GLYOXYLIC ACID CONDENSATION: A NEW FLUORESCENCE HISTOCHEMICAL METHOD FOR SENSITIVE AND DETAILED TRACING OF CENTRAL CATECHOLAMINE NEURONS

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THE HISTOCHEMICAL formaldehyde fluorescence method (FALCK, 1962; FALCK *et al.*, 1962; CORRODI and HILLARP, 1963, 1964) demonstrates central adrenergic neurons with high sensitivity and specificity. However, whereas the visualization of the cell bodies and axon terminals can be accomplished with the Falck–Hillarp method, demonstration of the non-terminal parts of the axons is, due to their very low amine concentration, usually not possible in intact and untreated adult animals. This requires localised mechanical or chemical lesions of the neuron in order to increase the intraaxonal amine concentration (ANDÉN *et al.*, 1966; UNGERSTEDT, 1971; JACOBOWITZ and KOSTRZEWA, 1971; SACHS and JONSSON, 1972).

Glyoxylic acid (GA) was recently introduced as a fluorescence histochemical reagent for biogenic monoamines and related compounds (AXELSSON *et al.*, 1972; BJÖRKLUND *et al.*, 1972; AXELSSON *et al.*, 1973), and in model experiments (AXELSSON *et al.*, 1973) it was demonstrated that this reagent has a considerably higher capacity than formaldehyde to form fluorophores with biogenic indolamines and catecholamines. When the GA method is applied to Vibratome sections of GA perfused brain tissue (LINDVALL *et al.*, 1973; LINDVALL and BJÖRKLUND, 1973; BJÖRKLUND *et al.*, 1973b) the catecholamine (CA) neurons are demonstrated with a sensitivity and richness in details that is superior to the standard Falck-Hillarp method. Thus, the GA method has proved extremely useful for sensitive and detailed neuroanatomical studies on CA neurons in the CNS. The following is a brief presentation of the principal features of the new method.

CHEMISTRY

The fluorescence induced from primary and secondary phenylethylamines and indolylethylamines in the GA method has been shown to be due to the efficient formation of highly fluorescent isoquinoline and β-carboline fluorophores, similar to the Falck–Hillarp formaldehyde method (BJÖRKLUND *et al.*, 1972, 1973a). The GA reaction proceeds in two steps (BJÖRKLUND *et al.*, 1972). In the first step, the phenylethylamine or indolylethylamine reacts with GA in an acid catalyzed Pictet-Spengler condensation yielding the 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid or 1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid, respectively, via a Schiff's base. These very weakly fluorescent compounds can be transformed into strongly fluorescent molecules in two alternative ways: via autoxidative decarboxylation to the 3,4-dihydroisoquinoline or 3,4-dihydro-β-carboline, or through a second, intramolecularly acid catalyzed reaction with another GA molecule to the 2-carboxymethyl-3,4-dihydroisoquinolinium or 2-carboxymethyl-3,4-dihydro-β-carbolinium compound.

The high fluorescence yields in the GA reaction can probably be referred mainly to the efficient formation of the strongly fluorescent 2-carboxymethyl-3,4-dihydro compounds through intramolecular acid catalysis exerted by the carboxyl group on the 1-carbon of the tetrahydroisoquinoline or tetrahydro-β-carboline molecules. The interested reader is referred to the paper by BJÖRKLUND *et al.* (1972) for further details and discussions on the reaction mechanisms.

