A HISTOCHEMICAL STUDY OF THE DISTRIBUTION OF OXIDATION AND HYDROLYTIC ENZYMES IN THE BRAIN OF THE ADULT PEROMYSCUS POLIONOTUS

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ABSTRACT

A HISTOCHEMICAL STUDY OF THE DISTRIBUTION OF OXIDATIVE AND HYDROLYTIC ENZYMES IN THE BRAIN OF THE ADULT PEROMYSCUS POLIONOTUS

by Don A. Hay

Four brain centers of wild mice (Peromyscus polionotus) were studied in detail by the use of histochemical procedures, i.e., the cerebral cortex, cerebellar cortex, superior colliculus, and the medulla oblongata. Seven NAD-linked dehydrogenases (α-glycerophosphate, isocitrate, glucose-6-phosphate, lactate, malate, alcohol, and glutamate dehydrogenases) and the carboxylic esterases were localized within these areas. The presence of enzymes (dehydrogenases) in regions of the brain or in individual cells of the brain indicated that either the glycolytic, citric acid, or perhaps the pentose pathway were utilized for respiration.

Upon removal from the cranium, some specimens of brain tissue were immediately transferred to tissue holders and frozen by quenching in acetone and solid-CO_2. Others were pre-fixed for 24 hours in cold formalin prior to freezing. This was necessary for esterase identification. Sagittal sections, eight to twelve microns in thickness, were prepared with a cryostat, and incubated in solutions containing either the monotetrazolium salt MTT, or the ditetrazolium salt

Nitro-BT. The former salt was used according to the procedures of Chason and Pearse (1961) and Thomas and Pearse (1961). The incubating solution containing Nitro-BT was prepared by the method of Hess, Scarpelli, and Pearse (1958). Only alcohol and glutamate dehydrogenases required Nitro-BT. Both the α-naphthyl acetate method (Pearse, 1960) and the indoxyl acetate method (Holt and Withers, 1952; Holt, 1958) for esterase identification were used. After incubation in air at 37°C, the sections were fixed in cold formalin, mounted in glycerine with or without a counterstain.

Enzyme distributions were studied with the light microscope, and with phase contrast. The level of enzyme intensity was evaluated on a subjective basis, with zero (0) representing no activity; one (1), weak; two (2), moderate; three (3), strong; and four (4), intense activity.

Enzyme activity (both oxidative and hydrolytic) was uniformly distributed in the cerebral cortex. However, the pyramidal cells in layers III and V exhibited slightly more activity.

The greatest oxidative enzyme activity in the cerebellar cortex was confined to the glomeruli cerebellosi. The abundant synapses of mossy and climbing fibers located in the glomeruli may be responsible for the high activity. Hydrolytic enzymes were most active within Purkinje cells.

Enzyme activity in the superior colliculi varied from layer to layer, but was most intense in the stratum griseum superficiale. The inferior colliculi did not exhibit

differential staining according to layers. Rather, metabolic activity was concentrated in a spherical regional nucleus. The stratified enzyme distribution found in the superior colliculi is thought to indicate a greater complexity in function.

Regional nuclei within the medulla oblongata exhibited all ranges of dehydrogenase activity. In some nuclei the neuropil was most active, whereas in others the neurons were. White fiber tracts remained non-reactive.

Very intense hydrolytic activity along capillaries was localized within pericytes (perivascular cells). Capillary endothelium contained moderate amounts of esterase activity. Oxidative enzyme activity could not be accurately assessed, as the capillaries and pericytes were almost imperceptible.

The caudate nucleus of the basal ganglia exhibited intense esterase activity, but only moderate levels of dehydrogenase activity identical to that in surrounding tissues.

Tissue sections incubated in Nitro-BT solutions lacking substrates were strongly positive. The distribution and intensity of the formazan deposits produced by alcohol and glutamate dehydrogenases were similar to those found in the control sections. Thus, enzyme localizations observed within brain sections must be considered inaccurate and possibly false.

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INTRODUCTION

Dehydrogenases. The dehydrogenases are oxidative enzymes that among other things, are involved in cellular respiration. Each of the enzymes consists of a substrate-specific protein which operates in conjunction with a co-enzyme, NAD, NADP, or FAD.* When the substrate and coenzyme are bound to the enzyme protein, hydrogen atoms are removed from the substrate and transferred to the coenzyme. Neither of the reduced pyridine nucleotides (NADH or NADPH) can react directly with oxygen. Thus, in order to be oxidized themselves, they must in turn reduce another substrate or depend upon specific enzymes which employ FAD as a prosthetic group. The flavoprotein then introduces the hydrogen atoms (or electrons) to the cytochrome system, and ultimately to molecular oxygen, with the production of water (White et al., 1959).

* The following abbreviations will be used throughout this paper: Nitro-BT, 2:2 di-p-nitrophenyl-5:5-diphenyl-(3:3-dimethoxy-4:4 bi-phenyline) ditetrazolium chloride; MTT, 3-(4:5 dimethylthiazolyl-2)-2:5-diphenyl tetrazolium bromide; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; α-GPDH, alpha-glycerophosphate dehydrogenase; Iso-CDH, isocitrate dehydrogenase (NAD linked); GDH, glutamate dehydrogenase; G-6-PDH, glucose-6-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; FAD, flavine adenine dinucleotide.

Within recent years, the methods of identifying and localizing enzymes in the brain centers of experimental animals have increased both in kind and in specificity. With the introduction of such highly specific tetrazolium salts as Nitro-BT (Tsou et al., 1956) and MTT (Pearse, 1957), it became possible to examine oxidative enzymes in ultra-thin sections of nervous tissue. The number of dehydrogenases that could be localized by employing MTT was increased by the modifications of Thomas and Pearse (1961).

As these new techniques came into use, investigators approached the analysis of nervous tissue in different ways. Wolfgram and Rose (1959) used Nitro-BT to show the intracellular distribution of NAD-linked dehydrogenases in neuroglia. Robins (1960) compared the distribution and intensity of enzymes in gray matter with that in white matter. Other investigators (Sidman, 1960; Romanul and Cohen, 1960; Chason, Gonzales, and Landers, 1963) contrasted the deposition of dehydrogenases in neurons and neuroglial cells. Brain regions have been mapped (qualitatively) for their enzyme content (Pope et al., 1956; Friede, 1959a, 1959b, 1959c, 1960, 1961; Thomas and Pearse, 1961; Tewari and Bourne, 1962a, 1962c; Lazarus et al., 1962; Friede, Fleming, and Knoller, 1963).

The significance of the enzymic distribution is that their presence is an indication of the prevailing type of energy cycle. Metabolic activity varies intracellularly

and from region to region, both in degree and derivation of energy (Pearse, 1957; Hess and Pearse, 1961; Friede and Pax, 1961; Friede and Fleming, 1962; Tewari and Bourne, 1962d; Nandy and Bourne, 1964a, 1964b; Takemori, 1965; Bartonicek and Lojda, 1966).

Esterases. Esterase identification was introduced by Nachlas and Seligman (1949). The substrate beta-naphthyl acetate was hydrolyzed, liberating naphthol which combined with a diazonium salt in the culture medium to form an insoluble dye at the site of enzyme activity. Gomori (1952) modified this by substituting α-naphthol, which was hydrolyzed by the same enzyme. Barrnett and Seligman (1951) and Holt (1952) found that esterases hydrolyzed indoxyl acetate to form indoxyl. The latter was oxidized to produce an insoluble dark blue indigo dye at the site of the enzyme activity. Utilizing these methods, as well as the modifications of Holt (1954, 1958), Holt and Withers (1952), and Delellis and Fishman (1965), the ester-splitting enzymes have been demonstrated in various regions of the nervous system (Pearse, 1956; Pearse, 1960; Fishman and Hayashi, 1962; David et al., 1962; Tewari and Bourne, 1962a, 1962b; Adams et al., 1963; Bartonicek and Lojda, 1964; Nandy and Bourne, 1966).

Objectives. The aforementioned investigations concerning the histochemical analysis of nervous tissue centered in such domesticated animals as albino rats, albino mice,

rabbits, cats and dogs. Occasional work was performed with monkeys and through post-mortem examination of humans.

It is the intent of the present study to show the existence and distribution of seven oxidative enzymes (dehydrogenases), as well as the esterases, within the brain centers of wild mice (Peromyscus polionotus). Both regional, intercellular, and intracellular localizations were examined in the cerebral and cerebellar cortices, the anterior colliculus and the medulla oblongata. Furthermore, any areas of the brain other than the preceding, where intense reaction occurred, were identified. The intensity of such reactions were evaluated on a subjective basis.

MATERIALS AND METHODS

Fully-grown, mature mice, Peromyscus polionotus of both sexes, were used in this investigation. All animals used for experimentation were raised by Dr. John A. King; they ranged in weight from 10.8 to 15.3 grams. The mice were sacrificed by transection of the upper cervical spinal cord while under a light ether anesthetic. The skin, underlying tissue, and the dorsal surface of the cranium were quickly removed, exposing the brain. After cutting each of the cranial nerves close to their point of exit from the skull, the brain was lifted out and put into cold physiological saline solution. Then each brain was bisected sagittally using a razor blade. Each half was placed on mouse liver attached to a microtome chuck (tissue holder). The lateral surface of the brain was in contact with the liver, thus leaving the inner brain surface exposed.

The tissue was simultaneously frozen and attached to tissue holders at -70°C using acetone and solid-CO_2. It was then transferred to a freezer and stored at -20°C. The length of the storage interval was approximately two hours.

The tissues were sectioned in the laboratory of Dr. R. A. Fennell, utilizing a Harris-International cryostat (refrigerated microtome). Fresh-frozen, unfixed brain was found to section best when the cryostat temperature was between -9°C and -12°C. Sagittal sections of the entire brain were cut at eight microns and occasionally at twelve microns in thickness.

When esterases were localized, the brain was fixed in formol-calcium for 24 hours prior to freezing it in the acetone-dry ice mixture. Under these conditions, it was necessary to keep the temperature of the cryostat at -25°C to -30°C.

Tissue sections were removed from the microtome blade by touching them with warm glass coverslips held with a rubber bulb suction pickup. All sections were then kept in the cryostat cabinet until cutting was completed.

The tissues were incubated in one of two ways, depending upon whether an incubating media for dehydrogenases or for esterases was to be used. For the identification of the former, the sections were covered with 0.1 to 0.2 ml of incubating media. Incubation continued for 30 minutes in air at 37°C. For the localization of the esterases, the tissues were placed in Columbia staining jars containing the incubating solution. The length of incubation varied from 30 minutes to several hours.

The incubating media used for the demonstration of α-glycerophosphate, glucose-6-phosphate, isocitrate (NAD), lactate, and malate dehydrogenases were prepared according to the methods of Chason and Pearse (1961), and Thomas and Pearse (1961). These workers utilized the cobalt-chelating system employing MTT introduced by Pearse (1957). Alcohol and glutamate dehydrogenases were identified using the method of Hess, Scarpelli, and Pearse (1958) in which Nitro-BT was used as an electron acceptor. Non-specific esterases were

studied using the α-naphthyl acetate method of Pearse (1960), and the indoxyl acetate method of Holt and Withers (1952), and Holt (1958).

Incubation (for dehydrogenases) was halted by rinsing the sections in distilled water and fixing them in formol-calcium for 15 minutes. After fixation, the tissues were either counterstained in Mayer's carmalum and then mounted upon slides and covered with glycerine jelly, or mounted in a similar manner unstained.

Considerable time and effort were spent in an attempt to use cresyl violet (Klüver and Barrera, 1953) as a counter-stain. It is highly specific for neurons and provides accurate identification of brain structures. However, only limited success was obtained due to the required differentiation of cresyl violet through several changes of 95% ethanol.

Control sections for each enzyme were incubated in media in which the substrate was omitted. Additional controls consisted of kidney and liver sections placed in the normal incubating media.

Enzyme reactions were studied in four areas of the brain; namely, the cerebral cortex, superior colliculus, cerebellar cortex and the medulla oblongata. As a means of examining the same specific locale of the cerebral cortex in each brain, the hippocampus was utilized as a landmark. Particular attention was given to that region of the cerebral cortex directly dorsal to the hippocampus. Because of its

relatively small size, the entire length of the anterior colliculus was analyzed. In the cerebellar cortex, all layers were subjected to scrutiny. Tissue in the medulla oblongata was examined at the level of the inferior olivary nucleus. All sections were taken from the first two millimeters of brain beginning at the midline and progressing laterally. This procedure reduced the possibility of variable staining reactions caused by regional metabolic differences.

RESULTS

A total of 1,087 slides was prepared for this investigation. Approximately 350 slides were randomly chosen for evaluation of enzyme distribution and intensity. Tissue sections were taken from four to thirteen mice for each enzymatic test. Frequently, several dehydrogenases were localized simultaneously in adjacent sections of the same mouse brain, since the stock solutions of MTT or Nitro-BT are made specific by the addition of appropriate substrates and coenzymes. Thirty mice were used in this study.

THE CEREBRAL CORTEX

Dehydrogenases

Alpha-glycerophosphate dehydrogenase (MTT) activity was relatively evenly distributed in moderate concentrations throughout the cortical tissue. Only that region (layer VI) closely associated with the corpus callosum presented a slightly reduced enzyme reaction (Table I).

Intracellular deposition was more distinct in the pyramidal cells of layers III and IV. Formazan granules were arranged in a perinuclear position (+2), with the nuclei showing no enzyme (α-glycerophosphate dehydrogenase) activity. The peripheral cytoplasm had considerably less activity. Neuroglial cells were weakly positive, but the exact intracellular localization of granules was not obtained, due to the masking effect of the formazan in the neuropil surrounding the cells.

Lactate and malate dehydrogenases present a distribution pattern very similar to that of α-glycerophosphate dehydrogenase (Fig. 1a). Formazan granules produced by lactate dehydrogenase were less abundant (+1) in layers I and VI, but were found in about the same concentration in all other layers (Tables I and II). Malate dehydrogenase activity was less intense only in layer VI. Intracellular distribution was identical, though slightly reduced in degree. Neuropil contained a moderate amount (+2) of formazan, but not in any particular orientation.

Glucose-6-phosphate and isocitrate (NAD) dehydrogenases did not exhibit any activity (Table II). Sections of kidney incubated in the same solutions presented weak-to-moderate (+1½) amounts of formazan in the cortical tissue, but little in the glomeruli. Sections of tissue incubated in solutions in which the substrate was omitted did not show enzyme activity.

Glutamate and alcohol dehydrogenases were identified by utilizing Nitro-BT as the tetrazolium salt. The distribution and intensity of reaction were quite similar for both enzymes (Tables I and II). A careful and thorough examination of tissue sections showed weak reactions (layer I) for glutamate dehydrogenase, whereas alcohol dehydrogenase activity was less intense. Glutamate dehydrogenase was usually localized along unmyelinated nerve fibers (Fig. 1b). The surrounding neuropil contained moderate and diffuse formazan deposits. The cytoplasm of pyramidal cells exhibited

a dark, diffuse reaction ($+2\frac{1}{2}$) closely associated with the nuclear membrane, along with distinct, medium-sized granules in the peripheral cytoplasm. Few granules were observed in the intermediate cytoplasm.

Alcohol dehydrogenase reactions were weak or absent in the outer layer of the cortex, increased to a maximum in layers IV and V, and then decreased to lower levels in layer VI (Table I). Unmyelinated nerve fibers were precisely outlined by small formazan granules. The basal region of layer I and much of layer VI showed reactive fibers in abundance.

