LABILE STORES OF MONOAMINES IN THE CENTRAL NERVOUS SYSTEM: A HISTOCHEMICAL STUDY[1]

B. CSILLIK[2] AND S. D. ERULKAR

Department of Pharmacology, University of Pennsylvania Schools of Medicine, Philadelphia, Pennsylvania

Accepted for publication June 17, 1964

Within the last year, there has become available a histochemical technique for the demonstration of tissue catecholamines (Falck, 1962; Carlsson *et al.*, 1962). Studies with this technique have shown that in the central nervous system of the rat, the main sites of catecholamine storage are the hypothalamus and caudate nucleus with occasional fibers seen in other areas (Carlsson *et al.*, 1962). These results agreed with those of M. Vogt (1954) who determined concentrations of norepinephrine (NE) and epinephrine in the central nervous system of cats and dogs using bioassay methods.

The present study began from the premise that the neurons of the lateral geniculate body of the cat would contain either catecholamines or 5-hydroxytryptamine (5-HT) or both, as synaptic transmission at this site could be suppressed after administration of large doses of lysergic acid diethylamide (LSD) (Evarts *et al.*, 1955; Bishop *et al.*, 1958). It became apparent, however, that there existed much catecholamine or 5-HT in the fibers not only of the lateral geniculate body, but also in many other areas of the central nervous system, including cortex, hippocampus, substantia nigra, and spinal cord. These results differed in degree with those of Carlsson *et al.*, and of M. Vogt. We found that our results agreed with theirs when we followed their procedures of removing the tissue from an animal which had been killed several minutes previously. On the other hand, if the tissues were removed from anesthetized living animals, a much more widespread distribution of fibers containing monoamine was apparent.

We have therefore postulated that there exists in the brain and spinal cord a widespread system which contains monoamines in only a labile form, as contrasted to the labile and stable storage forms postulated for the hypothalamus and peripheral autonomic system (Costa *et al.*, 1963; Axelrod, 1963).

METHODS. Fifteen cats and 28 albino rats were used. The animals were anesthetized by an intraperitoneal injection of 42 mg/kg sodium pentobarbital (Nembutal, Abbott). After removal of the calvarium, areas of the cortex (3 x 4 x 4 mm) were removed and frozen immediately with solid carbon dioxide. The hippocampus was exposed by removal of the cortex by gentle suction, and after a sample of the former had been obtained, the lateral geniculate body was exposed, removed, and frozen. Samples were fixed to chucks by physiological saline solution (0.85% NaCl). Sections of 10 μ were cut in a cryostat, placed on nonalbuminized slides and dried at room temperature for at least 1 hour, but for less than 3 hours. The slides were then incubated at 75°C for 1 hour in sealed Coplin jars containing 5 g paraformaldehyde (Trioxymethylene, Fisher).

In his original paper, Falck (1962) stated that cryostat sections cannot be used for the histochemical method to localize catecholamines. We found, however, that both in the central and peripheral nervous systems cryostat sections of samples fixed to chucks with physiological saline solution gave consistent results, provided they were dried thoroughly before the treatment with formaldehyde vapor. (See, *e.g.*, the adrenergic nerve fibers around blood vessels at the basal surface of the brain in fig. 1.) In our opinion, the main point here is the necessity to fix tissues with saline instead of distilled water (as usual), because the water from the molten ice surrounding the section might release catecholamines, which may account fo· Falck's failure to observe fluorescence in cryostat sections. After incubation, the sections were observed and photographed with or without mounting under darkfield illumination of a Leitz high pressure mercury lamp UV-microscope, equipped with BG-12, UG-1 and Schott 4-mm filters. Parallel sections were stained with cresyl violet to identify structures in visible light. The intensity of the fluorescence was greatest when the sections were observed unmounted, but

Received for publication March 30, 1964.
[1] Supported by Public Health Service Grants NB-02941 and NB-00282-11.
[2] Present address: Department of Anatomy, University Medical School, Szeged, Hungary.

