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# Histochemical studies on the distribution of alkaline and acid phosphatases and 5-nucleotidase in the cerebellum of rat

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## INTRODUCTION

Since quantitative biochemical investigations of a number of enzymes, including hydrolytic enzymes in the brain (Robins & Smith, 1953), show significant and consistent chemical differences between various parts of the same mass of grey matter, it seemed that a study of the histochemical distribution of enzymes in the brain would be of value. The present paper deals with the histochemical distribution of alkaline and acid phosphatases and 5-nucleotidase in the cerebellum of rat. Most of the previous studies (Wolf, Kabat & Newman 1943; Landlow, Kabat & Newman, 1942; Bourne, 1958) on the distribution of alkaline and acid phosphatases, though carried out at the histological level, involved the use of fixatives such as ethanol or acetone, which are detrimental to the architecture of the brain.

Naidoo & Pratt (1951), in examining the distribution of acid phosphatase in freeze-dried, paraffin embedded tissue, and Becker, Goldfischer, Shin & Novikoff, (1960) studying the localization of enzyme activities in the rat brain (both using Gomori's technique), demonstrated acid phosphatase at the cytological level in Purkinje cells, but detailed investigations on the distribution of this enzyme at the many synapses, and in the fibres and other cellular elements of the different layers have not been described.

The present study represents an attempt to record in some detail the distribution of alkaline and acid phosphatases and 5-nucleotidase both at intracellular and intercellular levels in various layers of the cerebellum using recent techniques, such as those of Burstone (1958) for alkaline and acid phosphatase and Wachstein & Meisel (1957) for 5-nucleotidase.

## MATERIAL AND METHODS

Normal, young rats were killed by decapitation. No attempt to kill the animals by anaesthesia was made because of the possible disturbance of the metabolic activity of the central nervous system. In all cases the entire cerebellum, after quick and careful removal, was quenched for 2–4 hr. by placing in close contact with dry ice at −70 °C. After quenching, the sections were cut in a cryostat at −20 °C. at 10 $\mu$. They were processed through Burstone's techniques for alkaline and acid phosphatases (1958) and 5-nucleotidase (Wachstein & Meisel, 1957). The incubation periods in both cases usually varied from $\frac{1}{2}$ to 4 hr. Some sections were incubated for 6–12 hr. The prolonged incubation only intensified the enzymic reactions, otherwise the distribution patterns were the same between the maximum and minimum

incubation periods. Supplementary preparations by Bonhag's mercury-bromphenol blue method for protein were also made (Pearse, 1960) for comparison with histochemical reactions.

## RESULTS

(1) *Acid phosphatase.* Of the four layers of the cerebellum, only the white matter was negative for this enzyme. The remaining three layers (viz. granular, Purkinje and molecular layers), showed enzymic activity to some degree (Pl. 1, figs. 1 and 3). (*a*) In the granular layer the granule cells and their dendrites were negative, while the glomeruli did not show any apparent localization (compare layers G, Pl. 1, figs. 1 and 3, with arrows *gc* and *gl*, Pl. 1, figs. 7 and 8). However in between the negative granule cells and glomeruli a distinct positive beaded reaction could be seen (areas *G*, Pl. 1, figs. 1 and 3); possibly this reaction is comparable to the blue dots mentioned by Brodal & Harrison (1948). It has been shown by neuroanatomists that in the granular layer mossy fibres branch and ramify extensively, and each ramification has a series of rosettes (Ramon y Cajal, 1911; Scheibel & Scheibel, 1954); thus the positive beads of acid phosphatase reaction on the mossy fibres suggest the localization of the enzyme at the synaptic endings as boutons and warty rosettes. (*b*) In the Purkinje layer the most conspicuous staining was shown in the cytoplasm of the Purkinje cells (arrows, Pl. 1, fig. 1). On examining these cells under high power, a cyclic activity of the acid phosphatase in the Purkinje cells became apparent. It was seen that whenever the nucleolus (arrows *Nu*, Pl. 2, figs. 9 and 10) touched the nuclear membrane, the enzymic activity was concentrated immediately around the nuclear wall (arrows *PC*, Pl. 2, fig. 9). However, when the nucleolus was in the central part of the nucleus, the enzymic activity appeared to have spread toward the peripheral part of the cytoplasm (Pl. 2, figs. 11 and 12). In fig. 11 it may be noted that the nucleolus lies in the central part and the enzymatic activity presents the appearance of a broad zone (arrow), leaving a more or less negative peripheral part. From other preparations (e.g. Pl. 2, figs. 12 and 13), an impression was gained that the negative peripheral part (as in Pl. 2, fig. 11) later on acquired enzymic activity. In such cases the nucleolus was never seen touching the nuclear wall, but invariably occupied a distinct central position (arrow *Nu*, Pl. 2, fig. 12). A rough estimate of the degree of enzymic activity was also available from those cells in which the entire cytoplasm showed enzymic activity. Thus in Pl. 2, fig. 12, the entire cytoplasm shows enzymic activity, the degree of staining is less intense and more or less distinct granules are traceable. On the other hand, in Pl. 2, fig. 13, also showing the general distribution of enzymic activity, the intensity of the reaction is of a much higher degree. The possibility and significance of such a variation will be considered in the discussion. Around the Purkinje cells some elements gave an intense reaction (Pl. 2, figs. 10, 12 and 13, unlabelled arrows). It was also apparent (Pl. 1, fig. 3) that no other cells, neither those intervening between Purkinje cells (e.g. the Bergmann cells) nor cells lying near the Purkinje cells, such as the basket and stellate cells and those of Lugaro and Golgi, were positive for acid phosphatase. Beside the Purkinje cells only the 'T' axons of the granule cells, showing a beaded appearance along the lengths of the fibres, stood out distinctly as positive structures (arrows *t*, Pl. 1, fig. 3). It may be mentioned that Estable (1923) described the existence of synaptic endings on the parallel

