted with anti-calcitonin antibodies in the same or consecutive section. Cells reacting with anti ACTH antibodies were found in 4 cases. They were scattered as single cells or small groups among largely prevalent non-reactive cells (Fig. 3). They were more numerous in case 4, associated with Cushing syndrome, and proved to be distinct from calcitonin cells.

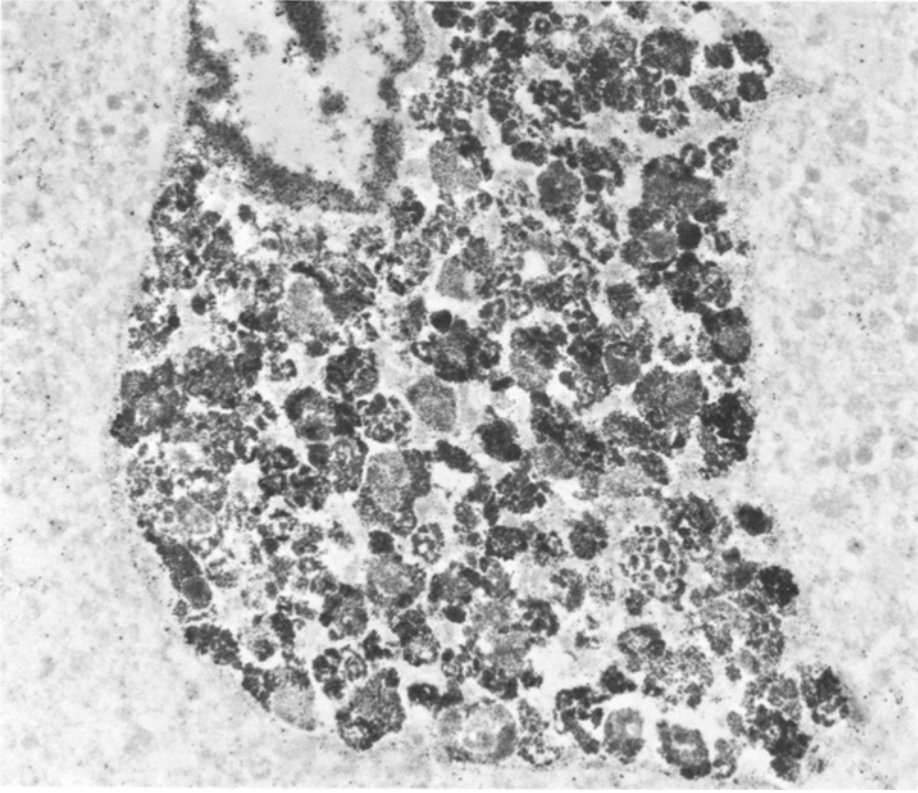


Fig. 4. Lipofuscin bodies heavily reactive with Masson's argentaffin reaction in a tumour cell of case 2. $\times 14,000$

Cells reacting with diazonium, argentaffin and formaldehyde-induced fluorescence reactions for 5HT were present in 2 cases. Microspectrofluorimetric analysis of the emission spectra of fluorescent neoplastic cells from case 8 showed a peak at $535\text{ m}\mu$, corresponding to that of 5HT. Tumour tissue from the same case was found to contain $16.2\ \gamma/\text{gr}$ of 5HT and $0.1\ \gamma/\text{gr}$ of 5HIAA. In case 2, besides weakly reactive 5HT cells, a few cells laden with heavily argentaffin and strongly fluorescent coarse granules were found; the latter cells also stained with Ziehl-Neelsen acid-fast technique while failing to react with diazonium technique. The reactivity of such cells was attributed to lipofuscins. Corresponding cells in haematoxylin-eosin sections showed yellow-brown granules in their cytoplasm.

Electron Microscopy

Most tumour cells showed endocrine granules. Poorly granular or non-granular cells occurred infrequently. Some of the latter showed well developed endoplasmic reticulum and Golgi complex, and possibly represented active tumour cells

