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# The Glyoxylic Acid Fluorescence Histochemical Method: a Detailed Account of the Methodology for the Visualization of Central Catecholamine Neurons

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*Summary.* This paper gives a detailed description of the glyoxylic acid fluorescence histochemical method as designed for the highly sensitive visualization of catecholamine neurons. In this method, the primary catecholamines, dopamine and noradrenaline, are efficiently converted to intensely fluorescent 2-carboxymethyl-dihydroisoquinoline derivatives in a well defined reaction with glyoxylic acid. The method is carried out on sections from fresh or glyoxylic acid-perfused tissue, which are immersed in a glyoxylic acid solution, dried, and then reacted either by heating at +100° C, or by glyoxylic acid vapour treatment at +100° C. The method has a high reproducibility, is rapid and convenient, and if desired, sections of good quality can be ready for fluorescence microscopy within half an hour after the sacrifice of the animal.

The glyoxylic acid method demonstrates central and peripheral dopamine- and noradrenaline-containing neurons with an extraordinary sensitivity and precision. The entire adrenergic neuron, including the non-terminal portions of the axon and sometimes also the dendrites, becomes fluorescent, making the method ideal for neuroanatomical tracing of central catecholamine pathways. The spectral characteristics of the glyoxylic acid-induced fluorophores have been investigated, and it is concluded that the catecholamine fluorophores can be identified and distinguished by microspectrofluorometry from those of other fluorogenic monoamines known to occur in the vertebrate brain.

## Introduction

The fluorescence histochemical formaldehyde (FA) method of Falck and Hillarp (Falck, 1962; Falck *et al.*, 1962; Corrodi and Hillarp, 1963, 1964) demonstrates central catecholamine (CA)-containing neurons with high sensitivity and specificity. In this method, the intraneuronal transmitter—dopamine (DA), noradrenaline (NA), or adrenaline—is converted to strongly fluorescent molecules by condensation with FA. There are, however, marked differences in transmitter content between various parts of the central adrenergic neuron—the cell body and the proximal dendrites containing only low and the non-terminal axon very low amine concentrations. In contrast, the terminal and paraterminal parts contain a very high proportion of the CA content of the nerve cell, localized in the so-called varicosities (Carlsson *et al.*, 1962; Fuxe, 1965a; Andén *et al.*, 1966). Thus, whereas the visualization of the cell bodies and axon terminals can be accomplished with the Falck-Hillarp FA method, demonstration of non-terminal parts of the CA axons is usually not possible in the CNS of intact and untreated adult animals. For this, the production of lesions of the axons is required in order to increase the intra-axonal amine concentration, which can be achieved either

mechanically (Dahlström and Fuxe, 1965), or chemically by means of 6-hydroxydopamine (Ungerstedt, 1971) or 6-hydroxy-DOPA (Jacobowitz and Kostrzewa, 1971; Sachs and Jonsson, 1972).

Recently, condensation with glyoxylic acid (GA) was introduced as a highly sensitive method for the fluorescence histochemical visualization of biogenic CAs, indolamines and structurally related compounds, GA exhibiting a considerably higher capacity than FA to form fluorophores from these substances in histochemical models (Björklund, Lindvall and Svensson, 1972; Axelsson, Björklund, Falck, Lindvall and Svensson, 1973; Björklund, Håkanson, Lindvall and Sundler, 1973). Subsequently, it was shown by Lindvall *et al.* (1973a, b) that sections (produced in a Vibratome instrument) of GA-perfused brains were ideal for the application of the GA method to central nervous tissue. With this type of tissue preparation, the GA method demonstrated the central CA-containing neurons with a remarkable sensitivity and precision. Thus, the CA stores of the entire axons, including their preterminal parts, and sometimes also the dendrites are readily demonstrated, making the GA method particularly useful for neuroanatomical studies of central CA neurons. This paper gives a detailed account of the technique that has been developed for the visualization of NA-containing and DA-containing neurons in the central and peripheral nervous system.

## Materials and Methods

*Substances Used.* Glyoxylic acid monohydrate was purchased from Fluka AG, Buchs, Switzerland. L-Dopa, dopamine hydrochloride, noradrenaline hydrochloride, 3-methoxytyramine hydrochloride, DL-normetanephrine hydrochloride, N-acetyl-5-hydroxytryptamine, N-acetyl-5-methoxytryptamine (melatonin), adrenaline bitartrate, N,N-dimethyltryptamine, N,N-dimethyl-5-hydroxytryptamine (bufotenin) oxalate hydrate, tryptamine hydrochloride, N-methyltryptamine, L-tryptophan, N-methyl-5-hydroxytryptamine oxalate, 5-hydroxytryptamine creatinine sulfate monohydrate, L-5-hydroxytryptophan, 5-methoxytryptamine, $\beta$-phenylethylamine, *p*-tyramine hydrochloride and *p*-octopamine hydrochloride were obtained from Aldrich, British Drug Houses, Calbiochem, Fluka, Hoffman-La Roche, Regis or Sigma. Human serum albumin was obtained from AB Kabi, Stockholm, Sweden.

*Tissue Experiments.* Brains and several peripheral organs of Sprague-Dawley rats (175–250 g body weight) were used. In some experiments, brains from young rats (about 9 days old; about 20 g of weight) were investigated. The tissues were either processed as whole mounts (iris, mesentery, sciatic nerve), or sectioned with a Vibratome® instrument (Oxford Instruments, San Mateo, California), as described in further detail in the Results and Comments section. Brain tissue from various regions was cut in frontal, sagittal and horizontal planes; section thickness refers to the scale of the instrument. When the MAO-inhibitor nialamide (Niamid®, Pfizer) was used, it was given i.p. in an amount of 300 mg/kg, 1–3 hrs before killing.

*Model Experiments.* The various substances were dissolved to a concentration of $10^{-3}$ M (for fluorescence intensity measurements) or 1 mg/ml (for spectral analysis) in a solution of human serum albumin in 0.1 M phosphate buffer (pH 7), containing 2% glyoxylic acid. The albumin concentration was 5% in the fluorescence intensity measurements and 2% in the spectral analyses. Before the substances were added to the GA-albumin solution, the pH had been adjusted with NaOH to 7.0. The solutions were sprayed into droplets on ordinary glass microscope slides (for intensity measurements) or on glass cover slips (for spectral analysis) and the droplets were allowed to dry. The dried droplets were then processed in various ways (see below).

*Fluorescence Microscopy and Microspectrofluorometry.* The fluorescence microscope used was equipped with Schott BG 12 or UG 1 as a primary lamp filter and Zeiss 41+47 or 44 as secondary barrier filters.

Fluorescence intensities and excitation and emission spectra of the fluorophores were recorded with a modified Leitz microspectrofluorometer (Björklund *et al.*, 1968a, b, 1972b). The fluorescence intensities were measured at maximal excitation and emission wave-lengths, and the values were corrected for the variable light flow from the exciting monochromator by means of a quantum counter device (Björklund *et al.*, 1968a). All spectra were corrected according to the procedures described previously (Björklund *et al.*, 1968a), and are expressed as relative quanta versus wave-length. In the recordings from droplet models, identically treated droplets free of added substances were used to obtain blank values. In the tissue recordings the blank spectra were obtained from the essentially non-fluorescent tissue adjacent to the specifically fluorescent structures.

## Results and Comments

### *1. Aspects of the Preparation and Treatment of Brain Tissue*

The GA procedure for central CA neurons is performed in five steps: a. Perfusion with a GA solution; b. Sectioning with the Vibratome instrument; c. Immersion of the sections in a GA solution; d. Drying; e. GA vapour treatment. Each individual step was investigated as follows in order to find optimal conditions for the visualization of central DA- and NA-containing neurons.

*a) Perfusion*: The animals were perfused with different amounts of an ice-cold GA solution. The perfusion solution was prepared by dissolving GA to various concentrations in an ordinary Krebs-Ringer bicarbonate buffer (initial pH 7.0), or in saline. The pH was thereafter adjusted to 7.0 by the addition of NaOH. In some experiments the GA perfusion was omitted, or it was replaced by perfusion with ice-cold buffer (pH 7.0), containing no GA.

*The time of perfusion* (directly related to the amount of medium perfused under constant pressure) was tested by using different volumes (from 20 ml up to 500 ml), of 2% GA solution. Up to a perfusion volume of at least 150 ml there was some improvement, both in the consistency of the tissue piece, making it easier to dissect and to section (see below), and in the fluorescence microscopical picture. Thus, the fluorescence morphology of the cell bodies (which are easily distorted in the sectioning procedure) improved markedly, many showing distinct margins and processes. As a routine procedure, a perfusion volume of 150 ml was chosen.

*The concentration of GA* in the perfusion solution also affected the consistency of the tissue and the quality of the fluorescence picture. Varying concentrations (from 0.1% up to 10%) of GA were given in a volume of 150 ml, and the sections were then immersed (see below) in a GA solution of the same composition as the perfusion medium. To avoid the precipitation that would otherwise occur, distilled water was used when dissolving 10% GA. The most prominent effect of increasing the GA concentration was a progressive improvement in the fluorescence morphology of the cell bodies (see above). Also, with a perfusion solution of up to about 2% GA, the dissection and the Vibratome sectioning were somewhat favoured. With the highest concentrations, however, the sectioning procedure was difficult, the tissue piece being rather sticky. Thus, after perfusion with 10% GA only very few sections with an even thickness and without too much foldings could be obtained. At present, 2% GA in the perfusion solution gives the most satisfactory results as regards both the fluorescence picture (see section 3 below) and the sectioning procedure.

*When the perfusion step was omitted*, the quality of the sections diminished. Due to the non-perfused brain tissue piece being rather soft, it was more difficult to get thin sections of an even thickness. Also, folds in the sections became more frequent and the general tissue fluorescence was somewhat higher. Nevertheless, the quality of the fluorescence picture of the CA-containing structures was quite good, both cell bodies (which often looked rather distorted), preterminals and terminals being visible.

*Perfusing the rat with cold buffer containing no GA* gave sections of useful quality, although the pieces from the GA-perfused brains were somewhat easier to section. The cell bodies were not so well preserved as in GA-perfused brains. The sectioning procedure was clearly easier than with non-perfused tissue and the folding of the sections could be kept at a minimum. From this it is evident that when non-GA perfused sections are required—*e.g.* for incubation experiments, (see below)—perfusion with cold GA-free buffer should be employed.

In some experiments, *the Krebs-Ringer bicarbonate buffer was replaced by 0.9% NaCl* in distilled water throughout the procedure (perfusion, sectioning, immersion; steps a, b, and c). In most cases, however, there was a rather rapid decrease in the quality of the sections with an increase in the general tissue fluorescence and a disintegration of the tissue sections. The fluorescence picture was in the first few sections similar to that of the procedure using Krebs-Ringer bicarbonate buffer but later became diffuse, and structures with a GA induced fluorescence could be identified only with great difficulty. Thus, the use of physiological saline does not seem recommendable at the present time.

*Perfusion with Formaldehyde.* When the rats were perfused with ice-cold 4% formalin according to Hökfelt and Ljungdahl (1972) the sectioning procedure was facilitated owing to the better fixation of the tissue. When further processed according to the GA method, the CA structures showed good fluorescence; however the quality of the fluorescence picture was not as high as in the GA-perfused sections.

