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TRYPTAMINE OR TRYPTOPHYL-PEPTIDES IN ENDOCRINE CELLS OF RABBIT GASTRIC ANTRUM

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Abstract—The pyloric gland area of the rabbit stomach contains argyrophil epithelial cells, probably of endocrine nature, exhibiting a formaldehyde-induced fluorescence with spectral properties different from those of the fluorophores of catecholamines and 5-HT but similar to those of the tryptamine and tryptophyl-peptide fluorophores. Formaldehyde treatment in the presence of ozone markedly improved the fluorescence yield in these cells as compared with formaldehyde alone; combined formaldehyde-HCl caused only moderate improvement. These features indicated the presence of a tryptophyl-peptide rather than tryptamine in the cells. This view was further supported by the failure to demonstrate tryptamine chemically in extracts of gastric mucosa.

INTRODUCTION

THE ARGYROPHIL cells of the stomach epithelium exhibit ultrastructural features typical of endocrine cells producing polypeptide or protein hormones.[1–3] Within the system of such gastric endocrine-like cells, several distinct types have been recognized on the basis of their different staining properties as well as the characteristic and distinguishing ultrastructure of their secretory granules.[4–7] The ability to produce and store various arylethylamines appears to be a common property of the gastric endocrine cells.[6,8] Thus, the rabbit stomach wall is rich in 5-hydroxytryptamine (5-HT), contained in a large system of cells which are argyrophil as well as argentaffin and chromaffin; these enterochromaffin cells are particularly numerous in the oxyntic gland area.[8,9] They are recognized histochemically by their intense yellow formaldehyde-induced fluorescence reflecting their content of 5-HT. A very small number of morphologically similar cells, exhibiting a strong green formaldehyde-induced fluorescence (typical of catecholamines), have been found to contain dopamine.[8] In addition, another gastric endocrine-like cell type, devoid of endogenous dopamine and 5-HT, has the capacity to produce and store such amines as shown, after administration of the precursor amino acids (i.e. 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan, respectively).[8,10] These "enterochromaffin-like" cells occur in fairly large numbers throughout the gastric mucosa, predominantly in the antrum (pyloric gland area). In untreated rabbits certain cells in the antrum were found to exhibit a very weak, formaldehyde- induced green-yellowish fluorescence. Because these cells show endogenous formaldehyde-induced fluorescence they have previously been classified as enterochromaffin cells.[8] The colour of the fluorescence emitted by these latter cells after standard formaldehyde treatment was not typical of either the catecholamine or 5-HT fluorophores and they were therefore referred to as a third type of enterochromaffin cells.[8] Recently, it was shown that tryptamine[11] and tryptophyl-peptides[12] form fluorophores

with formaldehyde in a modified Falck–Hillarp reaction, and preliminary cytospectrofluorometric analysis suggested that the formaldehyde-induced fluorescence of the enterochromaffin cells Type III might be due to the presence of tryptamine or tryptophyl-peptides.[13] This possibility has been investigated more extensively in the present study.

METHODS

Animals

The material included 120 rabbits of either sex, weighing 2·2–2·5 kg, and fed a standard diet of pellets (SAN-Bolagen, Sweden), turnips and tap water *ad lib.* All animals were killed by intravenous injection of air.

Methods for fluorescence microscopy

Formaldehyde condensation procedures. Tissue specimens were taken from the pyloric sphincter region, the pyloric gland area and the oxyntic gland area along the major curvature. The specimens were immediately frozen in a propane–propylene mixture cooled with liquid nitrogen, and then freeze-dried. The freeze-dried tissue specimens were treated in either of the following ways: (a) Exposure for 1 hr to gaseous formaldehyde generated from paraformaldehyde at +80° according to the standard histochemical method of Falck and Hillarp[14–16] as described in detail by Falck and Owman.[17] (b) Exposure for 1 hr to gaseous formaldehyde in the presence of ozone. Ozone was generated in the closed reaction vessel from a Tesla coil during 5–60 min of electrical discharge.[11] (c) Exposure for 1 hr to gaseous formaldehyde in the presence of HCl vapour, 100, 200 or 500 torr (mm Hg).[18] Following either of these treatments the specimens were embedded in paraffin and sectioned at 6 μ.[17] The above treatments were also carried out with protein droplet models and with deparaffinized sections from freeze-dried, paraffin-embedded but otherwise untreated tissue specimens.