Control sections containing Nitro-BT but no exogenous substrate consistently yielded the same distribution and intensity as that found for alcohol dehydrogenase. These sections exhibited both diffuse and distinct granular formazan depositions.

Esterases

Ester-splitting enzymes were found to be quite abundant in the cerebral cortex, but not homogeneous in distribution. Since there are no exact boundaries of the various layers, assessment of the intensities of enzyme reaction within particular strata is at best an approximation, i.e., an estimate of the overall activity. The molecular layer (layer I) was very weakly positive when indoxyl acetate was utilized, and negative with α-naphthyl acetate (Table III). The pericapillary cells (pericytes) found within the pia mater were intensely reactive (Fig. 2a).

The processes of neuroglial cells located in the layers exhibited randomly-distributed indigo crystals. In addition, much of the cellular material localized between neuronal and neuroglial cells showed less intense (+1-1½) reactions. The cytoplasm of medium-sized pyramidal cells of layer III exhibited moderate activity with indoxyl acetate (Fig. 2b), slightly less (+1) when α-naphthyl acetate was used as a substrate (Table III). No particular perinuclear distribution was apparent.

The most intense (+3) activity observed in the cortex, aside from that within the pericytes, was exhibited by the large pyramidal cells of layer V (Table III). Crystals of indigo were distributed throughout the cytoplasm and into the axon hillocks. Tissue near and adjacent to the corpus callosum exhibited a horizontal distribution of enzyme activity (+1). Neuroglial cells containing indigo crystals were dispersed throughout the corpus callosum, separated from one another by non-reactive tissue. All control sections were negative.

THE CEREBELLAR CORTEX

Dehydrogenases

The outer (molecular) layer of the cerebellum is comprised of a few small, stellate-shaped nerve cells and many unmyelinated nerve fibers. Dehydrogenase activity was moderate in intensity (+2) and uniformly distributed throughout this layer. Only the nuclei of the neurons were completely negative. Isocitrate, malate, and lactate (Fig. 3a)

dehydrogenase (MTT) reactions were weak, whereas alcohol (Nitro-BT) and α-glycerophosphate (MTT) dehydrogenases exhibited moderate (+2) activity (Fig. 3b). Glutamate dehydrogenase (Nitro-BT) reactions were more intense (+2½) than they were in either of the two preceding enzymes (Tables I and III). The presence of glucose-6-phosphate dehydrogenase was never shown when using MTT as the hydrogen acceptor, but Nitro-BT produced a diffuse (+1) formazan deposition.

All sections incubated in an MTT solution without the appropriate substrate were devoid of activity. Those controls immersed in a similar solution of Nitro-BT exhibited moderate degrees of activity (Fig. 3c).

Adjacent to the molecular layer is a single row of Purkinje cells. The cells are flask-shaped, with several dendrites extending into the molecular layer. Formazan deposition in the cytoplasm of cells was distinct, although the intensity of enzyme activity varied considerably, e.g., some dehydrogenase reactions were weak, whereas others were stronger (Tables I and II). Glucose-6-phosphate activity was not identified, and lactate and isocitrate dehydrogenases reacted weakly (+1). Most enzyme activity was concentrated in that portion of the cytoplasm near or adjacent to the molecular layer (Fig. 3d). The explanation for this distribution was quite apparent when the position of the nucleus was considered. It was found to be eccentrically located, leaving only a thin rim of cytoplasm adjacent to the granular layer. Weak to moderate amounts of formazan granules

(+1½) were produced by malate dehydrogenase. Alpha-glycerophosphate, alcohol, and glutamate dehydrogenase reactions were moderate in intensity. It is evident in Figure 3d that α-glycerophosphate reactions were intense in the cytoplasm and eccentrically localized due to the position of the nucleus. Likewise, an intense concentration of granules was observed at the cell periphery, in close association with the cell membrane (Fig. 4a).

With the exception of glucose-6-phosphate, which was negative, all dehydrogenase activity in the granular layer was limited to the glomeruli cerebellosi. The reactions were more intense than those identified in the molecular layer and in the Purkinje cells. The granule cells were devoid of enzyme activity. Thus, the overall appearance of formazan deposition is spotty, patchy, and therefore in direct contrast to the uniformity of distribution of enzyme reactions in the molecular layer (Figs. 4b, c, and d). Malate, isocitrate, and lactate dehydrogenases reacted weakly (Fig. 3a), whereas α-glycerophosphate, glutamate, and alcohol dehydrogenases produced moderate amounts of formazan (Figs. 3b and 4d). Dehydrogenase activity in the branches of myelinated fibers (arbor vitae) coursing through the cerebellum was not observed, although positive dehydrogenase reactions were seen in the cytoplasm of some neuroglial cells.

Esterases

The regional and cellular distribution of esterases as shown by the α-naphthyl acetate and indoxyl acetate methods

were essentially the same, although differences were found in the degree of enzyme activity (Table III). The molecular layer consistently presented more intense esterase reactions than those observed in the granular layer. The dye was quite evenly distributed in the former in both the scattered cellular elements and unmyelinated fibers (Fig. 5a). Enzyme activity in the granular layer was localized in the glial cells, the glomeruli, and the unmyelinated axons which run through this layer.

Esterase activity in the Purkinje cells was frequently strong (+3), although enzyme reactions in individual cells ranged from weak to intense. That portion of the cytoplasm closest to the molecular layer (including the axon hillock) exhibited the most intense stain (Figs. 5a, b, and c). Dendrites arise from this end of the Purkinje cell and then ascend into the molecular layer.

The rays of white matter (arbor vitae) passing through the cerebellum appeared to exhibit weak to moderate enzyme activity in all regions of the tissue. Closer examination revealed that the moderate staining should be attributed to interspersed glial cytoplasm and processes, and probably to synaptic endings of myelinated nerve fibers.

THE SUPERIOR COLLICULUS

Dehydrogenases

The zonal, or outer layer of the superior colliculus was devoid of all dehydrogenase activity, with the exception of weak glutamate dehydrogenase reactions. Immediately

adjacent to the above area is the thick stratum griseum superficiale, which exhibited a few myelinated fibers and nerve cell bodies. Tissues of this layer exhibited moderate amounts of formazan, following incubation in either α-glycerophosphate or malate substrate solution (Fig. 6a), whereas lactate dehydrogenase (MTT) activity was less intense (Tables I and II). A weakly positive enzyme reaction for glutamate and alcohol dehydrogenases (Nitro-BT) was visible in unmyelinated fibers. Occasional neurons exhibited weak perinuclear concentrations of formazan, whereas the rest of the cytoplasm exhibited less activity. All formazan granules in the stratum griseum were small, spherical, and distinct. The dehydrogenases specific for isocitrate and glucose-6-phosphate, respectively, were not identified here or in any other region of the superior colliculus.

Longitudinally-oriented fiber tracts within the deeper stratum opticum were responsible for moderate depositions of formazan following incubation in α-glycerophosphate, lactate, and glutamate substrate solutions (Fig. 6a). Alcohol and malate dehydrogenases were weakly active (Tables I and II). The granules within this region were half again as large as those identified in the stratum griseum, and were less abundant. Fiber tracts in this area exhibited moderate malate and glutamate dehydrogenase reactions along their periphery with less activity in the central portions.

The basal region of the layer referred to in the preceding paragraph exhibited multipolar neurons with weak

enzyme reactions. Examination of the cell bodies showed that lactate dehydrogenase was more concentrated in the perinuclear cytoplasm. Other dehydrogenases, e.g., α-glycerophosphate and alcohol dehydrogenases, were not identified in the cytoplasm adjacent to and surrounding the nuclei. Formazan deposits in low concentrations were localized in the peripheral cytoplasm. Enzymatic activity appeared to follow the outlines of the cell membrane.

The deepest layers, namely, strata lemnisci and album profundum, exhibited moderate enzyme reactions in the neuropil following incubation in α-glycerophosphate, lactate, malate, and glutamate dehydrogenase substrate solutions (Fig. 6b). Alcohol dehydrogenase yielded strong depositions of formazan granules. Commissural tracts consistently presented moderate activity along their periphery, and considerably less activity in the central region (Fig. 6b).

During the course of this study, it was noted that as one passed from superficial layers to the deeper layers of the superior colliculus, the size of the formazan granules increased. The small, fine granules were localized in layers I and II, whereas in the deeper layers the granules became progressively larger.

Control sections incubated in MTT without substrate did not exhibit formazan deposits, in contrast to tissue sections incubated in Nitro-BT, which were strongly positive (+2½). The distribution and intensity of enzyme activity in the controls closely paralleled that found in alcohol and glutamate dehydrogenases.

Kidney sections placed in the complete media (MTT) were weakly positive (+1½), whereas those in the control solution showed no enzyme reaction.

Esterases

The esterases presented a rather unique distribution in the superior colliculus. Intensity of enzyme reactions was greater than that observed in the inferior colliculus (Fig. 7a). Only the pericytes within the colliculi reacted with equal intensity (+4). It is evident in Figure 7a that the peripheral layer (zonal) exhibited the greatest degree of activity (+3), and that there was a gradual decrease in enzyme activity in the deeper layers (Table III). There was, however, one exception to the step-wise reduction in the intensity of enzyme reactions. The stratum album profundum showed strong, irregularly-distributed, enzyme-active areas (Fig. 7a).

Cytoplasm of neurons varied in the degree of staining (+1-3), and nuclei did not stain. The cytoplasm in contact with the nucleus showed slightly more activity than the peripheral cytoplasm.

The stratum griseum superficiale showed an abundance of large, circular areas in which enzyme reactions were not observed. Tissues adjacent to the circular areas exhibited moderate enzyme activity. Tissues which did not react consisted largely of myelinated nerve fibers, whereas reactive peripheral materials appeared to be sheathes of Schwann cells. The intensely active pericapillary cells were frequently

observed throughout the superior colliculi (Figs. 7a and b). Additional details are presented in a separate section.

Enzyme distribution was found to be identical with either of the methods cited in the preceding pages. The only variation in intensity was observed in the zonal layer, where α-naphthyl acetate gave a weak (+1½) reaction and indoxyl acetate gave a strong (+3) reaction. Esterase activity was weak to moderate in neuroglial cells and their processes.

THE MEDULLA OBLONGATA

Dehydrogenases

Considerable variation in the intensity of enzyme reactions, apparently correlated with the diversity of its components, was observed in tissues in the medulla. Regional nuclei, including neurons and surrounding neuropil, exhibited considerably different distribution patterns from the abundant fiber tracts which course throughout the area (Tables I and II, Fig. 8a).

Myelinated fiber tracts showed large formazan granules when examined for the presence of α-glycerophosphate, lactate, and malate dehydrogenases (MTT). It was found in this study that most of the tissue between the large granules was devoid of enzymatic activity. Glutamate, alcohol, and α-glycerophosphate dehydrogenases (Nitro-BT) presented a fine, diffuse formazan deposition within the myelinated tracts, varying from weak to intense in reactivity (Fig. 8b).

Isocitrate and glucose-6-phosphate dehydrogenases were not demonstrated in any area of the medulla. Large, branched, crystalline artifacts were observed occasionally when cresyl violet was used as a counterstain. The tissue adjacent to the branched particles completely lacked formazan granules. Sections that were stained in Mayer's carmalum did not exhibit artifacts or other types of granules. Kidney sections placed in the complete incubating medium exhibited a weak to moderate formazan deposition.

Neuropil in some regional nuclei showed more intense enzyme reactions than those found in general gray matter (Fig. 8b). This, however, was reversed in other nuclei. The reactivity of glutamate and α-glycerophosphate dehydrogenases produced moderate amounts of formazan granules in the neuropil, whereas malate, alcohol, and lactate dehydrogenases were only weakly active (Tables I and II).

Enzyme activity was usually associated with neurons. Neuroglial cells were difficult to identify and showed little activity. Neurons exhibited a perinuclear distribution that was moderately intense for both glutamate and alcohol dehydrogenases (Fig. 8c). Large concentrations of formazan granules were frequently noted in nerve cells whose nuclei were eccentrically positioned. Alpha-glycerophosphate dehydrogenase activity was usually uniform in distribution and weak in intensity when MTT was used, and in some instances perinuclear in position. When Nitro-BT was used as the hydrogen acceptor, the intracellular distribution was similar, but much more intense.

The perikarya of regional nuclei showed a few very fine formazan granules following incubation in lactate and malate substrate solutions. However, occasional solitary neurons widely scattered in the medulla exhibited considerable amounts (+2½) of malate dehydrogenase (Fig. 8d).

Tissue sections incubated without substrate were negative with MTT. Other controls were weakly to moderately positive when Nitro-BT was utilized (Fig. 9a). It was noted that in contrast to the light yellow color of the complete incubating media for lactate, malate, isocitrate, and glucose-6-phosphate dehydrogenases, an opaque, dark green colored incubation medium was seen subsequent to the addition of α-glycerophosphate. No precipitant occurred, and a differential distribution of reaction products was still observed.

Esterases

The demonstration of esterases by the α-naphthyl acetate and indoxyl acetate methods was similar in the distribution of the enzymes throughout the area. However, the intensity of the reaction was always less when using the former procedure, with the exception of the pericytes, which showed high activity with both methods (Table III).

Moderately-strong reactions were observed in the regional nuclei but the neurons were consistently strongly positive, while the surrounding neuropil was less intense (Fig. 9b). Neurons of the inferior olivary nucleus were much weaker than those in deeper nuclei. The esterase

distribution was observed to be perinuclear in solitary neurons when using the α-naphthyl acetate method (Figs. 9c and 9d). The peripheral areas of the cytoplasm normally exhibited less intense reactions. However, when indoxyl acetate was used as the substrate, the esterase activity was evenly distributed throughout the cytoplasm. Figures 9c and 9d show that axon hillocks and the proximal portion of the axons exhibited a moderate degree of activity which exceeded reactions in the peripheral cytoplasm, but was less intense than reactions in the perinuclear areas. Cellular nuclei were negative, but were somewhat obscured by the overlying darkly-staining cytoplasm (Fig. 9b).

Fiber tracts were very weakly active, with small branched granules appearing throughout. Neuroglial cells showed a weak-to-moderate reaction with indoxyl acetate, considerably less with α-naphthyl acetate. The pericapillary cells (pericytes) were always intensely positive, with only their nuclei remaining negative.

All controls were negative.

SPECIAL AREAS OF ENZYME ACTIVITY

Perivascular Cells (Pericytes)

While examining the brain centers in this study, it was not uncommon to observe large, irregularly-shaped cells in close proximity to small blood vessels (capillaries). These perivascular cells exhibited very intense esterase activity throughout their cytoplasm (Fig. 7b). The nuclei remained enzymically inactive, as was the case in all other types of cells examined. Pericytes follow the course of the capillaries and appear to be wrapped around the vessels, probably in contact with the endothelial membrane (Figs. 10a and 10b).

It was evident that the intense reactions were localized in pericytes and not in the endothelial cells (Figs. 10c and 10d). A thin rim of positively-staining cytoplasm was seen in the endothelial cells, but intensity of reactions was less pronounced than that in pericytes. Identification of pericytes as such was extremely difficult without the use of esterase incubating medium.

Choroid Plexuses

During the course of observations on the various brain centers, particular attention was given to the ventricles of the brain and their accompanying choroid plexuses. Comparison of the enzymatic activity within the epithelium of the choroid plexus with that in any other region, revealed that enzymes (both dehydrogenases and esterases) were equally as intense as the most active area, if not greater. Formazan

deposition was localized in the cytoplasm of short, cuboidal epithelial cells. Figures 11a and 11b illustrate the continuity between these cells and ependymal cells. It was also observed that although the ependyma did exhibit a positive enzyme reaction, the intensity of this reaction was found to be greater in the cuboidal cells which projected into the lumen of the ventricle and covered the choroid plexuses (Figs. 11c and 11d). Rarely were the neighboring ependymal cells seen to be equal in enzyme activity with the specialized covering of the choroid plexuses.

