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LOCALIZATION OF CATECHOL AMINES IN VISCERAL ORGANS AND GANGLIA OF THE RAT, GUINEA-PIG AND RABBIT

BY

B. C. S. HOLLANDS* AND S. VANOV†

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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Biologically active monoamines can be demonstrated in tissues by a fluorescence histochemical procedure (Eränkö, 1955; Falck, 1962; Carlsson, Falck & Hillarp, 1962; Falck, Hillarp, Thieme & Thorpe, 1962; Falck & Owman, 1965). This method allows accurate localization of certain catechol amines and tryptamines in completely dry tissue. These substances are condensed with formaldehyde to form intensely fluorescent and relatively stable products. The fluorescence can be excited in ultraviolet light and a fluorescence of characteristic wavelength observed in structures containing these monoamines with a microscope equipped for fluorescence work. The method has been extensively used to study the distribution of catechol amines in various tissues of different animal species (Falck, 1962; Carlsson *et al.*, 1962; Falck *et al.*, 1962; Dahlström & Fuxe, 1964; Norberg & Hamberger, 1964).

We have used the method to study the localization of catechol amines in some visceral organs and ganglia of the rat, guinea-pig and rabbit.

METHODS

Animals and freeze-drying procedure. The tissues studied were obtained from four female Wistar rats, four male guinea-pigs and three female rabbits. All animals were adult and kept on standard laboratory diets; they were killed by a blow on the head and were bled. Small pieces of tissue were rapidly excised for freeze-drying. These were mounted on to paper identification slips and rapidly frozen by immersion in dichlorodifluoromethane (Arcton 12, I.C.I.; Bell, 1952) which had been previously cooled in liquid nitrogen. The frozen tissues were dried in a Canalco tissue freeze-drying apparatus as follows: they were kept for 5 to 7 days at a temperature of $-35°$ C; 12 hr before termination of drying, the temperature was increased to $-20°$ C, and, finally, before breaking the vacuum the temperature was raised to 50° C to prevent condensation of water on the tissues. The specimens were transferred into a desiccator and cooled to room temperature. A vacuum better than 0.001 mm Hg was maintained throughout the drying period.

Treatment with formaldehyde. The freeze-dried specimens, other than those intended as controls, were exposed to formaldehyde vapour at a temperature of 80° C for a period of 1 hr. Paraformaldehyde powder (Merck) was used which had been previously equilibrated to a constant relative humidity of 25% by standing over a 57% v/v sulphuric acid solution.

Paraffin infiltration, embedding and microtomy. The formaldehyde-treated and the untreated control specimens were infiltrated with paraffin wax (56° C melting point) for 10 to 15 min under reduced pressure

* Present address: Wild Heerbrogg (U.K.) Ltd., 51 Church Street, Maidstone, Kent.
† Present address: Department of Pharmacology, School of Pharmacy, University of London.

before embedding in the same wax. The paraffin blocks were serially sectioned at 7 μ using a rotary microtome.

Sections were placed on clean glass slides and allowed briefly to flatten on a hot plate. A drop of liquid paraffin or Entellan (Merck) was placed on the sections and a cover glass was lowered; the slides were returned to the hot plate for a few minutes to allow the wax to dissolve in the mounting fluid.

Adjacent sections, to be stained by different methods, were mounted on to albuminized slides, dried at 37° C overnight, and stained either with cresyl fast violet, methylene blue, or haematoxylin and eosin.

Microscopy and photomicrography. A WILD Universal microscope lamp fitted with a 200-HBO high-pressure mercury vapour burner was used with Schott filters, 2 K.B.I. (heat absorbing) and a 3-mm-thick B.G. 12 for the excitation light source, which was reflected into the microscope by a surface-coated mirror. A microscope with a monocular tube was used and a Wratten 15 filter fitted in the ocular. Most observations and the photomicrography, except of sections stained to show morphological detail, were made with dark-field illumination, either using patch stops for low magnification or a Cardioid immersion condenser for high magnifications.

Photomicrographs were taken with an EXA I single-lens reflex camera on Scopix G (Gevaert) 35-mm roll film. Exposure times of 1 to 3 min were used.