Fluorescence microscopy. The sections were mounted in Entellan (Merck) and examined and photographed in a Zeiss standard fluorescence microscope equipped with BG 12 or UG 1 (Schott) primary filters (maximum transmission at 405 and 365 nm, respectively) and OG 4 or GG 9 (Schott) secondary filters.

Preparation of protein droplet models. Human serum albumin (final concentration 2 per cent) was added to aqueous solutions of tryptamine or tryptophyl-peptides (see Results) in various concentrations (0·03, 0·1, 0·25, 1·0, 5·0 and 25 mg free base per ml). The solutions were sprayed on histological cover slips and the microdroplets were allowed to dry at room temperature. The protein droplet models were then exposed to formaldehyde gas according to the procedures listed above.

Microspectrofluorometric analysis. The characteristics of the excitation and emission spectra of the formaldehyde-induced fluorescence observed in the gastric endocrine cells and in the protein droplet models as a result of the different histochemical treatments were analysed in a modified Leitz microspectrograph.[19] For the analysis, the protein droplet models and the tissue sections were carried on cover slips. A xenon high pressure lamp (Osram XBO 150) was used for recording the excitation spectra in a quartz optical system having a barrier filter with the edge at 510 nm. Emission spectra were recorded using the 365 nm lines of a high pressure mercury lamp (Osram HBO 200 W/2) as exciting light (no barrier filter). The curves were registered with

an x–y recorder, and the excitation and emission curves were corrected as previously described.[19] The values are expressed as relative quanta versus wavelength.

Methods for chemical analysis

Extraction of tryptamine from gastric mucosa and gastric content. The animals were deprived of food but not water for 24 hr. After sacrifice the stomachs were immediately taken out and placed on ice. The gastric mucosa was removed, washed carefully with 0·9% saline until clean and then blotted on filter paper. After weighing, the mucosa (pooled from four to five rabbits, usually 40–50 g wet weight) was homogenized in 5 vol. of ice-cold aqueous acetone (80%)[20] with an Ultra-Turrax homogenizer (Janke and Kunkel KG) and left at 0° overnight. Precipitated proteins were then filtered off, washed with 2 or 3 vol. (usually 100 ml) of acidified acetone (0·1 ml 2 N hydrochloric acid per 100 ml) and discarded. The acetone extract[20] was evaporated *in vacuo* until only an aqueous residue (usually 15–20 ml) remained. This extract was defatted with 2 × 30 ml of ice-cold diethylether. The aqueous extract was then evaporated to dryness. The dry residue was taken up in 2 ml 0·01 N hydrochloric acid, 0·5 ml 5 N sodium hydroxide was added, and tryptamine was extracted with 15 ml of benzene by shaking for 5 min.[21] The benzene phase was washed with 4 ml of 0·1 N sodium hydroxide for 1 min. Tryptamine (and residual tryptophan) was then transferred into an aqueous phase by shaking the benzene with 4 ml of 0·1 N sulphuric acid for 1 min.[21] In model experiments (without tissue or tissue extract) the recovery of authentic tryptamine (1–10 μg) was found to be approximately 70 per cent with the benzene extraction procedure. The corresponding value for tryptophan (1 mg) was less than 0·2 per cent 5-HT and 5-methoxytryptamine (1–10 μg) were not recovered in measurable amounts with this extraction procedure as could be established by fluorometry.[22]

