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Specific Methods for Detection of 5-Hydroxytryptamine in Carcinoid Tumors*

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Summary. The nature of the chromogenic material occurring in formalin-fixed biopsy specimens of carcinoid tumors has been studied by histochemical and microspectrofluorimetric methods. The most useful property of this material appears to be its yellow fluorescence which can be intensified by treating sections with formaldehyde gas, abolished by sodium borohydride reduction, and regenerated by subsequent formaldehyde gas treatment. This and other properties indicate that the chromogenic material is a mixture of non-fluorescent tetrahydro- and fluorescent dihydro-β-carbolines resulting from the condensation of 5-HT with formaldehyde. The borohydride-formaldehyde gas sequence provides a convenient technique for the demonstration of 5-HT in formalin-fixed biopsy specimens processed by routine histological methods. The fluorescence method for monoamines developed by Hillarp, Falck and coworkers requires fresh material for immediate freezedrying; it is however, more sensitive and should be used in preference when such material is available,

Zusammenfassung. Spezifische Methoden für die Bestimmung von 5-Hydroxytryptamin in Carcinoidtumoren.

Die Beschaffenheit des pigmentbildenden Materials, welches in Formalin-fixierten Biopsiematerial von Carcinoidtumoren vorkommt, wurde mit histochemischen und mikrospektrofluorometrischen Methoden untersucht. Die am meisten brauchbare Eigenschaft dieses Materials besteht in der gelben Fluorescenz, welche durch Behandlung der Schnitte mit Formaldehydgas gesteigert, durch Natrium-Bor-Wasserstoff-Reduktion aufgehoben und durch eine anschließende Formaldehydbedampfung wieder hergestellt werden kann. Diese und andere Eigenschaften ergeben, daß das pigmentbildende Material eine Mischung aus nicht fluorescierenden Tetrahydro- und fluorescierenden Dihydro-β-Carbolinen darstellt, die auf einer Kondensation von 5-Hydroxytryptamin mit Formaldehyd beruht. Die Borwasserstoff-Formaldehydbedampfungsmethode ist eine brauchbare Technik für die Demonstration von 5-Hydroxytryptamin in Formalin-fixierten Biopsienmaterial, welches mit den Routinemethoden der histologischen Technik bearbeitet worden ist. Die von Hillarp, Falck *et. al.* entwickelte Fluorescenz-Methode zum Nachweis von Monoaminen, welche lebensfrisches Material für die Gefriertrocknung erfordert, ist jedoch empfindlicher und sollte dann vorgezogen werden, wenn derartiges Frischmaterial für die Untersuchung verfügbar ist.

The term carcinoid tumor is currently used as a morphologic entity to designate tumors with a characteristic histologic appearance, occurring mainly in the small intestine and appendix, and believed to be derived from enterochromaffin cells. It is now well established that these tumors have the ability of synthesizing and storing 5-hydroxytryptamine (5-HT). It is apparent, however, that carcinoid tumors may differ with respect to the biologically active substances which they

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produce, and the site of tumor origin may determine its functional characteristics (Williams and Sandler, 1963). The classical carcinoid syndrome is mainly associated with metastasizing carcinoids of the small intestine (Thorsson, 1954). Gastric carcinoids have been reported to produce histamine and large amounts of the 5-HT precursor, 5-hydroxytraptophan (5-HTP) rather than 5-HT (Oates and Sjoerdsma, 1962), and bronchial adenomas of carcinoid type may secrete 5-HTP as well as 5-HT (Sandler *et al.*, 1961). Furthermore, bronchial adenomas of carcinoid type may be associated with pluriglandular adenomatosis and Cushings disease (Williams and Celestin, 1962).

The morphologic carcinoid spectrum may thus include tumors with the ability of synthesizing and storing of 5-HT, tumors with 5-HT synthesizing but limited or defective storage capacity, and tumors with neither of these properties, and thus possibly unrelated to the enterochromaffin cell. On the other hand, tumors without histologic resemblance to carcinoids may be 5-HT producing, such as bronchial carcinomas of oat cell type (Williams and Azzopardi, 1960). In order to classify and assess the biological character of tumors in this heterogeneous group, sensitive and specific methods for detection of 5-HT are essential.