Criteria applied in examination of material. Differentiation between specific and nonspecific fluorescence was made by comparison of formaldehyde-treated specimens and duplicate specimens where the formaldehyde-treatment had been omitted. The fluorescent structures were identified in adjacent stained sections.

Furthermore, the following criteria, recommended by Dahlström & Fuxe (1964), Norberg & Hamberger (1964) and Corrodi, Hillarp & Jonsson (1964), were applied:

1. Quenching of the fluorescence with water: a marked reduction, or even complete disappearance of the specific fluorescence was observed when sections were floated out on a water-bath at 45° C for about 5 min.

2. Reduction with sodium borohydride: treatment of the sections with 0.1% solution of sodium borohydride in 90% isopropanol caused almost complete disappearance of the specific fluorescence.

3. Treatment with reserpine (Serpasil, Ciba; 5 mg/kg, intraperitoneally): a guinea-pig was killed 12 hr after treatment and specimens of taenia coli, heart and trachea were taken for comparison with tissues obtained from an untreated guinea-pig.

4. Treatment with nialamide (250 mg/kg, intraperitoneally), followed 6 hr later by an injection of 200 mg/kg of (±)-3,4-dihydroxyphenylalanine (dopa): a rat treated in this way was killed 2 hr after the administration of dopa, and duodenum, ileum and inferior mesenteric ganglion were examined.

Nonspecific fluorescence. In addition to the specific green and yellow fluorescence, tissue autofluorescence and fluorescence due to artefacts and foreign bodies were present and had to be watched for.

Tissues examined. The following tissues were examined: rat duodenum, rat and guinea-pig ileum, guinea-pig trachea, rabbit distal colon (transverse sections), rabbit ileo-colic sphincter, taenia from the caecum of the guinea-pig (longitudinal sections), the edge of the guinea-pig right auricle, and the inferior mesenteric ganglion of the rat and rabbit, together with the adjacent blood vessels.

RESULTS

Table 1 summarizes the localization and the nature of the specific fluorescence seen in the tissues examined from the various animals. A more detailed description follows.

Duodenum (rat)

Specific fluorescence was present in the epithelium, sub-mucosa and the areas of Meissner's and of Auerbach's plexuses (Figs. 1 and 2).

Epithelium. Spindle-shaped cells present in the epithelium exhibiting strong yellow fluorescence (as produced by 5-hydroxytryptamine) were identified as enterochromaffin cells.

TABLE 1

NATURE AND LOCATION OF STRUCTURES HAVING SPECIFIC FLUORESCENCE IN TISSUES OF THE RAT, GUINEA-PIG AND THE RABBIT

Species	Tissue	Nature and location of the fluorescent structures: Green	Yellow
Rat	Duodenum	Plexuses Perivascular nerves	Enterochromaffin cells Scarce submucosal cells
	Ileum	Plexuses Perivascular nerves	Enterochromaffin cells
	Inferior mesenteric ganglion	Cell bodies, nerves Periarterial nerves	Mesenteric mast cells
Guinea-pig	Ileum Taenia coli	Plexuses Myenteric plexus Nerves along muscle fibres	Enterochromaffin cells
	Trachea	Nerves around blood vessels	
	Right auricle	Nerves along muscle or around blood vessels	
Rabbit	Distal colon	Plexuses Perivascular nerves	Enterochromaffin cells Cells in submucosa
	Ileo-colic sphincter	Plexuses Perivascular nerves	
	Inferior mesenteric ganglion	Cell bodies, nerves, peri-arterial nerves	Mesenteric mast cells

Sub-mucosa. Oval cells at the base of the villi also showed yellow fluorescence, but it was less intense than that of the enterochromaffin cells.

Meissner's plexus. Bright, green fluorescence (the colour corresponding to that of catechol amines), in the form of beads or small fibres, was associated with groups of ganglion cells of the plexus. Some of the nerve cell bodies were surrounded by fluorescent structures, but showed no fluorescence. The areas showing specific fluorescence were compared to areas in adjacent, stained sections; they corresponded to cells with a homogeneous protoplasm and elongated dense nuclei.