Tryptophan is considered the major contaminant in the chemical determination of tryptamine in tissue extracts.[21] Although in the model experiments only a small percentage of tryptophan was recovered with the benzene extraction procedure, the traces of this compound that remained may be critical since the tryptamine content of most tissues is extremely low[21,23,24] and the tryptophan content may be high. In addition, it has been suggested that a high lipid content of the extract may cause a higher recovery of tryptophan.[23,25] In attempts to eliminate tryptophan completely, extracted tryptamine was further separated from residual tryptophan by ion exchange chromatography. The sulphuric acid extract (see above) was passed through a Dowex 50-X4 column (200 mesh, column dimensions 13 mm^2 × 20 mm), equilibrated with 10 ml 0·2 M phosphate buffer, pH 7·0, and washed with 5 ml redistilled water. After passage of the extract (3 ml) the column was washed with 20 ml 0·1 M phosphate buffer, pH 7·0 and 5 ml redistilled water. The flow rate was 0·25 ml/min. Tryptamine was eluted with 5 ml 0·03 N ammonium hydroxide in a mixture of acetone and water (60% acetone); the eluate was then neutralized with N formic acid. After evaporation to dryness *in vacuo* and dissolution in 0·01 N hydrochloric acid the tryptamine content of the eluate was determined fluorometrically (see below). The recovery of added tryptamine was approximately 90 per cent with the ion exchange procedure. In model experiments this procedure was found to eliminate not only tryptophan but also residual 5-HT and 5-methoxytryptamine: none of these compounds were recovered in the tryptamine-containing eluate.

The capacity of this procedure (acetone extraction followed by benzene extraction

and ion exchange chromatography) to extract tryptamine selectively was tested by adding trace amounts (0·1–1·0 μc) of either tryptamine-2-^{14}C (25 mc/m-mole) or tryptophan-1-^{14}C (5 mc/m-mole) to the acetone homogenates. (All radiochemicals were purchased from New England Nuclear.) The recovery of these compounds was determined by liquid scintillation counting. Under these circumstances, the entire extraction and purification procedure was found to recover somewhat less than 1 per cent of tryptophan, while the recovery of authentic tryptamine was 30–40 per cent. By re-extracting the extract with benzene (see above) the recovery of tryptophan was reduced to about 0·1 per cent while that of tryptamine was about 20–30 per cent.

In a series of experiments (10 determinations on separate animals) the gastric content of freely fed rabbits was extracted (usually 10–15 g wet weight in each analysis) for tryptamine as described above.

Chemical assay of tryptamine. Three ml of the extracts from gastric mucosa and from gastric content were mixed with 0·3 ml 20 N phosphoric acid and 0·1 ml formalin (18%) in a stoppered glass tube, and placed in boiling water for 20 min. After adding 0·1 ml of 5% hydrogen peroxide heating was continued for 20 min. After cooling in a refrigerator for approximately 15 min, fluorescence was read in an Aminco–Bowman spectrophotofluorometer: excitation max. 390 nm, emission max. 460 nm (uncorrected instrumental values). The use of strong phosphoric acid is a modification of the original procedure of Hess and Udenfriend;[21] the fluorescence yield of authentic tryptamine is almost doubled by this modification whereas the spectral characteristics of the fluorophore are unaffected. Besides tryptamine, also tryptophan and *N*-methyltryptamine gave fluorescence with this procedure. 5-HT, 5-methoxytryptamine, melatonin, histamine, dopamine and tyramine gave no fluorescence.

RESULTS

Fluorescence microscopy

In sections from rabbit stomach specimens, treated according to the standard formaldehyde condensation technique,[17] the flask-shaped, yellow-fluorescent enterochromaffin cells were readily detectable (by excitation at 365 as well as 405 nm). Occasionally, also distinctly green-fluorescent enterochromaffin cells (best visible by excitation at 405 nm, secondary filter: OG 4) were observed. The yellow- and green-fluorescent cells have previously been identified as 5-HT and dopamine-containing, respectively.[8] Under the same histochemical and optical conditions (excitation at 365 nm), it was possible to demonstrate also a moderate number of similarly flask-shaped epithelial cells having a very faint formaldehyde-induced fluorescence, which appeared green-yellow in colour (although the faintness of the fluorescence made the colour difficult to define). These cells, which were argyrophilic (Fig. 1), were located mainly in the basal portion of the mucosa in the pyloric gland area and in the adjacent region of the oxyntic gland area, and have previously been referred to as enterochromaffin cells Type III.[8] Such weakly fluorescent cells could not be detected in any other region of the gastric mucosa.