The purpose of the present paper was to make some observations on the chromogenic material occurring in biopsy specimens of carcinoid tumors fixed in aqueous solutions of formaldehyde with histochemical and microspectrofluorometric methods, with the object of determining if its fluorescence can be used as a basis for a simple histochemical test for 5-HT, applicable on the routine material of histopathological laboratories. The results are compared with those obtained from another fluorescence method (Falck, Hillarp *et al.*, 1962) which has a well established mechanism, and high specificity and sensitivity. Some preliminary observations on the 5-HT-content of carcinoids of various locations are included, in order to demonstrate the value of fluorescence methods for detection of 5-HT in tumors with a known or suspected content of 5-HT.

Materials and Methods

The tumor material studied included carcinoids of the small intestine, appendix, stomach, colon and rectum as well as bronchial adenomas of carcinoid type. The material consisted of paraffin blocks from surgical biopsies which had been fixed in 4% formaldehyde solution and processed by conventional histological techniques. 6 randomly chosen carcinoids of the small intestine were used for the microspectro-fluorometric and histochemical studies (see below).

In 4 intestinal carcinoids, 2 rectal carcinoids and 1 gastric carcinoid, fresh material was obtained at operation and small tissue pieces were immediately quenched in liquid propane cooled by liquid nitrogen, freeze-dried in a Speedyvac-Pearse tissue dryer, treated with formaldehyde gas and embedded in paraffin in vacuo as described previously (Enerbäck, 1966).

Borohydride Treatment (Corrodi *et al.*, 1964). Sections deparaffinized with xylene were washed thoroughly in isopropanol. Optimal results (see below) were obtained with a 0.03% solution of sodium borohydride in 90% isopropanol for 5 minutes. The sections were washed again in 100% isopropanol and some of the sections were dried in an oven at 50° C for 30 minutes followed by 3 hours' treatment with formaldehyde gas obtained from paraformaldehyde equilibrated at 70% relative humidity (Hamberger *et al.*, 1964). Sections for fluorescence microscopy were washed in xylene and mounted in a xylene-Entellan® mixture. The ninhydrin reaction (Holcenberg and Benditt, 1961) was modified as suggested by Barka and Andersson (1963). A solution of 5% ninhydrin in isopropanol was

added to the sections which were then exposed to the vapours of acetic acid followed by treatment in an incubator at 110° C for 5 minutes. The sections were washed in isopropanol and mounted in xylene-Entellan®. Schmorls ferric ferricyanide reaction and diazo coupling with Garnet GBC were performed as described previously (Enerbäck, 1965).

Fluorescence Microscopy and Microspectrofluorometry. A Zeiss fluorescence microscope for illumination with transmitted light and a Leitz Orthoplan microscope for incident light illumination were used. The activating light was obtained from an Osram HBO 200 mercury burner and was filtered through a Schott BG 12 filter (transmitted light) or a Schott BG 3 filter plus an interference band filter with maximal transmission at 404 nm and half band width of 21 nm (Schott AG, Mainz, G.F.R.). The fluorescent light was filtered through barrier filters with 50% transmission at 470 or 500 nm.

Activation and fluorescence spectra were recorded with a microspectrofluorometer (Thieme, 1966) equipped with an Osram XBO 1600 xenon burner, motor driven activation and fluorescence grating monochromators, and an X-Y recorder. The spectra were recorded from 5 μ thick sections of carcinoid tumors. The measuring area had a diameter of about 5 μ and covered the cytoplasm of one tumor cell. The activation and emission spectra were compared with those obtained from models of 5-HT. These were prepared by dissolving 5-hydroxytryptamine creatinine sulphate (0.5 mg/ml) in 1% bovine serum albumin. 1 microliter drops were placed on object slides, dried in a dessicator over phosphorus pentoxide and treated with formaldehyde gas. The spectral data given are uncorrected instrumental values.

Results

Freeze Dried and Formaldehyde Gas Treated Specimens

The small intestinal carcinoids contained strongly fluorescent material yellow in color, and uniformly distributed in the cytoplasm of the tumor cells. The tumor stroma showed a very faint green or blue-green fluorescence. Enterochromaffin cells were numerous in the mucosal epithelium covering the tumor and showed a similar strong yellow fluorescence. The fluorescence was absent in specimens not treated with formaldehyde gas, and notably decreased after treatment with sodium borohydride. In sections treated with borohydride followed by formaldehyde gas much of the fluorescence was regained.

The gastric carcinoid and the two rectal carcinoids examined did not show any fluorescence at all. The intestinal and gastric mucosa lining the tumors, however, contained strongly fluorescent enterochromaffin cells.