fibres in the form of boutons and warty rosettes. These, in our preparations, gave a positive beaded appearance.

The molecular layer showed fine neuropil staining for acid phosphatase (areas *M*, Pl. 1, figs. 1 and 3). It appeared to possess a lesser degree of enzymatic activity than the granular layer (compare areas *M* and *G*, Pl. 1, figs. 1 and 3). Although in the granular layer the main elements, the granule cells and glomeruli, were negative for acid phosphatase, the synaptic knobs on the intervening fibres possessed enzymic activity to such a high degree that it superseded the fine granular reaction of the neuropil, although the reaction of the positive granules was similar to that of the neuropil of the molecular layer.

(2) *Alkaline phosphatase*: In the granular layer the reaction for this enzyme presented an identical picture to that obtained for acid phosphatase. The granule cells, including their dendrites, and the glomeruli were negative for alkaline phosphatase. Between these negative elements, beaded positive structures were distinctly marked out (area *G*, Pl. 1, fig. 2).

Generally the Purkinje cells, by contrast to the condition in acid phosphatase preparations, were negative with Burstone's technique for alkaline phosphatase (arrows, Pl. 1, fig. 2). In a few Purkinje cells a faint granular reaction was seen in the cytoplasm (Pl. 2, fig. 15). However, it was noticed that in alkaline phosphatase preparations with the Gomori method, the nucleolus was invariably positive. This was not found with the Burstone preparations. Sandler & Bourne (1960) have demonstrated that there is a complex of alkaline phosphatases, some of which hydrolyse only glycerophosphate. With Burstone's method a few positive elements, similar to acid phosphatase preparations, occupied the areas around the Purkinje cells (arrows, Pl. 2, fig. 15). As in acid phosphatase preparations, alkaline phosphatase activity was not shown by other cellular elements (as Bergmann, Lugaro, Golgi, basket and stellate cells). 'T' axons of the granular cells showed positive activity (arrows *t*, Pl. 1, fig. 2).

The molecular layer showed a very faint reaction for alkaline phosphatase and the enzymic activity was definitely of a very low degree compared to acid phosphatase reactions (compare areas *M*, Pl. 1, figs. 1 and 2). By comparing Pl. 1, figs. 1 and 2, it was clear that the blood vessels in alkaline phosphatase preparations possessed a very high degree of activity, whereas they were negative for acid phosphatase (note intensely positive blood vessel in the right corner of Pl. 2, fig. 15). By further comparing Pl. 1, figs. 1 and 2, with Pl. 1, fig. 8, it is apparent that in the molecular layer the cells (arrows, Pl. 1, fig. 8) and the fibres of the Bergmann cells (arrow *f*, Pl. 1, fig. 8) were negative for both enzymes.