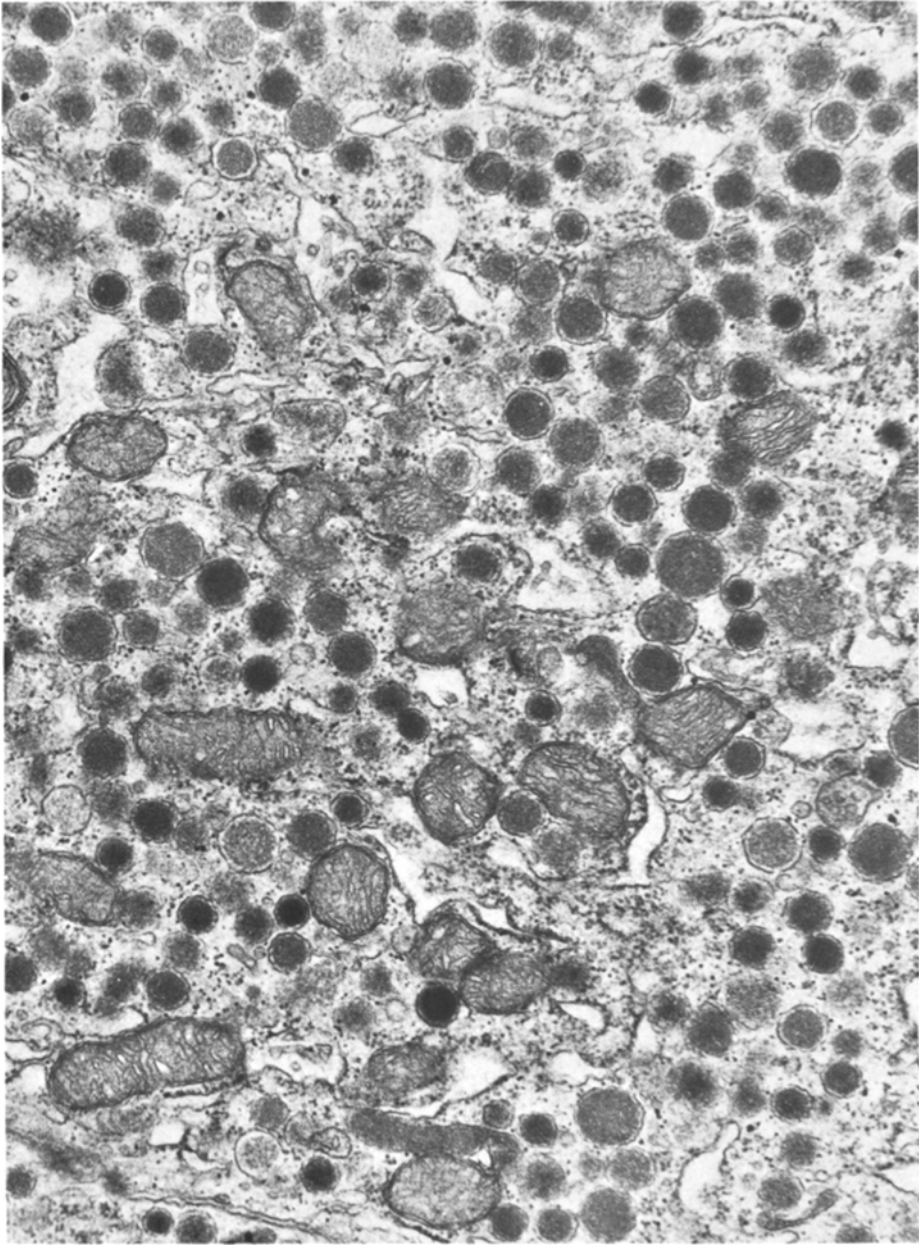


Fig. 5. C cell granules of case 3. $\times 28,000$

with defective hormone storage mechanism. Some cells of case 2 were filled with large, irregular, osmiophilic bodies which were strongly argentaffin (Fig. 4); these were interpreted as ceroid lipofuscin bodies. No secretory granules were identified in such cells. Ceroid bodies were found occasionally in granular tumour cells.

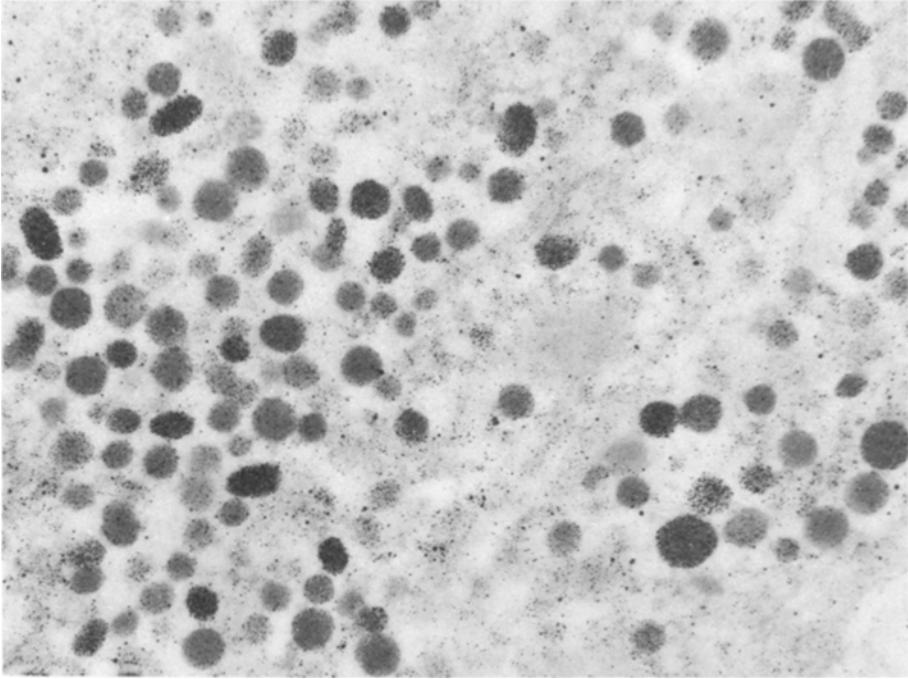


Fig. 6. Slightly argentaffin (5HT storing) secretory granules in a tumour cell of case 2. $\times 28,000$