Formalin Fixed Tumor Specimens

All 6 small intestinal carcinoids contained fluorescent material in the tumor cell cytoplasm of a similar color as that of the freeze-dried samples. The blue-green fluorescence of the tumor stroma and surrounding structures was however often intense, sometimes reducing the contrast in the fluorescence microscope.

Activation spectra recorded over the tumor cells showed activation maxima around 410 nm. To reduce the effect of the scatter, fluorescence spectra were read with the activation monochromator set at 400–412 nm. The resulting emission peaks varied between 510 and 520 nm. Fluorescence spectra recorded over the connective tissue stroma showed emission maxima at about 470 nm. A typical fluorescence spectrum is shown in Fig. 1. The emission and excitation spectra of the 5-HT models had a similar appearance, with activation peaks at 410 nm and emission peaks at 515–520 nm (Fig. 2).

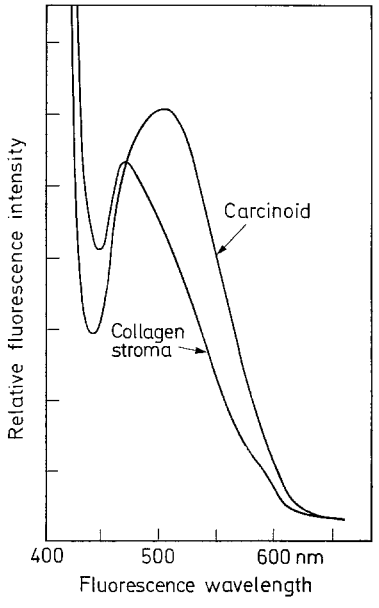

Fig. 1. Emission spectrum of a carcinoid tumor, fixed in aqueous formaldehyde and processed by routine histological procedures. Fluorescence maximum at 515 nm (uncorrected instrumental value)

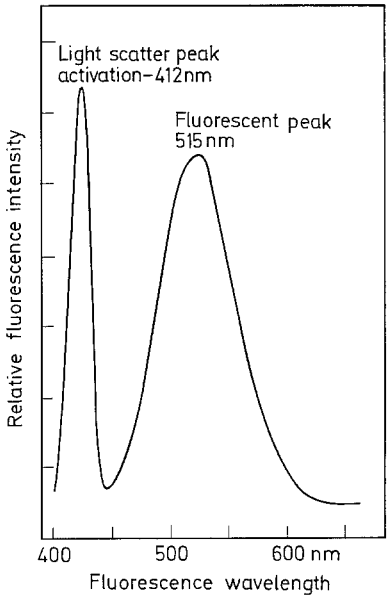

Fig. 2. Emission spectrum of 5-hydroxytryptamine in serum albumine, dried and treated with formaldehyde gas. Fluorescence maximum at 515 nm (uncorrected instrument value)

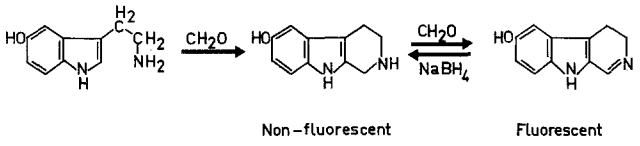

Fig. 3. The histochemical reaction between formaldehyde and 5-HT

Table 1. Effect of sodium borohydride and formaldehyde gas on the chromogenic material in carcinoid tumours. Sections from tissue blocks fixed in formalin solution

Treatment of tissue sections	Fluorescence	Ferric ferricyanide reaction	Diazo-coupling Garnet GBC	Ninhydrin reaction
None	+++	+++	++	++
$NaBH_4$ (0.03% in 90% isopropanol, 3 min)	±	+++	++	++
$NaBH_4$+CH_2O-gas (80°C, 3 hrs)	+++	+	++	±

The effects of sodium borohydride treatment and sodium borohydride followed by formaldehyde gas on the fluorescence, reducing properties, diazo coupling and ninhydrin reaction were studied with a semiquantitative grading on coded sections. The results are summarized in Table 1. Treatment with sodium borohydride reduced or abolished the fluorescence in all the examined carcinoids. The reducing property of the chromogenic material indicated by the ferric ferricyanide reaction, and the colour induced by ninhydrin were weaker or absent after treatment with formaldehyde gas in all tumors examined. The diazo coupling was unaffected by both sodium borohydride and formaldehyde gas treatment.