(3) *5-Nucleotidase*: The fundamental difference in the general pattern of enzymic activity between alkaline and acid phosphatases and 5-nucleotidase seems to lie in the reactions of the white matter. With the first two reactions the white matter was devoid of enzymic activity (area *W*, Pl. 1, fig. 3). In 5-nucleotidase preparations, however, not only was the white matter positive, but its enzymic activity exceeded that of the granular layer (compare areas *W* and *G*, Pl. 1, figs. 5 and 6). This was in complete contrast to the alkaline and acid phosphatase reactions. In many cases the fibres traversing the white matter (area *W*, Pl. 1, fig. 6) showed strong 5-nucleotidase activity.

In the granular layer the granule cells were negative for 5-nucleotidase (similar to alkaline and acid phosphatase), but surprisingly, the glomeruli were positive. If the negative glomerulus and surrounding granule cells of protein preparation (arrows *gc*, *gl*, Pl. 1, fig. 8) are compared with a similar unit in a 5-nucleotidase preparation (at and around arrow *gl*, Pl. 1, fig. 6) the positive nature of the glomerulus and negative reaction in granule cells for 5-nucleotidase becomes apparent. It is significant that in the granular layer no structures comparable to the positive beads of alkaline and acid phosphatase were found, nor were the 'T' axons of the granule cells apparent.

It has been clearly demonstrated that the synaptic endings both on the fibres in the granular layer as well as on the 'T' axons of the granule cells are negative for 5-nucleotidase. The Purkinje (*P*, Pl. 1, fig. 6) and the molecular layers (*M*, Pl. 1, fig. 5) showed the same degree of enzymic activity as the white matter. Another difference from acid and alkaline phosphatase preparations is that 5-nucleotidase is localized as large clumps of positive material (arrow *Nb*, Pl. 2, fig. 16) in the cytoplasm of the Purkinje cells. These clumps could be correlated in size and appearance with the pattern of Nissl material.

The significance of the differences between acid and alkaline phosphatase preparations and the intracellular distribution of 5-nucleotidase in Purkinje cells will be considered in detail later. Nevertheless, it may be noted here that the elements around Purkinje cells, positive for alkaline and acid phosphatases, (arrows, Pl. 2, figs. 10, 12, 13 and 15) were negative in 5-nucleotidase preparations (Pl. 2, fig. 16). The only similarity between alkaline and acid phosphatase and 5-nucleotidase preparations in the area around the Purkinje cells was in the fact in that all preparations Lugaro, Golgi, basket and stellate cells were negative for all these enzymes.

## DISCUSSION

Quantitative enzymic estimations of the cerebellum show that the molecular layer is apparently a metabolically more active tissue than the granular layer (Robins & Smith, 1953).

Our present observations and those of Becker *et al.* (1960) are in disagreement with the quantitative estimation by Robins & Smith (1953), of acid phosphatase in the molecular layer. In biochemical estimations they found 50% more acid phosphatase in the molecular layer than in the granular layer. The disparity in our results may be due rather to the large number of granules in the neuropil of the molecular layer as compared to the number of synapses in the granular layer, than to the quantity of enzyme in the individual granules and synapses. Histochemically, acid phosphatase is more intensely localized in the synapses of the granular layer than in the granules of the neuropil of the molecular layer.

The virtual absence of acid phosphatase in the white matter in our preparations (*W*, Pl. 1, fig. 3) and the prominent staining in the granular layer (areas *G*, Pl. 1, figs. 1 and 3) supports the biochemical observations of Robin & Smith that acid phosphatase activity is higher in the granular layer than in the white matter.

Since the molecular layer is primarily composed of dendrites of Purkinje cells, as well as granule cell axons, and basket cells, it is natural to expect that the enzymic

equipment in the main body of the Purkinje cells would determine the presence or absence of any enzyme in the dendrite. Our preparations have shown that acid phosphatase is found prominently in Purkinje cells and therefore the possibility exists that it would be present in their dendrites; and therefore it should be seen in the molecular layer. On the other hand, alkaline phosphatase is often absent from Purkinje cells (arrows, Pl. 1, fig. 2), therefore it is not unexpected to find it absent in their dendrites and the molecular layer would not, in this case, show an alkaline phosphatase reaction. This is demonstrated in Pl. 1, fig. 2.