*b) Sectioning*: The tissue piece was sectioned with the Vibratome instrument and, during this procedure, immersed in the ice-cold Krebs-Ringer bicarbonate buffer. The low temperature (between 0 and $+5°$ C) proved to be essential. Otherwise, the CA fluorophores diffused away from the nervous structures, and in addition, the tissue piece softened, making the sectioning much more difficult.

The sectioning procedure and the quality of the sections were influenced by several other factors as well: 1. *The consistency of the tissue,* which was affected by the time of perfusion and the concentration of GA in the perfusion solution (cf. above). 2. *The size of the tissue piece.* Brain tissue pieces as large as frontal sections through the adult rat diencephalon and telencephalon could be sectioned. The thickness of the dissected tissue piece could, depending on the surface area, be up to 5–6 mm. 3. *Type of tissue.* Certain areas of the brain were markedly easier to section than others. Homogeneous brain regions such as cerebral cortex, caudate nucleus, and diencephalon in the sagittal plane were easiest while regions with a heterogeneous build-up, *e.g.* heavily myelinated regions such as the lower brain stem, and regions including large ventricle spaces such as hypothalamus in the frontal plane, were more difficult.

The possibility of obtaining thin sections of high quality was influenced by all these different factors. It was found that 30–35 μ thick sections were useful for

the study of innervation density and denervation effects, because they could be obtained from all brain areas and in all planes of section. However, in the most favourable areas and in the most favourable planes (see above), sections could be obtained with a thickness down to 20 μ. A thickness greater than about 40 μ was not useful since the opacity of the thicker sections decreased the contrast and, finally, disguised the CA structures.

*c) Immersion*: In this step the sections were immersed in an ice-cold GA solution and then transferred to glass microscope slides. The following factors proved to be of importance for the quality of the fluorescence picture: 1. During the immersion procedure the sections had to be kept below the surface of the GA solution in order to avoid the gradual destruction that otherwise occurred; 2. The time of immersion could not exceed 3–5 min. If the sections were kept for a longer time in the GA solution they started to disintegrate, and the fluorescence intensity in the CA-containing structures decreased. When sections contained a lot of myelinated bundles (as in sections from the lower brain stem), the time of immersion had to be particularly carefully supervised because these sections so easily tear apart; 3. The concentration of GA in the immersion solution proved to be of great importance for the fluorophore-forming reaction. Thus, when the immersion step was omitted, or when low concentrations of GA were used (0.1%), the CA fluorescence was much lower and only scattered terminals and cell bodies could be seen. The optimal GA concentration was found to be about 2%; any further increase in the concentration was not accompanied by an observable improvement of the fluorescence picture; 4. The effect of the pH of the immersion solution on the fluorescence microscopical picture was tested by using a 2% GA solution, adjusted to pH 3, 7 or 9 by the addition of NaOH. Clear differences were noted between sections that had been soaked in an acid solution when compared with those that had been immersed in neutral or alkaline solutions. The former sections contained very few fluorescent structures, and the CA fluorophores showed clearly non-quinoidal (acid) spectra (see below). In addition, these sections were not so well preserved and sometimes they disintegrated during the immersion. Optimal results, with reference to both the fluorescence picture and the morphology of the sections, were obtained with the neutral GA solution. With respect to the fluorescence picture, similar results were also obtained with sections that had been immersed in an alkaline GA solution. However, after immersion in a GA solution of pH 9 the sections were more difficult to handle. They often adhered to the glass rod and tended to fold too much when transferred to the microscope slides.

*d) Drying*: The sections were dried in a two step procedure: first under the warm air-stream from a hair-dryer during about 15 min, and then in a dessicator *in vacuo* over fresh phosphorous pentoxide over-night. Omitting the first step, or using air of room temperature, caused diffusion of the fluorophores from the nervous structures. It thus proved necessary to use warm air in order to get the optimum, distinct appearance of the fluorescent structures. If the sections were examined in the fluorescence microscope immediately after this first drying step, *i.e.* less than half an hour after the animal was killed, a fluorescence picture of high quality was noted. This was also achieved in the delicate systems of the cerebral cortices, which are particularly difficult to visualize with present standard techniques.

*e) GA Vapour Treatment*: In this step, the sections were exposed to GA vapour as described in paragraph 2.5.1 below. In some experiments the GA vapour treatment was omitted and the sections were either heated during 6 min at +100° C, or examined directly after the two-step drying procedure (2.4).

The quality of the fluorescence microscopical picture of *sections that had been neither heated nor GA vapour treated* was high. Thus, the general tissue fluorescence was notably low and the CA-containing fibres were demonstrated with high precision and good, though not optimal, sensitivity; thus, for example, non terminal axons were well visible. *Sections that had been heated*, without GA, showed a similar fluorescence picture. However, there seemed to be an increase in the fluorescence intensity of some fibre systems, *e.g.* in the cerebral cortex. In sections treated as above, the CA fluorophores showed clearly quinoidal (neutral) spectra (see below).

*GA vapour treatment of the sections* induced a stronger fluorescence in the CA containing structures than that found in sections not exposed to gaseous GA. When the influence of GA concentration (100, 300, and 500 torr GA were tested) and reaction time (1–10 min were tested) on the fluorescence picture were studied, it was found that treatment with 300 torr GA for 2 min gave optimum results. With longer reaction time and/or increased GA concentration, there was an increase in the background fluorescence with no further increase in the GA induced CA fluorescence. After the exposure to GA vapour there was a partial conversion of the CA fluorophores to the non-quinoidal (acid) form. The relative amounts of fluorophore found in this form were dependent on the reaction conditions, *e.g.* the GA concentration in the reaction vessel (see below).

### *2. Summary of Procedure for Central CA Neurons*

The following procedure for central CA neurons was devised, and is currently in use in our laboratory.

2.1. Rats are anaesthetized with Brietal® (40 mg/kg, i.p.; Lilly), and then perfused via the ascending aorta (adult animals) or the left ventricle (young animals) with an ice cold (0 — + 5° C) GA solution. This is prepared by dissolving 2% GA in a Krebs-Ringer bicarbonate buffer (initial pH = 7.0; composition in g per litre: NaCl 6.923; KCl 0.354; $CaCl_2 \cdot 6\ H_2O$ 0.278; $KH_2PO_4$ 0.162; $MgSO_4 \cdot 7\ H_2O$ 0.294; $NaHCO_3$ 2.100; with 1.8 g/l glucose added). The buffer was saturated with a mixture of 95% $O_2$ and 5% $CO_2$. On the addition of GA the solution becomes acid, and the pH has therefore to be adjusted back to 7.0 with NaOH. The perfusion is performed at high pressure using a syringe of suitable volume. Adult animals are perfused with 150 ml and young animals with 10 ml of this solution.

2.2. The brains are rapidly taken out, cooled in the buffer and the desired piece (maximally 5–6 mm thick) is dissected out. The specimen is then glued to the holder of the Vibratome, and sectioned. Ordinary razor blades, divided in the middle, are used; they are changed frequently (at least a new blade for every new tissue piece) in order to get optimum results. During the sectioning procedure, the tissue piece is immersed in the oxygenated, pure Krebs-Ringer bi-

carbonate buffer (pH about 7.0) kept at a temperature between 0 and +5° C by non-corroding metal bars, cooled to a very low temperature in a solid carbon dioxide-ethanol mixture. These metal bars are placed in the buffer trough and changed at suitable intervals. The sectioning is performed at a vibration rate of 6–7 scale units and a feeding speed of 1–3 scale units. Sections down to 20 μ can be obtained in successful cases; as a standard sections about 30–35 μ thick should be used. The tissue piece will remain in an acceptable condition in the cool buffer for a maximum of 3–4 hrs, during which time 15–20 good sections are usually obtained. It is recommended to change the buffer in the trough for oxygenated buffer with regular intervals.

2.3. The sections are then transferred with a blunt glass-rod to an ice-cold 2% GA solution (pH 7.0, prepared as above). During this immersion, which should be continued for about 3–5 min, the sections have to be kept below the surface. Thereafter, the sections are transferred to glass microscope slides (for fluorescence microscopy) or to cover slips (for spectral analysis) in the following way: The section is picked up on the glass rod, which is then dipped into the solution so that the section partly floats out on the surface. The slide is now put below the section and the edge of the section is allowed to attach on the glass. The section is then spread out on the slide by gently lifting it out of the bath. In this way, folding of the sections is minimized. Excess buffer is removed from the slides with a filter paper. Care should be taken not to stretch or tear the sections on the slide.

2.4. The sections are dried in a two-step procedure: First, the slides are put under the warm air-stream from a hair-dryer for about 15 min. Second, the glasses are kept overnight *in vacuo* at room temperature, in a dessicator containing fresh phosphorous pentoxide. The sections can be kept in darkness in the dessicator for up to 1 week with no harmful effects on the quality of the fluorescence picture.

2.5. The sections are reacted either by GA vapour treatment or by heating, according to the following:

*2.5.1. GA Vapour Treatment.* The equipment used is illustrated in Fig. 1, and is operated in the following way: The GA treatment of the specimens is carried out in a 1 l glass vessel ("reaction vessel") provided with a multisocket lid connected to a manometer. Two grams of GA, dried in a dessicator over phosphorous pentoxide for about 24 hours, is heated at +100° C for at least 1 hr in a 1 l vessel ("GA vessel") equipped with a valve (to allow for expansion of the air in the vessel) and connected with the reaction vessel. The two vessels are placed together in the oven, which is kept at +100° C.

The following steps are followed when performing the GA treatment: The microscope slides are prewarmed in the oven for about 3 min (together with the rack in which they are contained) to prevent condensation of GA onto the slides when they are introduced into the reaction vessel. The slides are then placed in the reaction vessel, which is evacuated with a vacuum pump. Hot GA-saturated air (from the GA-vessel) is introduced into the hot reaction vessel to a partial pressure of 300 torr (mm Hg). Hot air (from the oven) is then allowed into the reaction vessel until atmospheric pressure is reached. Thus, the temperature is approximately +100° C throughout the reaction. After 2 min, the specimens are taken out of the reaction vessel.

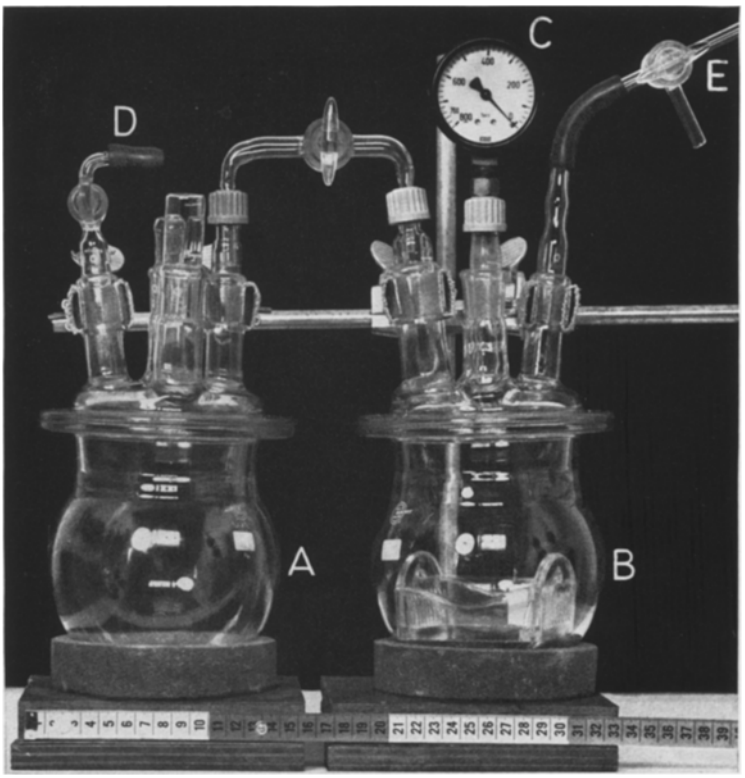


Fig. 1. Equipment used for the GA vapour treatment of the specimens. *A*, 1-litre vessel (GA vessel) containing 2 g glyoxylic acid monohydrate. *B*, 1-litre vessel (reaction vessel) containing the specimens. *C*, manometer recording the pressure in the reaction vessel, *D*, valve to allow for expansion of the air in the GA vessel. *E*, connection to a mechanical vacuum pump

*2.5.2. Reaction through Heating.* The sections are put in a rack and placed in the oven at + 100° C for 6 min.