Furthermore, intensity of enzyme reaction was irregularly distributed throughout the epithelium. Figures 11c and 12 illustrate the variability in activity between closely associated portions of choroid plexuses. Esterases, as well as glutamate and alcohol dehydrogenases, showed the most intense activity, while lactate and α-glycerophosphate dehydrogenases were moderately active. Similar evaluations of the remaining enzymes were not made due to insufficient numbers of suitable sections.

The Basal Ganglia

In the ventral portion of the forebrain, laterad to the midline, and just anterior to the optic chiasma, an intensely positive reaction for esterases was observed. Reactions were usually localized in the nuclear components of the basal ganglia, i.e., in the caudate and lenticular nuclei. The intensity of reactions decreased laterally from the midline and disappeared about three millimeters from the

center (Figs. 13a, b, c, and d). The overall shape of the enzymatically-active tissue was that of an inverted comma when viewed in sagittal sections (Fig. 13b).

The esterase activity, as shown by the α-naphthyl acetate and the indoxyl acetate esterase methods, differed only in the intensity of reaction, not in the distribution (Figs. 13b and 14a). The former method always produced an intense (+4) reaction, whereas the latter consistently showed moderate activity.

The tail of the caudate nucleus extends directly ventrally to the genu of the corpus callosum, and is just anterior to the anterior commissure (Fig. 13c). This highly reactive tissue extends from the choroid plexuses of the lateral ventricles (Fig. 14a) to the ventral surface of the brain, just anterior to the root of the optic chiasma (Fig. 13d).

It was apparent, when viewed under greater magnification, that not only the neurons but the surrounding neuropil stained intensely (Figs. 14b and 14c). The nuclei of the nerve cells were again negative of all enzymatic activity. None of the dehydrogenases exhibited any similarity in distribution or in intensity of reaction within the basal ganglia.

DISCUSSION

THE CEREBRAL CORTEX

Dehydrogenases

The significance of the distribution and intensity of oxidative enzymes may best be understood if one realizes that the level of activity of these enzymes is an approximate indication of the intensity of oxidative metabolism.

The similarity in enzyme distribution and intensity among the NAD-dependent dehydrogenases indicates that both the glycolytic and the citric acid energy cycles exist within cortical tissue of the mice used in this study. The predominance of axonal and dendritic fibers in the molecular layer (layer I) was responsible for the reduced enzyme activity in that area. This investigator found some enzymatic reactions to be greater in perikarya than in neuropil. Although this observation appears to be directly contradictory to the findings of Robins (1960), it is in agreement with the observations of Thomas and Pearse (1961), and Chason *et al*. (1963). Friede *et al*. (1963) found that both distributions existed not necessarily for the same dehydrogenase but for different ones. Their work indicated that some enzymes of the citric acid cycle (SDH and MDH) predominate within neuropil, whereas the glycolytic enzyme lactate dehydrogenase prevailed in perikarya (of pyramidal cells).

Indeed, when cortical tissue was incubated for the demonstration of alcohol and glutamate dehydrogenases, unmyelinated fibers in layers I and VI were conspicuous (Fig. 1b). Neither of the aforementioned enzymes can be identified with MTT as the electron acceptor, only with Nitro-BT. It should be noted also that such enzymically-active fibers could not be demonstrated for any dehydrogenase utilizing MTT.

Few nerve cell bodies were observed until deeper strata were reached. The intensity of enzymatic activity remained consistent throughout the layers, even when considering enzymes of different energy cycles. The slight variation between cortical layers and between enzymes made it difficult to arrive at any significant conclusions with respect to differential metabolic activity (Tables I and II). Thomas and Pearse (1961) obtained similar results using rat cerebral cortex.

The pyramidal cells of layers III and V were only slightly more active than the neighboring neuropil, as is evident with lactate dehydrogenase in Figure 1a. This investigator was unable to demonstrate the existence of the pentose-pathway enzyme glucose-6-phosphate dehydrogenase, nor of NAD-dependent isocitrate dehydrogenase, though other workers have (Friede et al., 1963). Kidney sections incubated in the complete isocitrate medium exhibited typical formazan granules, which indicates that this enzyme is either non-functional within the cerebral cortex or in concentrations

insufficient for detection. Kidney sections immersed in glucose-6-phosphate medium were barely reactive, making this data inconclusive.

It was shown in the preceding pages that five dehydrogenases were identified by the cobalt-chelation method using MTT, i.e., α-glycerophosphate, glucose-6-phosphate, isocitrate (NAD-dependent), lactate, and malate dehydrogenases. All control sections placed in media lacking the substrate were negative. Alcohol and glutamate dehydrogenases were demonstrated with the use of Nitro-BT. However, control sections immersed in such media exhibited considerable formazan deposition of both varieties (diffuse and granular). Rinsing tissue sections in dilute (15%) alcohol or in acetone did not decrease the activity. Since this is of considerable significance, a thorough discussion of this phenomenon is merited. However, due to the fact that similar results were obtained in the other brain centers covered in this study but not yet mentioned, discourse on the subject will be deferred until a later section.

Esterases

If one observes Figure 2a, it becomes apparent that the esterases were stratified in their distribution. Glial metabolic activity is responsible for the slight reaction seen in the molecular layer of the cerebral cortex. The small triangular nerve cells within the second layer and the pyramidal cells in layers III and V contain moderate amounts of esterases, as is evident in Figure 2b. It should

be understood, however, that much of this activity in the neuropil is actually at the point of synapse of nerve fibers (Pearse, 1956; Tewari and Bourne, 1962b and 1962e).

However, analysis of the reactivity within nerve cell bodies of the mouse cortices in this study definitely show more activity than in the surrounding neuropil. This finding can still be compatible with the results reported above if one considers the method of esterase identification, i.e., the formation of indigo from indoxyl acetate. Pearse (1956) pointed out that "this (method) shows not only non-specific esterases, but also specific cholinesterase and pseudo-cholinesterase". Thus, it is entirely possible and indeed quite probable, that different enzymes are responsible for the variation in results.

Unless the chemical situation (such as optimum pH) is considered, and unless specific inhibitors are employed at certain concentrations, comparison of esterases in different brain regions and in different species will be futile.

Although the pH was kept constant (7.3) in this study, inhibitors like E 600 were not used extensively. Therefore, esterase activity between different cell types, e.g., glial cells versus pyramidal cells, may be due to different esterases.

The intensely reactive, elongated structures in figure 2a are pericytes. This fibroblastic-type cell is always associated with capillary networks throughout the various brain centers. They will be considered in detail in a later section.

Dehydrogenases

The molecular layer was found to present a relatively uniform enzyme distribution for all of the dehydrogenases. As this layer contains few nerve cells but many unmyelinated nerve fibers, it was not surprising to find metabolic activity relatively low (moderate). Other authors (Friede et al., 1963) have reported a similar distribution of oxidative enzyme activity within this layer, with Lazarus et al. (1962) finding metabolic activity to be equal in both neuropil and perikarya. Friede and Pax (1961), noting a direct correlation between the location of mitochondria and citric acid cycle enzymes, found that both mitochondria and enzyme activity were more abundant in the deeper half of the molecular layer.

Of the dehydrogenases considered in this study, only isocitrate (NAD) exhibited weak enzyme reactions, while glucose-6-phosphate dehydrogenase was entirely non-reactive. Since positive controls using kidney sections could not be obtained for the latter enzyme, no conclusions can be made regarding its distribution.

Metabolic activity in Purkinje cells frequently equaled or exceeded that found in the molecular layer. In the case of lactate dehydrogenase, Purkinje cells were difficult to distinguish from the adjacent molecular layer, as their activities were identical. In the main, however, enzyme reactions in Purkinje cells agreed with the findings of

other investigators (Tewari and Bourne, 1962e; Chason, Gonzalez, and Landers, 1963; Friede and Pax, 1961). Considerable dehydrogenase activity was apparent, but varied from cell to cell in intensity.

Thomas and Pearse (1961) were unable to demonstrate α-glycerophosphate dehydrogenase activity within Purkinje cells, whereas this worker found moderate formazan depositions with the same enzyme, when using either MTT or Nitro-BT (Figs. 3b, d, and 4a-c). In addition, there was, if anything, a drop in enzyme activity in the immediate vicinity of the Purkinje cells. In contrast, Thomas and Pearse (1961) reported considerable activity. It should be noted that Friede and Pax (1961) found decreased numbers of mitochondria and reduced oxidative enzyme reactions in this same area.

Occasional sections showed an increased metabolic activity at the junction of axons and their respective Purkinje cells. This was especially the case with the large, unmyelinated fibers which enter the molecular layer.

In the granular layer, utilization of either the glycolytic or the citric acid cycle enzymes was confined to the glomeruli cerebellosi, which, according to Friede and Pax (1961), "represent the synaptic areas of the mossy and climbing fibers and the dendrites of the granular cells". The actual level of metabolic activity within these areas was found to be higher than in any other tissue of the cerebellar cortex. These results are in agreement with

those of many other authors (Friede, 1960; Friede and Pax, 1961; Thomas and Pearse, 1961; Lazarus et al., 1962; Tewari and Bourne, 1962d and 1962e).

White matter coursing through the cerebellum (arbor vitae) contained little oxidative enzyme activity, although the perikarya of glial cells was faintly positive. It should be noted, however, that a diffuse, positive reaction was observed when Nitro-BT was used, but none with MTT.

Esterases

The distribution of esterases in the four layers of the cerebellar cortex presents a considerably different picture than that described for the dehydrogenases. The molecular layer remained homogeneous in appearance, but it exhibited a more intense enzyme reaction, with only the nuclei of scattered cells being negative.

Purkinje cells contained varying amounts of stain. Even adjacent cells were observed at opposite extremes of metabolic activity. Tewari and Bourne (1962b) reported similar findings, and were of the opinion that the cells were exhibiting cyclic activity. In spite of the variations, the perikarya as a whole was more reactive than in any other layers of the cerebellum.

The granular layer was much less conspicuous. Granular cells still were without enzyme activity, and the glomeruli were considerably weaker in their esterase content. Myelinated fibers were observed throughout the granular layer, showing continuity both with the Purkinje cell layer

and the deeper white matter tracts. Figure 5a shows that the esterase activity in the fibers manifests itself in the form of tiny beads of naphthol. This is in agreement with the results of Tewari and Bourne (1962b). They explain the beaded appearance as representing the localization of the enzyme at synaptic endings. These same workers (1962e) felt that much of the esterase activity observed in the molecular layer and in the glomeruli cerebellosi is actually at points of synapse.

The arbor vitae of the cerebellar cortex exhibited esterase activity only along a few fibers, and then just at synaptic endings and in glial cytoplasm.

THE SUPERIOR COLLICULUS

The following brief review of anatomical and functional aspects of the superior and inferior colliculi is presented so that the reader might understand more easily the significance of enzymatic distribution differences.

It has been determined that the colliculi (both superior and inferior) receive the terminations of most of the optic nerve fibers, and at the same time have become massive auditory reflex centers (Zeman and Innes, 1963). In the mouse the inferior colliculi are considerably smaller than the superior, whereas in man both are nearly alike in size. This is due primarily to a decrease in the functional importance of the superior colliculus in man, and therefore results in a decrease in its size.

Since the colliculi are important reflex centers, they receive many different kinds of fibers coming from the spinal cord, medulla oblongata, and the forebrain. Some of these fibers terminate in the inferior colliculi, while others divide, sending a branch into the superior colliculus. Still other fibers send a branch into the colliculi, but continue up further in the brain. Those fibers ending in the colliculi are thought to form the chief reflex auditory pathway, while those traveling up into higher centers of the brain form the cortical pathway. By means of this latter tract, auditory impulses pass into consciousness and into the auditory cortex (Zeman and Innes, 1963).

The superior colliculi differ basically from the inferior colliculi because of divergence during their development. That is, the inferior colliculus increased in size but not in complexity as the auditory paths increased. The superior colliculus exhibits a secondary differentiation due to the migration of neurons during embryonic development to form peripheral layers. According to Huber and Crosby (1943), this "layering....favors localization and permits specificity of reception and projection of impulses".

Dehydrogenases

The inferior colliculi exhibit no differential staining but rather a moderate degree of oxidative enzyme activity concentrated in a sphere identical to regional nuclei found elsewhere in the brain. Synaptic neurons are probably responsible for the activity.

The stratification described above is evident when the distribution of the oxidative enzymes is considered. Little activity is observed in the peripheral (zonal) layer, which is comprised of fibers that turn and course downward into the lower layers. Directly below the zonal layer, the stratum griseum superficiale exhibits a moderate degree of metabolic activity. Much of this layer is comprised of unmyelinated fibers, their synapses, and an occasional neuron. Since the superior colliculus is a reflex center with fibers from various parts of the brain synapsing here, it is entirely possible that synaptic endings might be responsible for much of the oxidative enzyme activity observed here. When studying synaptic areas in the olfactory bulb of the rat, Nandy and Bourne (1966) found that synapses were much more metabolically active than the nerve cell bodies.

Before accurate conclusions can be made, further analyses are necessary. A possible avenue of investigation would involve comparison of enzymes and their intensities between layers of the superior colliculi and areas of known synaptic endings, e.g., glomeruli cerebellosi and portions of the olfactory bulb. Although a detailed examination was not conducted in this study, the enzyme activities listed in Tables I and II for the colliculi and the glomeruli indicate the plausibility of such an undertaking.

Enzyme reactions were gradually reduced in intensity in the deeper layers, as entering optic fibers are encountered

and as ascending systems are received and correlated with optic and cortical terminals. It seemed to this worker that as the number of myelinated fiber tracts increased, the intensity of oxidative enzyme activity decreased. It was also noted that small, spherical formazan granules in the outer layers were replaced by larger, irregularly-shaped granules that were fewer in number. The latter were very similar in size and shape to those found in the arbor vitae of the cerebellum, and in other concentrations of white matter. Therefore, perhaps the increased amounts of lipids found within myelin cause the formation of large aggregates of formazan, drawing smaller granules from their point of origin.

Esterases

The stratification observed in the superior colliculus with dehydrogenases was not quite so apparent with esterases. The two peripheral layers were strongly reactive, with a reduction in activity occurring in the stratum opticum. Koelle (1954) reported strong cholinesterase activity in this layer, attributing it to neurons which synapsed directly with afferent motor neurons. Hydrolytic activity in the adjacent stratum lemnisci was intense, due perhaps, to a concentration of synapses from large commissural fiber tracts.

The superior colliculi possessed considerably more esterase activity than the inferior colliculi (Fig. 7a). Although the former structures were known to be more complex

due to the inward migration of neurons during embryonic development, there have been few reports of a distinct predominance in their (superior colliculi) esterase activity. Extensive enzymatic study is necessary before the functional significance of such a difference is completely understood.

THE MEDULLA OBLONGATA

Dehydrogenases

Regional nuclei differed considerably in the location of dehydrogenase activity, and consequently, of metabolic activity. In the inferior olivary nucleus, both α-glycerophosphate and glutamate dehydrogenases (Nitro-BT) were active primarily in the neuropil, whereas formazan deposits were not identified in many neurons. In contrast, other nuclei showed the same two enzymes along with malate dehydrogenase concentrated in the perikarya of neurons with only diffuse deposits in neuropil. This variety in localization of both glycolytic and citric acid cycle enzymes is in agreement with the findings of Friede and Pax (1961), Thomas and Pearse (1961), and of Friede *et al.* (1963).