Auerbach's plexus. An abundance of green fluorescent material was observed in this plexus. Some of the fluorescent structures could be identified as nerve fibres and were like the fibres seen in the myenteric plexus, but were more numerous. In addition, large fluorescent masses were present. At low magnifications they appeared homogeneous (Fig. 1) but at a higher magnification showed an uneven distribution of the fluorescent material (Fig. 2). Nonfluorescent areas within the mass appeared to correspond to ganglion cell bodies, and the fluorescent areas appeared to correspond to a different type of cells. The fluorescent nerve fibres were arranged in two ways: some followed the direction of the circular muscle layer, whilst others penetrated the muscle layer towards the submucosal plexus.

Arteries. The arteries were surrounded by bright green fluorescent spots or small fibres encircling the adventitia.

Pancreas. In a number of preparations where a piece of pancreas had been included, specific fluorescence was only seen in the adventitia of the arteries.

Ileum (rat and guinea-pig)

The fluorescent material seen in the ileum was essentially similar in nature and distribution to that of the rat duodenum, but the ileum contained more enterochromaffin cells and less fluorescence in the plexuses than the duodenum.

Taenia coli (guinea-pig)

The taenia coli contained, in addition to the longitudinal muscle layer, the adjacent connective tissue with the myenteric plexus. The taenia coli of the guinea-pig was the intestinal tissue richest in green fluorescent structures (Figs. 3 and 4). An abundance of intensely fluorescing nerve fibres was seen in the muscle layer proper (Fig. 3). The nerve fibres followed the direction of, and were in close proximity to, the muscle fibres. A few nerve fibres were seen in close relationship to blood vessels. The connective tissue underlying the muscle contained groups of elongated or oval green fluorescing structures of different sizes (Fig. 4). At high magnification it could be seen that these masses contained intensely fluorescent areas, and dark patches. A methylene blue-stained adjacent section (Fig. 5), corresponding to the area photographed in Fig. 4, shows that the fluorescent regions (F) corresponded to groups of ganglion cells and of cells with dark, elongated nuclei, all incorporated in a homogeneous ground mass.

Ileo-colic sphincter (rabbit)

The smooth muscle of the sphincter contained large fluorescing structures of a bizarre shape and similar to those seen in the plexuses of the duodenum. They appeared to consist of nerve cell bodies closely associated with some other cells and surrounded by the green fluorescent material. Processes which showed bright green fluorescence could be seen projecting from the central mass and were evidently nerve fibres.

Distal colon (rabbit)

The nature and distribution of the fluorescent material in the colon was similar to that of the rat duodenum (Fig. 6).

Trachea (guinea-pig)

The trachea showed less specific fluorescence than all other tissues examined. Sparsely scattered nerve fibres exhibiting green fluorescence were found in the perivascular spaces. A distinct line of yellow green fluorescence which was attributed to mucus was present on the surface of the ciliated epithelium.

Heart, right atrium (guinea-pig)

The right atrium of the heart showed a rich adrenergic innervation indicated by a dense and intricate network of brightly fluorescing green fibres. These nerve fibres were evenly distributed and were in close proximity to the muscle fibres. A few nerve fibres were found in the perivascular connective tissue.

Inferior mesenteric ganglion (rat and rabbit)

The ganglia were excised together with the associated blood vessels and connective tissue. The majority of the cell bodies in the ganglion of both the rat and rabbit showed faint but distinct green fluorescence (Figs. 7 and 8). Green fluorescent beaded structures, characteristic of nerve fibres, were irregularly distributed in the ganglion. These nerve fibres did not show a particular association with the nerve cells (" basket-like " synaptic contacts), such as has been described for the cat sympathetic ganglia (Hamberger, Norberg &

Sjöqvist, 1964). At one pole of the rat ganglion a very bright area of green-yellow fluorescence was observed. This appeared indistinct because of the high intensity of fluorescence, and it was not possible to identify its structure. It is likely to have been chromaffin tissue (Muscholl & Vogt, 1958).

The large mesenteric vessels, particularly the artery, showed bright green fluorescence of the adventitia. Bundles of periarterial sympathetic nerves were fluorescing slightly less intensely. Scattered mast cells were found in the vicinity of the rat ganglion, and emitted an intense yellow fluorescence.