*2.5.3. Rapid Procedure.* The sections are mounted and analysed directly after the first step of the drying procedure (2.4, above), *i.e.* after 15 min under the hair dryer. In this way sections are available for microscopy within half an hour after dissection.

2.6. The sections are mounted in liquid paraffin and examined in a fluorescence microscope equipped with Schott BG 12 as primary lamp filter, and Zeiss 41 + 47, as secondary barrier filters. For details of the fluorescence microscopical and microspectrofluorometric techniques, the reader should consult, *e.g.*, the paper by Björklund *et al.* (1972 b).

2.7. The disturbing background fluorescence which gradually develops if the mounted, GA vapour treated sections are kept at room temperature, is largely avoided by storing them in a freezer at a temperature of about − 20° C. In this way, the fluorescence microscopical picture of the sections can be preserved for several months.

### *3. The Fluorescence Microscopical Picture of Central CA Neurons*

With the present GA technique, described in Paragraph 2 (above), DA- and NA-containing neurons become strongly fluorescent, emitting a blue fluorescence that appears blueish-green with the filter setting used (see Materials and Methods). This differs from the brownish-yellow, weak and variable fluorescence induced in the indolamine-containing neurons—primarily seen in rats pretreated with the MAO-inhibitor nialamide. The CA fluorescence differed also from the indolamine fluorescence in being notably stable upon irradiation with ultraviolet or blue-violet light. Thus, in its present design, the GA method appears to be essentially selective for DA- and NA-containing structures. As will be discussed in more detail below (Paragraph 6) this is most obvious when the sections are reacted through heating (2.5.2, above). No significant effect of nialamide pretreatment on the fluorescence induced in CA-containing structures could be detected subjectively in the fluorescence microscope. In the control, non-GA-treated, sections from non-perfused tissue, no fluorescent catecholamine or indolamine structures were observed. In the following, some essential features of the fluorescence appearance of the various parts of the central CA neuron will be described.

*a) Cell Bodies*: In the GA treated sections, as in Vibratome sections processed according to the formaldehyde technique (Hökfelt and Ljungdahl, 1972), the morphology of the CA-containing cell bodies was of variable quality. Thus, the fluorescence often covered the nucleus, and the cell bodies sometimes looked distorted and had a diffuse outline. The cellular fluorescence was, however, in many cases considerably stronger than that observed in freeze-dried, FA-treated specimes. This was the case, *e.g.*, with the diencephalic cell groups, A 11 and A 13, located in the caudal thalamus and the medial part of the zona incerta, respectively (Björklund and Nobin, 1973). These cells, which in FA-treated specimens are weakly fluorescent and often only partly visible, had a medium to strong fluorescence in the GA-treated specimens (Fig. 2A). In young animals the perikaryal fluorescence was often considerably stronger than in adults (*cf.* Loizou, 1972), and also the quality of the fluorescence morphology was higher. Consequently, for studies in the rat on the distribution and morphology of CA cell bodies within the CNS using the GA method, young animals (*e.g.* 1–2 weeks old) can be recommended.

Cell processes could in favourable sections be followed for considerable distances from the cell bodies. In almost all CA neuron systems analysed the entire axon—from the cell body up to the terminals—was fluorescent, although the fluorescence in the initial segment of the axon was often rather weak. Interestingly, a GA-induced fluorescence was also observed in the dendrites of many CA cells. Most often only the proximal parts of the dendrites were clearly fluorescent. A notable exception to this has been discovered in the substantia nigra, where the presumed dendrites of the DA-containing cells in the pars compacta (group A 9 of Dahlström and Fuxe, 1964) could be traced for long distances. These processes were seen to project into the pars reticulata, giving rise to a loose network with small, mostly spindle-shaped varicosities quite regularly spaced along the fibres (Björklund and Lindvall, 1974).

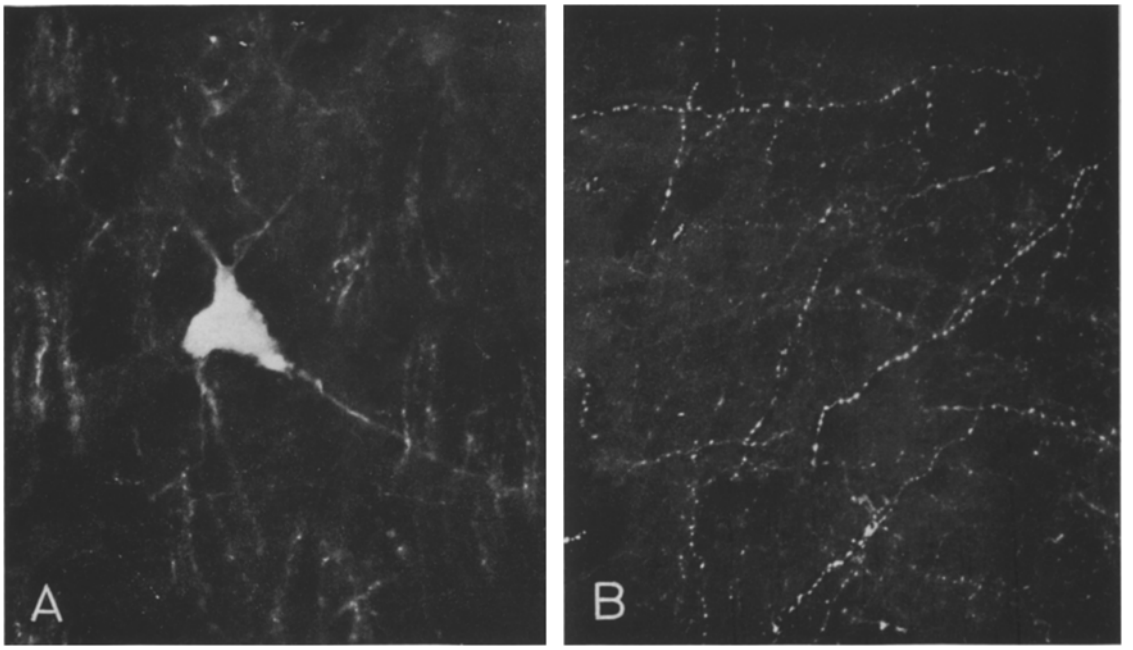


Fig. 2. A. Fluorescent cell body (belonging to the A11 cell group) in the caudal thalamus, situated among the fascicles of preterminal fibres of the dorsal catecholamine bundle. ×295. B. Detail of axons belonging to the medial forebrain bundle (horizontal section) at the level of the dorsomedial hypothalamic nucleus. Collaterals are seen to leave the bundle in medial and lateral direction. ×190. Treatment: Perfusion: 2% GA, 150 ml; Immersion: 2% GA, pH 7.0; GA vapour treatment: 300 torr GA, 2 min, +100° C

*b) Non-terminal Fibres*: The main CA bundles—previously described with the Falck-Hillarp FA method in conjunction with lesions—could be followed from their cell bodies of origin up to the terminal areas in intact, untreated adult animals. Moreover, due to the lack of diffusion, the detailed fluorescence morphology of the individual fibres could be observed. This has proved to be of great value in neuroanatomical work, since axons of the same origin were found to exhibit characteristic morphological features, and in many cases the axonal morphology of different systems is clearlys different and distinguishable in the fluorescence microscope (Fig. 3A–C and 4). This can be used to trace individual systems of axons and axon terminals also in regions and bundles where different systems occur intermingled with each other. In the following paragraphs some

Fig. 3. Non-terminal catecholamine axons in different areas of the rat brain. A. Ascending catecholamine axons with a smooth appearance in the pons ×450. B. Frontal section through the bundle of commissural locus coeruleus axons running ventral to the nuclei. ×430. C. Sagittal section through the medial forebrain bundle at the level of the supraoptic commissures. Axons of the characteristic locus coeruleus appearance are recognized. ×430. Treatment as in Fig. 2

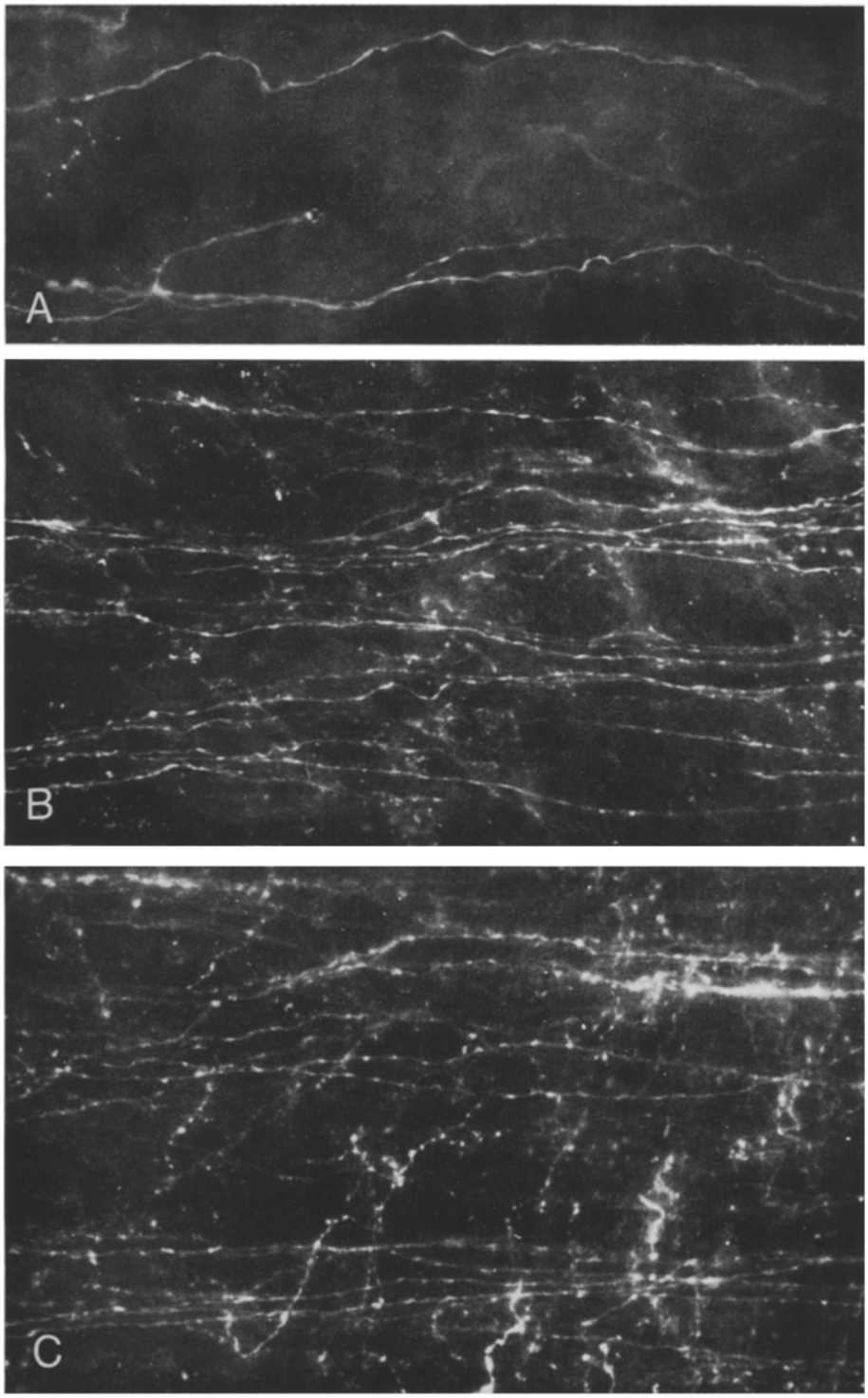


Fig. 3A—C

characteristics of the major axonal systems will be exemplified. For further information the reader should consult the recent paper by Lindvall and Björklund (1974).