The fluorescence intensity of the Type III cells was markedly enhanced (best seen with excitation at the 365 nm lines and with GG9 secondary filter) when the formaldehyde condensation reaction was carried out in the presence of ozone (Fig. 1). When the formaldehyde condensation of the specimens was carried out in the presence of HCl vapour, the fluorescence intensity was also enhanced, but less markedly than

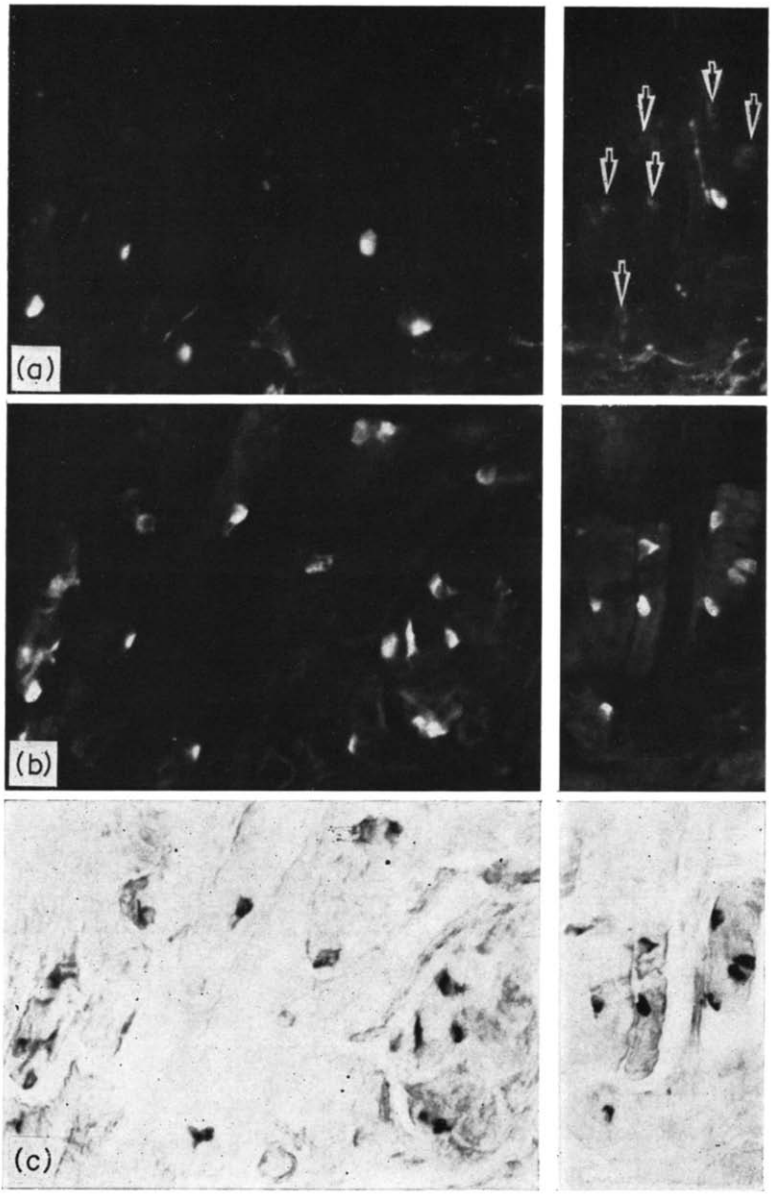

FIG. 1. Fluorescence histochemical demonstration of Type III cells in rabbit antrum, × 190 (left), × 245 (right). (a) *Standard formaldehyde treatment.* Intense yellow fluorescence has developed in 5-HT-containing enterochromaffin cells, green fluorescence in scattered sympathetic nerve terminals. A weak, greenish fluorescence is seen in some cells referred to as Type III cells (arrows). (b) *Formaldehyde–ozone treatment* of the same sections. An intense fluorescence is now seen in a fairly large number of Type III cells in the mucosal epithelium. The fluorescence of the 5-HT cells is retained whereas that of the sympathetic nerve terminals is lost. (c) Subsequent *silver staining.* Argyrophil reaction over all fluorescent epithelial cells.

