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SHORT COMMUNICATION

A simple method for the serial sectioning of fresh brain and the removal of identifiable nuclei from stained sections for biochemical analysis[1]

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STUDIES of the distribution in the brain of putative transmitters and of the enzymes involved in their synthesis and degradation provide important information concerning sites where these substances might play a role in synaptic transmission. The anatomical resolution of such studies is limited by the sensitivity of the assay techniques employed and by the procedures used to dissect the brain. With recent increases in the sensitivity of radiochemical methods for assaying a number of putative transmitters and their associated enzymes, a need has arisen for a method for the rapid and reproducible dissection of small regions of the brain. We report here a simple method for serially sectioning fresh brain and dissecting small visually identifiable nuclei from these sections. In addition, we present evidence that, at least in certain cases, these sections can be lightly stained with methylene blue prior to dissection without interfering with subsequent biochemical measurements. Such staining allows the visualization of many nuclei which are not easily identifiable in unstained sections.

METHODS AND RESULTS

Procedure for sectioning brain and dissecting individual nuclei

Rats (200–300 g) are anesthetized with Equi-Thesin (4 mg/kg; Jensen Salsbery). The following operations are then performed in a cold room (4°C). Animals are rapidly perfused through the left ventricle of the heart with 100 ml of ice-cold Krebs–phosphate (0°C). The brain is removed from the skull and placed for a minute or two in a beaker filled with the cold buffer. Serial sections are cut using a McIlwain tissue chopper (Mickle Laboratory Engineering Co.) (McILWAIN & BUDDLE, 1953) in which the cutting stage has been replaced by a Petri dish containing a layer of wax (Fig. 1). The brain is blotted, attached to a piece of filter paper by a few drops of Loctite Quickset Adhesive IS12 (Loctite Ireland Ltd.) and pinned to the layer of wax. After cutting a few sections the position of the brain relative to the blade can be readjusted, if necessary, to ensure that the brain is cut in a frontal plane.

Sections of 200 μm or more thickness (we usually use 400 μm sections) are cut one at a time by manually regulating the speed control knob of the tissue chopper. When the brain is firm and the speed at which the blade moves is kept slow, the cut sections adhere to the blade (Fig. 1) from which they can be easily removed with a moist brush. The sections are then placed on glass slides and kept on ice in the cold room until they are dissected. The entire procedure from the injection of the anesthetic until the forebrain is completely sectioned takes approx 15 min.

While the McIlwain tissue chopper has been traditionally used to produce fairly crude brain 'slices', we have found it also suitable for cutting complete frontal sections which are highly suitable for dissection under a dissecting microscope (Fig. 1). Perfusing the brain with ice-cold buffer and sectioning it in a cold room seem to be essential features of the procedure as suitable sections could not be obtained from unperfused brains or from perfused brains at room temperature. In two experiments, using thermister electrodes, we found that perfusion produced a drop of about 20°C in the thalamus after 2–3 min. For sectioning of larger brains, such as the cat brain, the blade holder of the tissue chopper can be modified to allow for a larger cutting surface. In such cases, single-edged razor blades are used instead of double-edged blades due to the greater rigidity of the former.

Although the dissection is not performed in the cold room, care is taken to keep the sections cold (though not frozen). For this purpose we have constructed a simple metal box which fits onto the stage of the dissecting microscope and which can be filled with dry ice. The glass slides containing the sections are placed on the box and illuminated from below through an aperture in the center of the box. This type of illumination greatly aids in the visualization of discrete nuclei. The nuclei are then easily removed using dissecting knives. In order to isolate a particular nucleus in its entirety it must usually be dissected from several sections. The tissues are kept on ice in a covered ice bucket until the dissection is completed, weighed in the cold room to minimize drying due to evaporation, and stored at −20°C in small glass homogenizers until they are assayed. Alternatively the nuclei can be frozen directly and their protein content determined later.