Most tumours showed prevalence of endocrine cells filled with medium-sized (150–250 nm), moderately osmiophilic, round granules with or without a very thin clear space in between the core and the membrane; such granules showed a slight diffuse reactivity with Grimelius' silver and no reactivity with Masson's argentaffin reaction (Figs. 5, 8). These cells were considered to be calcitonin C cells, since 1) there was a close resemblance of their granule size, structure and argyrophilia to those of the normal human (Chan and Conen, 1971; Teitelbaum et al., 1971; Hill et al., 1973) or animal C cells (Vassallo et al., 1971a) and 2) there was a close correspondence with an apparently predominant population of calcitonin cells found in light microscopically examined specimens of the same tumours stained immunohistochemically.

Cells with medium-sized (197 ± 47 nm), round or slightly elongated granules with a fairly osmiophilic, intensely argyrophil and slightly argentaffin content enveloped by an evident membrane, were numerous in case 2 (Figs. 6, 7). Owing to their argentaffinity, these cells were interpreted as the ultrastructural equivalent of the 5HT-storing cells observed in light microscopy specimens. Their granules resembled those of C cells in size but were more osmiophilic and argyrophil. Similar cells were found by conventional electron microscopy of case 8, also showing 5HT cells in light microscopy specimens.

In all tumours, some cells were found whose granules were so different from C cells with regard to size, shape, osmiophilia, inner structure or silver reactivity, as to suggest their separation from C cells, even allowing for some