METHODOLOGY

For a detailed methodological description of the GA fluorescence method applied to Vibratome sections the reader should consult the paper by LINDVALL and BJÖRKLUND (1973). The principal steps of the GA procedure are as follows: Adult rats are perfused via the ascending aorta with ice-cold 2% GA in a Krebs–Ringer bicarbonate buffer. The pH of the perfusion solution is adjusted to 7·0 with NaOH. Fifty ml is perfused during about 3 min; thereafter the brains are rapidly dissected out and cooled in the buffer. Pieces of brain tissue are then cut on a Vibratome® instrument (Oxford Instruments, San Mateo, Calif., USA) in 30 μm thick sections, according to the principles introduced by HÖKFELT and LJUNGDAHL (1972). During the sectioning procedure, the tissue piece is immersed in Krebs–Ringer bicarbonate buffer (pH 7·0) kept at a temperature between 0 and +5°C by metal blocks cooled to a very low temperature by a dry ice–ethanol mixture. These metal blocks are changed at suitable intervals. The sections are immersed for 5 min in the ice-cold GA perfusion medium and then transferred to glass microscope slides. Excess buffer is removed with a filter paper, and the sections are then put under the warm air-stream from a hair-dryer for 15 min. The sections are kept overnight *in vacuo* in a desiccator containing fresh phosphorous pentoxide. All sections are then treated with GA for 2 min according to the procedure described by LINDVALL and BJÖRKLUND (1973). Briefly, this GA treatment is performed as follows: 2g GA monohydrate (dried over phosphorous pentoxide for about 24 hr) are heated at +100°C for at least 1 hr in a closed vessel connected to the reaction vessel, which is placed together with the GA vessel in the oven. After evacuation of the reaction vessel with a vacuum pump, hot GA saturated air (from the GA vessel) is introduced into the hot reaction vessel—containing the tissue specimens—to a partial pressure of 300 torr (mm Hg). Hot air is then let into the reaction vessel to atmospheric pressure. Thus, the temperature is approximately +100°C throughout the reaction. As controls, non-GA treated sections from non-perfused brains are used. The sections are mounted in liquid paraffin and examined in a fluorescence microscope equipped with Schott BG 12 as primary lamp filter and Zeiss 50+47 as secondary barrier filters.

COMMENTS

When the GA method is applied according to the description above dopamine-containing and noradrenaline-containing neurons become strongly fluorescent, whereas the fluorescence induced in indolamine-containing structures is lower and more variable, and the present procedure does not appear useful for the visualisation of intraneuronal indolamines. The CA-containing neurons emit a green fluorescence, which differs from the brownish-yellow fluorescence induced in the indolamine-containing cells. In the control non-GA treated sections from non-perfused tissue, no fluorescent monoamine structures are observed.