Since the dissection procedure was developed to facilitate the study of the distribution of enzymes involved in neurotransmitter synthesis, we sought to determine whether any changes in these enzymes occurred as a result of the procedure itself. For this purpose the activities of two such enzymes—choline acetyltransferase and glutamate decarboxylase—were compared in hippocampi removed either *in toto* from the whole brain after decapitation or from a series of frontal sections prepared as described above. The two enzymes were measured by slight modifications of the assays described by FONNUM (1969) and ROBERTS & SIMONSEN (1963) respectively. No significant differences were found in the activity of either enzyme suggesting that no deterioration had occurred as a result of the different aspects of the procedure (i.e. anesthesia,

[1] Part of this work has been presented to The Physiological Society (BEN-ARI & ZIGMOND, 1975).

TABLE 1. LACK OF EFFECT OF STAINING ON THE ACTIVITY OF VARIOUS ENZYMES AND ON THE MEASUREMENT OF PROTEIN CONTENT

	Control	Stained	$\frac{\text{Stained}}{\text{Control}} \times 100$
Choline acetyltransferase activity (μmol ACh/g/h) (n = 25/3)	17.7 ± 0.9	17.0 ± 0.9	98.0 ± 4.4
Glutamate decarboxylase activity (μmol CO_2/g/h) (n = 20/4)	7.81 ± 0.29	7.43 ± 0.3	94.0 ± 3.4
Tyrosine hydroxylase activity (nmol Dopa/g/h) (n = 27/3)	179.2 ± 8.8	184.1 ± 10.8	105.3 ± 5.8
Protein content measured by the Lowry method $\left(\frac{\text{Protein (g)}}{\text{Wet weight (g)}} \times 100\right)$ (n = 7/1)	11.7 ± 1.2	10.7 ± 0.3	91.4 ± 8.4

Frontal sections were bisected on the midline and half of the section was stained for 10 s with 0.2% methylene blue and then washed with Krebs–phosphate buffer. The brain region to be studied was then removed from the control and stained hemi-sections and weighed on a torsion balance. Choline acetyltransferase activity, glutamate decarboxylase activity and protein content were measured in control and stained sections through the hippocampus. Tyrosine hydroxylase activity was measured in the caudate–putamen. Number of sections studied and the number of animals from which these were removed are shown (N). The units of measurement are given for each assay. The data are also expressed as the ratio of the stained to the control samples taken from each section. The data are all expressed as the mean values ± S.E.M.

perfusion, sectioning the brain, dissecting individual nuclei).

Use of methylene blue to increase the anatomical resolution of the dissection procedure

To increase further the anatomical resolution and reliability of the dissection procedure, sections were stained with a 0.2% solution fof methylene blue (Vital Stain, BDH Chemicals U.K.) made up in Krebs–phosphate buffer. Since the methylene blue often precipitated out of the buffer upon standing, the solution was left stirring overnight before use. In addition, if during the experiment any precipitate was detected in the staining dish, a fresh portion of stain was used. Sections were stained at room temperature for about 10 s and then washed twice in ice-cold buffer. This light staining allowed the identification of certain nuclei, such as the basolateral, basomedial or anterior lateral nuclei of the amygdala (see BEN-ARI *et al.*, 1976) which could not be seen in unstained sections.

In order to evaluate whether this staining procedure interfered with the subsequent biochemical measurements we wished to make, we studied the effect of the procedure on the estimation of the activities of choline acetyltransferase, glutamate decarboxylase and tyrosine hydroxylase. For this purpose, individual frontal sections were bisected at the midline and half of the section was stained with methylene blue. The brain region to be studied was then removed from the stained and control hemi-sections and frozen for subsequent biochemical analysis. Choline acetyltransferase and glutamate decarboxylase were assayed in the hippocampus by the methods described above. Tyrosine hydroxylase activity was measured in the caudate–putamen by the method of HENDRY & IVERSEN (1971). The tissue was homogenized in 25 vol of 5 mM-Tris (pH 6) containing 0.1% Triton X-100. Tetrahydrobiopterin was used as the cofactor (1.5 mM) and the final concentration of tyrosine was 51 μM.