In the Purkinje cells we found acid phosphatase located in the form of rounded granular bodies, with Burstone's technique. Becker *et al.* (1960), using the Gomori procedure, also located acid phosphatase in such cytoplasmic bodies, which they presumed to be lysosomes. It was noted in our preparations that whenever the nucleolus touched the nuclear wall, acid phosphatase positive bodies were concentrated around the nucleus. This suggests that some sort of metabolic intercourse is proceeding between the nucleolus and the perinuclear concentration of acid phosphatase positive bodies. However, when the nucleolus was located in the central part of the nucleus we observed cells in which the acid phosphatase reaction appeared in some as a central broad zone or accumulation of granules leaving the peripheral part clear, and in others it was spread throughout the entire cytoplasm. Presumably the observations of Becker *et al.* (1960) that 'lysosomes' are randomly distributed in the cytoplasm relates only to the distributive stage seen in our preparations. Our results show that the myelinated axons of the Purkinje cells are negative for acid phosphatase. Such a view is also shared by Becker *et al.* (1960).

In our histochemical studies on spinal ganglion cells (Tewari & Bourne, 1962*a*) we failed to see a cyclic arrangement of acid phosphatase activity such as occurs in Purkinje cells. On the other hand, we clearly established a perinuclear concentration of mitochondria followed by general distribution throughout the cytoplasm in spinal ganglion cells. The oxidative enzymes followed the pattern of mitochondrial distribution. From our experience in spinal ganglion studies and from the fact that mitochondria are numerous in Purkinje cells and are situated around the nucleus (Tewari & Bourne, unpublished data), the possible occurrence of acid phosphatase in the mitchondria of Purkinje cells should be considered. The proper designation of the acid phosphatase positive bodies around the nucleus is difficult because many neurones, particularly in the spinal ganglion cells (Tewari & Bourne, 1962*b*) also show a concentration of Golgi bodies in the perinuclear zone at some stage and therefore, the possibility exists that the acid phosphatase contained in the Golgi bodies may be reacting with Burstone's technique. However, since our results show rounded bodies and not a reticulum, the assignment of such bodies to the Golgi apparatus seems inappropriate. In view of the studies made by many workers on the localization of acid phosphatase in lysosomes it seems most likely that these are the structures that we have observed in our acid phosphatase preparations.

The most interesting result of the present study is the localization of acid and alkaline phosphatase at synapses, both at the mossy fibres in the granular layer and at the 'T' axons of the granule cells. Simple esterase preparations also show a similar pattern of localization (Pl. 1, fig. 4; Tewari & Bourne, 1962*b*).

Bourne (1958), using Gomori's method for alkaline phosphatase in the brain of

the rat, suggested that the possible role of phosphatases in synaptic transmission should be considered. The two functions of phosphatases which would be represented by the presence of these enzymes at blood brain barriers and at synaptic sites may be perhaps reconciled by the suggestion that in each case they are responsible for alteration of permeability or supply of free energy. It may be noted here that contrary to our findings in spinal ganglion cells, the alkaline phosphatase is not concentrated at the periphery of the Purkinje cells. Most of the Purkinje cells (including the nucleolus which is negative with Burstone's technique and positive with the Gomori method) are negative except for isolated instances showing faintly positive granules distributed in the cytoplasm. This Bourne (1958) also noticed and suggested that the Purkinje cells may work in relays, interspersing periods of activity with periods of quiescence. Bourne also noticed increased hydrolytic activity in senescent cells and it is possible that Purkinje cells which show a slight degree of alkaline phosphatase activity in the cytoplasm of our preparations may be reaching the senescent stage.

The localization of 5-nucleotidase in the white matter and the molecular layer with the enzymic activity in the glomeruli of the granular layer is interesting from many respects. It has been seen that the white matter gives a very intense reaction for 5-nucleotidase (area *W*, Pl. 1, fig. 6). Futhermore, the localization of this enzyme would not be unexpected in view of the recent claim that the 'protein fraction of white matter is not an enzymically inert substance' (Robins & Smith, 1953). The complete absence of 5-nucleotidase from the synapses of all fibres of the granular layer of 'T' axons of the granule cells is unlike the distribution of alkaline and acid phosphatase. Therefore it seems, likely that 5-nucleotidase is not concerned in synaptic transmission. The localization of 5-nucleotidase in the glomeruli of the granular layer, however, resembles a similar localization of oxidative enzymes and simple esterase (Tewari & Bourne, 1962*c*). Furthermore, the localization of 5-nucleotidase in the same region as Nissl-like structures, reported by us for the first time, may indicate a close relationship between the two.