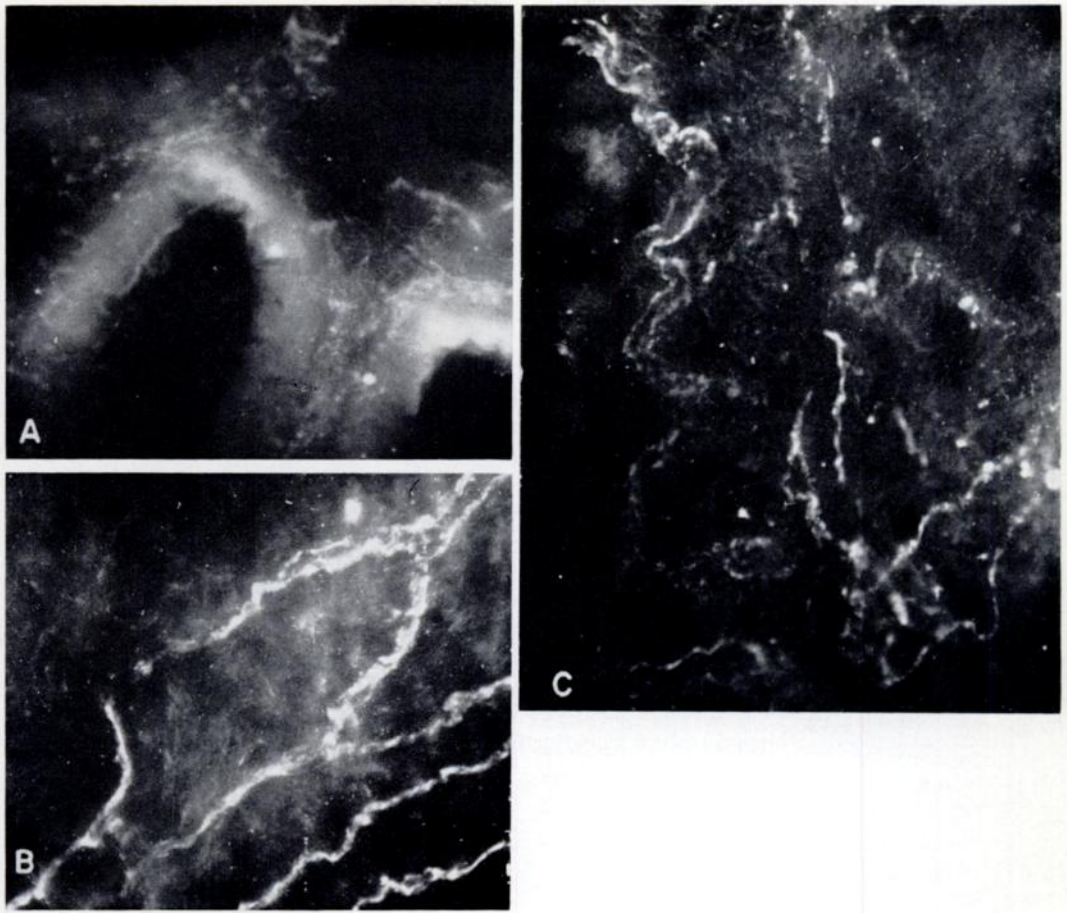

FIG. 1. Nerve fibers in the walls of blood vessels at the basal surface of the brain.
Specimens removed 10 minutes after death of the animal. × 650. a) Cross section; b and c) tangential sections.

in these cases the specimens contained blue bubble-like artifacts (fig. 2) which crowded the field. When the sections were mounted in Entellane (Merck), the artifacts were eliminated, but at the same time the intensity of the fluorescence was diminished (fig. 2).

RESULTS. 1. *Lateral geniculate body.* In the cat, the perikarya of the neurons in the lateral geniculate body possessed a granular pigment material which displayed a red autofluorescence even in sections not treated with paraformaldehyde vapor. This distinguished the cell bodies clearly from the neuropil. In sections taken from living, anesthetized animals, however, nerve fibers with a characteristic beaded appearance, which possessed a greenish-yellow fluorescence, could be identified throughout the dorsal nucleus (fig. 3). These fibers were abundant in the dorsal nucleus, but sparsely distributed in the ventral nucleus. It was impossible to determine whether these fibers were going to or from the cell bodies. In some cases they could be seen wrapped around the nerve cell (fig. 3) as if making synaptic contact. No greenish-yellow fluorescence (suggesting the presence of monoamines) was ever detectable in the cell bodies of the lateral geniculate nucleus. The fluorescent nerve fibers among and around the nerve cells (referred to in the following descriptions as "intrinsic" fibers) differed from the sympathetic fibers which surrounded blood vessels, in that the latter were more strongly fluorescent and not so beaded. The color of the fluorescence, however, was the same in both