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with the formaldehyde-ozone treatment (see Fig. 4). After formaldehyde-ozone or formaldehyde-HCl the colour of the fluorophore was clearly green-yellow (excitation at 365 nm, GG9 for secondary filter). Regardless of the histochemical procedure used, the Type III cells were only demonstrable in the pyloric gland area and in the immediately adjacent portion of the oxyntic gland area.

It may be noted that many of those enterochromaffin cells, which were yellow after formaldehyde or after formaldehyde-HCl, displayed a clearly reddish fluorescence after formaldehyde-ozone treatment.

Administration of L-DOPA resulted in the appearance of an intense green formaldehyde-induced fluorescence (standard Falck–Hillarp procedure, excitation at 405 nm, OG4 secondary filter) in a large number of argyrophil cells devoid of dopamine and 5-HT in untreated animals. The cells were located basally in the mucosal epithelium of all regions in the stomach. The green fluorescence probably reflected the presence of dopamine.[10] These green-fluorescent cells were morphologically indistinguishable from the enterochromaffin cells and have therefore been named "enterochromaffin-like".[8] Such enterochromaffin-like cells were most numerous in the pyloric gland area as were the Type III cells. However, it was not possible to discern the very weak endogenous fluorescence of the Type III cells after the injection of L-DOPA, suggesting that these Type III cells are included among the cells taking up L-DOPA.

Microspectrofluorometric analysis

Standard formaldehyde treatment. The Type III cells, which were found predominantly in the pyloric gland area, had a very low fluorescence intensity. Therefore, only a few of these cells permitted microspectrofluorometric analysis

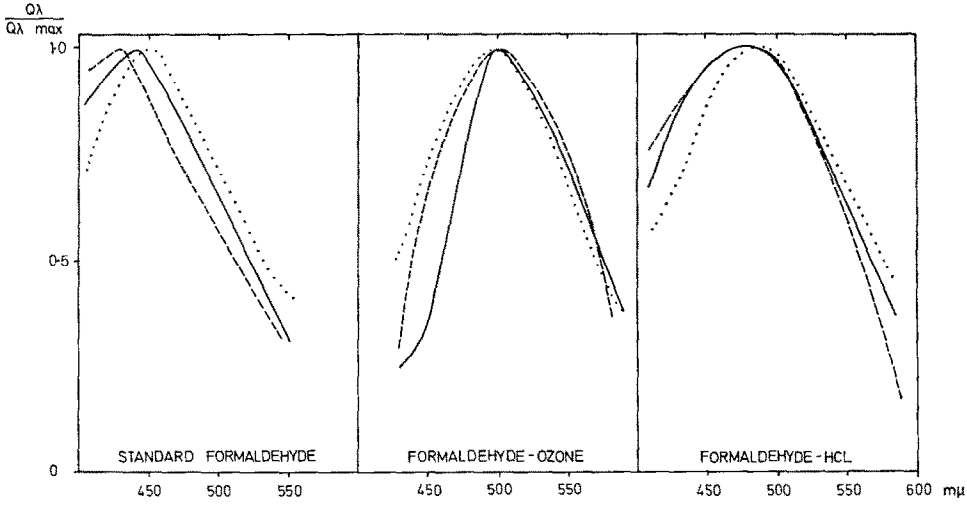

FIG. 2. Fluorescence emission spectra of gastric Type III cells (——) and protein microdroplet models containing authentic tryptamine (– – – –) or tryptophyl-glycine (· · · ·). The protein droplets were prepared from solutions containing 0·03 mg/ml of tryptamine or 0·5 mg/ml of tryptophyl-glycine. Ozone concentration corresponded to a generation time of 20 min and the HCl concentration was 300 torr.

after the standard formaldehyde treatment. Excitation was maximal at 365 nm (range 350–370 nm) and the corresponding value for the emitted light (Fig. 2) was at 430 (420–440) nm.