Comments

The formaldehyde condensation method developed by Hillarp, Falck and coworkers (Falck, 1962; Falck, Hillarp *et al.*, 1962) provides a sensitive and specific means of detecting 5-HT in tissue specimens. The method requires freeze-dried tissue samples and is based on a condensation between formaldehyde and 5-HT resulting in the formation of a strongly fluorescent compound. The mechanism of this reaction has been extensively studied and the fluorophor derived from 5-HT has been shown to be a dihydro-β-carboline (Corrodi and Hillarp, 1963, 1964). The formation of the fluorescent compound is catalyzed by protein and requires dry or almost dry conditions in order to proceed. Treatment with sodium borohydride results in the conversion of the fluorescent dihydro-β-carboline to the non-fluorescent tetrahydroderivative. The fluoresent compound can be regenerated by renewed treatment with formaldehyde gas (Corrodi *et al.*, 1964). The reaction proceeds according to Fig. 3. The spectral characteristics of the fluorescence have been studied. The fluorophor resulting from 5-HT has been

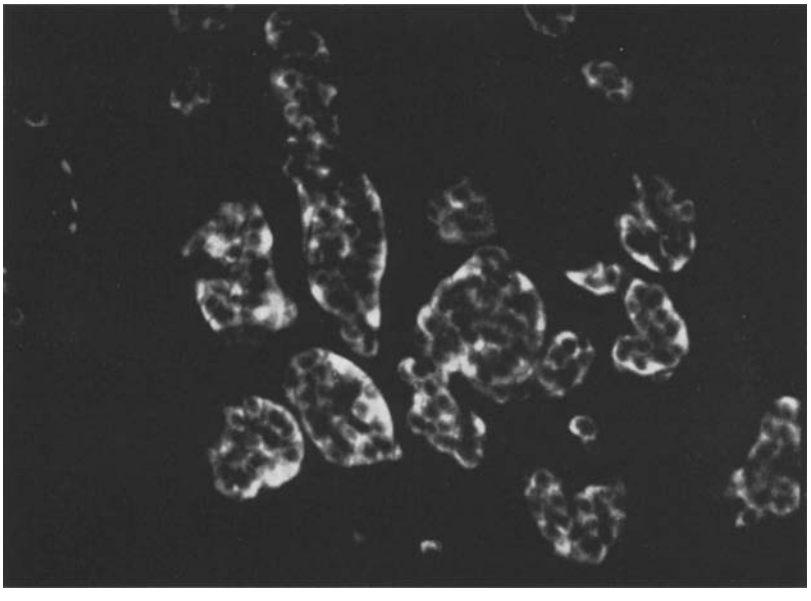

Fig. 4. Fluorescence photomicrograph of a carcinoid tumor, freeze-dried and treated with formaldehyde gas. Yellow fluorescence of the tumor cells. Magnification ×250

reported to have an activation maximum at 410 nm and an emission peak at 520 nm (corrected spectral data, Casparson *et al.*, 1966). The sensitivity of the method is high, indicated by the fact that the small amounts of 5-HT occurring in rat mast cells can be demonstrated with this method and quantitatively measured by microspectrofluorimetric techniques (Ritzén, 1967).

The finding of yellow fluorescence in the small intestinal carcinoids similar to that of enterochromaffin cells in the freeze-dried and formaldehyde gas treated tissue samples is thus indicative of the presence of 5-HT. The specificity of the fluorescence was checked by borohydride treatment and renewed treatment with formaldehyde gas. The gastric and rectal carcinoids examined did not exhibit any fluorescence, in contrast to enterochromaffin cells in the same tissue specimens. Due to the high sensitivity of the method this finding probably indicates that these tumors lacked 5-HT storing capacity. These preliminary data demonstrate the value of this method for demonstration of 5-HT in tumors. The method requires specialized technical equipment and fresh tumor material must be obtained as quickly as possible during surgery for quenching down to −170° C. The method can therefore only be used when the presence of 5-HT in the tumor is known or suspected.