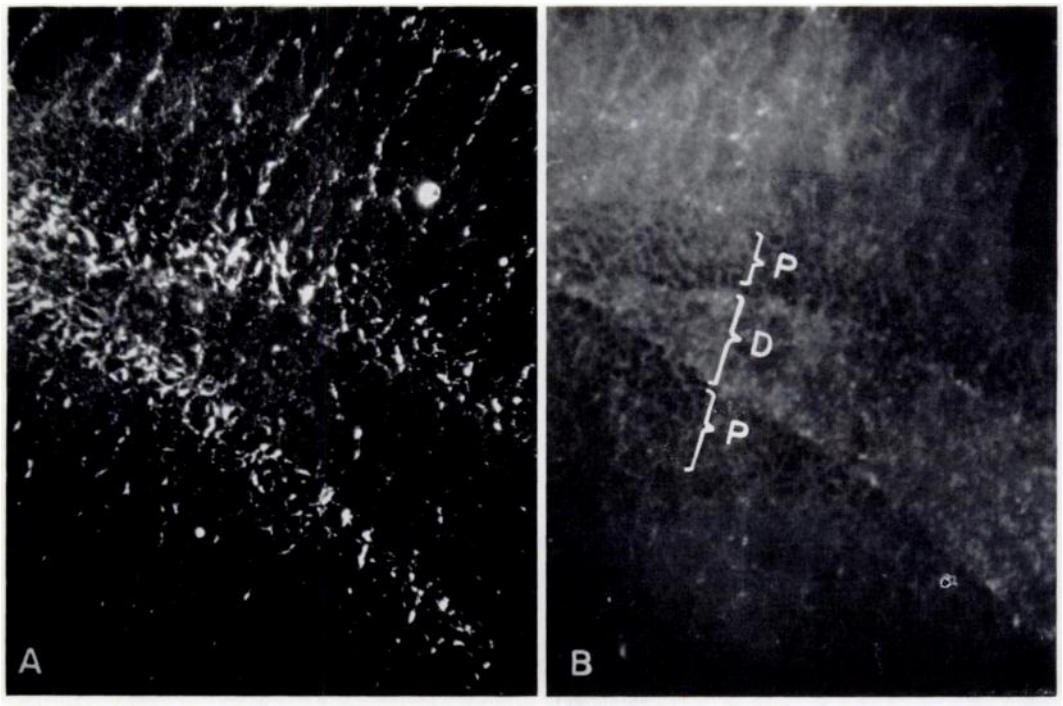

FIG. 2. Sections of rat hippocampus made after removal of the specimen from a living anesthetized animal. × 120.
A. Fluorescence seen before mounting the section in Entellane. B. Diminution of fluorescence after mounting the section in Entellane. P = pyramidal layer; D = apical dendritic layer.

types of fiber. Furthermore, if the sections were taken after the death of the animal, the fluorescence remained in the perivascular sympathetic fibers, but not in the intrinsic fibers. Figures 3D and 3E show that fluorescence of these fibers persisted with normal strength and distribution in two cats after enucleation of both eyes 11 days previously. In these cats, control sections of the optic nerve, stained by the Bodian silver proteinate method, showed almost complete degeneration of nerve fibers, although a few thin fibers appeared to have remained undegenerated.

Similar findings concerning fluorescent fibers were obtained in the rat lateral geniculate nucleus. The fluorescence was no longer present in fibers from animals killed by either decapitation or by an overdose of sodium pentobarbital. Sections taken from either side of the same brain of the anesthetized animal showed that within 1 minute of the cessation of heart beat there occurred a slight diffusion of the fluorescence resulting in loss of the beaded appearance and a definite diminution of the fluorescence in the fibers themselves. Seven minutes after the heart had stopped, the fluorescence disappeared from the fibers. This pattern of disappearance of fluorescence also was seen in the sections taken from the lateral geniculate nucleus of the decapitate animal.

2. *Cortex.* The precrueiate gyrus and lateral gyrus of the cat and equivalent sensory areas in the rat were examined for monoamines in samples obtained from living animals. In Layer 1 the majority of the fluorescent fibers crossed parallel to the surface, but immediately below this layer the fibers coursed irregularly through the remaining gray matter of the cortex. As in the lateral geniculate body, these fibers appeared to make synaptic contact with cell bodies in the cortex (figs. 4B and C), but it was impossible to determine this unequivocally. No monoamine-fluorescence was seen in the cell bodies, although an autofluorescent pigment was usually present. No greenish-yellow fluorescence was ever seen in the white matter.