Myelinated fibers usually did not show dehydrogenase activity. When MTT was used, the fibers occasionally exhibited large, angular, irregularly distributed formazan granules. In contrast, when using Nitro-BT the formazan deposition was indistinct and not clearly granular. The fibers exhibited a diffuse, gray hue. With either tetrazolium salt, granules were not found consistently in glial

cells, or in a distinctively beaded fashion, nor were they just on the periphery of the tracts. Therefore, due to the variability in distribution from one tissue section to the next, both localizations are thought to be false.

Esterases

Esterase activity varied greatly among regional nuclei, but the inferior olivary nucleus consistently exhibited the weakest hydrolytic activity. In most nuclei, however, the neuropil remained non-reactive. The perikarya of many neurons showed moderate concentrations of esterases in a perinuclear position. The occasional solitary hyperactive cells seen within the medulla also exhibited this perinuclear distribution. In addition, the hydrolytic activity appeared to be continuous with that surrounding the axis cylinder (Fig. 9c).

SPECIAL AREAS OF ENZYME ACTIVITY

Pericytes

Throughout the brain centers considered in this study, large cells identified as pericytes were observed. They were extremely reactive to esterase incubating medium, but when using dehydrogenase solutions they were difficult to identify and therefore to evaluate. Pearse (1956) discussed the enzymatic activity of pericytes and concluded that although these cells were intensely reactive to indoxyl acetate, an actual esterase was not being shown. Rather, he stated that "the esterase of the pericytes is not a true esterase but an intracellular peptidase or cathepsin".

Though found to be distinct from the endothelial cells, pericytes appear to be wrapped about the vessel. Farquhar and Hartman (1956), and Donahue and Pappas (1961) have reported the specific location of these cells, noting that they are external to the endothelium but are surrounded by a basement membrane which splits to enclose them.

Landers, Chason, Gonzalez, and Palutke (1962) considered the possibility that pericapillary cells were of reticuloendothelial origin, but could detect none of the properties related to such cells, i.e., phagocytosis or acid phosphatase activity. Therefore, they concluded that on the basis of "morphologic relationships, enzymatic activities, and lack of phagocytic ability....that these cells are a modified form of smooth muscle". It was not implied, however, that pericytes were necessarily contractile in function. Thus, at the present time, the natural activity of these cells remains unknown.

Choroid Plexus

Ependyma and choroid plexuses consistently exhibited intense dehydrogenase and esterase activity. Hess and Pearse (1961) found mitochondrial α-glycerophosphate dehydrogenase activity to be high in the ependyma and choroid plexuses. Lazarus et al. (1962) reported similar findings for both lactate and glucose-6-phosphate dehydrogenases. Alpha-glycerophosphate, lactate, glutamate, and alcohol dehydrogenase activity of the choroid plexus epithelium consistently exceeded that of the adjacent ependymal cells, irrespective of the definite continuity of the two.

When working with white laboratory mice, rats, guinea pigs, and golden hamsters, Bartonicek and Lojda (1966) obtained essentially the same results. Furthermore, they supported the work of Thomas and Pearse (1961), who found that the luminal half of the ependyma was more reactive than the basal portion. This investigator was neither able to confirm the luminal enzyme concentration nor refute its existence. The majority of the plexuses examined contained a relatively uniform distribution of formazan granules. Because of the orientation of these oxidative enzymes, Thomas and Pearse (1961) contended that the ependymal cells probably secrete substances into the ventricular lumen.

Choroid plexus epithelium within the same ventricle varied considerably in the degree of enzyme activity, but each epithelial cell contained granules that were evenly distributed throughout the cytoplasm. This agrees with the observations of Bartonicek and Lojda (1966), who felt that this variation in cell activity actually indicated different phases of metabolic activity. The capillaries surrounded by the choroid plexus epithelium were not enzymatically reactive which is similar to the results of Bartonicek and Lojda (1966), and Felgenhauer and Stammler (1962).

When utilizing α-naphthyl acetate or indoxyl acetate as substrates for esterase activity, a strong positive reaction was observed. This is in agreement with the findings of Fishman and Hayashi (1962) and Bartonicek and Lojda (1964). The distribution did not differ significantly from

that exhibited by the dehydrogenases, as the present study utilized the same animal species throughout. However, Bartonicek and Lojda (1964) found that the type of hydrolytic enzyme detected varied considerably from species to species, but not among members of the same species. The presence of the several oxidative and hydrolytic enzymes is a strong indication that the choroid plexuses play an important role in the formation of cerebrospinal fluid.

Basal Ganglia (Corpus Striatum)

The basal ganglia were found to be exceedingly rich in esterase activity, whereas the surrounding tissue exhibited only weak hydrolytic activity. Only the choroid plexuses rivaled this area in the intensity of esterase reaction. Examination of NAD-linked dehydrogenases in the same area showed moderate enzyme activity which was no different than that in adjacent tissues. That is, the caudate and lenticular nuclei were not unique in the intensity of their oxidative enzyme activity. This is in contrast to the findings of Thomas and Pearse (1961), which indicated a very high dehydrogenase activity in two components of the basal ganglia (putamen and pallidum). In the present study, glycolytic activity within this region was considered to be moderate, as both α-glycerophosphate and lactate dehydrogenases (MTT) produced moderate amounts of formazan. The same situation existed for enzymes of the citric acid energy cycle, in that malate (MTT), alcohol, and glutamate (Nitro-BT)

dehydrogenase activities were observed, but no greater than that found in other tissues of the telencephalon. The possible utilization of the pentose pathway in this area could not be ascertained, since glucose-6-phosphate dehydrogenase could not be demonstrated.

Examination of the esterase-active tissue in the basal ganglia indicated that both perikarya and neuropil stained with equal intensity. The circular, non-reactive areas were apparently the individual nuclei. The basal ganglia contain fiber connections, making this region a relay center, as it appears to be in rats (Zeman and Innes, 1963). At the present time, the exact significance of a concentration of highly-active esterases in this region is not clear.

NOTHING DEHYDROGENASE REACTIONS

During this investigation, seven NAD-linked dehydrogenases were studied for their distribution and their intensity in the various brain centers. Five of these enzymes were analyzed by using MTT at pH 7.2 as the electron acceptor. Three enzymes, α-glycerophosphate, alcohol, and glutamate dehydrogenases, were studied by means of Nitro-BT (also at pH 7.2). The activity of α-glycerophosphate dehydrogenase was examined by using both of the tetrazolium salts (in separate tissue sections). Just Nitro-BT was utilized with the other two enzymes.

Distinct formazan deposits were produced with the addition of appropriate substrates. In addition to speci-

fically-localized granules, a diffuse positive reaction was observed. However, sections immersed in incubating medium (Nitro-BT) without exogenous substrates exhibited considerable amounts of formazan granules. These deposits were equal in intensity and similar in distribution to both alcohol and glutamate dehydrogenase activities. Therefore, the accuracy of certain enzyme localizations reported herein is subject to question, and the possibility of false localization must be considered.

Zimmermann and Pearse (1959) reported similar findings with other NAD-linked dehydrogenases. By using particular inhibitors, they found that sulfhydryl groups (SH) apparently reduced NAD or NADP to NADH and NADPH, respectively. If the appropriate diaphorases are present in the tissue, these substances can pick up and transfer electrons directly to the tetrazolium salt, with the production of formazan. Adding support to this concept are the findings of Racker (1955) and Nachlas, Walker, and Seligman (1958), which indicated that the final localization of formazan appeared to resemble the pattern formed by the histochemical reactions for the diaphorases.

Thomas and Pearse (1961) reported similar distribution patterns in nerve tissue for glutamate dehydrogenase and control solutions containing Nitro-BT. Pearse and Hess (1961) concluded that the great affinity (substantivity) of Nitro-BT for lipoprotein structures was responsible for this "nothing dehydrogenase" reaction.

The recent efforts of Shaw and Koen (1965) have shown that alcohol dehydrogenase may be the major agent of "nothing dehydrogenase" activity. They determined that pure alcohol dehydrogenase reduced the tetrazolium salt in the absence of added substrate. Furthermore, liver extracts with no substrate were electrophoretically compared to pure alcohol dehydrogenase. The incubating medium contained ethyl alcohol and no substrate. The resulting protein bands were identical. The sulfhydryl reagent parahydroxy-mercuri-benzoate completely inhibited the appearance of the bands both with-and-without substrate. However, Shaw and Koen (1965) were unable to explain the basis for the hydrogen (electron)-transferring behavior of alcohol dehydrogenase in the absence of added substrate.

Thus it appears that both experimental and control sections incubated in Nitro-BT may actually be presenting the localization of endogenous alcohol dehydrogenase. Results obtained during this investigation support this conclusion.

SUMMARY

Oxidative and hydrolytic enzyme localizations and intensities were studied within the central nervous system using slide histochemical methods. Fresh-frozen sagittal sections of brain were prepared from mice of the species Peromyscus polionotus. The NAD-dependent dehydrogenases, i.e., α-glycerophosphate, glucose-6-phosphate, isocitrate, lactate, and malate were identified by using the monotetrazolium salt MTT. Two other NAD-linked enzymes, alcohol and glutamate dehydrogenases, were analyzed with the use of Nitro-BT. Esterases were localized by both the α-naphthyl acetate and the indoxyl acetate methods.

Four brain centers were examined in detail; namely, the cerebral cortex, cerebellar cortex, superior colliculus, and the medulla oblongata. Pericapillary cells, choroid plexuses, and the basal ganglia were also observed in detail.

In the cerebral cortex, amidst relatively uniform dehydrogenase distribution, slightly greater enzyme activity was noted in the neurons of layers III and V. Enzymes of both the glycolytic and the citric acid energy cycles were present. Very little enzyme activity was obtained for isocitrate dehydrogenase (NAD-dependent), none for glucose-6-phosphate dehydrogenase. Hydrolytic enzyme activity was centered in the perikarya of pyramidal cells in layers III and V.

In the cerebellar cortex, glomeruli cerebellosi showed the strongest dehydrogenase activity, but Purkinje cells were also prominent, exhibiting moderate activity. The high reaction within the glomeruli was correlated with a concentration of synaptic endings in the same areas. Purkinje cells contained the greatest esterase activity, whereas glomeruli were considerably reduced.

The stratum griseum superficiale, an intermediate layer in the superior colliculus and known to contain abundant synapses, exhibited high metabolic activity. The formazan distribution was stratified, indicating considerable complexity, in contrast to the inferior colliculus, where layering was absent. The superior colliculus showed strong esterase activity, whereas little was observed in the inferior colliculus. Commissural tracts deep within the colliculus (in the stratum lemnisci) exhibited strong hydrolytic enzyme activity.

Metabolic activity in the medulla oblongata was primarily localized in the regional nuclei, but varied greatly in intensity. In some nuclei the perikarya was highly active; in others, the neuropil was predominant. Scattered throughout the general gray matter of the medulla were solitary cells that were intensely active for esterases.

Esterase activity within pericytes was extremely high, but distinct enough to show moderate levels in the endothelium. Oxidative enzyme activity was difficult to ascertain within these cells.

Choroid plexus epithelium exhibited wide variations of dehydrogenase activity in the same ventricle with the same enzyme. It was concluded that this indicated a fluctuation in the metabolic activity. The distribution and intensity of oxidative and hydrolytic enzymes remained consistent between different ventricles of the same mouse and between ventricles of different mice of the same species.

The basal ganglia showed very strong esterase activity especially in the caudate and lenticular nuclei. The dehydrogenases were moderately active in these nuclei and also in the surrounding tissues, which made differentiation of the separate areas almost impossible.

Results obtained when using Nitro-BT must be regarded as inaccurate and possibly false. Control sections were quite positive, and similar in all respects to the localization given by alcohol dehydrogenase. Thus the highly-lipoprotein character of brain tissue appears to cause a "nothing dehydrogenase" reaction when Nitro-BT is employed.

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Table 1. Enzymatic Activity of Brain Regions

Dehydrogenases of the Glycolytic Energy Cycle*

Region	Alpha-GPDH			ADH			LDH		
	A	B	C	A	B	C	A	B	C
Cerebellar Cortex									
Purkinje cells	2	0-4	(13)	2	1-3	(5)	1	0-2	(6)
Molecular layer	2	0-3	(13)	2	0-3	(5)	1	0-2	(6)
Granular layer	2	0-4	(13)	2	0-3	(5)	1½	0-2	(6)
White matter	0	0-1	(13)	0	0-2	(5)	0	0-1	(6)
Cerebral Cortex									
Layer No. I	2	0-3	(9)	0	0-1	(4)	1	0-2	(8)
Layer No. II	2	0-3	(9)	1	0-2	(4)	2	0-2	(8)
Layer No. III	2	0-3	(9)	1½	0-2	(4)	2	0-2	(8)
Layer No. IV	2	0-3	(9)	2	0-2	(4)	2	0-2	(8)
Layer No. V	2	0-3	(9)	2	0-2	(4)	2	0-2	(8)
Layer No. VI	2	0-3	(9)	1½	0-3	(4)	1	0-2	(8)
Corpus Callosum	1	0-2	(9)	0	0-1	(4)	1	0-2	(8)
Medulla Oblongata									
Inf. Olivary Nucl.	½	0-2	(9)	1	0-1	(5)	0	0-2	(4)
Neurons	1	0-4	(9)	1	0-3	(5)	0	0-3	(4)
Tracts	1	0-1	(9)	2	1-2	(5)	1	0-2	(4)
Neurophil	2	0-2	(9)	1	0-2	(5)	1	0-2	(4)
Superior Colliculus									
Zonal layer	0	0-2	(11)	0	0-1	(5)	0	0	(6)
Stratum Griseum	2½	0-3	(11)	1	0-1	(5)	1½	0-2	(6)
Stratum Opticum	2	0-3	(11)	1	0-3	(5)	2	0-2	(6)
Stratum Lemniscus and Al. Profundum	2	0-3	(11)	3	0-3	(5)	2	0-2	(6)

* The number in Column A represents the average intensity of enzymatic activity for the designated structure, based on a subjective evaluation by this investigator: 0, no activity; 1, weak activity; 2, moderate activity; 3, strong activity; 4, intense activity.

The value in Column B is the range of enzymatic activity.

The number within the parentheses (in Column C) indicates the number of mice analyzed for each enzyme.

Table 2. Enzymatic Activity of Brain Regions

Dehydrogenases of the Krebs Cycle*

Region	IDH			GDH			MDH		
	A	B	C	A	B	C	A	B	C
Cerebellar Cortex									
Purkinje cells	1	0-2	(8)	3	0-4	(3)	1½	0-2	(6)
Molecular layer	1	0-2	(8)	2½	0-3	(3)	1	0-1	(6)
Granular layer	1	0-2	(8)	3	0-4	(3)	1½	0-2	(6)
White matter	0	0	(8)	0	0-1	(3)	0	0-1	(6)
Cerebral Cortex									
Layer No. I	0	0-1	(8)	1	0-1	(4)	2	0-3	(6)
Layer No. II	0	0-1	(8)	1	0-1	(4)	2	0-2	(6)
Layer No. III	0	0-1	(8)	2	0-3	(4)	2	0-2	(6)
Layer No. IV	0	0-1	(8)	2	0-3	(4)	2	0-2	(6)
Layer No. V	0	0-1	(8)	2	0-3	(4)	2	0-2	(6)
Layer No. VI	0	0-1	(8)	2½	0-3	(4)	1	0-2	(6)
Corpus Callosum	0	0-1	(8)	1	0-1	(4)	1	0-1	(6)
Medulla Oblongata									
Inf. Olivary Nucl.	0	0	(6)	1	0-3	(4)	0	0-2	(4)
Neurons	0	0	(6)	1½	0-3	(4)	0	0-2	(4)
Tracts	0	0	(6)	0	0-1	(4)	½	0-1	(4)
Neuropil	0	0-1	(6)	2	0-3	(4)	1	0-2	(4)
Superior Colliculus									
Zonal layer	0	0-1	(8)	1	0-3	(5)	0	0-1	(5)
Stratum Griseum	0	0-1	(8)	1½	1-3	(5)	2	0-3	(5)
Stratum Opticum	0	0-1	(8)	2	0-2	(5)	1	0-2	(5)
Stratum Lemniscus and Al. Profundum	0	0-1	(8)	2	0-2	(5)	2	0-2	(5)

* For explanation of symbols, see Table 1.