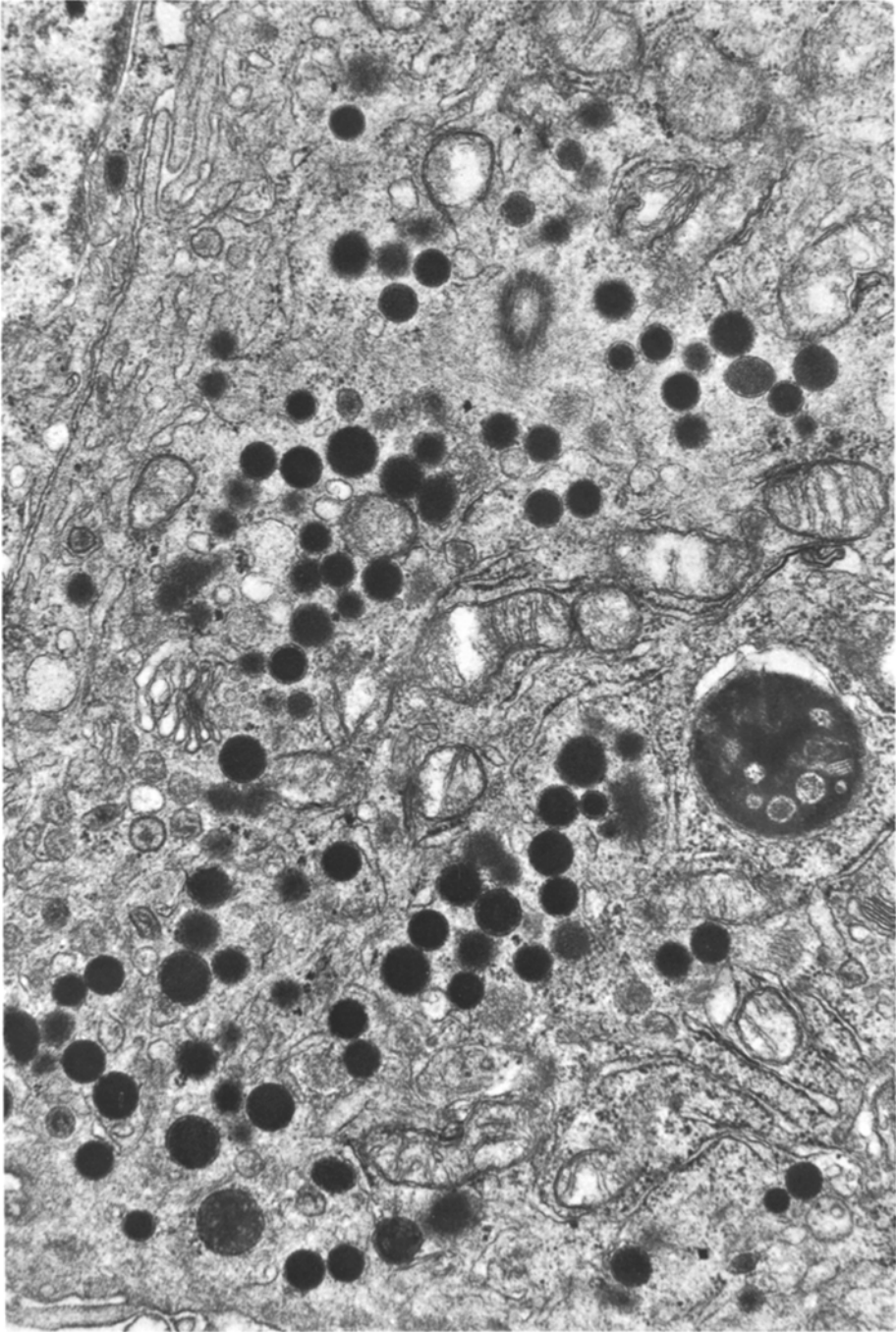


Fig. 7. Case 2: conventional electron microscopy of a cell likely corresponding to the argentaffin cell of fig. 6. $\times 28,000$