Tryptamine and tryptophyl peptides (L-tryptophyl-L-glycine and L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide) in albumin droplet models were maximally excited at 370 nm and showed a concentration-dependent shift in the emission maximum (Fig. 3) from 430 (0·03 mg/ml) to 500 nm (at 5–25 mg/ml).

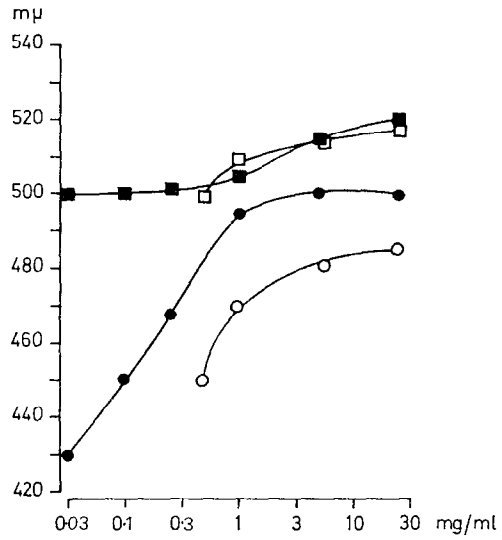

FIG. 3. Formaldehyde (circles) and formaldehyde-ozone (squares) induced fluorescence of tryptamine (filled) and tryptophyl-glycine (open) in protein droplets. Concentration-dependent shift of emission maximum.

The fluorescence of the yellow enterochromaffin cells, detectable in both the pyloric and the oxyntic gland area, was maximally excited at 385 nm (380–390); the emission maximum was at 540 nm (520–550). These spectral characteristics agree fairly well with those of enterochromaffin cells in other species and with the formaldehyde-induced fluorophore of 5-HT.[8,26]

Formaldehyde-ozone treatment. The presence of ozone markedly enhanced the fluorescence yield of the cytoplasmic fluorophore in the Type III cells and of tryptamine and the above-mentioned tryptophyl-peptides in protein droplet models (Fig. 4). The marked increase in the fluorescence intensity of the Type III cells after this combined treatment provided ample opportunities for exact cytospectrofluorometric analysis: the fluorophore induced by formaldehyde-ozone was maximally excited at 370 nm (365–380 nm). The emission maximum (Fig. 2) was at 505 nm (ranging from 495–510 nm). With the combined formaldehyde-ozone treatment the tryptamine and tryptophylpeptide fluorophores in the protein droplet models showed emission maxima (Fig. 3) varying from 500 to 505 nm (at concentrations between 0·03–1 mg/ml) to 520 nm (at concentrations above 5 mg/ml). The excitation maximum remained constant at about 370 nm for all concentrations of the compounds.