The non-terminal fibres within *the dorsal CA bundle* (Ungerstedt, 1971), which originates in NA cell bodies within the locus coeruleus and ascends through the diencephalon and telencephalon to innervate, above all, many regions of the cortex, the hippocampus and the thalamus (Ungerstedt, 1971; Olson and Fuxe, 1972; Lindvall, Björklund, Nobin and Stenevi, 1974; Lindvall and Björklund, 1974), showed strong fluorescence in the GA treated sections. Most axons had a beaded appearance, with closely and rather regularly spaced spindle-shaped fluorescent swellings along the entire length of the fibre, up to the terminal regions where the axonal morphology changed. The smooth intersegments between these varicosity-like enlargements were clearly visible but showed a lower fluorescence intensity (Fig. 3B). This locus coeruleus type of axon could also be identified when intermingling with other axons in the medial forebrain bundle (Fig. 3C) and when sweeping dorsally and caudally within the cingulum to innervate certain parts of the cerebral cortex (Fuxe *et al.*, 1968; Lindvall, Björklund and Moore, 1974).

Collateral branching was common from the locus coeruleus neurons, which are known to give rise to both ascending and descending projections, as well as projections to the cerebellum (see Ungerstedt, 1971). In the mesencephalon the ascending locus coeruleus axons gave off collaterals towards the colliculi and the geniculate bodies, and in passing the fasciculus retroflexus and the stria medullaris some of these axons were seen to give off collaterals dorsally towards thalamic nuclei (*cf.* Lindvall *et al.*, 1974b). This directly demonstrates the rich collateralization of the locus coeruleus neurons previously postulated from indirect evidence by Olson and Fuxe (1971) and Ungerstedt (1971). It seems possible that one locus coeruleus neuron could give branches not only to the cerebral and the cerebellar cortex, as has been described previously (Olson and Fuxe, 1971), but also to mesencephalic, metathalamic and thalamic areas, and perhaps also—via the descending collaterals—to medullary and spinal cord levels.

Within *the medial forebrain bundle* several types of CA-containing axons were observed in addition to the locus coeruleus axons: One type of fibre that was extremely delicate and fine-varicose could be followed from the DA cells of the substantia nigra and adjacent cell groups in the ventromedial tegmentum to the caudate nucleus (constituting the nigro-striatal pathway; Fig. 4) and to the limbic forebrain regions (constituting the meso-limbic pathway). These axons were arranged in fascicles, and had the same fluorescence appearance along their entire length; when ramifying into terminal and paraterminal branches the axons changed their morphology. Other fibres in the medial forebrain bundle, probably originating in pontine and medullary cell groups, appeared thicker with more prominent varicosity-like enlargements. These fibres demonstrated

Fig. 4. Sagittal section through the primarily dopamine-containing nigro-striatal pathway in the lateral hypothalamus. The preterminal axons which are likely to be dopamine-containing are arranged in fascicles and have an extremely delicate varicose appearance. × 195. Treatment as in Fig. 2

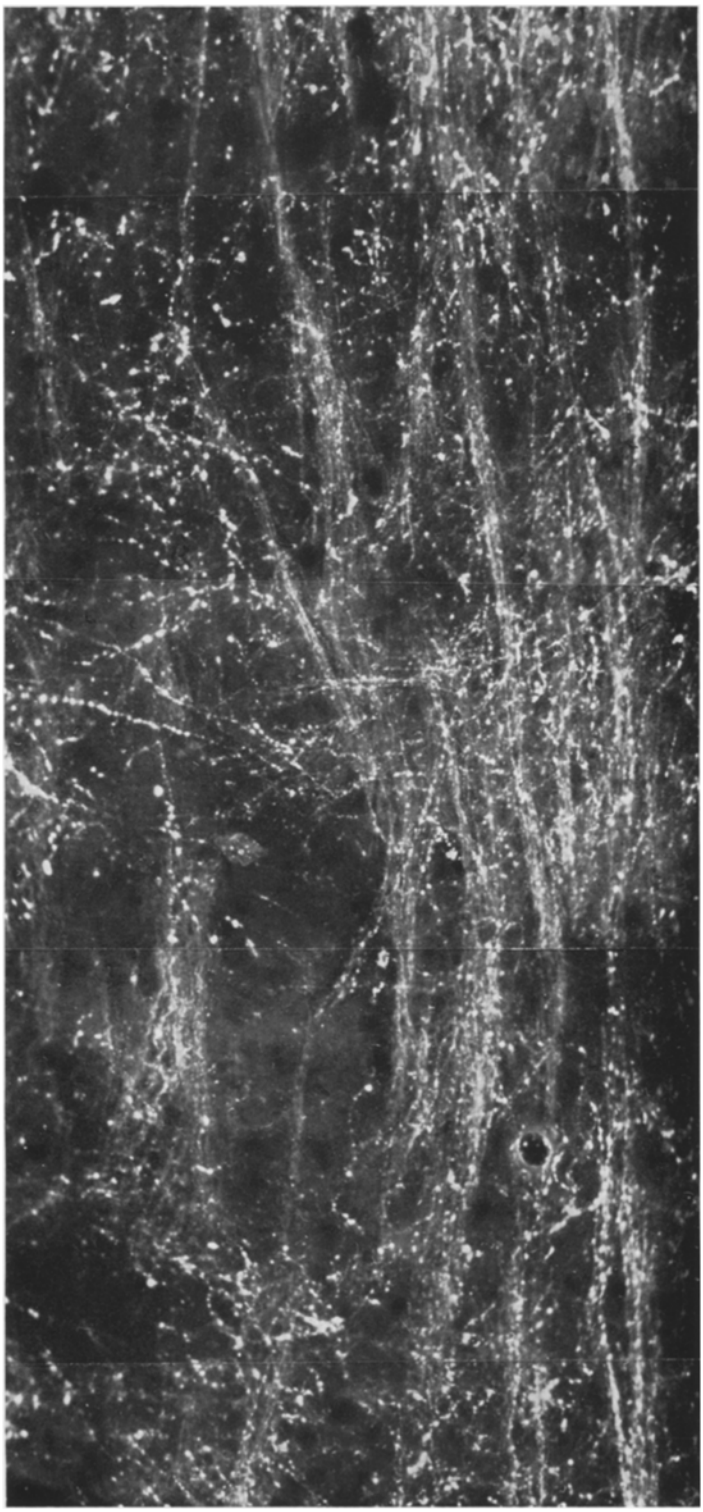

Fig. 4

an irregularly spaced, frequent beading with coarse, ovoid to round varicosity-like enlargements of unregular size and readily observable intervaricose parts. Axons of this type were, *e.g.*, seen to leave the medial forebrain bundle in a medial direction and to give rise to dense networks of CA-containing terminals in several medial hypothalamic nuclei. Some of these axons deviated from the bundle, but most of them were collaterals from axons continuing further rostrally (Fig. 2B).

The present GA method also demonstrated two previously unknown CA systems in the diencephalon (Lindvall *et al.*, 1974b; Björklund, Lindvall and Nobin, 1974). One system originated in cells in the mesencephalic periaqueductal gray substance and in the periventricular zone of the caudal thalamus. Most of the fibres in this so-called *dorsal periventricular CA bundle* had a characteristic, smooth appearance with long intervaricose segments. These axons formed a periventricular system in the hypothalamus and the thalamus, contributing to the adrenergic innervation in both hypothalamic and midline and medial thalamic nuclei. The second diencephalic system disclosed by the GA method formed an *incerto-hypothalamic CA fibre system* with a characteristic fluorescence appearance in the zona incerta; the posterior, dorsal, anterior and preoptic hypothalamic areas; and in the dorsomedial hypothalamic nucleus. These axons—having low-fluorescent, closely spaced fine varicosities and only partly visible intervaricose segments—most probably originate in the A 11 and A 13 DA cells (Björklund and Nobin, 1973) to terminate diffusely in the zona incerta and in dorsal and anterior hypothalamic regions.

From the description above it is evident that the GA method visualizes both known and previously unknown CA-containing axonal pathways, and this raises the question of whether *all* non-terminal axons in the rat brain are revealed by the GA method. Of the known CA axon systems there is possibly one exception: the preterminal axons of the tubero-hypophyseal DA neuron system in the hypothalamus (see Hökfelt and Fuxe, 1972; Björklund, Moore, Nobin and Stenevi, 1973) could not be identified with certainty. It thus seems probable that these fibres contain concentrations of the transmitter too low to be visualized even with the more sensitive GA-technique. It is also possible, however, that the failure to observe these axons is due to the very dense and confused fluorescence picture of this region in the GA-treated specimens.