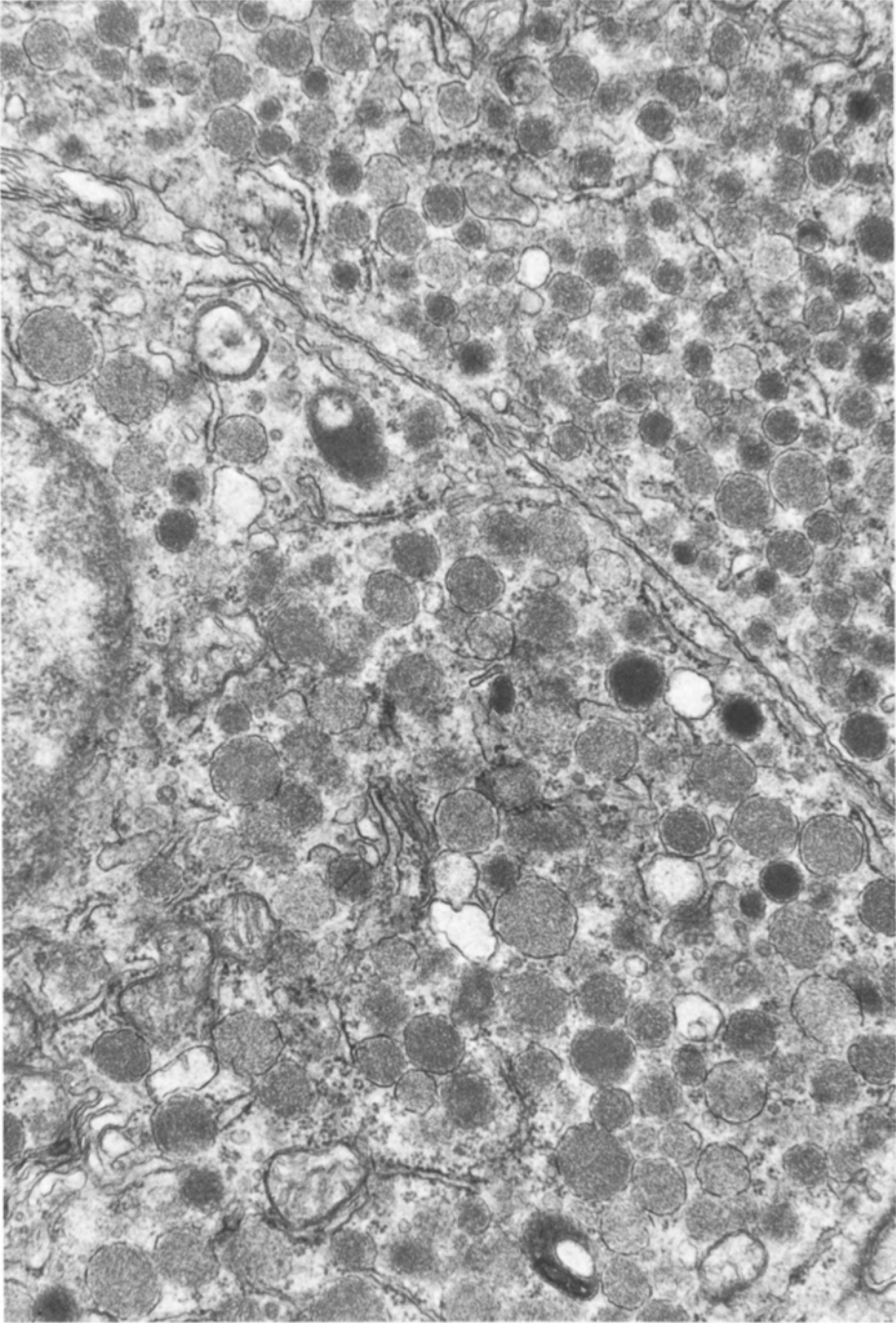


Fig. 8. Large granules of a type 1 cell (D cell?) and medium-sized granules of a C cell (top right) in tumour case 3. $\times 28,000$