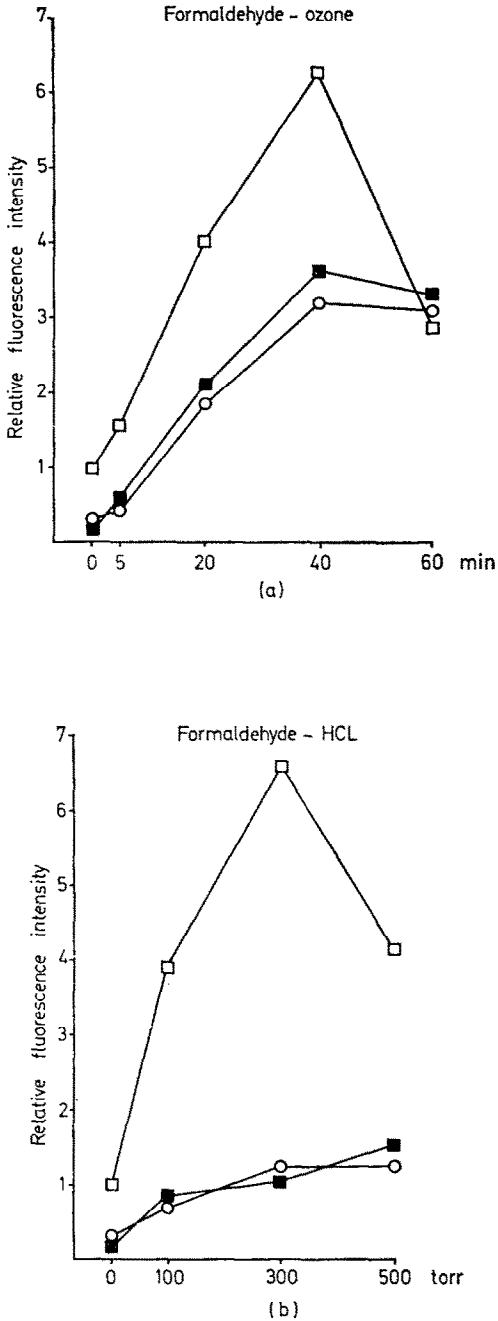

FIG. 4. Yield of formaldehyde-induced fluorescence in gastric Type III cells (○——○) and in models containing authentic tryptamine (□——□) and tryptophyl-glycine (■——■) as affected by various amounts of ozone (a) and HCl (b). Measurements were performed in the microspectrograph. Ozone concentration is expressed as time of ozone generation, HCl concentration as partial pressure in the reaction vessel. The microdroplet models were prepared from solutions containing 1 mg/ml of the compounds. Fluorescence intensity of tryptamine after formaldehyde alone is given as 1.

Certain enterochromaffin cells developed a red fluorescence as a result of the combined formaldehyde-ozone treatment. These cells were found to have excitation maximum at 395 nm (380–410 nm) and emission maximum at 575 nm (560–590 nm). The spectral characteristics of the enterochromaffin cells remaining yellow-fluorescent were the same as after the standard formaldehyde treatment.

Formaldehyde-HCl treatment. After this treatment the fluorescence intensity of the Type III cells was higher than after the standard formaldehyde procedure, though not as high as with the formaldehyde-ozone treatment (Fig. 4). Excitation was maximal at 370 nm (360–370 nm), and the corresponding value for the emission (Fig. 2) was 480 nm (range 470–485 nm).

When studied with protein droplet models, the formaldehyde-HCl treatment which induced intense fluorescence with tryptamine gave only moderate fluorescence with the tryptophyl-peptides (Fig 4). With both tryptamine and the peptides there was a concentration-dependent shift in the emission maximum from 450 nm (0·03 mg/ml) to 510 nm (at a concentration of 5–25 mg/ml). The fluorophores were maximally excited at about 370 nm irrespective of the concentration.