*c) Terminal and Paraterminal Axons*: CA-containing terminal and paraterminal axonal ramifications, demonstrable with the Falck-Hillarp FA technique (see Fuxe, 1965b), were also observed in the GA treated Vibratome sections. The smooth, intervaricose segments of the terminal fibres were most often clearly visible. The fluorescence picture was extremely distinct and without any signs of diffusion, and consequently the morphology of the individual terminal fibre could be studied. This was particularly evident, *e.g.*, in the caudate nucleus, where the fluorescence was distinct and confined to very delicate, smooth and varicose fibres (identical with the terminals of the nigro-striatal DA system) and some scattered fibres with coarser varicosities (possibly identical with NA-containing terminals). Also in other terminal areas where the fluorescence appears diffuse after FA treatment according to the Falck-Hillarp method—such as the nc. accumbens, the olfactory tubercle and the median eminence of the hypothalamus—the GA induced fluorescence was distinct. It should be noted, however,

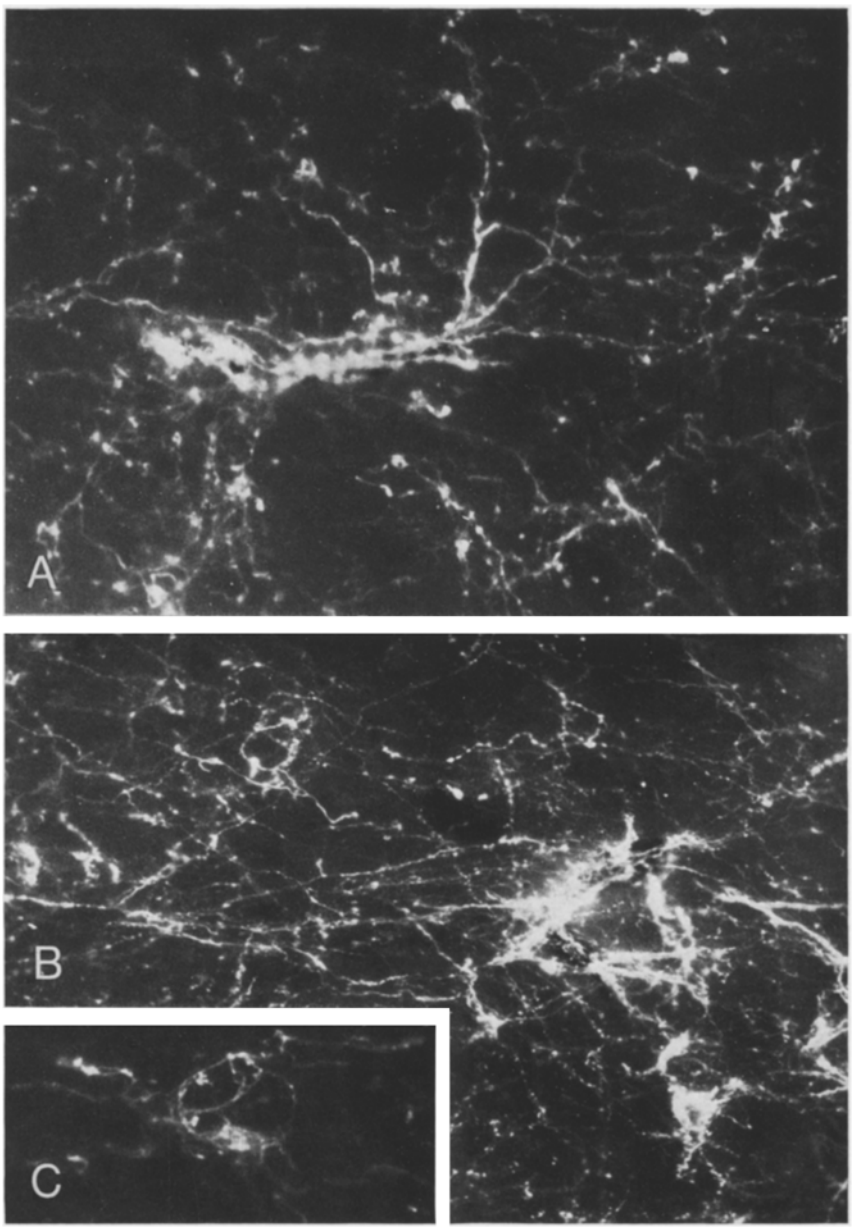


Fig. 5. Arrangements of terminal and paraterminal catecholamine axons in the lateral septal nucleus (A and B) and in the amygdaloid region (C). In A (×740) many, predominantly smooth, fluorescent fibres can be traced towards a septal neuron, giving rise to numerous brightly fluorescent varicosities and clusters of varicosities (not well resolved in the picture) in close apposition to the perikaryon and a presumed dendrite of the septal neuron. B (×270) demonstrates branching, predominantly smooth, fluorescent fibres forming dense, intensely fluorescent terminal arrangements around several cell processes, probably dendrites, in the area. In C (×660) a loose, basketlike arrangement is seen in connection with a number of smooth, fluorescent fibres. Note the irregular arrangement of the varicosities. Treatment as in Fig. 2

that the median eminence-pituitary region—due to its unfavourable superficial position at the bottom of the third ventricle—was particularly difficult to section in the Vibratome.

The morphology of the CA fibres within the terminal areas was, in most cases, in agreement with earlier descriptions of the fluorescence appearance of CA terminal axons (see, *e.g.*, Fuxe, 1965a). Thus, the majority of fibres showed abundant varicosities of varying sizes and shapes spaced more or less irregularly along the fibre. However, in some areas, for example in the lateral septal nucleus and in the amygdaloid region, the morphology of the fluorescent fibres was more heterogeneous, and apart from fibres with the classical terminal appearance (see above), many smooth axons and axons with only few varicosity-like enlargements could be observed (Fig. 5A–C). The, often spherical, fluorescent beads were sometimes arranged two or three together on an otherwise smooth fibre, but they could also occur singly. Entirely smooth fibres were also detected; they were often arranged in such a manner, *e.g.* around neuronal perikarya and dendrites, as to suggest that they, despite lacking typical terminal varicosities, made synaptic contacts on these cells.

With the present technique, delicate varicose axon terminals are demonstrated in many areas where no or few such fibres have previously been detected. This is the case, for example, in the thalamus, hypothalamus and the cortical regions. Many thalamic areas, which appear sparsely innervated by CA fibres in specimens processed according to the standard Falck-Hillarp FA method, were found to be richly supplied with such fibres in the GA-treated sections (Lindvall *et al.*, 1974b), and (as noted above) a previously unknown fibre system was revealed in the zona incerta and the dorsal and anterior hypothalamus (Björklund *et al.*, 1974b). In the cerebral cortex, extensive terminal systems hitherto not described have been demonstrated in *e.g.* the frontal and anterior limbic regions (Lindvall *et al.*, 1974a). Thus, in layers 1–3 of the anterior limbic cortex a very dense aggregation of extremely delicate, varicose fibres with a fluorescence appearance of some resemblance to that of the terminals within the caudate nucleus was observed (Fig. 6). In fact, it has been shown that this terminal system is most probably dopaminergic and has its cell bodies of origin in the substantia nigra (Björklund *et al.*, 1974a). The new axonal systems revealed by the GA method are now being studied in further detail, and descriptions of their morphology are in press or under preparation (Lindvall *et al.*, 1974a, b; Björklund *et al.*, 1974a, b; Lindvall and Björklund, 1974).

Of particular interest for neuroanatomical tracing was the finding that terminal fibres of the same origin, but localized in different brain areas, often had very similar fluorescence appearance. This was the case with *e.g.* most of the axons within the cerebral cortex, cerebellar cortex, and the thalamus, which have a common origin in the locus coeruleus (Lindvall *et al.*, 1974a, b). A classification of terminals, on the basis of their fluorescence appearance in the GA method, thus seems possible and should provide a very useful tool for the identification of projections from one and the same group of cells in different brain regions, as well as for the distinction between different terminal systems in one and the same brain region (see Lindvall and Björklund, 1974).

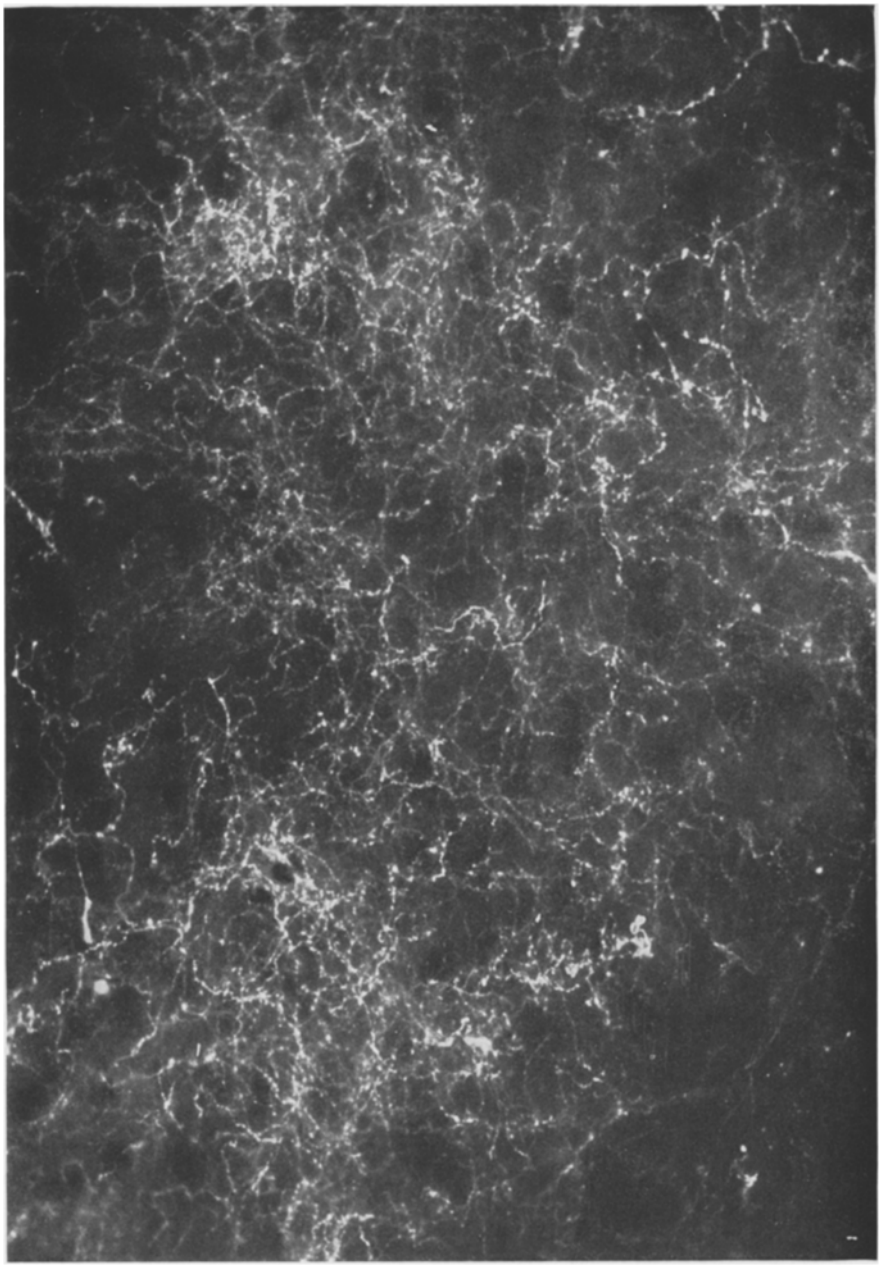

Fig. 6. Horizontal section through the anterior limbic cortex (layers 2–3) of a rat subjected to a bilateral, complete electrolytic destruction of the locus coeruleus 14 days before sacrifice (for details on the extension of the lesion, see Lindvall *et al.*, 1974b). A dense system of delicate, fine-varicose terminal axons can be seen. ×330. Treatment as in Fig. 2

*4. Aspects of the Preparation and Treatment of Peripheral Tissue*

The principles devised for the demonstration of central adrenergic neurons were also applicable to peripheral tissue, and peripheral sympathetic nerves could also be visualized, with high sensitivity and a richness of detail, using the GA-Vibratome technique described above. However, owing to the less favourable consistency, the sectioning of non-nervous tissue was, in most cases, much more difficult than that of brain tissue. Whole mount preparations (iris, mesentery and sciatic nerve were tested) proved very suitable for the GA method (Fig. 7 A and B). In this application, the thin tissues from non-perfused or GA-perfused rats were immersed in the 2% buffered GA solution (pH 7) for 3–5 min. The specimens where then spread out on microscope slides as whole mounts, dried, and reacted with GA vapour as described in Paragraph 2, above. The fluorescence picture of the sympathetic axons and axon terminals was comparable with that obtained under optimal conditions in the standard FA method. The results were the same irrespective of whether the tissue was taken from GA-perfused or non-perfused animals, and good results were also obtained when the GA vapour treatment (Paragraph 2.5.1) was replaced by heating for 6 min at $+100°$ C (Paragraph 2.5.2).