Effects of previous treatment with drugs

Reserpine. An almost complete disappearance of the specific fluorescence was noted in the heart, taenia coli, trachea and ileum of the guinea-pig treated with one dose of reserpine. However, occasional enterochromaffin cells showed fluorescence, but their number was greatly diminished.

Nialamide and dopa. Sections of duodenum from the rat treated with nialamide and dopa did not show a marked alteration of the specific fluorescence, but some increase in tissue autofluorescence was observed. The acini of the pancreas included in the sections were swollen and some of them showed intense yellow-green fluorescence.

The nerve cell bodies of the inferior mesenteric ganglion were more intensely fluorescent than the cells of an untreated animal. An increase in the intensity of fluorescence was also observed in the periarterial nerves of the mesenteric blood vessels.

DISCUSSION

Our observations that the duodenum of the rat, the distal colon and the ileo-colic sphincter of the rabbit and the taenia coli of the guinea-pig contain an abundance of specific green fluorescence suggests that the adrenergic innervation of these tissues is rich. However, Norberg (1964) found that the smooth muscle layer of the small intestine and the colon of both rat and cat contained few or no fluorescent fibres. He observed that the adrenergic postganglionic fibres terminated at the intramural nerve cells to form synaptic contacts. He was, however, unable to identify an autonomic ground plexus around the smooth muscle fibres. Norberg is of the opinion that the sympathetic postganglionic nerves do not directly innervate the smooth muscle and that " the sympathetic inhibition of intestinal motility is probably mediated indirectly by an effect on the postganglionic parasympathetic neurons."

In a number of sections from the rat duodenum, rabbit colon, and particularly from the taenia coli of the guinea-pig, a great number of fluorescent fibres were seen adjacent to the smooth muscle fibres. The number of the fluorescent fibres exceeded the number of blood vessels and for the majority of them an association with blood vessels was not seen. Only a few of the fluorescent fibres were closely associated with small arterioles and capillaries. It is therefore possible that these fibres directly innervate the smooth muscle and are not vasomotor fibres. A similar distribution of fluorescent nerve fibres was found in the guinea-pig atrium, where these fibres form an extensive network closely associated with the muscle fibres. The number of fibres would seem to eliminate the possibility of their being vasomotor nerves only, and their close proximity to the muscle fibres suggests that they are directly innervating the smooth musculature.

Fig. 1. Distribution of fluorescence in the rat duodenum: M, mucosa; e, enterochromaffin cells; P, plexuses; a, artery. ×52.

Fig. 2. Higher magnification of Fig. 1 showing detail of fluorescent structures: SP, Meissner's plexus; AP, Auerbach's plexus; e, enterochromaffin cells; M, mucosa; SM, submucosa; C, circular muscle; L, longitudinal muscle. ×104.

Fig. 3. Fluorescent nerve fibres in the muscle of the guinea-pig taenia coli. ×222.

Fig. 4. Fluorescent structures in the connective tissue (C) of the guinea-pig taenia coli: M, mucosa. ×74.

Fig. 5. Methylene blue-stained section of Fig. 4: M, muscle; F, fluorescent structures. ×119.

Fig. 6. Fluorescent structures in the rabbit distal colon. Note that fluorescent fibres are close to muscle fibres. ×121.

Fig 7. Fluorescent structures in the rat inferior mesenteric ganglion. Note the intensely fluorescent area. ×133.

Fig. 8. Distribution of fluorescence in the rabbit inferior mesenteric ganglion. ×81.

This and other photomicrographs are of freeze-dried and formaldehyde-treated tissues which, except for Fig. 5, were photographed with dark field ultraviolet illumination.