The currently used histochemical tests for detection of 5-HT in carcinoid tumors are based on some simple properties of the chromogenic material occurring in the tumor cell cytoplasm in tissue specimens fixed in aqueous formaldehyde and processed by conventional histological procedures. The most popular methods appear to be the argentaffin reaction (indicating reducing properties), and diazo-coupling reactions (indicating aromatic hydroxyl groups). Such methods may

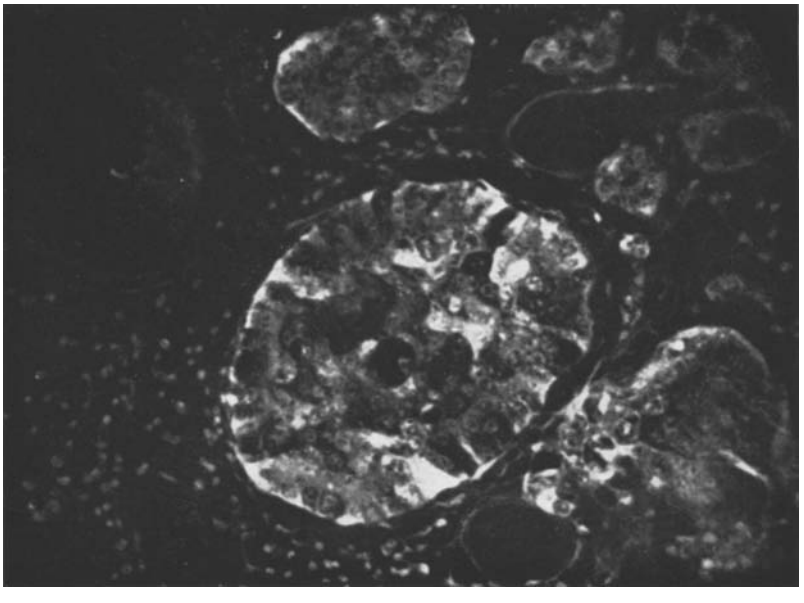

Fig. 5. Fluorescence photomicrograph of a carcinoid tumor, fixed in aqueous formaldehyde and processed by routine histological procedures. Yellow fluorescence of the tumor cells. Magnification ×250

be of assistance in classifying carcinoid tumors but more specific and sensitive methods would be disirable. The ninhydrin reaction introduced by Holcenberg and Benditt (1961) appears to be a more specific procedure than those mentioned above. These investigators performed model experiments with β-carboline derivatives and found that the organge colored compound resulting from the treatment of enterochromaffin cells with ninhydrin and acetic acid could be induced by tetrahydro-β-carboline. The model experiments indicated that the chromogenic material in enterochromaffin cells fixed in formalin solutions was of this type.

It has been known for many years that enterochromaffin cells exhibit a yellow fluorescence in formalin-fixed tissue specimens (Erös, 1932; Barter and Pearse, 1953, 1955). This fluorescence may be used as a basis for a simple diagnostic test for 5-HT applicable to the routine material of histopathological laboratories. Yellow fluorescence similar to that of enterochromaffin cells has been reported to be a constant finding in carcinoids of the appendix and small intestine (Enerbäck, 1965). The method does not seem to have come into general use, however, possibly owing to difficulties in distinguishing the fluorescence due to 5-HT from the sometimes strong autofluorescence of many proteins, such as collagen. The specificity of the fluorescence has not been determined and the nature of the chromogenic material, responsible for the fluorescence has not been established.

The finding reported here and those reported previously indicate that carcinoids of appendix and small intestine fixed in formalin solution contain yellow

fluorescence in the cytoplasm of the tumor cells. This fluorescence appears to have the same spectral characteristics as models of 5-HT in a protein layer treated with formaldehyde gas. The activation and emission spectra reasonably agree with the corrected data reported by other investigators for enterochromaffin cells, mast cells and models of 5-HT (Ritzén, 1967). The findings thus strongly indicate that the fluorophore occurring in carcinoid tumors fixed in aqueous solutions of formaldehyde and processed by conventional histological techniques is identical to that in material treated according to the Falck-Hillarp technique. The fact that the fluorescence could be extinguished by borohydride treatment and regenerated by subsequent formaldehyde gas treatment is a further indication of the specificity of the fluorescence. The background autofluorescence is often of a considerable intensity in the formalin-fixed material. This fluorescence is, however, not affected by the borohydride treatment. The ninhydrin reaction was reduced or abolished after treatment of the sections with formaldehyde gas but unaffected by the borohydride treatment. This finding is in agreement with the model experiments presented by Holcenberg and Benditt (1961), indicating that the ninhydrin reactions is specific for tetrahydro-β-carbolines.