In samples of the cortex obtained from animals killed 10 minutes previously (either by

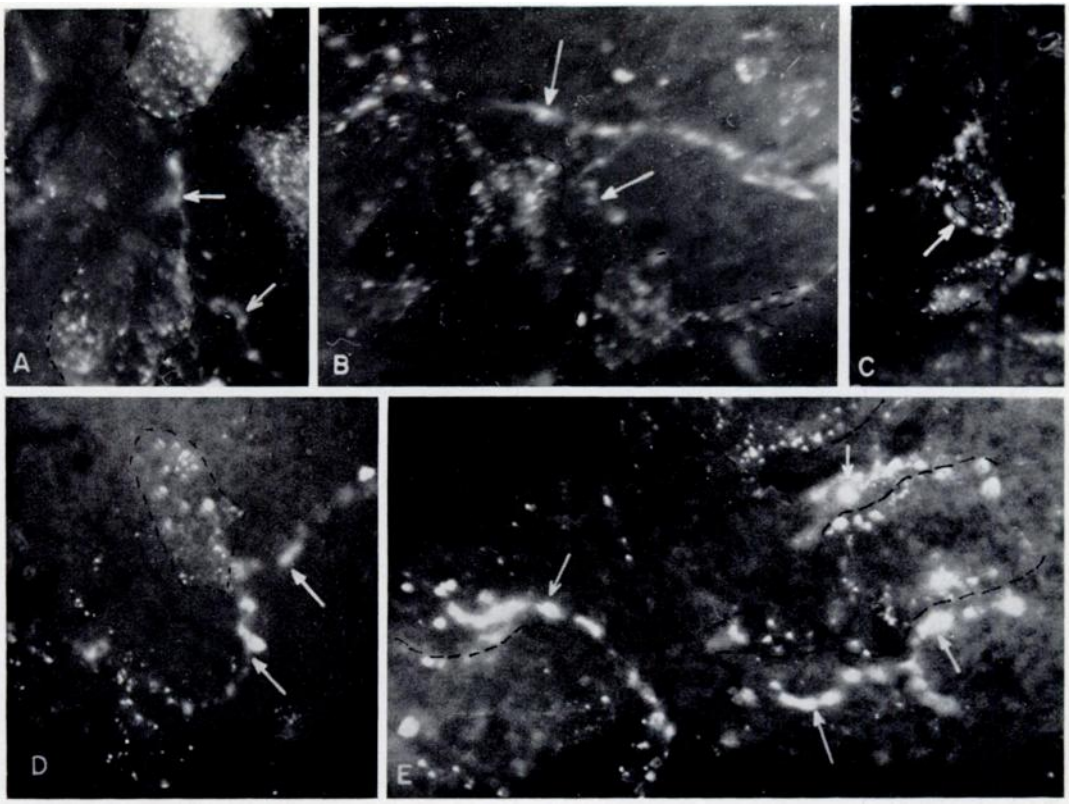

FIG. 3. A, B. Cat lateral geniculate body removed from living anesthetized animal. Dashed lines outline cells which show autofluorescence, arrows point to "intrinsic" fibers which have beaded appearance. × 700.

C. Rat lateral geniculate body from living animal. × 600. D, E. Sections taken from living anesthetized cat, 11 days following bilateral enucleation. Fluorescence appears essentially the same as that seen in sections taken from normal cat (A and B). × 700.

decapitation, or with an overdose of Nembutal), only occasional fluorescent fibers could be seen, and a diffusion of the fluorescent material into the neuropil was apparent.

3. *Hippocampus.* Sections of the hippocampus were obtained only from rats. In the apical dendritic layer numerous fluorescent fibers were seen, scattered irregularly, but no fluorescent fibers were seen in the pyramidal layer. Fluorescence was seen in the basilar dendritic layer, but this was not as strong as that seen in the apical dendritic layer (fig. 2). Only a few fibers remained fluorescent 10 minutes after death, in contrast to the abundant fluorescent fibers seen in sections taken from the living anesthetized animal.

4. *Spinal cord.* Fluorescent fibers with labile monoamines were seen in the gray matter of the cord, but not in the white matter. Fibers (fig. 4D) coursed across both interneurons and motoneurons, in some cases appearing to make synaptic contact. These fibers were not seen following death of the animal.

5. *Substantia nigra.* The only locus at which we have found cell bodies which contained a labile store of monoamines was in the substantia nigra. Figures 5 and 6 show the bright fluorescence in the large nerve cells of the magnocellular division of the substantia nigra at diencephalic level of the right side, obtained from the living anesthetized rat. Figures 6D and E show the fluorescence to have almost disappeared from the equivalent cells of the substantia nigra of the left side taken 10 minutes after death of the same animal. Interestingly, we failed to detect significant fluorescence in many of the axons leading away from the cells, although, as figure 6A shows, some of their processes did