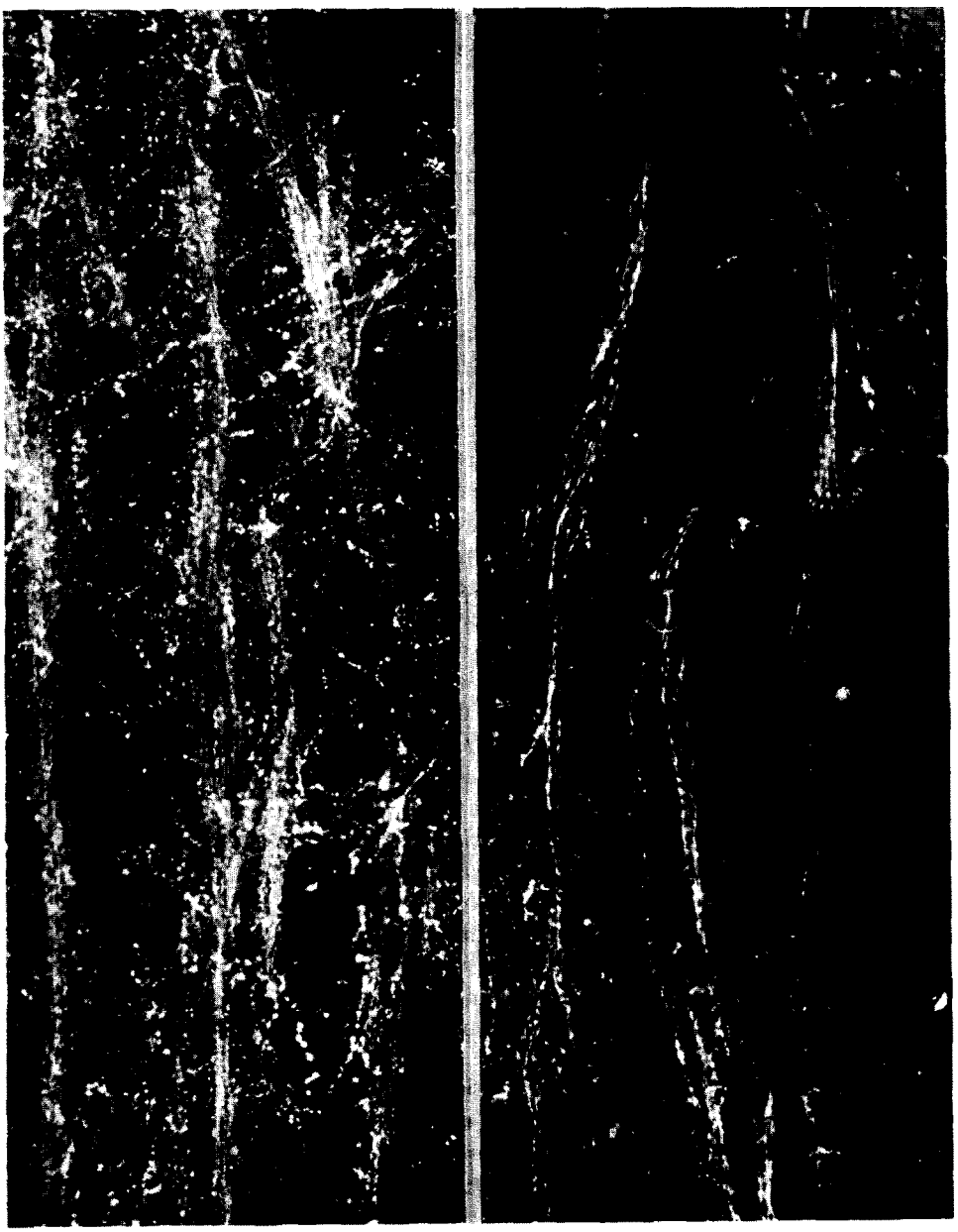

FIG. 1.—Bundles of non-terminal catecholamine axons in the rat brain. (top) Sagittal section through the primarily dopamine-containing nigro-striatal pathway in the lateral hypothalamus. The axons which are likely to be dopamine-containing have an extremely delicate varicose appearance. ×210. (bottom) Sagittal section through the noradrenaline-containing dorsal catecholamine bundle at the level of the interpenduncular nucleus. ×210.

The fluorescence microscopical picture obtained in the GA-treated sections is characterized by an extreme sensitivity and precision in the visualization of the central CA neurons. The high sensitivity is valid above all for the axonal part of the central adrenergic neuron. Thus, the individual CA fibres can in most cases be followed from their cell bodies of origin all the way to the terminal area. This is e.g. the case for the CA axons within the nigro-striatal pathway (Fig. 1), the medial forebrain bundle and the dorsal CA bundle (Fig. 1). Moreover, systems of non-terminal axons not previously described have been demonstrated in many areas of the brain.

Systems of CA-containing axon terminals demonstrable with the Falck-Hillarp technique (FUXE, 1965) are observed also in the GA treated sections. In most terminal fibres the smooth intervaricose segments are clearly visible despite their low fluorescence intensity. With the present technique many areas show a more prominent adrenergic innervation than observed earlier, and furthermore in many areas considered to be practically devoid of CA-containing structures the new method has revealed abundant CA terminal systems. Thus, in the thalamus, whose CA innervation previously has been considered insignificant in comparison with that of the hypothalamus (FUXE, 1965; FUXE *et al.*, 1969), prominent supplies of delicate varicose axon terminals are demonstrated (BJÖRKLUND *et al.*, 1973b).

The usefulness of the GA method for the demonstration of adrenergic cell bodies is limited because of their variable morphology in the Vibratome sections. The fluorescence often covers the nucleus and the cell bodies sometimes have a diffuse outline. The cellular fluorescence is, however, in many cases stronger than in formaldehyde-treated, freeze-dried specimens.

The high precision and richness in details in the visualization of the CA neurons are most probably due not only to the increased sensitivity (see above), but also to the absence of diffusion in the GA treated sections. Thus, the fluorescence in the caudate nucleus is distinct and confined to very delicate, smooth and varicose fibres. In addition, scattered fibres with coarser varicosities are observed, possibly identical with noradrenaline-containing terminals. In the median eminence, where the fluorescence also often appears diffuse after formaldehyde treatment, each of the very delicate, beaded palisade fibres can be traced through the external layer up to the surface. The richness in details of the CA-axons in the GA treated sections is further demonstrated in e.g. the septal nuclei, where a fine structural picture is obtained of the terminals located around neuronal perikarya and dendrites.

CONCLUSIONS

In the GA method, the intraneuronal CA:s are very efficiently transformed into strongly fluorescent molecules in a well-defined reaction with GA. When the method is applied to sections of GA perfused brain tissue, the CA neurons are demonstrated with a sensitivity and richness in details that is superior to the standard formaldehyde method. Thus, the individual CA fibres can be followed from their cell bodies to the terminal area and the detailed morphology of the axon can be studied. Consequently, the GA method seems to be *ideal for studies on the details of organization of the CA neuron systems in the CNS.*

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