It is evident that the GA method offers an easy, rapid, and highly reproducible way of demonstrating also peripheral adrenergic nerves. This seems to be of special value when a comparison between central and peripheral CA neurons is desired, *e.g.* for morphological or experimental studies. Also, in our hands, the GA method has been more reproducible and easier to standardize.

*5. Uptake of Catecholamines in Vibratome Sections of Central Nervous Tissue*

If fresh brains or brains perfused with buffer alone are used in the Vibratome procedure the tissue sections will still be viable, and thus it should be possible to carry out various kinds of dynamic *in vitro* experiments prior to their processing for fluorescence microscopy. For this purpose the Vibratome sections would serve as substitutes for brain slices, which have been widely used for, *e.g.*, metabolic and functional studies in isolated tissues under *in vitro* conditions (see Elliott, 1969; Farnebo, 1971; Hamberger, 1967). With respect to the CA neurons, the Vibratome sections should be of particular interest for studies on the neuronal uptake and retention of exogeneous amines. In this regard the Vibratome sections are probably more ideal than thicker slices because of the shorter diffusion distances in the thin sections (*cf.* Farnebo, 1971). In the present experiments we investigated the usefulness of the GA-Vibratome method for studies on the uptake of CAs into CA axons. We were particularly interested to test whether the use of selective membrane pump blockers, such as desipramine (see Hamberger, 1967; Horn *et al.*, 1971), could offer possibilities for a differentiation between DA and NA neurons in the fluorescence microscope. The experimental design of Hamberger (1967) was essentially followed. Accordingly, brains from rats treated with reserpine plus nialamide were used, and the sections were incubated in DA, with or without desipramine present, and they were then processed according to the GA technique.

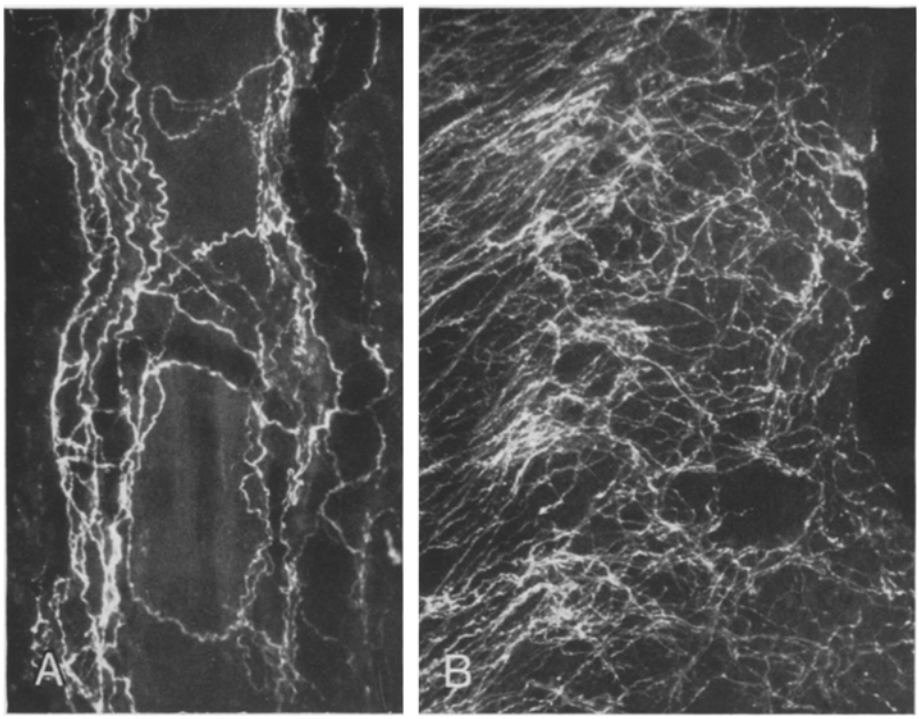


Fig. 7. A. Stretch-preparation of rat mesentery. Vascular plexus around small arterioles and a vein. ×170. B. Stretch-preparation of rat iris. The adrenergic ground plexus of the dilator (left) and the plexus of terminals in the sphincter (right) are seen. ×170. Treatment as in Fig. 2

*a) Procedure*: All rats used in these experiments were pretreated with reserpine (Serpasil®, Ciba-Geigy; 5 mg/kg, 12–18 hrs before killing) and nialamide (300 mg/kg, 1–3 hrs before killing).

The rats were anaesthetized as above and then perfused via the ascending aorta with 150 ml of the ice-cold, neutral Krebs-Ringer bicarbonate buffer. After sectioning (as described in Paragraph 2) the sections (about 35 μ thick) were collected in the buffer trough and then transferred with a glass rod to glass beakers (25 ml volume) containing 10 ml Krebs-Ringer bicarbonate buffer, saturated with a mixture of 95% $O_2$ and 5% $CO_2$, and with 1.8 g/l glucose added (usually 2 sections to each beaker). The sections were preincubated in a metabolic shaker at +37° C for 15 min; in some experiments desipramine (desipramine-HCl; Ciba-Geigy) at a concentration of $10^{-5}$ M was present during the incubation. DA was then added to a final concentration of $5\times10^{-5}$ M, $10^{-5}$ M, $5\times10^{-6}$ M, $10^{-6}$ M or $5\times10^{-7}$ M. The incubation was then continued for another 5, 20, or 45 min. The control sections were incubated in Krebs-Ringer bicarbonate buffer alone during the same time. To the incubation medium ascorbic acid (0.2 mg/ml) and EDTA (0.05 mg/ml) were added. After incubation the sections were immediately transferred to the GA immersion bath and then processed as described in Paragraph 2, above.

*b) Uptake of DA after Reserpine-Nialamide Pretreatment*: The efficiency of the reserpine treatment to deplete the endogeneous CA stores was controlled in the sections incubated in buffer alone; in these sections no fluorescent CA structures were detectable. The uptake and accumulation of DA in the CA axons were detected in the DA-incubated sections as a return of fluorescence in the CA fibres. At all DA concentrations tested optimal results (as refers to fluorescence intensity and morphology of the sections) were obtained after about 20 min. No or only very weak fluorescence was observed after incubation for 5 min. After 20 min incubation there was a pronounced increase in the CA fibre fluorescence, but no further increase was clearly observable in the sections incubated for 45 min. During this longer incubation time the sections started to disintegrate, and the fluorescence picture was often somewhat diffuse.

With all DA concentrations tested ($5\times10^{-7}$ M — $5\times10^{-5}$ M) the uptake—as revealed by the intensity of the GA-induced fluorescence—was markedly higher in presumed DA axons, a finding that is in agreement with previous biochemical and fluorescence histochemical findings (Hamberger, 1967; Snyder and Coyle, 1969). Thus, already at the lowest concentration, $5\times10^{-7}$ M, DA terminals in, e.g., nc. caudatus-putamen, amygdala, and the olfactory tubercle exhibited a strong fluorescence, whereas in presumed NA terminal areas in, *e.g.*, hypothalamus and the sensory-motor cortex only some few fibres had regained a faint fluorescence. With increasing concentration there was a progressive increase in the fluorescence of both DA and NA fibre systems. Thus, after incubation for 20 min in $10^{-6}$ M — $5\times10^{-5}$ M DA a distinct fluorescence was demonstrable in many terminals in hypothalamic and thalamic nuclei. In the sensory-motor cortex a faint fluorescence was observed in abundant varicose fibres, most probably identical with the locus coeruleus NA terminals.

Significant extraneuronal DA uptake was detected as a rise in the general background fluorescence of the section after incubation in $10^{-5}$ — $5\times10^{-5}$ M DA.

*c) Differentiation between DA and NA Neurons in the Fluorescence Microscope*: Desipramine is an inhibitor of neuronal CA uptake that is about 1000 times more potent on the uptake into NA neurons than into DA neurons (Horn *et al.*, 1971). This selective blocking effect of desipramine has been utilized for both biochemical and histochemical differentiation between DA and NA terminals in the CNS (Fuxe *et al.*, 1967; Cuello *et al.*, 1973). In the present experiments we tested whether a blockade of the DA uptake into the NA neurons with desipramine would allow a selective visualization of DA neurons in the DA-incubated Vibratome sections.

In these experiments the DA incubation ($10^{-6}$ M, 20 min) was performed in the presence of $10^{-5}$ M desipramine. According to Hamberger's (1967) observations no uptake of DA into NA structures is demonstrable fluorescence histochemically with the Falck-Hillarp FA technique under these conditions. This was confirmed in the GA treated sections in, *e.g.*, hypothalamus and cerebral cortex. In contrast, the DA uptake into the DA fibres in the nc. caudatus-putamen was seemingly unaffected by desipramine, and here, a distinct, intense fluorescence was obtained in the dense DA terminal network. An unaffected DA uptake was also noted in the system of delicate fibres in the anterior limbic cortex (Lindvall *et al.*, 1974a), which have been proposed to be projections from DA cells in the substantia nigra (Björklund *et al.*, 1974a).

This strongly supports the idea that Vibratome sections (from reserpine and nialamide treated animals) incubated in a CA in the presence of desipramine can be used for a direct differentiation between DA and NA neurons in the GA-treated sections, the NA neurons being non-fluorescent after this procedure.

### *6. Cytofluorometric Analysis*

#### a) Model Experiments

*Fluorescence Yields.* Table 1 lists the relative fluorescence yields of a number of phenylethylamine and indolylethylamine derivatives in histochemical models which were prepared to simulate the perfusion—immersion procedure used on tissue. For this purpose, 2% GA was dissolved in the amine-containing protein solution which was used to make the histochemical models. Two types of treatment were tested: heating for 6 min at +100° C; and GA vapour treatment (300 torr) for 3 min at +100° C (Paragraph 2.5, above). When the GA-containing models were heated alone, only DOPA and the primary CAs, DA and NA, gave strong fluorescence. The fluorescence induced from the CAs was about 5–6 times higher than that obtained in the standard FA reaction (1 hr at +80° C; see Björklund *et al.*, 1972b). No or very weak fluorescence was obtained from the secondary CA, adrenaline, from methoxylated phenylethylamines, and from all indolamines tested. Thus, under these reaction conditions—designed to simulate the conditions under which the GA-perfused and GA-immersed sections are reacted through heating, see Paragraph 2.5.2, above—the GA method appears to be highly selective for NA, DA and DOPA, with respect to amines known to occur in the mammalian CNS.