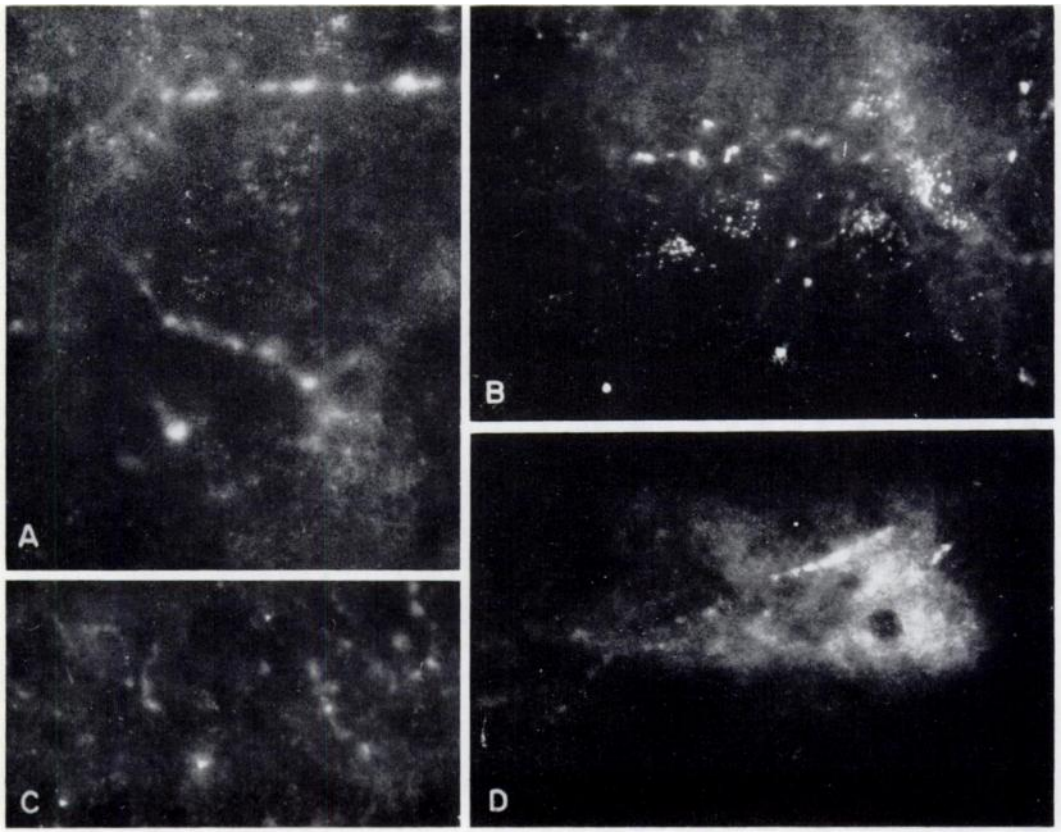

FIG. 4. A. Beaded appearance of fluorescent fibers in rat thalamus. × 800.
B. Fibers seen in cat cortex coursing through layers 3 or 4. Cells showing autofluorescence can also be seen. × 800. C. Fibers in rat cortex. × 800. D. Spinal cord of cat. Beaded fluorescent fiber seen crossing anterior horn cell which shows autofluorescence. × 70.

exert strong fluorescence. In these same sections taken from the dead animal, the hypothalamic nerve fibers and cells showed the same strong fluorescence as in the living animal.

Preliminary experiments suggested that some of the cells of the red nucleus may also have shown fluorescence, but the results in these cases were equivocal.

6. *Other procedures.* In an effort to obtain more precise data on the chemical nature and sites of synthesis of this labile substance, further procedures were carried out:

A. *Bilateral adrenalectomy* was performed on two rats, 3 and 9 days prior to the histochemical investigation. This procedure did not evoke any alterations in the fluorescence of the nerve fibers at sites previously mentioned or in the cells of the substantia nigra.

B. *Bilateral removal of the superior cervical ganglion* performed on two rats 6 days prior to histochemistry also failed to alter the fluorescence of the intrinsic nerve fibers at all sites investigated. These included lateral geniculate body, cortex, hippocampus, and substantia nigra. On the other hand, the sympathetic (perivascular) fibers surrounding blood vessels showed complete absence of fluorescence due to the degeneration of the nerve fibers themselves.

C. *Reserpine* was administered to two cats in an initial dose of 5 mg kg, and followed by a second dose of 2.5 mg kg 24 hours later. The animals were killed 24 hours after the second injection. Histochemical investigation showed that all the greenish-yellow fluorescence disappeared, both of sympathetic (perivascular) and intrinsic nerve fibers at all sites investigated. No change was observed in the red autofluorescence of the lateral geniculate neurons.