The findings presented here and those reported previously thus indicate that formalin-fixed tumors with 5-HT storing capacity contain a chromogenic material composed of a mixture of tetrahydro- and dihydro-β-carbolines. The fluorescent dihydroderivative may be converted to the non-fluorescent tetrahydroderivative with sodium borohydride. The sodium borohydride-formaldehyde gas sequence thus provides a simple and specific means of identifying 5-HT in specimens of carcinoid tumors processed by routine histological procedures. It must be realized, however, that a diffusion of 5-HT from its storage sites probably occurs to some extent during the fixation in the aqueous solution of formaldehyde. Absence of fluorescence does therefore not exclude 5-HT storing potentials of a tumor. The method is currently used at our laboratory on all tumors showing histological structures suggesting a carcinoid nature. 30 hitherto examined carcinoids of the appendix and small intestine have all exhibited fluorescence compatible with a content of 5-HT. Of 29 examined bronchial adenomas of carcinoid type only 3 contained such fluorescent material. 4 examined carcinoids of the colon and rectum and one gastric carcinoid did not exhibit any fluorescent material.

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References

Barka, T., Andersson, P. J.: Histochemistry. Theory, practice and bibliography. New York-Evanston-London: Harper & Row 1963.

Barter, R., Pearse, A.G.E.: Detection of 5-hydroxytryptamine in mammalian enterochromaffin cells. Nature (Lond.) **172**, 810 (1953).

Barter, R., Pearse, A. G. E.: Mammalian enterochromaffin cells as the source of serotonin (5-hydroxytryptamine). J. Path. Bact. **69**, 25 (1955).

Caspersson, T., Hillarp, N. Å., Ritzén, M.: Fluorescence microspectrophotometry of cellular catecholamines and 5-hydroxytryptamine. Exp. Cell Res. **42**, 415 (1966).

Corrodi, H., Hillarp, N.- Å.: Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 1. Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6, 7-Dimethoxyisochinolinderivaten und Formaldehyd Helv. chim. Acta **46**, 2425 (1963).

Corrodi, H., Hillarp, N.- Å.: Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. Helv chim. Acta **47**, 911 (1964).

Corrodi, H., Hillarp, N.- Å., Jonsson, G.: Fluorescence methods for histochemical demonstration of monoamines. 3. Sodium borohydride reduction of the fluorescent compounds as a specificity test. J. Histochem. Cytochem. **12**, 582 (1964).

Enerbäck, L.: Mast cells in carcinoid tumours. Distribution, histochemical properties and 5-hydroxytryptamine content. Acta path. microbiol. scand. **64**, 491 (1965).

Enerbäck, L.: Mast cells in rat gastrointestinal mucosa. 4. Monoamine storing capacity. Acta path. microbiol. scand. **67**, 365 (1966).

Erös, G.: Eine neue Darstellungsmethode der sogenannten „gelben" argentaffinen Zellen des Magen-Darmtraktes. Zbl. allg. Pathol path. Zbl. Anat. **64**, 385 (1932).

Falck, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol. scand. **56**, Suppl. 197 (1962).

Falck, B., Hillarp, N.- Å., Thieme, G., Torp, A.: Fluorescence of catechol amines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. **10**, 348 (1962).

Hamberger, B., Malmfors, T., Sachs, Ch.: Standardization of paraformaldehyde and of certain procedures for histochemical demonstration of catecholamines. J. Histochem. Cytochem. **13**, 147 (1964).

Holcenberg, J., Benditt, E. P.: A new color reaction for tryptamine derivatives. Lab. Invest. **10**, 144 (1961).

Oates, J. A., Sjoerdsma, A. A.: A unique syndrome associated with secretion of 5-hydroxytryptophan by metastatic gastric carcinoids. Amer. J. Med. **32**, 333 (1962).

Ritzén, M.: Cytochemical identification and quantitation of biogenic monoamines. A microspectrofluorimetric and autoradiographic study. Thesis, Stockholm, 1967.

Sandler, M., Scheuer, P. J., Watt, P. J.: 5-Hydroxytryptophan secreting bronchial carcinoid tumour. Lancet **1961 II**, 1067.

Thieme, G.: A versatile device for microscopic spectrofluorimetry. Acta physiol. scand. **67**, 514 (1966).

Thorson, Å. H.: Studies in carcinoid disease. Acta med. scand. **161**, Suppl. 334 (1954).

Williams, E. D., Azzopardi, J. G.: Tumours of the lung and the carcinoid syndrome. Thorax **15**, 30 (1960).

Williams, E. D., Celestin, L. R.: The association of bronchial carcinoid and pluriglandular adenomatosis. Thorax **17**, 120 (1962).

Williams, E. D., Sandler, M.: The classification of carcinoid tumours. Lancet **1963 I**, 238.

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