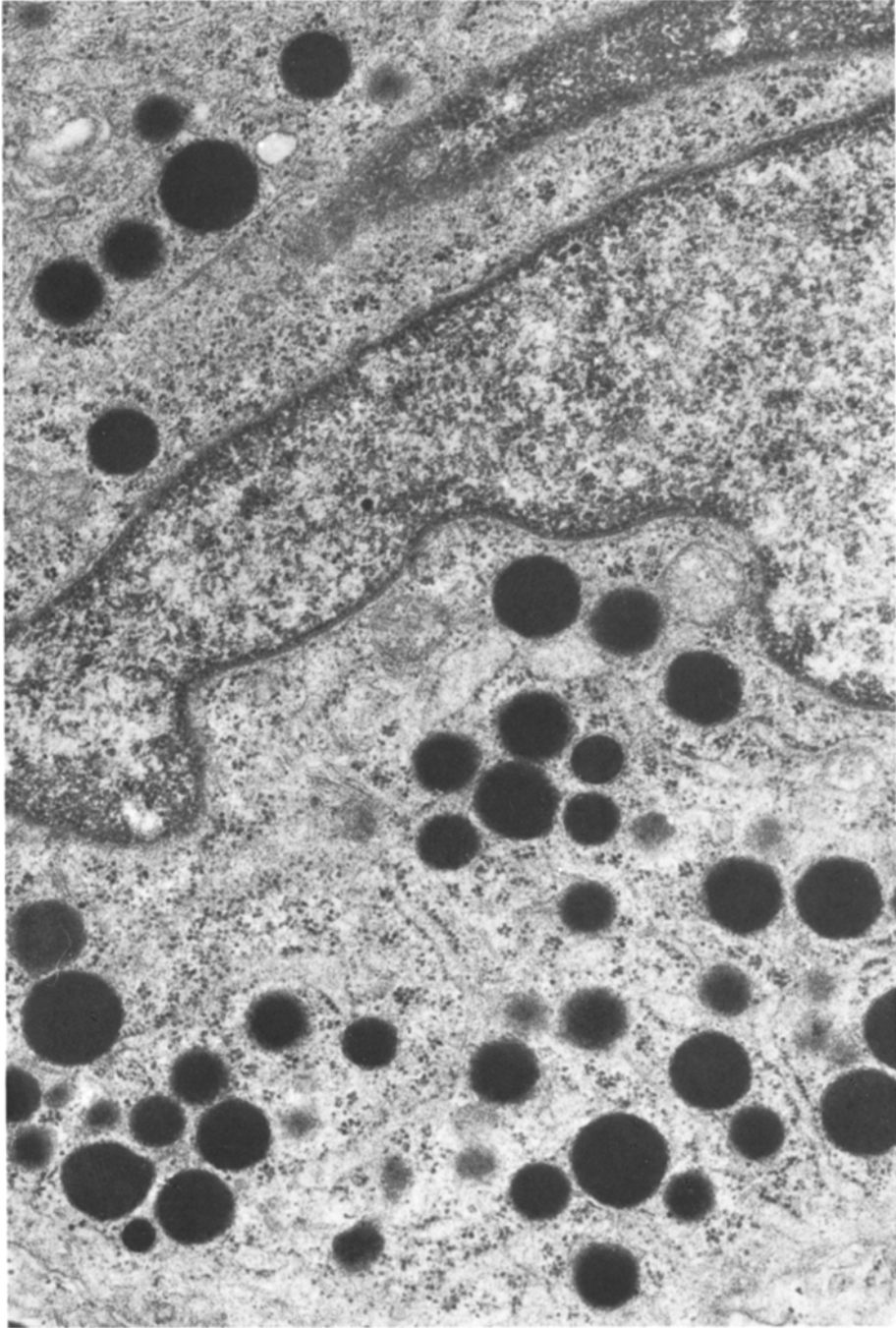


Fig. 9. Type 2 cell (ACTH-MSH cell?) in tumour 4. $\times 30,000$

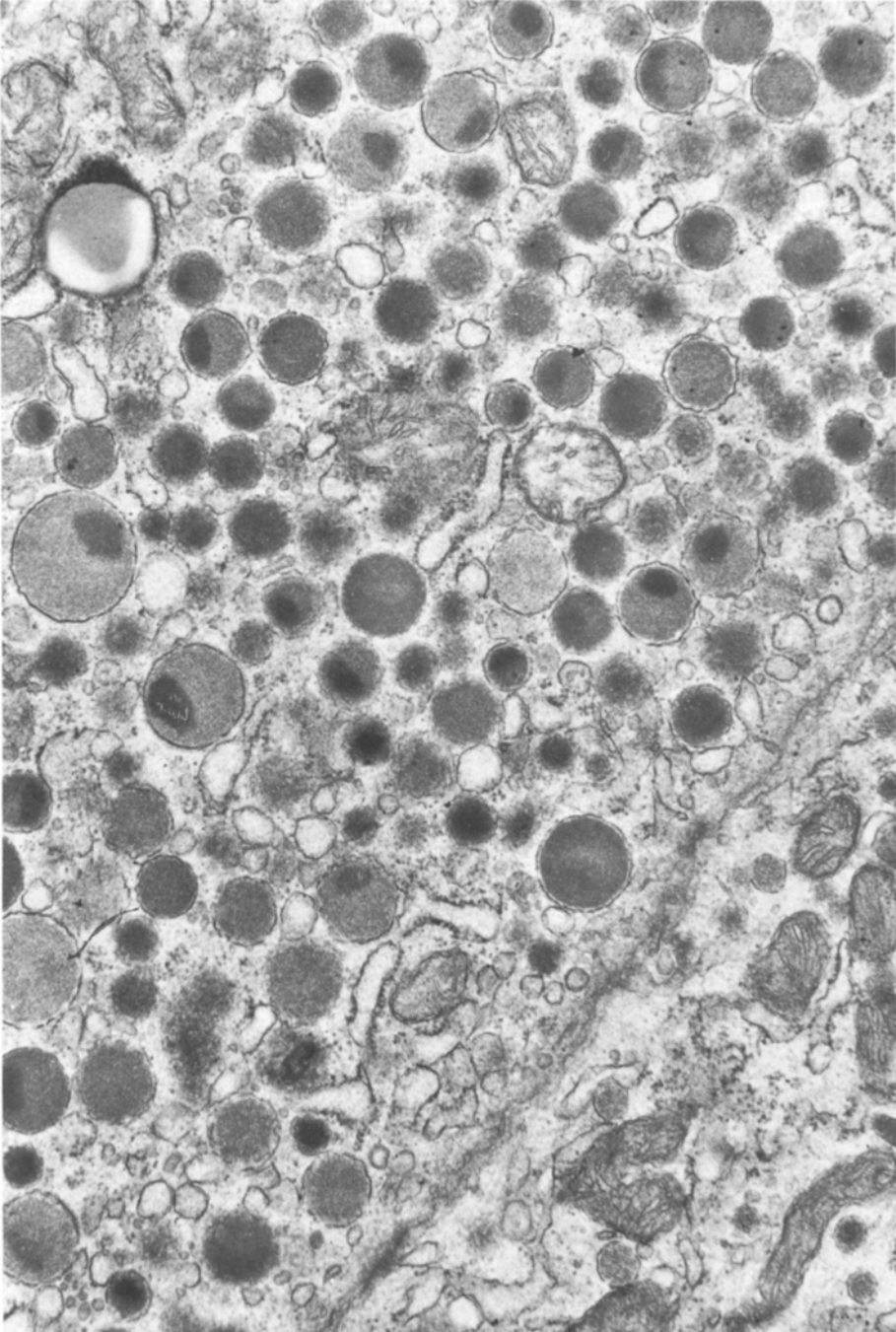


Fig. 10. Case 3: type 2 cell granules with inner osmiophilic bodies and part of a type 3 cell with few small granules in the lower right corner. $\times 28,000$