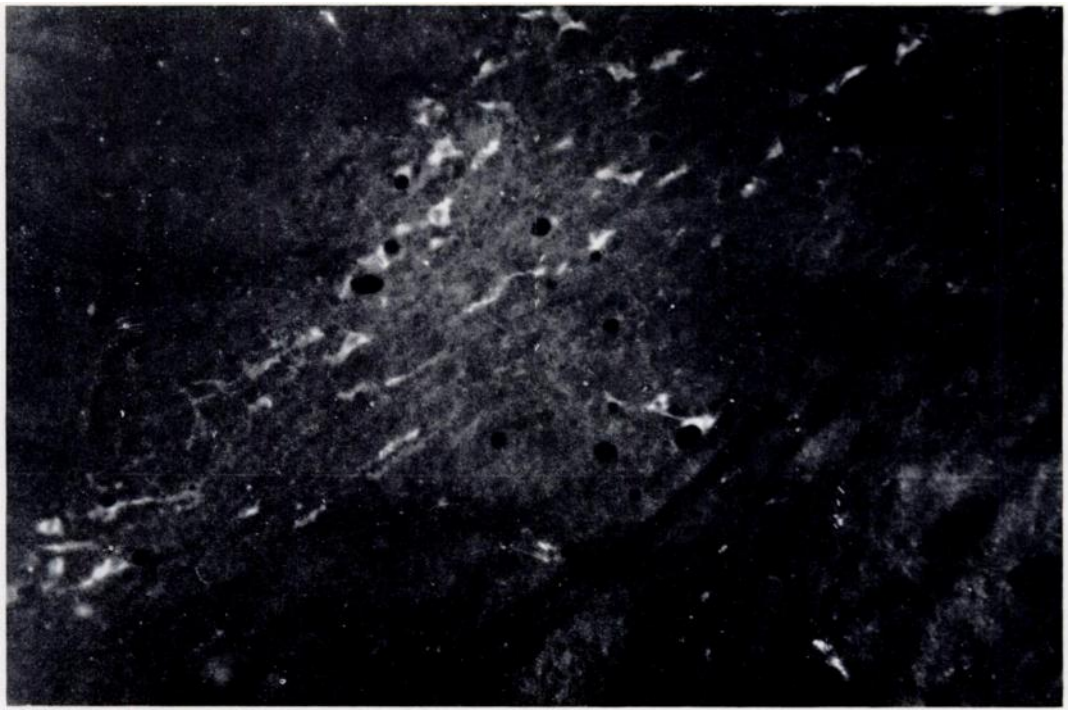

FIG. 5. Fluorescence of cell bodies and fibers of the magnocellularis division of the substantia nigra of the rat; specimen removed from living anesthetized animal. × 140.

D. *α-Methyl-meta-tyrosine* given to two rats in single doses of 400 mg/kg intraperitoneally caused complete absence of fluorescence in both sympathetic and intrinsic fibers 24 hours after administration.

Both the reserpine and the α-methyl-*meta*-tyrosine treated animals showed considerable behavioral depression 24 hours after the administration of these drugs.

DISCUSSION. The first question which arises is whether this greenish-yellow fluorescence which could be seen in nerve fibers of tissues taken from living, anesthetized animals does indeed represent the presence of monoamines. The color of the fluorescence was identical to that seen at the peripheral adrenergic nerve terminals and to that seen in the hypothalamus. The fact that reserpine administration caused complete disappearance of this fluorescence would indicate that it could be either 5-HT or NE. Norepinephrine is most likely the substance since α-methyl-*meta*-tyrosine caused its complete disappearance. If it is indeed NE, it may be in a differently bound form to that seen in the hypothalamus and in peripheral structures, but we cannot exclude the possibility that this fluorescence was caused by some compound with chemical properties similar to NE. It is unlikely that the substance was dopamine, for Carlsson *et al.* (1962) have stated that most of the fluorescence due to the presence of dopamine in the caudate nucleus of the mouse persisted after administration of α-methyl-*meta*-tyrosine. We would like to say here, however, that it is exceedingly difficult to identify the amine on the basis of color alone. Carlsson *et al.* (1962) report "... a clearly observable green to yellow-green or green-yellow to yellow fluorescence." True it is that they state that "The very fine smooth fibers with a more yellowish fluorescence may well contain 5-HT, however," but the main difficulty in our results lay in deciding how much green and how much yellow was present. Carlsson and his colleagues also mention the presence of cell bodies containing red-brown autofluorescent material, and this was similar to that found in the lateral geniculate body cells. This material does not, they conclude, contain catecholamines.