Staining sections with 0.2% methylene blue for about 10 s had no significant effect on the activities of any of these enzymes nor on determination of protein content by the method of LOWRY *et al.*, 1951 (Table 1).

DISCUSSION

Until recently, detailed information on the localization in the brain of putative neurotransmitters and their associated enzymes has been derived largly from histochemical methods such as the histofluorescence method for norepinephrine and dopamine (DAHLSTROM & FUXE, 1965) or the histochemical method for cholinesterase (SHUTE & LEWIS, 1967). Such methods, while achieving a great deal of anatomical precision, are not suitable for obtaining quantitative information concerning the concentrations of these substances in various parts of the brain. Quantitative biochemical studies on the other hand have mainly been performed on rather large areas of the brain. In addition it is often difficult to determine exactly what parts of the brain are included in these studies. However, it is now possible using highly sensitive radiochemical assay methods together with a dissection procedure which allows the visualization and removal of individual brain nuclei to achieve a great deal more anatomical precision in such studies.

While our results suggest that light staining with methylene blue may be generally useful in neurochemical studies, control experiments are obviously required if the method is to be used for measuring other substances or if a higher concentration of stain or longer staining time is used. We have found for instance, that the activity of tyrosine hydroxylase is partially inhibited if brain sections are stained with a very high concentration of methylene blue (e.g. 5% rather than 0.2% as we normally use) or for a long time. Similarly, AUGUSTINSSON (1948) reported that addition of 4×10^{-4} M-methylene blue to brain homogenates *in vitro*