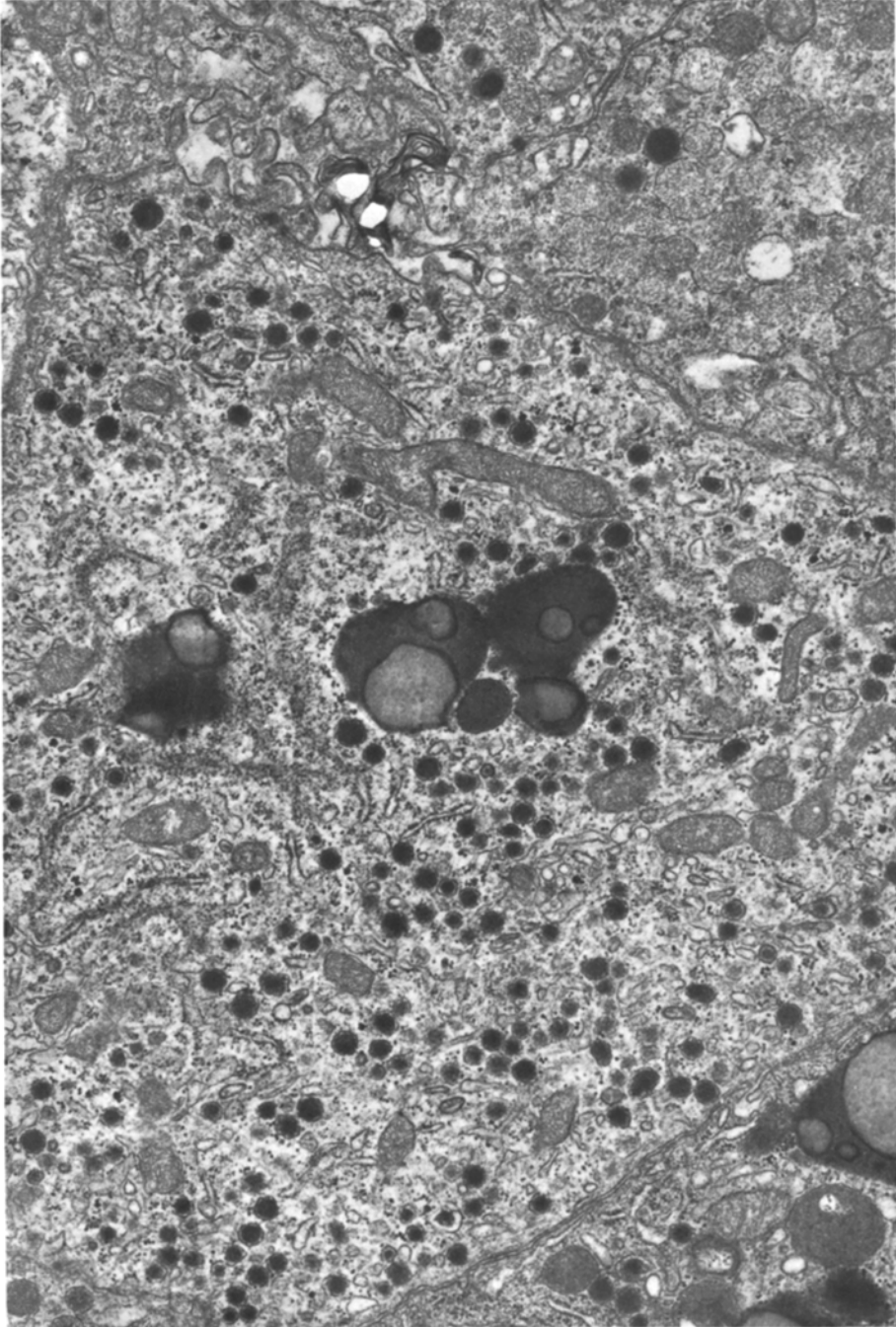


Fig. 11. Type 3 cell with small granules; type 1 cell with large granules in the top right corner. Case 6. $\times 30,000$

polymorphism, occasionally shown by tumour C cells. Among these non-C cells of TMC, cells with large poorly osmiophilic granules (type 1), cells with large dense granules (type 2) and cells with small granules (type 3) were distinguished. *Type 1 cells* showing large (250–350 nm), poorly dense, granules with tightly fitting membrane were found in 6 cases, being abundant in 3 cases (Figs. 8, 11). They closely resembled the D cells of human gastrointestinal mucosa and pancreas (Vassallo et al., 1971 b and 1972), known to store somatostatin (Polak et al., 1975; Rufener et al., 1975; Canese and Bussolati, 1977). As a whole the number and distribution of type 1 cells corresponded well with those of somatostatin immunoreactive cells.

Type 2 cells with large (200–350 nm), electron dense, round to fairly irregular granules were found in 5 cases (Fig. 9). Their number and distribution corresponded roughly to those of ACTH immunoreactive cells; they resembled ultrastructurally the ACTH-MSH cells of the human pituitary (Solcia et al., 1977). Large granules of case 5 and some of case 3 showed double inner structure with a dense body immersed in a less dense matrix (Fig. 10).

Type 3 cells with small granules (100–150 nm) occurred in 6 tumours, but were always scarce. Their fairly argyrophil non-argentaffin granules often showed a thin halo of less osmiophilic material in-between the core and the enveloping membrane (Figs. 10, 11). The cytoplasm showed well developed Golgi complex and reticulum, filaments and microtubules.

As a whole, amyloid deposits were more frequently and intimately associated with C cells than with “non-C” cells.

Discussion

In keeping with previous histochemical (Bussolati et al., 1969; Solcia et al., 1970) and ultrastructural findings (Gonzales-Licea et al., 1968; Meyer and Abdel-Bari, 1968) our results show that calcitonin C cells are a prominent and distinctive feature of TMC. In our opinion the histochemical and ultrastructural demonstration of C cells should be considered a fundamental diagnostic tool for TMC, to be added whenever possible to the study of histological structure, amyloid deposits and reactivity with granule staining techniques like Grimelius' silver, lead haematoxylin or masked metachromasia.

The histochemical findings described above suggest the occurrence of at least two types of non-C endocrine cells in TMC, storing somatostatin and ACTH or closely related molecules. Ultrastructural findings suggest the presence of at least three types of endocrine cells likely to be interpreted as non-C cells: type 1 cells, probably corresponding to somatostatin immunoreactive cells, type 2 cells possibly corresponding to ACTH immunoreactive cells, and type 3 cells with no histochemical counterpart. In contrast with C cells, not one of these cells was represented in all cases studied. In some instances they accounted for a small minority of the whole tumour cell population; in case 7 they were absent. However, as a whole they formed an important tumour cell population in at least half of the cases investigated.