When GA vapour treatment was performed on the droplet models, there was a dramatic increase in the fluorescence yield from most substances tested, and there was also a moderate increase in the yields from DA and DOPA (Table 1). A striking effect was obtained on the fluorescence yields from the indolamines tested, and also the methoxylated phenylethylamines, 3-methoxytyramine and normetanephrine, became fluorescent. Strong fluorescence was induced not only from primary and secondary indolamines (such as tryptamine, tryptophan, 5-hydroxytryptamine, 5-methoxytryptamine, and N-methyltryptamine) but also from the tertiary indolamine (N, N-dimethyltryptamine) and the N-acetylated indolamines (N-acetyl-5-hydroxytryptamine and melatonin). The fluorescence intensities obtained from the primary and secondary indolamines after the GA vapour treatment was several times higher than that obtained in the standard FA reaction. It was particularly interesting that the N-acetylated and the tertiary indolamines, which give no observable fluorescence upon FA treatment, showed strong fluorescence after GA treatment; the fluorescence yield from melatonin and from N, N-dimethyltryptamine being of the same order as that obtained from NA in the FA method. No or very weak fluorescence was induced from phenylethylamines lacking a cyclization-promoting hydroxy-group in 3-position (see Corrodi and Jonsson, 1967), *i.e.* phenylethylamine, *p*-tyramine and *p*-octopamine.

These observations indicate that, under conditions simulating the procedure in which the GA-perfused and GA-immersed sections are reacted with GA vapour (Paragraph 2.5.1), the sensitivity of the GA method for primary CAs increases

but the selectivity is partially lost. On the other hand, this procedure now provides very interesting possibilities for the visualization of other biogenic monoamines, particularly a wide variety of indolylethylamines, including N-acetylated and tertiary indolylethylamines. However, when the GA method is performed according to the procedure described in this paper, indolamine neurons exhibit only weak and variable fluorescence; and according to our present experience it seems that the procedure in its present form, has a high sensitivity for only DA and NA neurons. As well be described below, this conclusion is partly derived from microspectrofluorometric analyses of the GA-induced fluorophores in the rat CNS.

*Spectral Characteristics.* The fluorescence excitation and emission peak maxima of the GA-induced fluorophores of some biogenic phenylethylamines and indolylethylamines are given in Table 1. The spectra were registered microspectrofluorometrically from dried albumin droplet models containing GA (see Materials and Methods), *i.e.* under conditions simulating the GA perfusion-immersion procedure. As in the fluorescence intensity measurements described above, the models were reacted in one of two ways: either they were exposed to GA vapour (300 torr) at $+100°$ C for 3 min (2.5.1), or they were heated at $+100°$ C for 6 min (2.5.2).

*After reaction through heating alone,* the fluorophores of DA, NA and DOPA —being the only compounds yielding strong fluorescence after this treatment— showed indistinguishable spectra, with excitation and emission maxima at 415 and 475–480 nm. These spectra are characteristic for the 2-carboxymethyl-3,4-dihydroisoquinoline fluorophores—formed from the primary CAs and DOPA in the reaction with GA—in their neutral, quinoidal state (Lindvall, Björklund and Svensson, 1974). These fluorophores exhibit a pH-dependent tautomerism similar to that of the FA-induced CA fluorophores (Jonsson, 1966; Björklund *et al.*, 1968a, 1972a; Lindvall *et al.*, 1974c). Thus, at neutral pH—as in the GA-containing specimens, just after heating—the fluorophores are in their *neutral, quinoidal form* (main excitation peak at 415 nm); and at acid pH, such as obtains after GA vapour treatment (see below), they are in their *acid, non-quinoidal form* (main excitation peak at 370–380 nm). The CA and DOPA fluorophores can also be converted into their acid, non-quinoidal form by exposure to HCl vapour, and, furthermore, this treatment provides a possibility for the spectral differentiation between NA on one hand and DA and DOPA on the other (Lindvall *et al.*, 1974c), according to the principles previously described by Björklund *et al.* (1968a, 1972a) for the FA-induced CA fluorophores. Thus, after about 5 sec of HCl treatment, there is a primary shift of the main excitation peak of all the CA and DOPA fluorophores from 415 nm to 370–380 nm. Upon prolonged treatment (about 10–15 min at room temperature) there is a further shift of the excitation peak of the NA fluorophore down to 330 nm, whereas those of the DA and DOPA fluorophores remain unchanged at 370–380 nm. This latter spectral shift has been obtained, so far, only in models; when the prolonged HCl treatment was applied to the Vibratome sections it resulted in a marked diffusion of the amines from their neuronal stores, making spectral analysis impossible.

*After GA vapour treatment* a greater number of biogenic monoamines give strong fluorescence, and on the basis of their emission peak maxima they can be separated into four groups (Table 1): *Group A* (emission maxima at 430 nm)

Table 1. Fluorescence yields and spectral characteristics of a number of biogenic phenylethylamine and indolylethylamine derivatives after glyoxylic acid treatment[a]. The fluorescence intensities are expressed with the intensity obtained from noradrenaline and dopamine in the standard formaldehyde reaction as 100[b]

| Substance group[c] | Substance | Heating[d] | | | GA vapour treatment[e] | | |
|---|---|---|---|---|---|---|---|
| | | Relative fluorescence yield | Exc. max. (nm)[f] | Em. max. (nm)[f] | Relative fluorescence yield | Exc. max. (nm)[f] | Em. max. (nm)[f] |
| Group A | 1. N,N-dimethyltryptamine | 0 | | | 115 | (340)370 | 430 |
| | 2. N,N-dimethyl-5-hydroxytryptamine (bufotenin) | 0 | | | 35 | (340)370 | 430 |
| Group B1 | 3. 3-methoxy-4,β-dihydroxyphenylethylamine (normethanephrine) | 0 | | | 35 | 330 | 470 |
| | 4. N-methyl-3,4,β-trihydroxyphenylethylamine (adrenaline) | 10 | | | 45 | 335 (370) | 485 |
| Group B2 | 5. 3,4-dihydroxyphenylethylalanine (dopa) | 130 | 415 | 480 | 570 | 330 and 380 | 480 |
| | 6. 3,4-dihydroxyphenylethylamine (dopamine) | 670 | 415 | 475 | 810 | 330 and 375 | 460[g] |
| | 7. 3,4,β-trihydroxyphenylethylamine (noradrenaline) | 460 | 415 | 475 | 445 | 330 and 370 | 460[g] |
| | 8. 3-methoxy-4-hydroxyphenylethylamine (3-methoxytyramine) | 0 | | | 80 | 330 and 370 | 470 |
| | 9. N-acetyl-5-hydroxytryptamine | 0 | | | 60 | (335) 370 | 480 |
| | 10. N-methyltryptamine | 0 | | | 215 | 370 | 485 |
| Group C | 11. tryptamine (β(3-indolyl)ethylamine) | 25 | | | 305 | 370 | 495 |
| | 12. tryptophan | 0 | | | 375 | 370 | 500 |
| | 13. N-methyl-5-hydroxytryptamine | 0 | | | 80 | 370 | 500 |
| | 14. N-acetyl-5-methoxytryptamine (melatonin) | 0 | | | 80 | (335) 370 | 500 |
| Group D | 15. 5-hydroxytryptamine | 10 | | | 125 | 375 | 520 |
| | 16. 5-hydroxytryptophan | 0 | | | 130 | (320) 380 (410) | 530 |
| | 17. 5-methoxytryptamine | 15 | | | 115 | 375 | 530 |
| Other compounds | 18. phenylethylamine | 0 | | | 0 | | |
| | 19. 4-hydroxyphenylethylamine (p-tyramine) | 0 | | | 5 | | |
| | 20. 4,β-dihydroxyphenylethylamine (octopamine) | 0 | | | 25 | | |

[a] The droplet models were made from solutions containing $10^{-3}$ M (for fluorescence intensity measurements) or 1 mg/ml (for spectral analyses) of the substances.

[b] Treatment at +80°C for 1 hr with paraformaldehyde equilibrated in air of about 50% relative humidity, according to the Falck-Hillarp method (see Björklund *et al.*, 1972b).

[c] Classification based on the spectral characteristics after GA vapour treatment.

[d] Reaction by heating (+100°C, 6 min).

[e] Reaction by GA vapour treatment (300 torr, +100°C, 3 min).

[f] Figures indicate peak values ± 5 nm. Figure within brackets indicates the position of a low peak or a shoulder in the spectrum.

[g] At higher concentrations there is a shift of the emission peak maximum to longer wavelengths (about 485 nm).

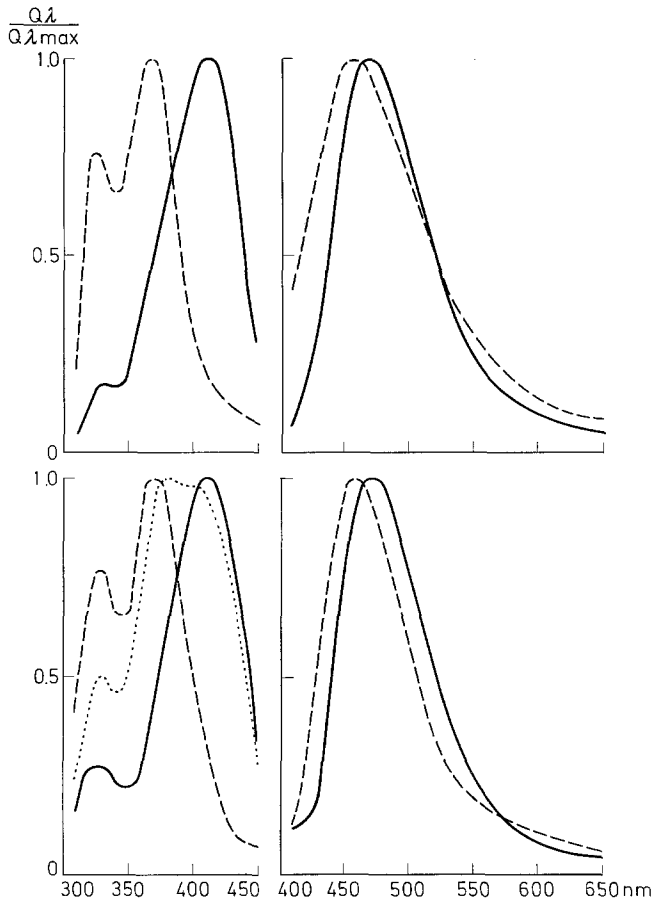


Fig. 8. *Top.* Excitation (left) and emission (right) spectra of noradrenaline in GA-containing protein droplets after reaction through heating alone (+100° C, 6 min; ———), and after GA vapour treatment (+100° C, 3 min, 300 torr; — — —). *Bottom.* Excitation (left) and emission (right) spectra recorded from CA-containing structures in GA treated Vibratome sections. (Treatment: Perfusion: 2% GA, 150 ml; Immersion: 2% GA, pH 7.0; GA vapour treatment: 300 torr GA, 2 min, +100° C). With this type of treatment most spectra demonstrate the fluorophores in their neutral, quinoidal form (———). Spectra showing the fluorophores in the acid, non-quinoidal form (— — —) can also be recorded, as well as spectra indicating a mixture of the two forms of fluorophore (...)

comprises N, N-dimethyltryptamine and bufotenin. *Group B* (emission maxima at 460–485 nm) includes normetanephrine, adrenaline, DOPA, DA, NA, 3-methoxytyramine, N-acetyl-5-hydroxytryptamine and N-methyltryptamine. *Group C* (emission maxima at 495–500 nm) includes tryptophan, tryptamine, N-methyl-5-hydroxytryptamine and melatonin. *Group D* (emission maxima at 520–530 nm) comprises 5-hydroxytryptophan, 5-hydroxytryptamine and 5-methoxytryptamine.