Table 3. Enzymatic Activity of Brain Regions

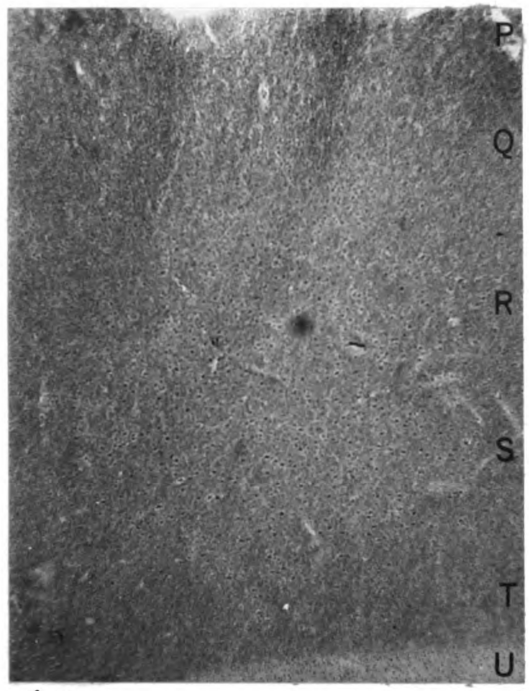
Carboxylic Esterases*

Region	Indoxyl Acetate Esterase			α-Naphthyl Acetate Esterase		
	A	B	C	A	B	C
Cerebellar Cortex						
Purkinje cells	3	0-4	(4)	2	1-3	(5)
Molecular layer	2	1-3	(4)	2	0-2	(5)
Granular layer	0	0-2	(4)	1	0-2	(5)
White matter	2	0-3	(4)	0	0-1	(5)
Cerebral Cortex						
Layer No. I	1	0-2	(4)	0	0-2	(5)
Layer No. II	2	0-2	(4)	0	0-1	(5)
Layer No. III	2	0-3	(4)	1	0-3	(5)
Layer No. IV	1	1-2	(4)	1	0-2	(5)
Layer No. V	3	1-3	(4)	2	0-3	(5)
Layer No. VI	2	1-3	(4)	0	0-2	(5)
Corpus Callosum	0	0-1	(4)	0	0-1	(5)
Medulla Oblongata						
Inf. Olivary Nucl.	2	1-4	(4)	1	0-3	(6)
Neurons	3	3-4	(4)	2	0-3	(6)
Tracts	0	0-1	(4)	0	0-2	(6)
Neuropil	2	1-3	(4)	1	0-2	(6)
Superior Colliculus						
Zonal layer	3	0-3	(4)	1½	0-4	(6)
Stratum Griseum	2	1-3	(4)	2	0-3	(6)
Stratum Opticum	1	1-2	(4)	1	0-2	(6)
Stratum Lemniscus and Al. Profundum	2	1-3	(4)	2	0-3	(6)

* For explanation of symbols, see Table 1.

Fig. 1a. Cerebral cortex. Lactate dehydrogenase activity. The symbol P represents layer I; Q, layers II and III: R, layer IV; S, layer V; T, layer VI; U, corpus callosum. No counterstain. MTT.; x 130.

Fig. 1b. Cerebral cortex. Glutamate dehydrogenase activity localized in non-myelinated nerve fibers. No counterstain. Nitro-BT.; x 520.



1 a

b

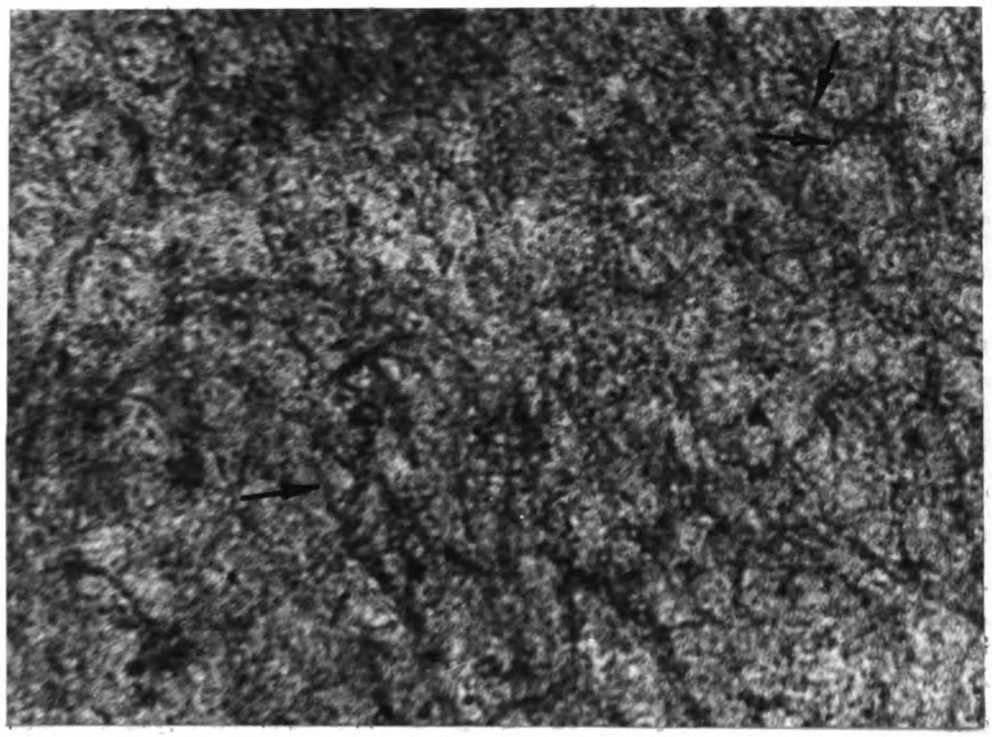
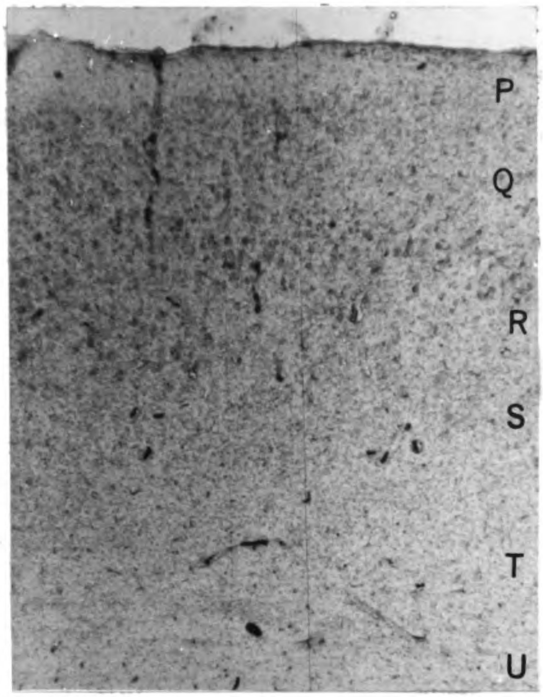


Fig. 2a. Cerebral cortex. Esterase activity. See Fig. 1a for explanation of symbols. The most intense activity is exhibited by pericytes (black structures). Mayer's carmalum counterstain. Indoxyl acetate; x 48.

Fig. 2b. Cerebral cortex. Esterase activity in layers I, II, and III (See Fig. 1a for explanation of symbols). Mayer's carmalum counterstain. Indoxyl acetate: x 130.



2 a

b

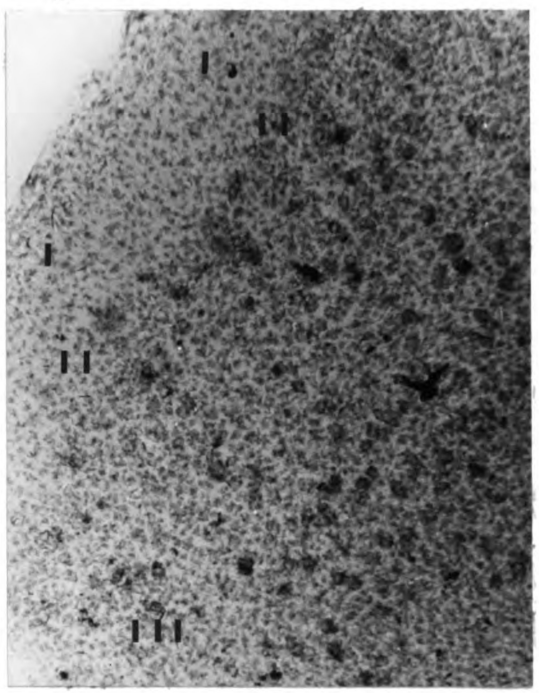


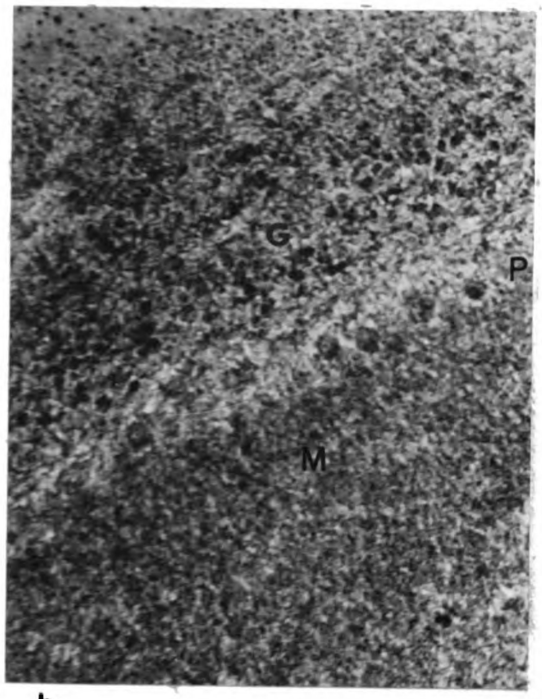
Fig. 3a. Layers of the cerebellar cortex. Lactate dehydrogenase activity in the molecular (M) layer and in the granular (G) layer. Counterstained in Mayer's carmalum. MTT.; x 130.

Fig. 3b. Layers of the cerebellar cortex. α-glycerophosphate dehydrogenase. The letter P designates the Purkinje cell layer. See Fig. 3a for explanation of other symbols. Mayer's carmalum. MTT.; x 520.

Fig. 3c. The cerebellar cortex. "Nothing dehydrogenase" activity; control section, substrate lacking. The letter W represents fiber tracts of white matter. See Fig. 3a and 3b for explanation of the other symbols. No counterstain. Nitro-BT.; x 130.

Fig. 3d. Purkinje cells bounded by the granular and the molecular layers. Note the eccentric distribution of the cytoplasm (arrow) in the Purkinje cells. α-glycerophosphate dehydrogenase. Mayer's carmalum. MTT.; x 520.

3 a



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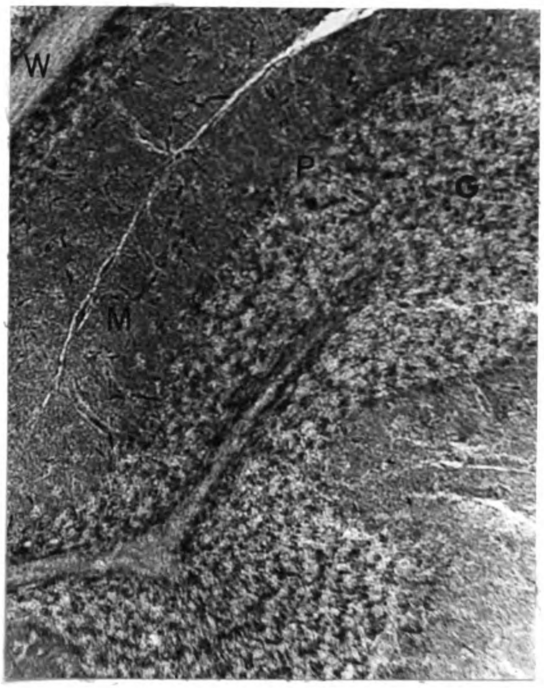

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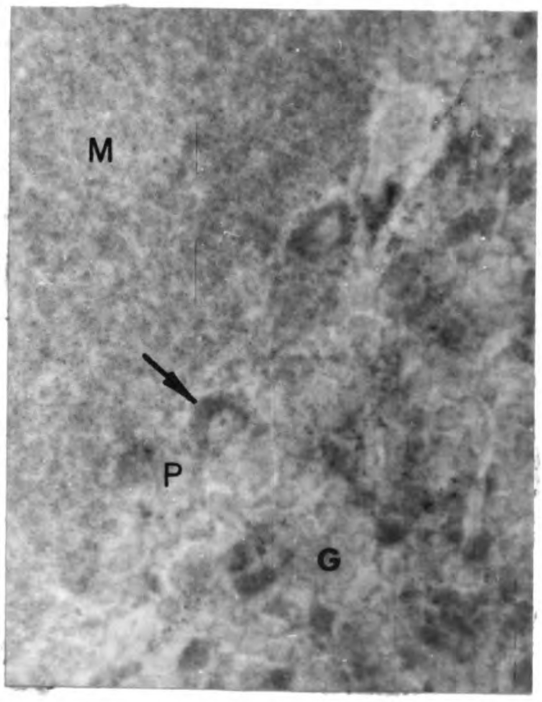
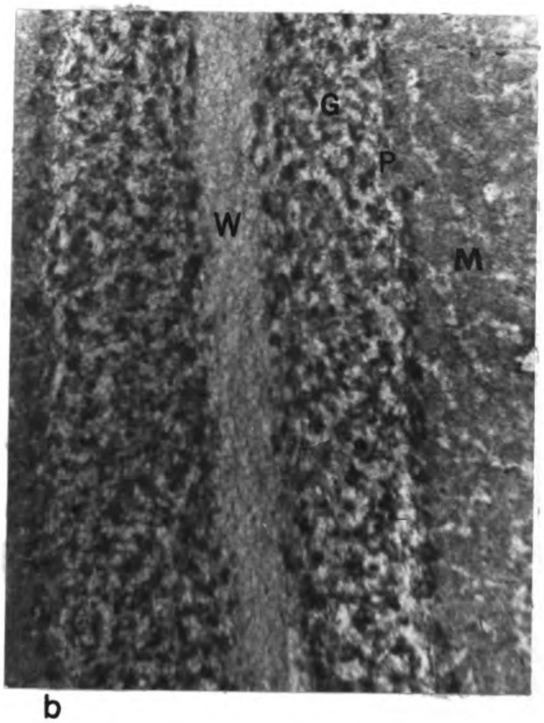


Fig. 4a. Purkinje cell containing α-glycerophosphate dehydrogenase. Note the intense perinuclear distribution, as well as the increased peripheral activity (arrows). Mayer's carmalum. MTT.; x 1330.