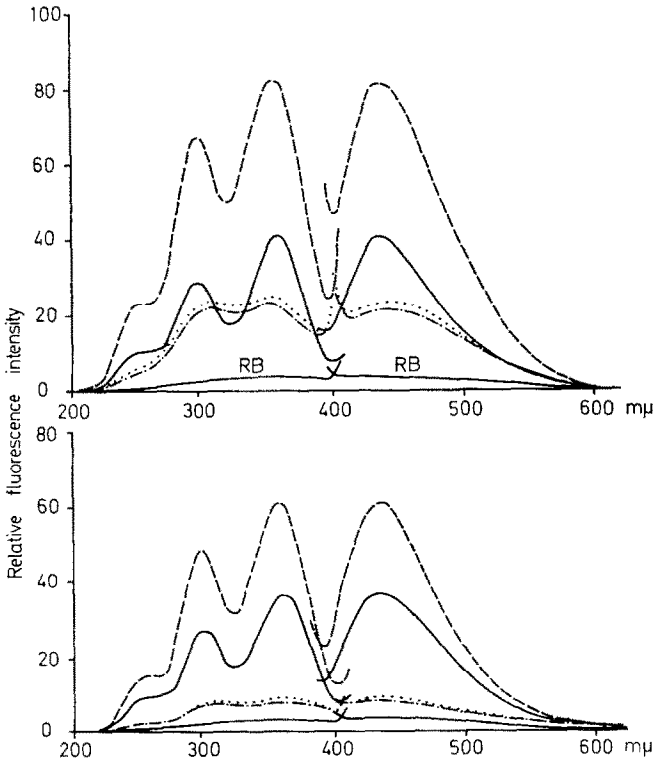

FIG. 5. Excitation (left) and emission (right) spectra of tryptamine-like compounds in extracts of 15 g gastric content (– – – –) and 12 g of mucosa from rabbit antrum (·—·—·) and 25 g from oxyntic gland area (· · · ·) after condensation with formaldehyde in the presence of hydrogen peroxide. Standard, 0·5 μg tryptamine (——). Reagent blank, RB. Extracts prepared by homogenization in acetone, followed by benzene extraction and ion exchange chromatography (top). Recovery of added [^{14}C] tryptamine 35 per cent. The extracts were re-extracted with benzene (below). Recovery of [^{14}C] tryptamine 30 per cent.

Chemical analysis

Attempts to demonstrate tryptamine in gastric mucosa. Extracts were prepared by homogenization in acetone, followed by benzene extraction and ion exchange chromatography (see Methods). The extracts of the gastric content and of the gastric mucosa contained components giving formalin-induced fluorescence similar to that of authentic tryptamine (Fig. 5). These components were uniformly distributed in the gastric mucosa in that the concentration in the pyloric gland area was similar to that in the oxyntic gland area. When these extracts were re-extracted with benzene (see Methods), the tryptamine-like component of the gastric content was recovered (corresponding to a tryptamine concentration of 0·5–1·0 μg/g of gastric content) whereas that of the gastric mucosa was lost (Fig. 5). It may thus be concluded that tryptamine is either absent from the gastric mucosa of the rabbit, or present in concentrations below 0·05–1·0 μg/g, which is the threshold value for the chemical detection of tryptamine with the method and amount of tissue used.

DISCUSSION

Fluorescence microscopy has previously demonstrated that the gastric mucosa of the rabbit contains one large population of enterochromaffin cells storing 5-HT, and another much smaller population storing dopamine.[8] In addition, it is possible to demonstrate an extensive system of morphologically similar (enterochromaffin-like) cells, displaying an intense green, formaldehyde-induced fluorescence (probably due to dopamine[10]) after administration of L-DOPA to the animal. Some of these latter cells were visible also without L-DOPA pretreatment by their faint green-yellow, formaldehyde-induced fluorescence (for this reason they have previously been referred to as enterochromaffin cells Type III)[8]. In the present study it was observed that when the formaldehyde condensation reaction was carried out in the presence of ozone, the fluorescence yield of these cells was markedly improved. The cytoplasmic fluorophore was similar to the fluorophores of tryptamine and tryptophyl-peptides[11–13] in its spectral characteristics, both after the conventional formaldehyde condensation reaction and after condensation in the presence of ozone. The fluorescence intensity of authentic tryptamine was markedly enhanced when the formaldehyde reaction was carried out in the presence of HCl vapour.[11,18] In this respect, the cytoplasmic fluorophore of the Type III cells was different: as with the tryptophyl-peptides[12] the fluorescence intensity was only moderately improved by combined formaldehyde-HCl treatment.

Chemical (fluorometric) analysis of gastric mucosa failed to reveal any measurable quantities of tryptamine. In the gastric content, however, tryptamine-like compounds —possibly identical with tryptamine formed by bacterial decarboxylation of tryptophan—were demonstrated.

From the present results it is suggested that the endogenous formaldehyde-reactive fluorogenic compound in the amine-forming gastric endocrine cells, previously referred to as enterochromaffin cells Type III,[8] is a tryptophyl-peptide (rather than tryptamine). Similar fluorogenic components have recently also been demonstrated in the calcitonin-producing cells of the cat and pig thyroid gland.[27]

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