If we compare our results with those obtained

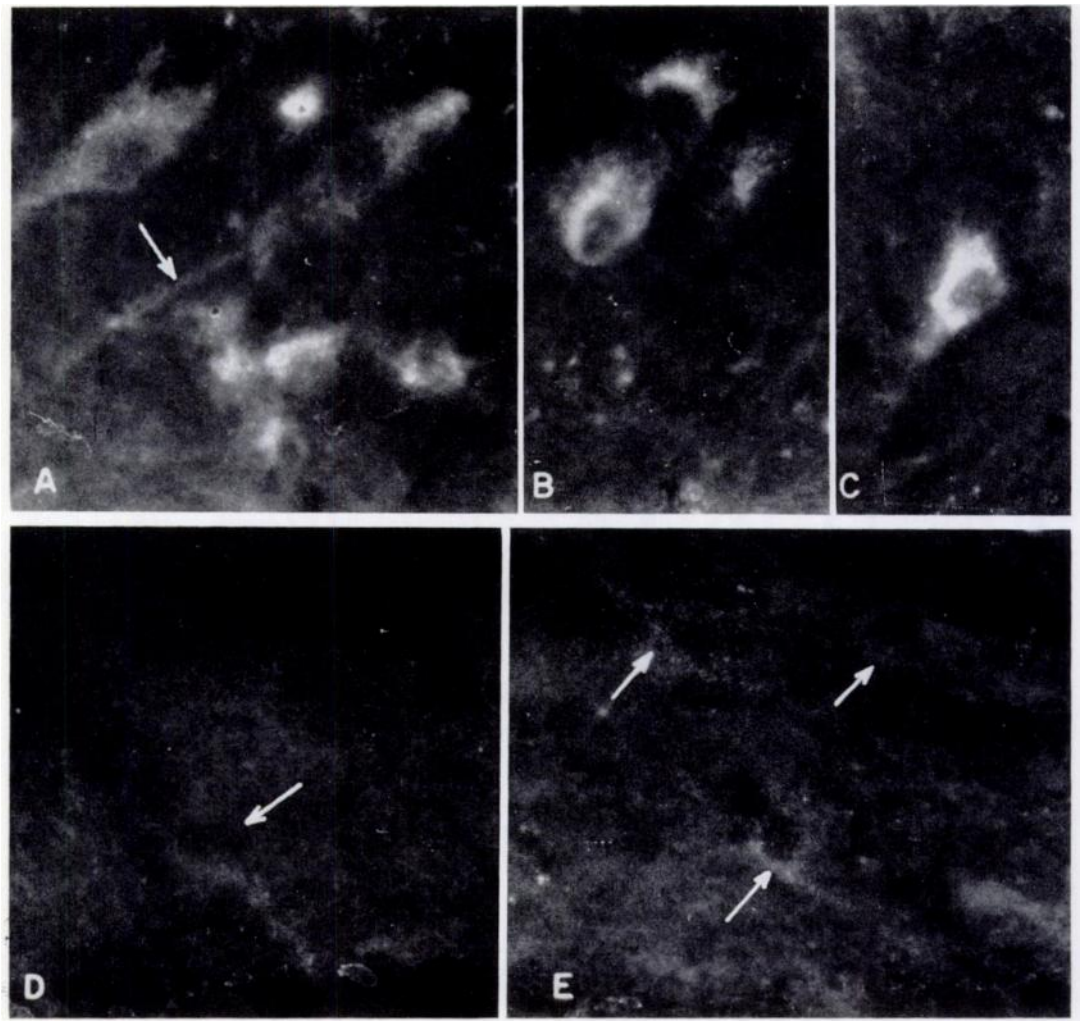

FIG. 6. A, B, C. Fluorescence in cells of right substantia nigra removed from living anesthetized rat. × 750.
D, E. Loss of fluorescence from cell bodies of left substantia nigra, after removal of the specimen 10 minutes after death. Same animal as that used for A, B, and C. × 750.

by M. Vogt (1954), striking differences can be seen in NE content for different parts of the central nervous system. For instance, her results conclusively demonstrated relatively low values of NE in the lateral geniculate nucleus and in the gray matter of the hippocampus. Her results agree with those of Carlsson *et al.* (1962), and it therefore appears that both her measurements and theirs were on the stable store of NE, whereas our results include labile NE.