Fig. 4b and 4c. Layers of the cerebellar cortex. Note the patchy appearance of enzyme within the granular layer (G). α-glycerophosphate dehydrogenase. No counterstain. Nitro-BT.; 4b, x 130; 4c, x 260.

Fig. 4d. Cerebellum. Glutamate dehydrogenase, with no counterstain. Nitro-BT.; x 260.

4 a



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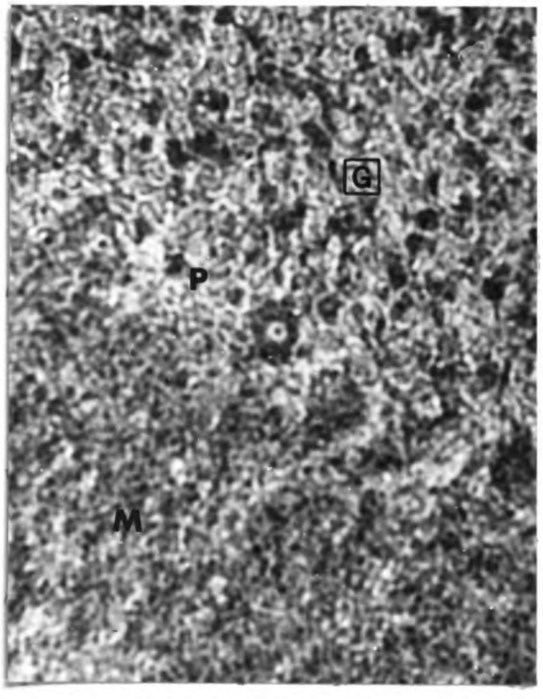

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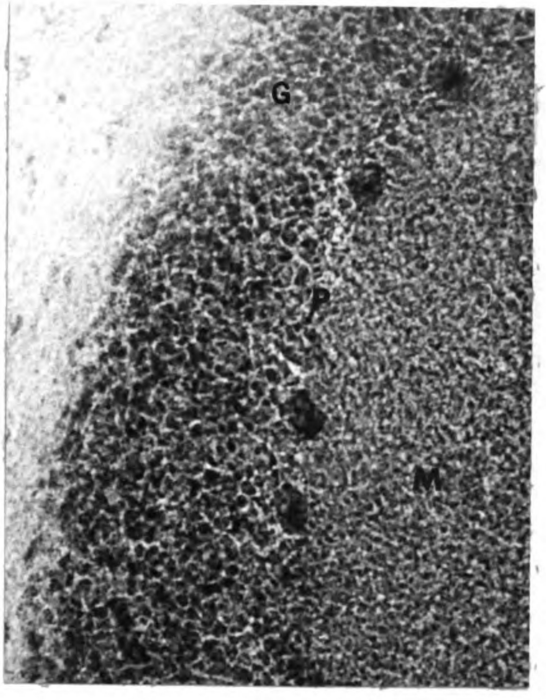
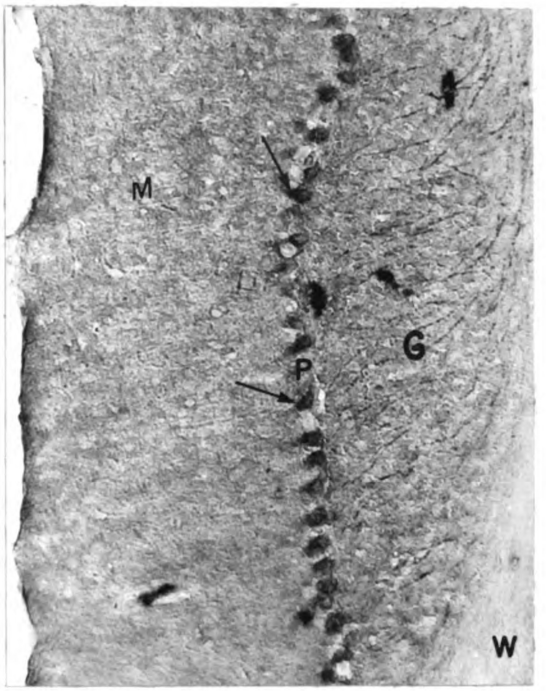


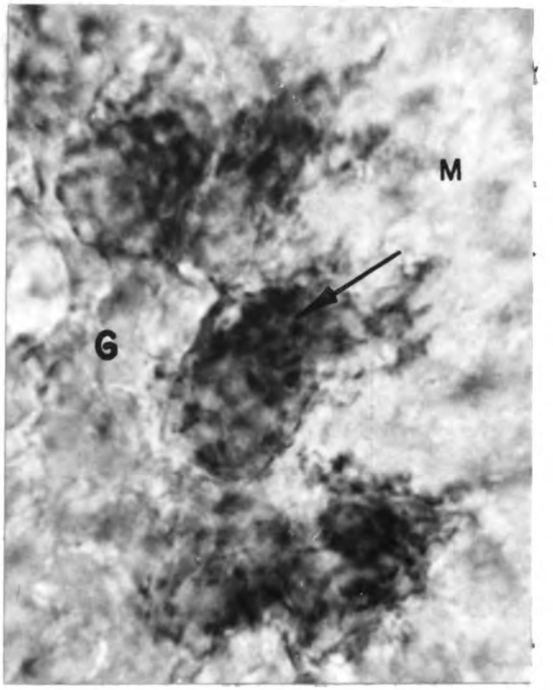
Fig. 5a. Esterase activity in the layers of the cerebellar cortex. See Fig. 3c for explanation of the symbols. Counterstained in Mayer's carmalum. α-naphthyl acetate; x 130.

Fig. 5b. Purkinje cells. The esterase activity is concentrated in that portion of the cytoplasm nearest the molecular layer (M). Counterstained in Mayer's carmalum. Indoxyl acetate; x 520.

Fig. 5c. Layers of the cerebellar cortex. See Fig. 3c for explanation of the symbols. Esterase activity. Indoxyl acetate; x 130.



5 a



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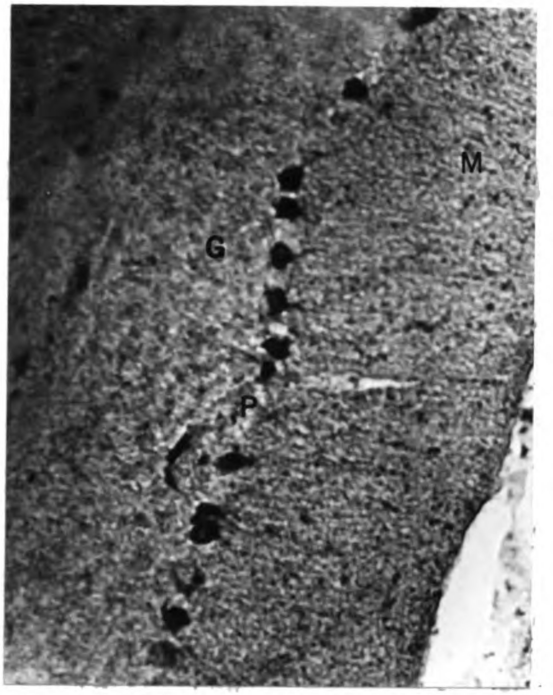
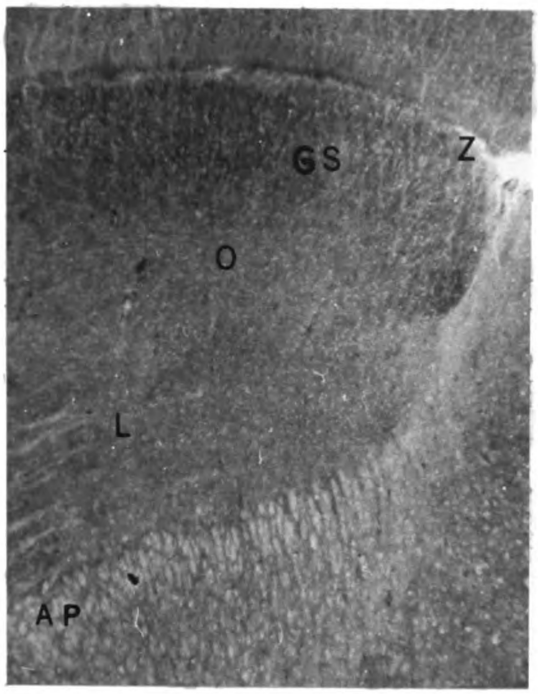


Fig. 6a. Superior colliculus. Glutamate dehydrogenase. Z represents the zonal layer; GS, the stratum griseum superficiale; O, the stratum opticum; L, the stratum lemnisci; AP, the stratum album profundum (containing commissural tracts). The tissue above the zonal layer is the ventral aspect of the cerebral cortex. No counterstain. Nitro-BT.; x 48.

Fig. 6b. Superior colliculus. α-glycerophosphate dehydrogenase activity. The distinct line coursing diagonally across the upper-right corner is the surface of the colliculus in contact with the cerebral cortex. See Fig. 6a for explanation of symbols. No counterstain. Nitro-BT.; x 83.



6 a

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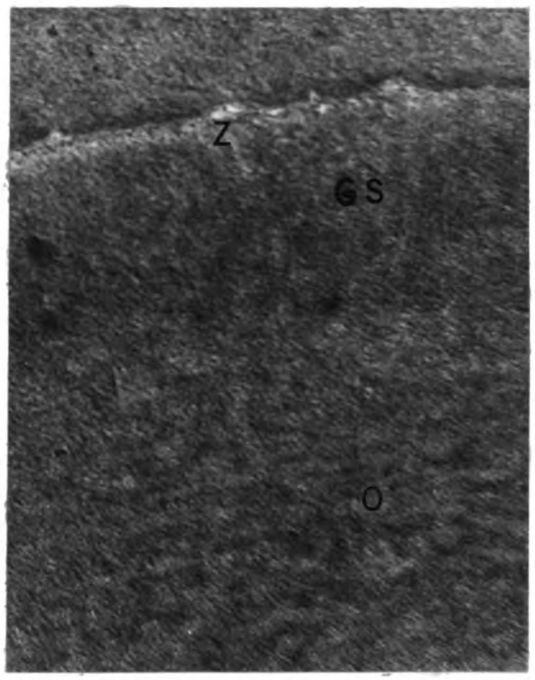
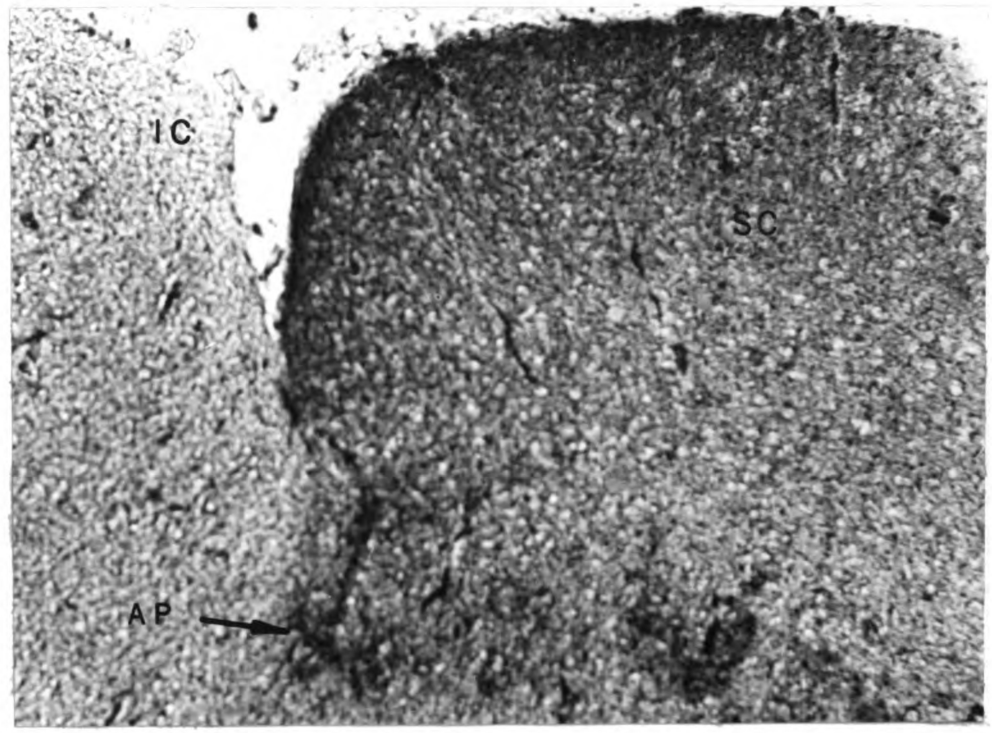


Fig. 7a. The superior colliculus (SC) and the anterior half of the inferior colliculus (IC). Note the greater esterase activity within the superior colliculus. The stratum album profundum (AP) exhibits a strong enzyme reaction. No counterstain. α-naphthyl acetate; x 130.

Fig. 7b. The superior colliculus (SC) and part of the inferior colliculus (IC). Note the intense esterase activity exhibited by pericytes (see arrows). Counterstained in Mayer's carmalum. Indoxyl acetate; x 83.



7 a

b

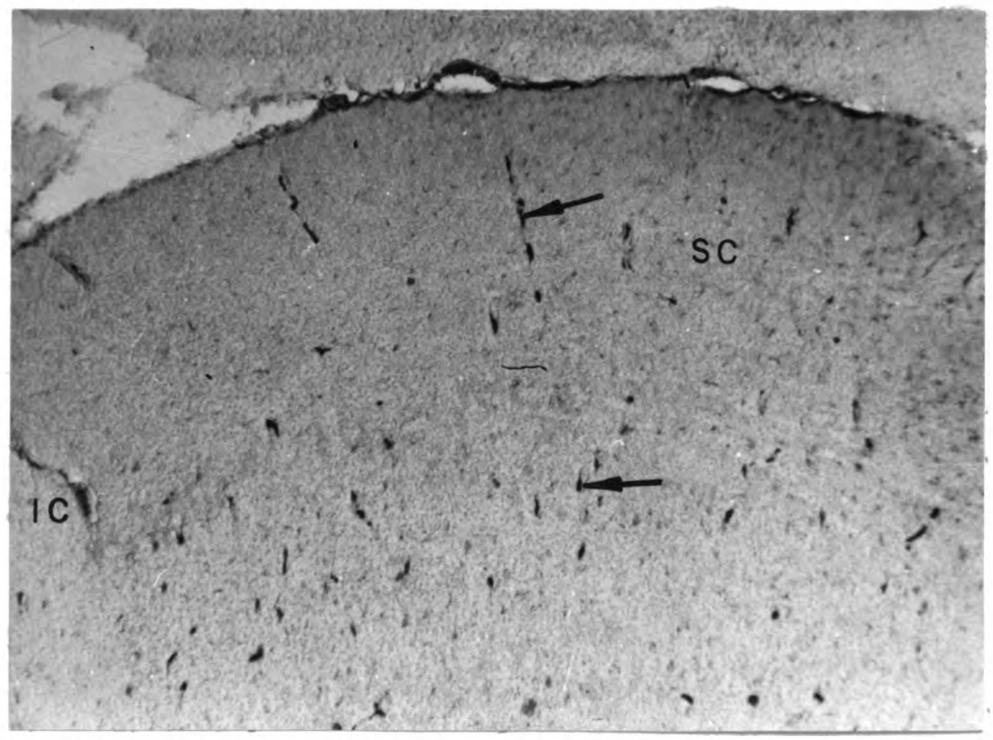
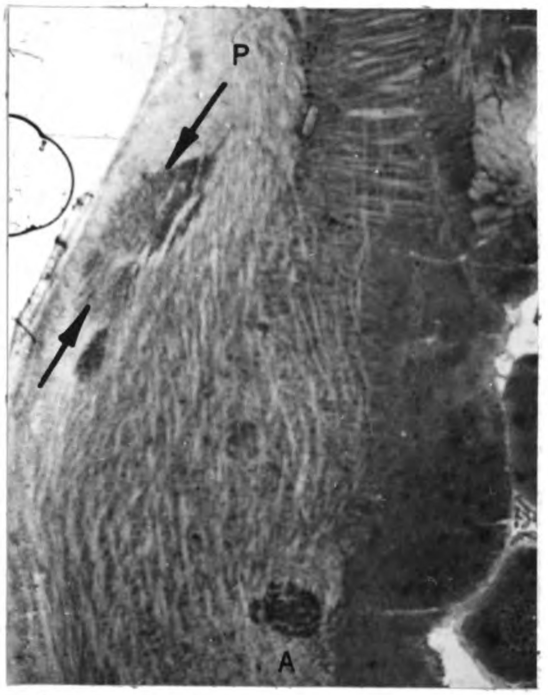


Fig. 8a. Medulla oblongata in sagittal section. α-glycerophosphate dehydrogenase. The arrows point out the inferior olivary nucleus. The letters A and P indicate the anterior and posterior ends of the tissue in the photograph, respectively. A portion of the choroid plexus of the fourth ventricle is visible in the lower-right corner of the picture. No counterstain. Nitro-BT.; x 24.