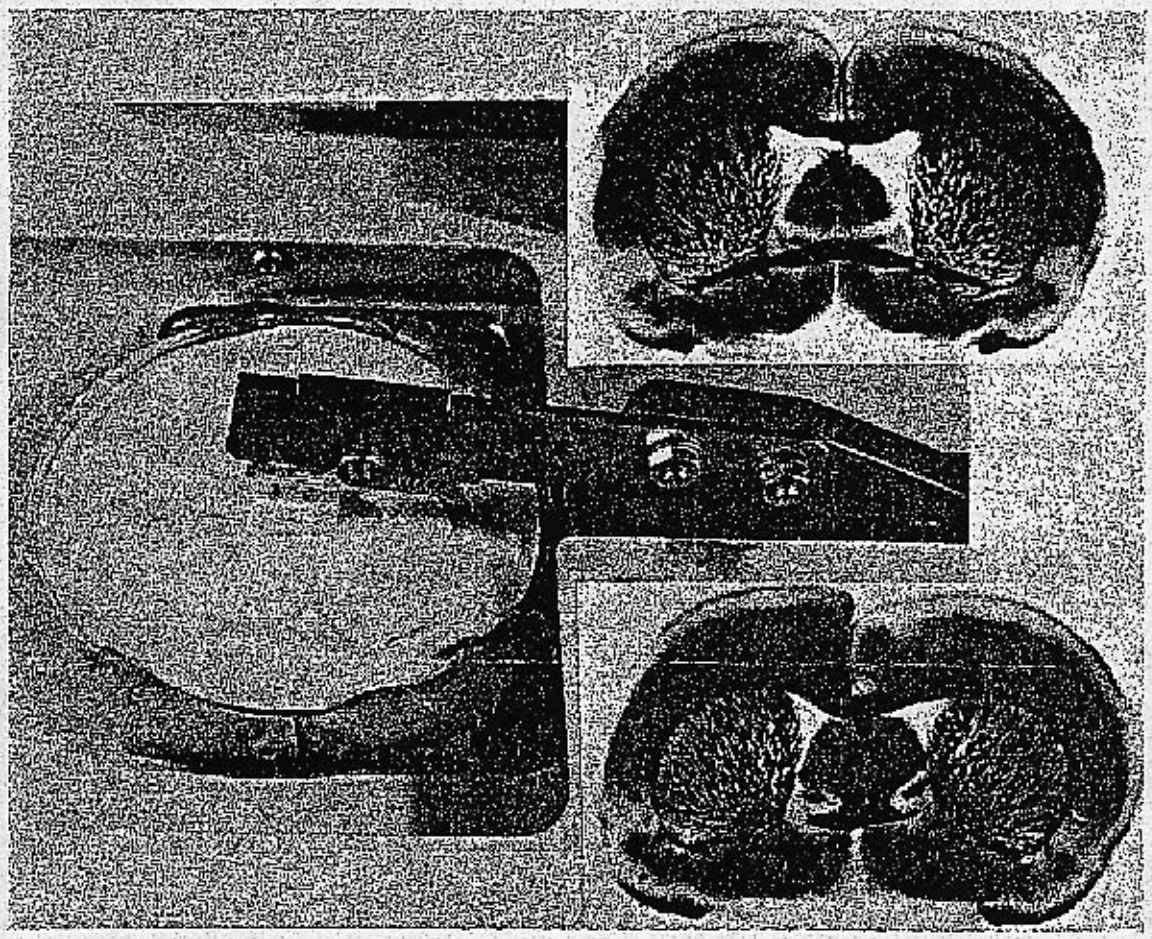

FIG. 1. Procedure for sectioning fresh brains. The figure shows the modified McIlwain tissue chopper in which the cutting stage has been replaced by a Petri dish containing a layer of wax and several pieces of filter paper. Rats are perfused with cold buffer and the brain removed from the skull. The brain is then attached by its dorsal surface to the top piece of filter paper with an adhesive and also pinned to the layer of wax. Frontal sections (200–400μm) are cut one at a time through the brain. As can be seen the individual sections stick to the cutting blade from which they can be carefully removed. The sections are homogeneous in thickness and show a great deal of anatomical detail particularly when illuminated from below. Two sections prepared in this manner are shown on the right side of the figure. These sections have not been stained.

can inhibit the activity of acetylcholinesterase. This, however, is a much higher concentration of stain than would be produced by the procedure we have described. We have estimated spectrophotometrically (E at 665 μm) that after dipping sections for 10 s in a 0.2% solution of methylene blue the concentration of stain in a 2% tissue homogenate (such as we use in the choline acetyltransferase assays) was less than 8×10^{-7} M. It should be emphasized in addition that these mild staining conditions are adequate for the visualization of discrete nuclei in various regions of the brain (see for instance, BEN-ARI *et al.*, 1976).

An alternative approach which has been used for dissecting individual brain nuclei for biochemical analysis is to remove a portion of each nucleus from frozen sections using stainless steel cannulae. This method has been used by EIK-NES & BRIZEE (1965) to determine the distribution in the dog brain of injected [^{3}H]corticosterone and more recently by Palkovitz and his co-workers to measure the distribution of a variety of putative neurotransmitters, enzymes and releasing factors (PALKOVITZ, 1973; PALKOVITZ *et al.*, 1974*a*, *b*). (JACOBWITZ (1974) has adapted this cannula technique for use with fresh sections cut with a vibratome.

While having a possible advantage in the study of compounds which are highly labile post-mortem, the use of frozen sections has two main disadvantages. First it entails a considerable loss of visibility of anatomical landmarks compared to the use of fresh sections. Second, frozen tissue cannot be used for certain types of *in vitro* studies, such as studies on transmitter uptake, since cell membranes are disrupted by freezing and thawing tissue. The use of cannula to dissect nuclei also presents certain problems. Since most nuclei are irregular in shape, only a portion of the nucleus will be removed by a cannula. However, since the distribution of substances within a particular nucleus may not be homogenous, this could result in a biased estimation of the concentrations of a substance in the entire nucleus. We have recently found, for instance, a 5-fold difference in choline acetyltransferase activity in different parts of the lateral posterior nucleus of the amygdala (Ben-Ari *et al.*, in preparation). Thus to obtain an accurate measurement of the content of a substance in a particular nucleus, the nucleus should be dissected in its entirety. On the other hand, rostro–caudal differences within a nucleus can be studied using serial sections. The method we have proposed here can be used for either purpose.

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