Within Group B, the adrenaline and normetanephrine fluorophores (Group B 1) can be differentiated from those of the other compounds (Group B 2), on the basis

of their excitation spectra, directly after the gaseous GA treatment. The primary CAs and DOPA can easily be distinguished from the other amines in Group B2 as these latter substances show essentially no fluorescence after the type of histochemical reaction where the GA vapour treatment is omitted (see above and Table 1). Moreover, the DA, NA, and DOPA fluorophores exhibit the characteristic, pH-dependent shift in their excitation spectra described above. After GA vapour treatment, the CA fluorophores are in their acid non-quinoidal form, exhibiting a main excitation peak at 370–380 nm (Table 1). A short exposure of the sections to (dry) ammonia vapour at room temperature will transform them into the neutral, quinoidal state, having a peak maximum (415 nm) that is clearly distinguishable from that of the other compounds in Group B2.

b) Tissue Experiments

In the following, the spectral identification of the GA induced fluorophores from DA and NA in the CNS will be discussed. As pointed out above, the pH of the environment strongly influences the spectral characteristics of the CA fluorophores. It has been found that, in the tissue, the tautomeric equilibrium between the acid and the neutral (non-quinoidal and quinoidal) states of the isoquinoline fluorophores depends primarily on the pH of the GA immersion solution, and the GA vapour treatment (time of reaction and GA concentration). Immersion of the tissue sections in an acid GA solution (pH 3) gave clearly acid, non-quinoidal spectra from the CA fluorophores (excitation maxima at 370–375 nm, emission maxima at 460 nm). On the other hand, when the sections had been soaked in a neutral GA solution, the handling of the specimens after this step determined the equilibrium of the fluorophores. Thus, sections that were examined directly after the drying procedure, or after being heated without GA vapour, contained only CA fluorophores in the neutral form (excitation maxima at 415 nm; emission maxima at 475 nm). Exposure to GA vapour involved a partial conversion of the CA fluorophores to the non-quinoidal (acid) form. In most CA containing structures one type of fluorophore was clearly predominant; the spectral analysis giving either neutral or acid spectra. However, spectra that indicated a mixture of the two forms of fluorophore were also recorded (Fig. 8).

With the type of GA vapour treatment used in the standard procedure (described in paragraph 2 above), most spectra demonstrated the fluorophores in their neutral form. When increasing the time of reaction and/or the GA concentration in the reaction vessel, there was a concomitant transformation of the fluorophores into the acid state, and after the most vigorous treatment tested (500 torr, 10 min) acid spectra were most often recorded.

One important aspect of the tautomerism of the GA-induced CA fluorophores is the sensitivity of the method. In case the microscope is arranged for illumination close to the excitation maximum of the fluorophore—such as is the case particularly in microscopes having band interference filters and dicroic mirrors (*e.g.* the Leitz' Ploem-Opak system)—a mixture of the two forms of the fluorophore will result in a decrease in the sensitivity. For this reason it is recommendable to perform the GA method so that the CA fluorophores will occur almost completely in the neutral form.

As indicated by the model experiments, the tautomeric shift in the excitation spectrum (between 415 nm and 370–380 nm) is, in conjunction with the emission spectrum, a very useful spectral characteristic of the CA and the DOPA fluorophores that will distinguish them from those of other biogenic phenylethylamines and indolylethylamines that are known to yield fluorescence in the GA method (see Table 1).

### 7. *Specificity*

The mechanisms of the reactions between GA and primary CAs and indolamines have been studied in detail and the identity of their fluorescent products are known (Björklund *et al.*, 1972c; Lindvall *et al.*, 1974c; Svensson, Björklund and Lindvall, 1974). It has been shown that the reactions underlying the fluorophore formation in the GA method are principally the same as in the FA method (Corrodi and Hillarp, 1963, 1964; Corrodi and Jonsson, 1967; Björklund *et al.*, 1973a). Thus, the first step is a Pictet-Spengler cyclization reaction, yielding the very weakly fluorescent 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid or 1,2,3,4-tetrahydro-$\beta$-carboline-1-carboxylic acid. This is followed by the formation of highly fluorescent products along two different pathways: either by autoxidative decarboxylation to 3,4-dihydro-derivatives, or by intramolecularly catalysed reaction with a second GA molecule to yield the corresponding 2-carboxymethyl-compounds. Under present conditions the latter pathway is predominating. The fluorophore-forming reactions proceed very efficiently in the present technique, the yield of fluorophores from the CAs being almost quantitative (Lindvall *et al.*, 1974c).

On the basis of this knowledge of the GA-monoamine reactions and the identity of the fluorescent products, the *histochemical tests of specificity* in the GA method should aim at a characterization of a) the conditions of fluorescence development, and b) the nature of the fluorophore.

*a) Conditions of Fluorescence Development.* It is a basic requirement that the fluorescence is GA-induced, thus not occurring in tissue not exposed to GA. A fluorescence due to a primary CA (DA or NA) or DOPA will develop during the drying procedure of the GA-perfused and GA-immersed sections, and it will become stronger after either the subsequent heating (6 min at $+100°$ C) or the subsequent GA vapour treatment (2 min at $+100°$ C). Fluorogenic monoamines other than the primary CAs will become significantly fluorescent only after the GA vapour treatment, and it is clear that the fluorescence yield from these amines will be much dependent on the conditions of the GA vapour treatment and perhaps of other steps in the procedure as well. Our experience from the rat CNS (with or without MAO inhibition), however, is that only DA and NA will yield significant fluorescence in the brain sections when the GA method is performed according to the procedure described in the present paper.

*b) Characterization of the Fluorophore.* This is best done with microspectrofluorometric analysis, and—as described above, Paragraph 6—the excitation and emission spectra of the CA fluorophores will distinguish them from other biogenic monoamines known to yield fluorescence in the GA method. In the fluorescence microscope, both the colour of the CA fluorescence (blue or blueish green, depending on the filter setting), and the intensity of the fluorescence at

different activation wavelengths, should be used as general screening tests. The pH-dependent tautomeric shift in the excitation spectra of the CA fluorophores is of special value for their characterization. As described in Paragraph 6, this shift is best studied in the microspectrofluorometer. However, since the acid and the neutral forms of the CA fluorophores have excitation maxima that are close to two of the main lines in the emission spectrum of the mercury lamps (the 365 and the 405 nm lines, respectively), the tautomeric shift can be directly registered in the fluorescence microscope. This is observed as a change in the relative intensity of the fluorescence with the 365 or the 405 nm lines; the acid form fluorescing much stronger at 365 nm, and the neutral form much stronger at 405 nm. As this shift can easily be obtained through brief exposures of the sections to (dry) HCl or $NH_3$ vapours, such exposures can be recommended as a general specificity test for CA-containing structures which does not require access to microspectrofluorometry. For this purpose the fluorescence microscope should be equipped with lamp filters that allow for a change in the illumination of the section, *e.g.* Schott BG 12—for relatively high transmission at 405 nm—and UG1 for relatively high transmission at 365 nm.

In conjunction with the histochemical tests, the use of *pharmacological tests*, such as have been used extensively in the applications of the Falck-Hillarp FA method (see Corrodi and Jonsson, 1967; Björklund *et al.*, 1972b), can be recommended. Such tests involve, for example, disappearance of the fluorescence after depletion of the amine with reserpine or α-methyl-*m*-tyrosine treatment, or after amine synthesis inhibition, *e.g.* with α-methyl-*p*-tyrosine. When working with previously non-characterized systems great care should be taken to ascertain the CA nature of the induced fluorescence, and this should always involve microspectrofluorometric analyses.

## Discussion

The new GA method possesses a number of attractive features. It has a high sensitivity for CA neurons; it has a high precision, demonstrating the CA structures with a great richness of detail, and the procedure is relatively simple and quick. Normally, the sections are ready for microscopy the day after they are made, but if desired, sections of good quality can be obtained within 1/2–1 hour. The method is, thus, unusually fast; even quicker than, for example, the so-called smear technique (Olson and Ungerstedt, 1970), which is often used to obtain rapid results in the Falck-Hillarp FA method, particularly in cortical areas where this method otherwise is fairly capricious. Furthermore, in the GA-Vibratome technique the adrenergic neurons are demonstrated without the loss of morphology that goes with the smearing procedure.

Experiences gained so far indicate that the GA method is very well reproducible and rather easy to standardize and that variations in the outcome of the method—*e.g.* during different times of the year or during different weather conditions—are not obvious. Such variations are quite disturbing and sometimes deleterious in the FA method and it thus seems that the GA method is one way of getting round such problems. The high reproducibility of the GA method is

probably due, at least in part, to the fact that the fluorophore-forming reactions are very little dependent on the humidity. This is in contrast to the FA reaction, where the amount of water is critical for the outcome of the technique.

The most attractive field of application for the GA method is, undoubtedly, in neuroanatomical studies of central DA and NA neurons. For this purpose it appears to be superior to any other available method. This is, first, because of its high sensitivity, which has made possible the visualization of the entire axons and has revealed several previously unknown axonal systems in the brain. Second, the very distinct and precise picture of the CA-containing structures allows observations on details of the systems such as axonal morphology, branching patterns, terminal arrangements—information that has proved to be of very great value in neuroanatomical tracing (*cf.* Lindvall and Björklund, 1974). Third, incubation of Vibratome sections (taken from rats pretreated with reserpine and nialamide) in DA, after blockade of the uptake into NA neurons with desipramine, seems to allow a selective demonstration of central DA neurons with the GA method. Finally, the quality of the Vibratome sections—lacking cracks and other freeze-drying artifacts—contributes to the achievement of good structural integrity.

It is obvious, however, that the GA method applied to Vibratome sections is not convenient for all types of fluorescence histochemical work on CA neurons. One limitation of the technique is its capacity: from each brain usually only one piece can be sectioned (unless more than one microtome is used), and, so far, a means of preserving brain pieces for longer time before sectioning has not been discovered. This also means that in experiments where many animals are killed at the same time there is, at present, no way of keeping the brains for later sectioning. In such cases freeze-drying and subsequent storage in paraffin blocks is much more convenient. On specimens processed in this way the GA method has not given results that are better than those of the Falck-Hillarp FA method, and as the penetration of GA in tissue is very limited, the GA vapour treatment has to be done on tissue sections. For this reason the FA treatment, performed *en bloc*, would in most cases be more convenient for freeze-dried specimens. In this context it should be pointed out that brain tissue from GA-perfused animals can be freeze-dried and processed according to the Falck-Hillarp FA procedure (see Björklund *et al.*, 1972b) with good results. This has been done in our laboratory: thus, where one region of an animal has been processed according to the GA-Vibratome technique, other regions have been freeze-dried and processed according to the FA method. This is of particular advantage in lesion work where both the area of the lesion and several terminal areas have to be analysed in each brain.

Another very useful application of the GA method is on whole mount preparations, which can be obtained from many peripheral organs (see, *e.g.*, Falck, 1962, and Furness and Malmfors, 1971). The specimens are then soaked in a cold GA solution of neutral pH and processed for fluorescence microscopy as described in Paragraph 4. Although so far not tested, this should be useful also for the study of CA neurons in culture, the GA method probably representing a sensitive, rapid and convenient technique for this purpose.

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