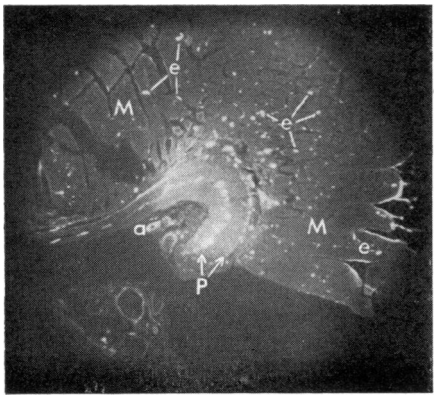

FIG. 1

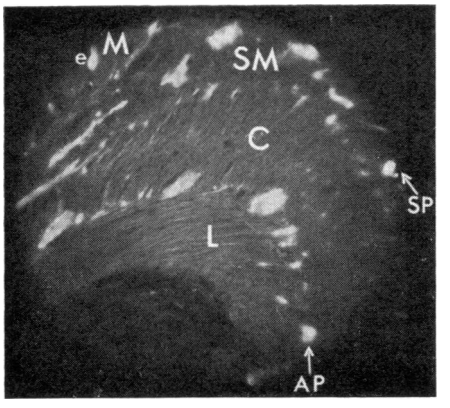

FIG. 2

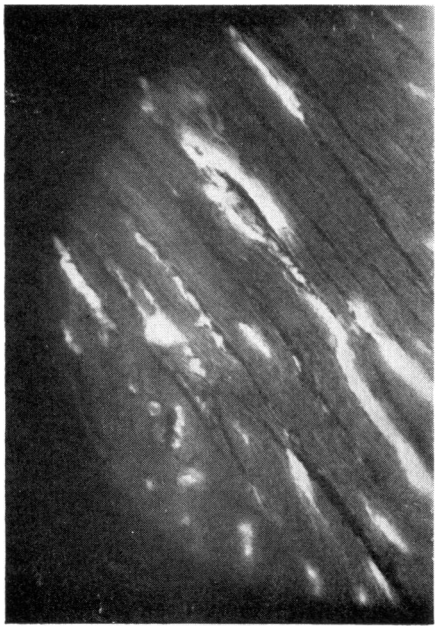

FIG. 3

FIG. 4

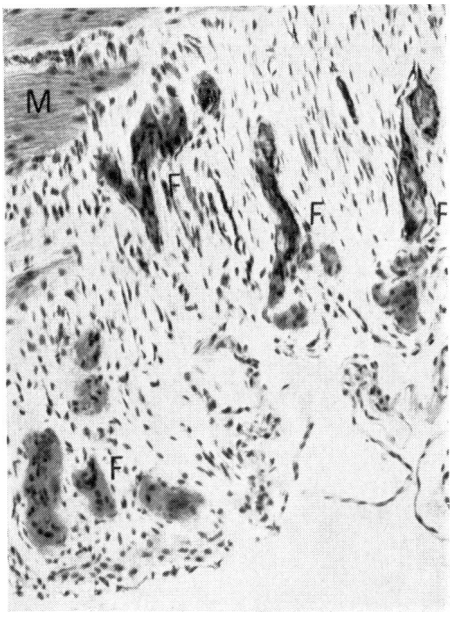

FIG. 5

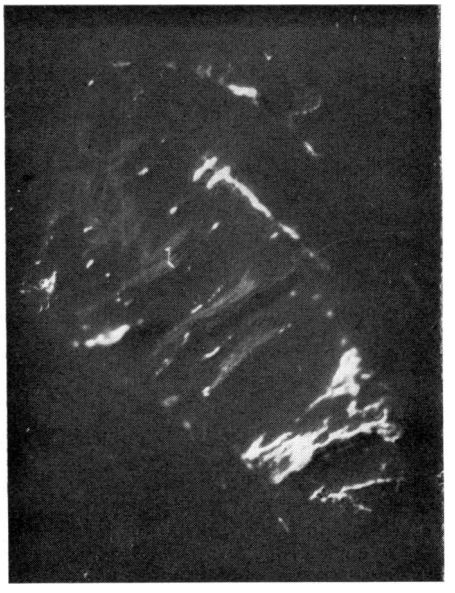

FIG. 6

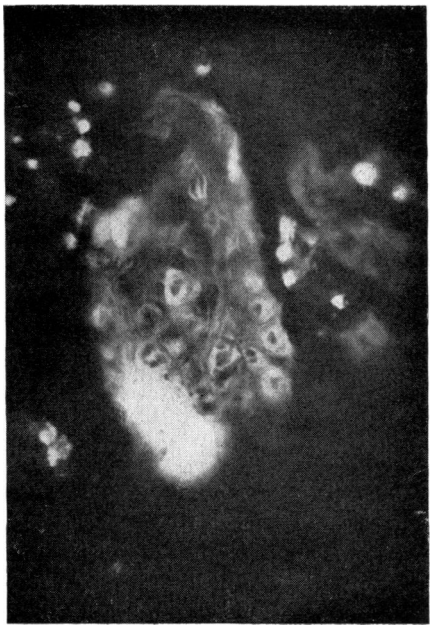

FIG. 7

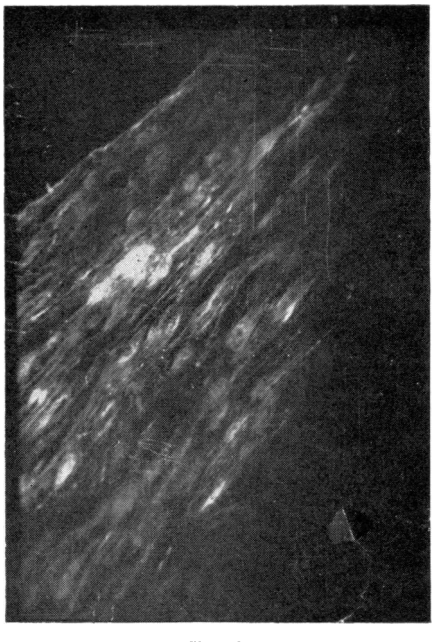

FIG. 8

In view of the abundance of catechol amine-containing structures in the intestinal and cardiac tissues studied, it is of interest to note that nicotine and dimethylphenylpiperazinium exert a sympathomimetic action on these tissues. Thus, either of these drugs can cause relaxation of the rat duodenum (Levy & Michel-Ber, 1953), the rabbit distal colon (Gillespie & MacKenna, 1960), rabbit ileo-colic sphincter (Jarrett, 1962), taenia coli of the guinea-pig (Weiss, 1962; Burnstock, Campbell & Rand, 1965), and stimulation of guinea-pig atria (Giotti, 1954). These authors agree that an adrenergic mechanism via a release of catechol amines is involved. If this view is accepted, the results presented in this communication indicate that there is sufficient catechol amine-containing material in these tissues from which nicotinic drugs may release catechol amines. Nicotine may act on the adrenergic nerve terminals or on intramural inhibitory neurones, as suggested by Burnstock *et al.* (1965) for the guinea-pig taenia coli. It is also possible that the sympathomimetic actions of nicotine on various tissues are exerted through different mechanisms. For example, the smooth muscle of guinea-pig trachea, in spite of the fact that it has few adrenergic structures, relaxes in response to nicotine (Hawkins & Paton, 1958).