We should, however, also consider the possibility that the presence of the fluorescence in the fibers was due to the release of labile monoamines by the barbiturate anesthetic. We feel that this possibility is unlikely, not only in view of the widespread distribution of the fluorescence, but also in view of the similarity of the temporal relationships of the disappearance of the fluorescence in decapitated rats and in those killed by an overdose of sodium pentobarbital. One minute after death in both cases, there occurred a diffusion of the fluorescence and diminution in intensity of the fluorescence in the fibers, although some fluorescence did persist. This, however, disappeared within 7 minutes.

The diffuse distribution of the fluorescent fibers throughout the central nervous system raises the question where the origin of this substance lies. First, it may be derived from the well-known central stores of NE such as the hypothalamus and the area postrema. It cannot be derived to any significant extent from the adrenal gland, for the fluorescence persisted after adrenalectomy. Secondly, it may be synthesized in the neurons of the fibers in which it is seen. Finally, the fact that this labile substance was detected only in the somata of the magnocellular division of the substantia nigra (and possibly in the cells of the red nucleus) strongly suggests that these sites may be involved in either the production, storage, or breakdown of this substance.

What may be the function of this labile NE? There was no conclusive evidence in these experiments that this labile NE was present in synaptic structures (*e.g.*, boutons) and the possibility of its acting as a neurohumoral transmitter at all sites seems to us to be remote. In the lateral geniculate body virtually complete degeneration of the optic nerve resulted in no diminution of this substance, so it is unlikely to be a transmitter from the optic nerve to the cells of the lateral geniculate body. It would be interesting to see whether transneuronal degeneration of the cells of the lateral geniculate body could have caused a diminution in the amount of the substance present. The loss of synaptic transmission following administration of LSD indicates that catecholamines or 5-HT are involved in the transmission, and it may be that these fluorescent fibers belong to the internuncial neurons operating with these transmitter substances in this nucleus. It was striking that those drugs which caused loss of the fluorescent material also caused significant behavioral depression in these animals. Although it has been stated (Costa *et al.*, 1963) that α-methyl-*meta*-tyrosine administration elicits no sedation, the large doses used in this study may account for the difference in the results.

It seems more reasonable to us that the labile monoamines are present in dendrites, which may provide a background level of excitability, and influence the initiation of activity in the neurons. The support of this idea is the finding that this labile store of monoamines was localized to the dendritic layers in the hippocampus. Spencer and Kandel (1961) have shown that it is in these layers that prepotentials originate which cause some depolarization in the pyramidal neurons and may trigger activity. It may well be, therefore, that this labile store of monoamines is responsible for dendritic activity, and thus for a background of excitation for many cells in different areas of the central nervous system.

SUMMARY

The distribution of tissue monoamines in cat and rat central nervous systems has been studied by histochemical techniques. Samples of the brain taken from living anesthetized animals showed that monoamines were present in cells of the substantia nigra, and in fibers of the cortex, hippocampus, lateral geniculate body, spinal cord, hypothalamus and caudate nucleus. In brain samples from previously killed animals, monoamines were only present in the hypothalamus and caudate nucleus. This suggests the presence of a labile store of monoamines with a more widespread distribution than has hitherto been described. The functional implications of this widespread distribution are discussed.

ACKNOWLEDGMENTS. We wish to thank Dr. G. B. Koelle for his kindness and help throughout this study. We are also grateful to Dr. J. Sprague and Mrs. Mary Etherington for their help in the protargol staining.

REFERENCES

AXELROD, J.: The Formation, Metabolism, Uptake and Release of Nor-adrenaline and Adrenaline in Monoamines, Elsevier Publ. Co., New York, 1963.

BISHOP, P. O., FIELD, G., HENNESSEY, B. L. AND SMITH, J. R.: J. Neurophysiol. **21:** 529, 1958.

CARLSSON, A., FALCK, B. AND HILLARP, N. A.: Acta physiol. scand. **56:** suppl. 196, 1962.

COSTA, E., GESSA, G. L., KUNTZMAN, R. AND BRODIE, B. B.: The effect of drugs on storage and release of serotonin and catecholamines in brain. in Pharmacological Analysis of Central Nervous Action, Proceedings of First International Pharmacological Meeting, ed. by W. D. M. Paton, vol. 8, Pergamon Press, Oxford, 1963.

EVARTS, E. V., LANDAU, W., FREYGANG, W., JR. AND MARSHALL, W. H.: Amer. J. Physiol. **182:** 594, 1955.

FALCK, B.: Acta physiol. scand. **56:** suppl. 197, 1962.

SPENCER, W. A. AND KANDEL, E. R.: J. Neurophysiol. **24:** 272, 1961.

VOGT, M.: J. Physiol. **123:** 451, 1954.