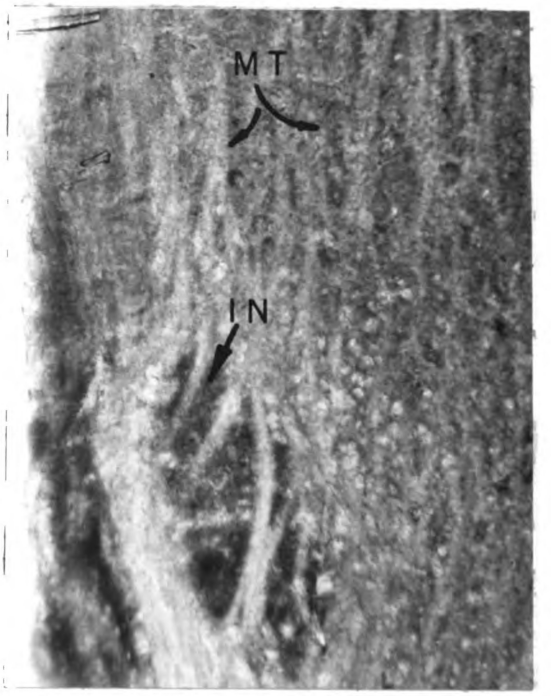
Fig. 8b. Ventral portion of the medulla oblongata, at the level of the inferior olivary nucleus (IN). Note myelinated fiber tracts (MT). Glutamate dehydrogenase. Nitro-BT.; x 130.

Fig. 8c. Neurons of a small regional nucleus within the medulla oblongata. Note the perinuclear concentration of glutamate dehydrogenase (arrows). No counterstain. Nitro-BT.; x 570.

Fig. 8d. Hyperactive neurons within the medulla, exhibiting malate dehydrogenase activity. Mayer's carmalum. MTT.; x 260.



8 a



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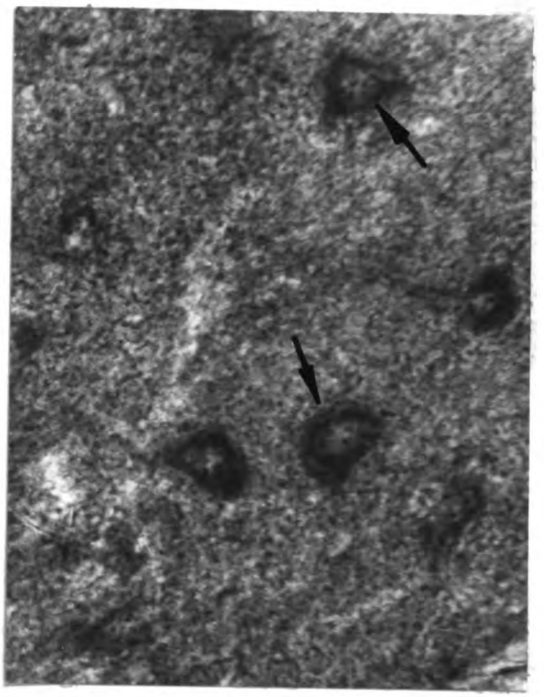

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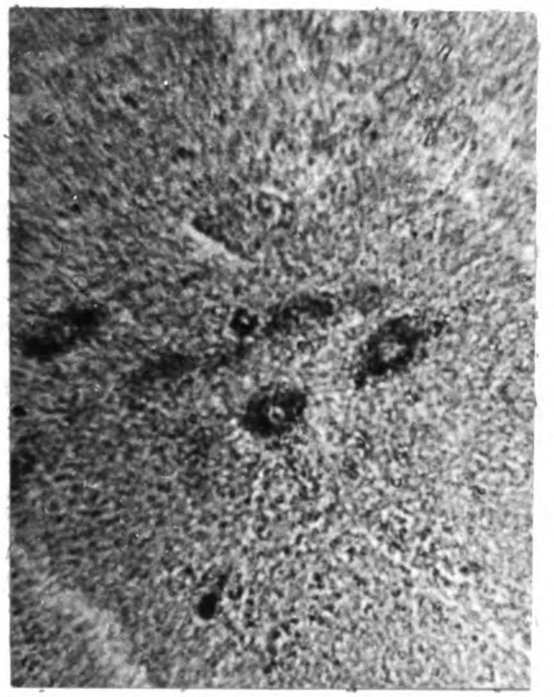
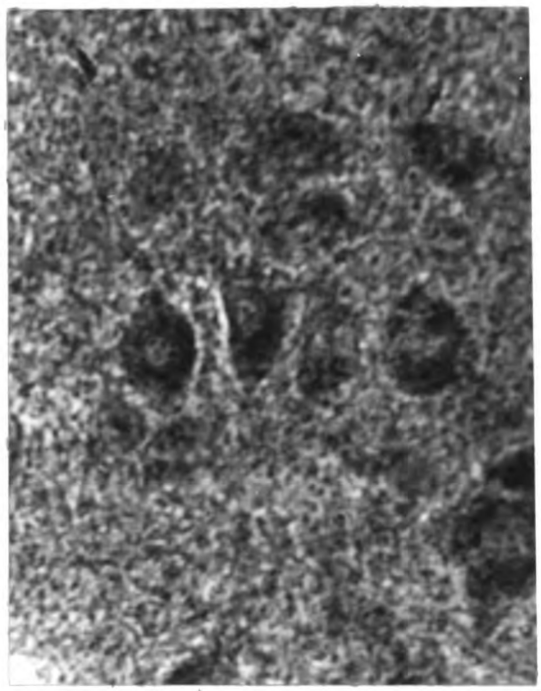


Fig. 9a. Regional nucleus within the medulla oblongata. "Nothing" dehydrogenase activity. Control section; substrate absent from the incubating media. No counterstain. Nitro-BT.; x 570.

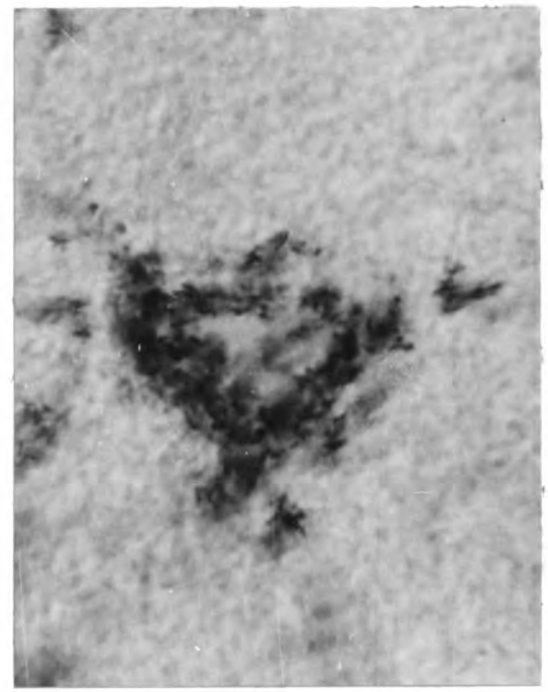
Fig. 9b. A neuron in the medulla oblongata exhibiting intense esterase activity. Mayer's carmalum counterstain. Indoxyl acetate; x 1330.

Fig. 9c. Solitary hyperactive neuron of the medulla. Esterase. No counterstain. α-naphthyl acetate; x 1330.

Fig. 9d. The same neuron shown in 9c, but photographed while under phase contrast. Note the increased esterase activity on the periphery, probably on the cell membrane (arrows). α-naphthyl acetate; x 1330.



9a



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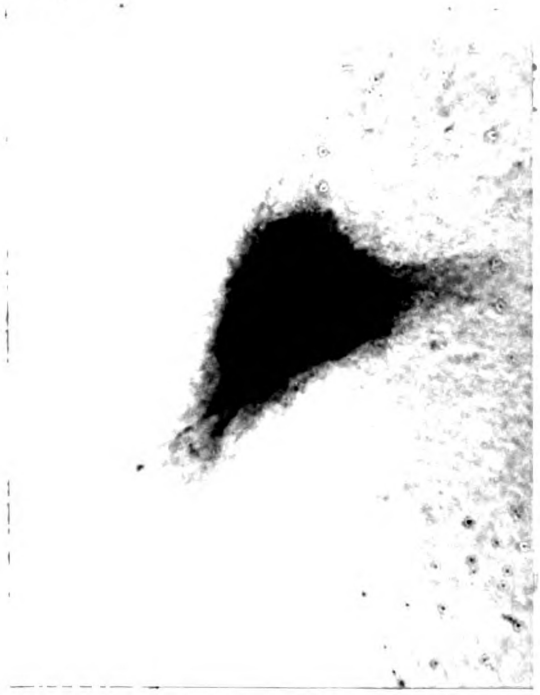

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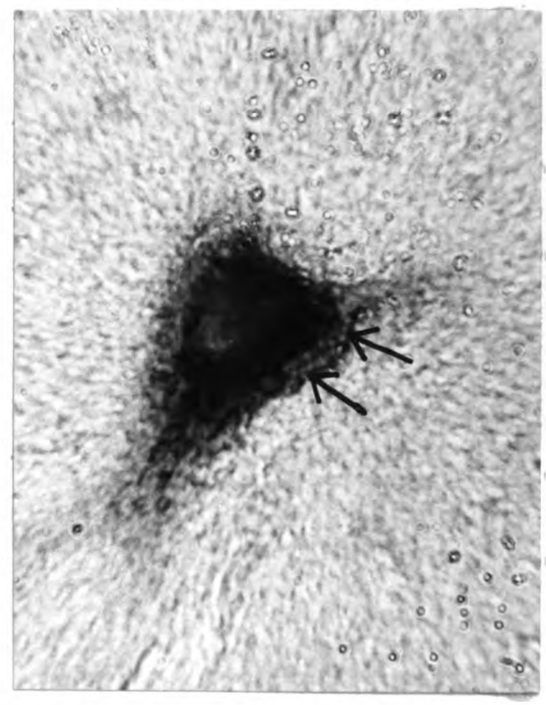
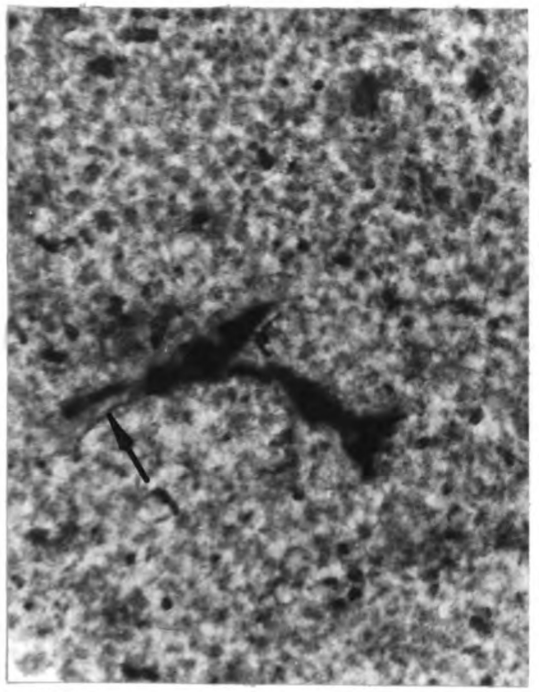


Fig. 10a. Pericytes at a capillary junction. Note portion of capillary membrane (endothelium) by arrow. Esterase. Mayer's carmalum. Indoxyl acetate; x 570.

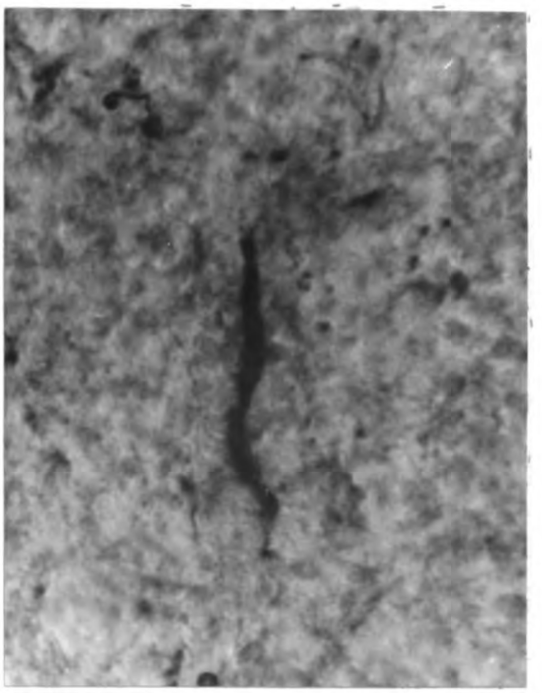
Fig. 10b. Pericyte. Esterase. Indoxyl acetate; x 570.

Fig. 10c. Pericyte. Arrows delineate endothelial esterase activity. Mayer's carmalum. Indoxyl acetate; x 1330.

Fig. 10d. Pericyte. Note endothelial nucleus (arrow) made visible by the surrounding esterase activity of the cytoplasm. Mayer's carmalum. Indoxyl acetate; x 1330.