The majority of the nerve cell bodies seen in the inferior mesenteric ganglion of the rat and rabbit exhibited a specific fluorescence characteristic for noradrenaline. The intensity of this fluorescence was increased by previous treatment with nialamide and dopa. Synaptic contacts of preganglionic terminals with the postganglionic neurones such as have been described by Hamberger *et al.* (1964) have not been found in this study, which may indicate a species difference between the cat on one hand and the rabbit and rat on the other hand.

SUMMARY

1. Formaldehyde-condensed monoamines were localized in the rat duodenum, ileum and inferior mesenteric ganglion; guinea-pig ileum, taenia coli, trachea and heart; rabbit ileo-colic sphincter, distal colon and inferior mesenteric ganglion, by a fluorescent histochemical procedure.

2. Catechol amines, characterized by a green fluorescence, were seen as beads, fibres or larger fluorescent structures. In the intestinal plexuses, taenia coli and heart there was an abundance of fluorescent fibres, many of which ran along the muscle fibres. The trachea showed sparsely scattered fibres within the perivascular spaces. The adventitia of the arteries contained small beaded fluorescent fibres.

3. In the inferior mesenteric ganglia weakly fluorescing nerve cell bodies were present in addition to intensely fluorescent nerve fibres. The fibres did not show a particular association with the cells.

4. 5-Hydroxytryptamine, characterized by yellow fluorescence, was observed in enterochromaffin cells and in mesenteric mast cells.

5. It is suggested that the sympathomimetic action of nicotine or dimethylphenylpiperazinium on the heart and intestine is produced by the release of noradrenaline.

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