The identity of type 1 cells and somatostatin immunoreactive cells is suggested by their parallel distribution in different tumours and by the close

ultrastructural similarity of type 1 cells with the D cells of the gut (Vassallo et al., 1971 b) which are known to store somatostatin (Rufener et al., 1975; Canese and Bussolati, 1977). Moreover, somatostatin immunoreactive cells have been detected in the rat (Hökfelt et al., 1975) and rabbit thyroid (Fontana et al., 1977) and cells resembling D cells ultrastructurally have been found in the rabbit thyroid (Fontana et al., 1977). It seems noteworthy that the largest number of somatostatin immunoreactive cells and type 1 cells were found in two multiple tumours with familiar incidence (cases 1 and 6). No distinctive functional signs were noticed in patients bearing such tumours.

Type 2 cells have been found in 5 cases. They resembled ultrastructurally the ACTH-MSH cells of the human pituitary and pituitary adenomas associated with Cushing syndrome (Landolt, 1975; Racadot et al., 1975; Solcia et al., 1977). These cells are probably related to ACTH immunoreactive cells, detected histochemically in at least 4 of the 5 TMCs. Only one of our TMCs showed large numbers of both ACTH immunoreactive cells (case 4; see also Bussolati et al., 1973) and cells resembling ultrastructurally the ACTH-MSH cells; it seems interesting that only this tumour was associated with Cushing syndrome. According to a recent review (Bordi et al., 1976), Cushing syndrome has been reported in only 30 cases of TMC; however, increased plasma levels of cortisol have been found in several TMC patients lacking the Cushing syndrome (Hill et al., 1973). These patients may well correspond to our cases with low numbers of ACTH cells in tumour sections and no Cushing syndrome. Moreover, some TMC may produce biologically inactive ACTH immunoreactive peptides, as found in a recent case (Birkenhäger et al., 1976).

Type 3 cells we found in TMC resembled, ultrastructurally, some cells with small haloed granules described in human bronchial mucosa (Bensch et al., 1965a; Lauweryns et al., 1970; Capella et al., 1977a) and many bronchial carcinoids (Bensch et al., 1965b; Hage, 1973; Gabrielli et al., 1977), in human carotid body and some related tumours (Capella and Solcia, 1971; Capella et al., 1977b) and in human adenohypophysis and some related tumours (Solcia et al., 1977). The function of such cells remains obscure. Comparable cells were present in a thyroid tumour—interpreted as a non-chromaffin paraganglioma—which lacked amyloid and C cells and was associated with bilateral carotid body tumours (Haegert et al., 1974).

The endocrine cells storing 5HT we found in two TMCs, must be distinguished from heavily argentaffin cells filled with lipofuscin granules and lacking secretory granules, also observed in one such TMC. The latter cells might correspond to the heavily argentaffin and chromaffin cells described by Ljungberg (1970, 1972) in TMC and non-tumour thyroid tissue of TMC bearing patients; similar cells have been found in some carotid body tumours (Grimley and Glenner, 1967; Capella and Solcia, 1971). Despite the presence of 5HT-storing endocrine cells we failed to detect in the two TMCs cells which fully reproduced the ultrastructure of enterochromaffin (EC) cells, the usual 5HT cells of the gut and gut related tissues (Solcia et al., 1975).

Besides calcitonin, C cells of the sheep, goat, horse and bat store 5HT (Falck et al., 1964; Solcia and Sampietro, 1968; Gershon and Nunez, 1970). Although C cells of human non-tumour thyroid lack 5HT (Solcia and Sampietro,

1968), the C cell nature of 5HT-storing cells in our two TMCs cannot be entirely ruled out. Secretion of 5HT by these tumour cells might contribute to the diarrhea and flushing found in some TMC patients. It seems noteworthy that the thyroid tumour reported by Horwath et al., (1972), which showed cells reproducing the ultrastructure of gut EC cells, apparently lacked amyloid and C cells and showed many glycogen-rich non-endocrine cells resembling those of ultimobranchial cell rests in non-tumour thyroid (Stoeckel and Porte, 1970).

An heterogeneous population of granular cells has been found by De Lellis et al. (1977) on ultrastructural examination of human thyroids with "C cell hyperplasia". Cells resembling our C, type 1 and type 3 cells were shown in the micrographs. However, only two types of cells were recognized by the authors, who interpreted them as two variants of calcitonin cells; they did not investigate the occurrence of somatostatin cells, which have been recently detected in the human thyroid by Yamada et al. (1977).

In conclusion, although the presence of C cells is to be considered as the most distinctive feature of TMC, non-C cells occur often and may be so important both quantitatively and functionally that to consider TMC merely as a "calcitoninoma" or "C cell tumour" seems an oversimplification of the matter. The more comprehensive and less committal term "thyroid medullary carcinoma" seems preferable in many cases.

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