10a



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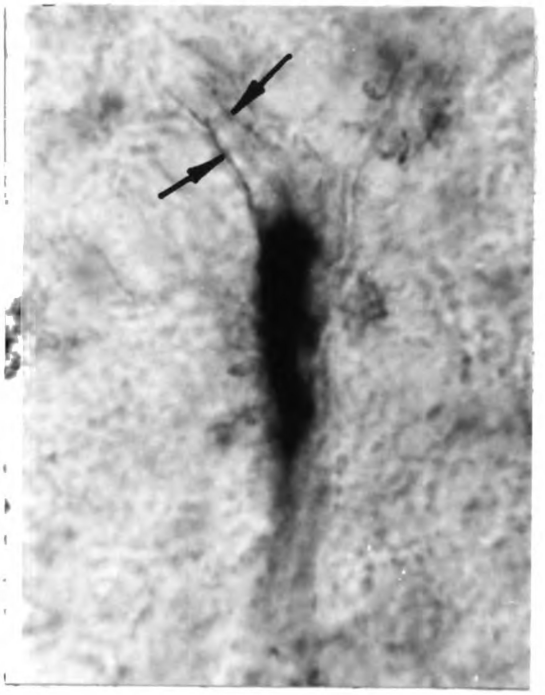
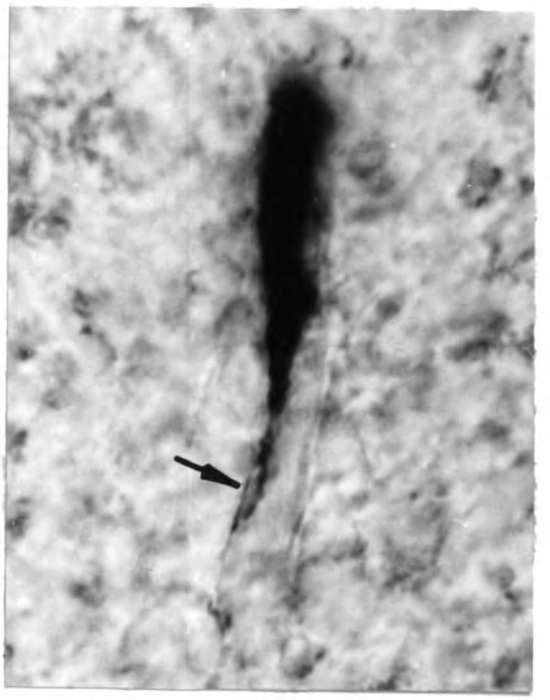




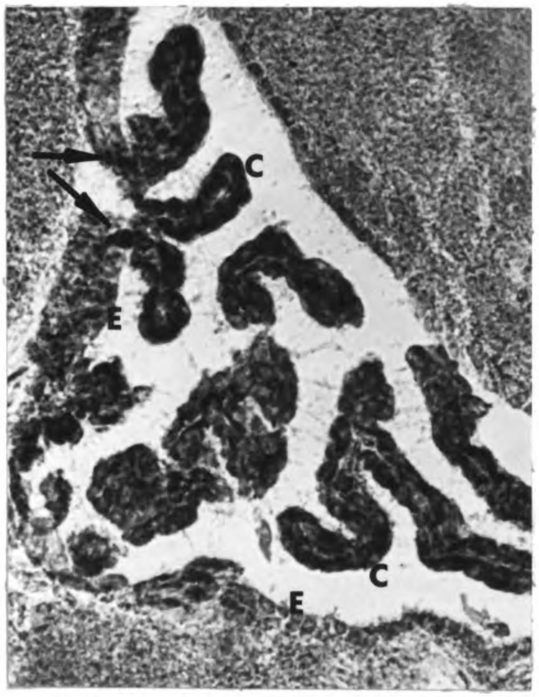
d

Fig. 11a. Glutamate dehydrogenase activity within the choroid plexus of the fourth ventricle. The arrows point out the continuity between the ependyma (E) and the cuboidal epithelium (C) covering the blood vessels. No counterstain. Nitro-BT.; x 260.

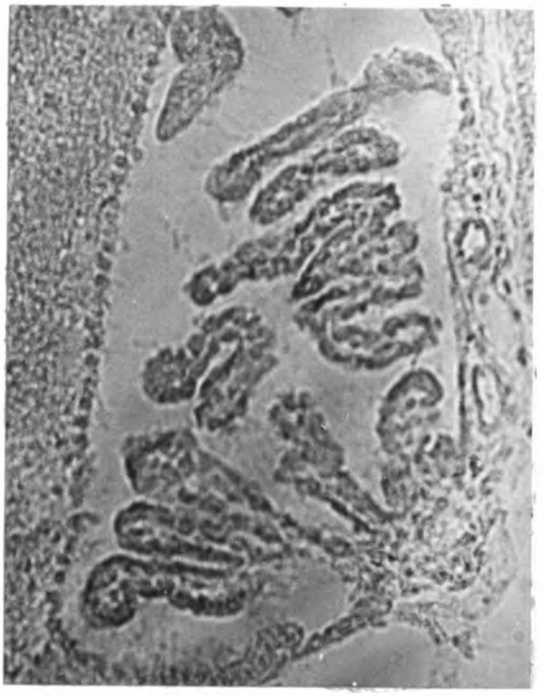
Fig. 11b. Choroid plexus of the fourth ventricle. Lactate dehydrogenase activity. Mayer's carmalum. MTT.; x 130.

Fig. 11c. Choroid plexus of the fourth ventricle. α-glycerophosphate dehydrogenase. No counterstain. Nitro-BT.; x 260.

Fig. 11d. Choroid plexus of the lateral ventricle. Esterase. Mayer's carmalum. Indoxyl acetate; x 130.



11 a



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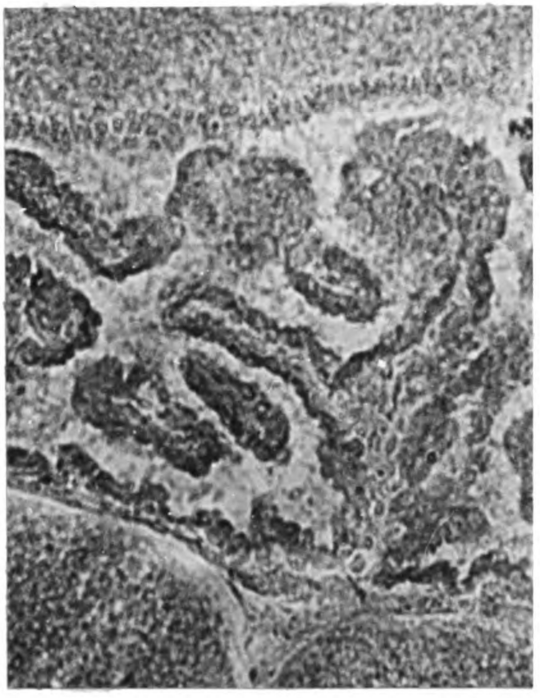

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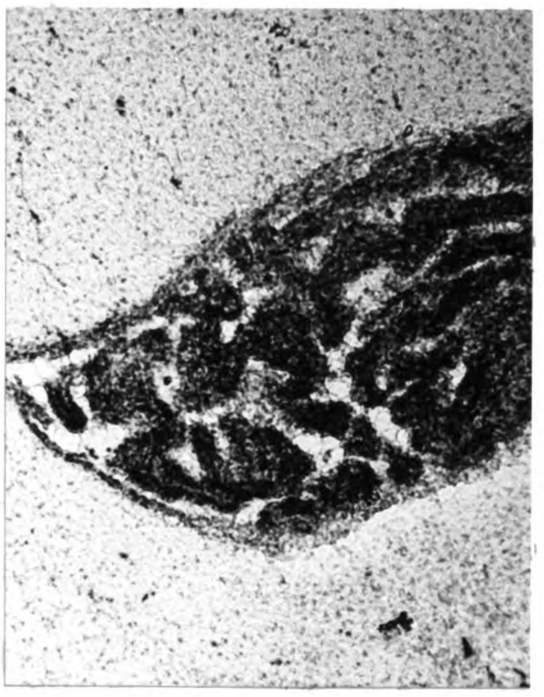
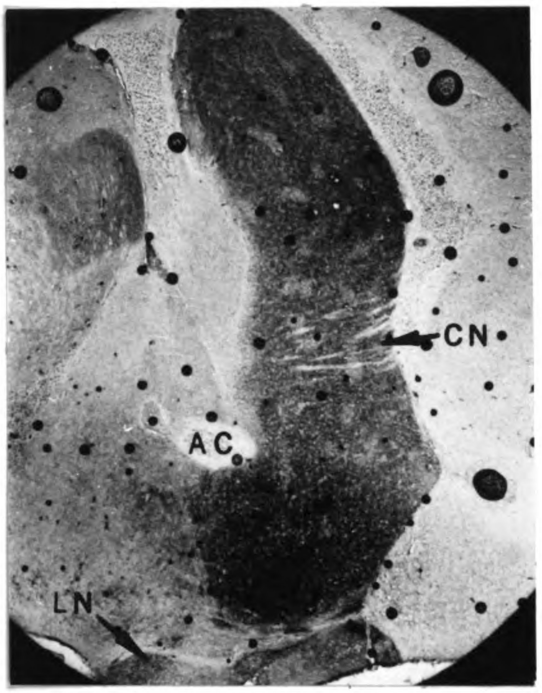


Fig. 12. Lactate dehydrogenase activity within the choroid plexus of the fourth ventricle. Note the variation in intensity between different portions of the same plexus. Counterstained with Mayer's carmalum. MTT.; Phase contrast, x 570.

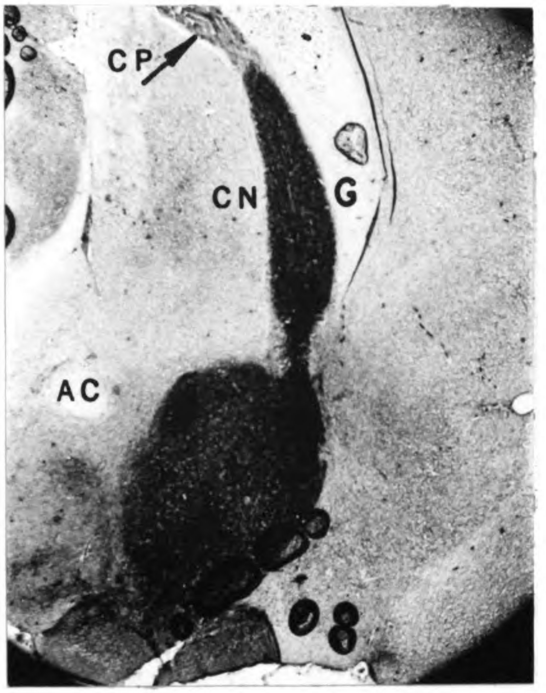
12

Figs. 13a-d. The caudate and lenticular nuclei (neostriatum) of the basal ganglia. In sagittal section. Figures 13a-d show the distribution of esterase activity from the medial limit of the ganglia to its lateral boundary. The following letters represent their respective structures: AC, anterior commissure; CN, caudate nucleus; CP, choroid plexus of the lateral ventricles; G, genu of the corpus callosum; LN, lenticular nucleus; OC, optic chiasma; T, thalamus. All sections represented were counterstained in Mayer's carmalum. α-naphthyl acetate; x 24.

(The right side of each photograph is the anterior end of the tissue shown, the left side is the posterior end.)



13 a



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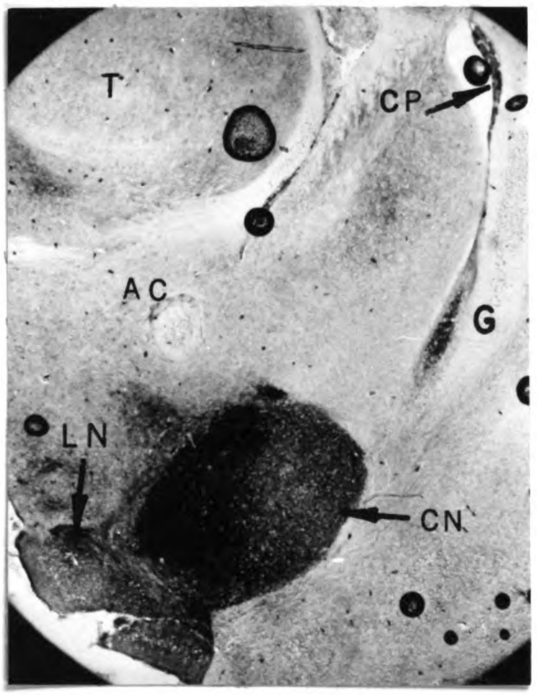

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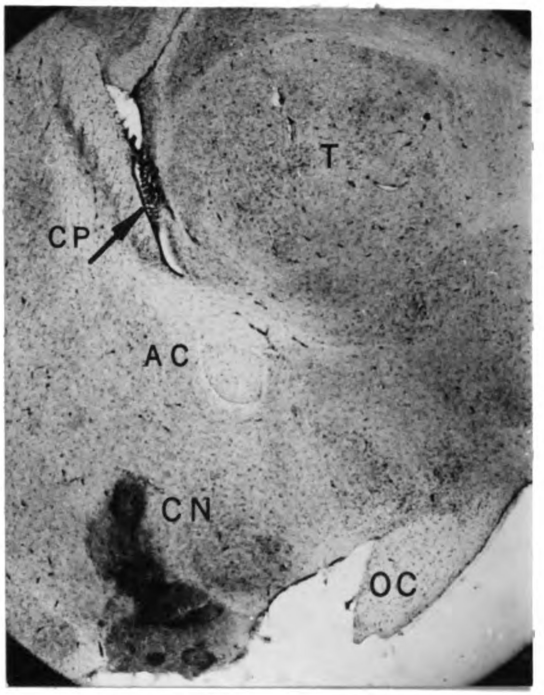
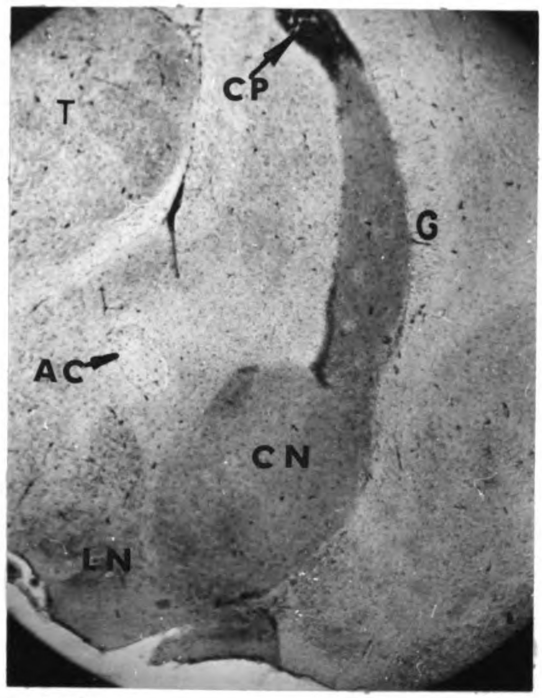


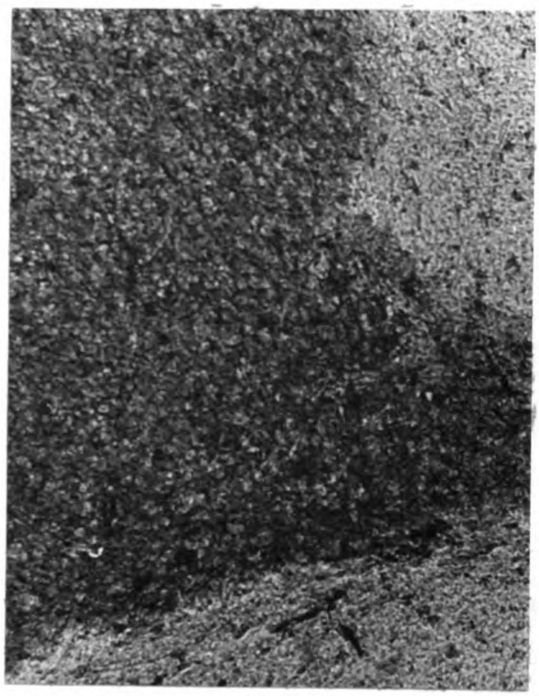
Fig. 14a. Esterase activity in the caudate and lenticular nuclei. For explanation of symbols, see Figures 13a-d. No counterstain. Indoxyl acetate; x 24.

Fig. 14b. Esterase activity in the caudate nucleus. Mayer's carmalum. α-naphthyl acetate; x 130.

Fig. 14c. Caudate nucleus. Esterase activity. Nerve cell nuclei are negative. Cells at the periphery of the nucleus (arrows) exhibit reduced activity. α-naphthyl acetate; x 260.



14 a



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