Finally, it may be stated that in alkaline and acid phosphatase preparations (and also in simple esterase studies; see Pl. 2, fig. 14), various positive elements can be seen. A detailed consideration of the nomenclature of these elements has been discussed elsewhere (Tewari & Bourne, 1962*b*), but it is sufficient to say here that such structures may be either the synaptic knobs of the 'T' axons or the spines of the branchlets of the Purkinje dendrites (Fox & Barnard, 1957). This study again stresses the possible role of phosphatases in synaptic transmission. Since 5-nucleotidase preparations do not show such structures around the Purkinje cells, the participation of 5-nucleotidase in synaptic transmission seems remote. On the other hand, it can be speculated that 5-nucleotidase may be concerned in the metabolic activity of the Nissl substance since it appears to be localized in these bodies.

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## EXPLANATION OF PLATES

### PLATE 1

Figs. 1–6 are fresh frozen sections; figs. 7 and 8 fixed paraffin sections.

Fig. 1. Acid phosphatase reaction (Burstone technique: AS-BI phosphate, red violet LB salt). Note the intense cytoplasmic reaction in the Purkinje cells (arrows), positive beaded structures in the granular layer (*G*), negative granular cells and glomeruli and also granular reaction in the neuropil of the molecular layer (*M*). ×340.

Fig. 2. Alkaline phosphatase reaction (Burstone technique: AS-MX phosphate, red violet LB salt). Note more or less negative reaction in the Purkinje cells (compare with fig. 1). Identify positive bead-like structures situated in between the negative granular cells and the glomeruli of the granular layer (*G*) and also on 'T' axons of the (arrows *t*) granular cells. Molecular layer (*M*) without any distinct neuropil reaction (compare with area *M* of fig. 1). Blood vessels can be seen intensely stained. ×340.

Fig. 3. Acid phosphatase reaction (Burstone technique: AS-BI phosphate, red violet LB salt). Note negative white matter (*W*), intensely positive Purkinje layer (*P*), positive beaded structures in the granular layer (*G*) as well as 'T' axons or parallel fibres (arrows *t*) of the granular cells. Compare the reaction on the parallel fibres (arrows *t*) of figs. 2 and 3. ×340.

Fig. 4. Simple esterase reaction (Burstone technique: AS-D acetate + fast Blue RR salt). Distinguish the granular layer (*G*) showing a positive beaded structure and glomeruli (*gl*) and also negative granular cells. Purkinje layer with positive cytoplasmic reaction in Purkinje cell and molecular layer (*M*) illustrating positive structures on 'T' axons of the granular layer (arrows *t*) and granular neuropil reaction. Compare with figs. 1–3. ×340.

Fig. 5. 5-Nucleotidase reaction (Wachstein and Meisel). Note intense reaction in the white matter and the molecular layer (*M*). Distinguish the negative granular cells and the moderately stained glomeruli (as seen at arrow *gl*) in the granular layer (*G*). ×340.

Fig. 6. 5-Nucleotidase reaction (Wachstein and Meisel). Identify white matter (*W*) with deeply stained fibres; granular layer (*G*), strong positive glomeruli (*gl*) and negative granular cells. Purkinje layer (*P*) with intense reaction in the Purkinje cells. Compare one unit of the positive glomerulus (*gl*) and negative granular cells around it with a similar unit showing positive glomerulus (arrow *gl*) and granular cells (arrow *gc*, fig. 8). ×340

Fig. 7. Carnoy fixed; mercury bromphenol blue method for proteins. Note the blue granular reaction for protein in the nuclei of the granular cells (arrows *gc*) and the glomeruli (*gl*). ×1360.

Fig. 8. Carnoy fixed; mercury bromphenol blue method for proteins. Distinguish the cells of Bergmann (arrows *Bc*) and the Purkinje cells of the Purkinje layer (*P*), neuronal elements (unlabelled arrows) and the processes of the Bergmann cells (arrow *t*) of molecular layer (*M*); and the granular cells (arrows *gc*) and the glomeruli (arrow *gl*). ×340.

## PLATE 2

(Fresh frozen sections 10$\mu$: figs. 9–13 are acid phosphatase reactions (Burstone technique, AS-phosphate, red violet LB salt).)

Fig. 9. Note the perinuclear concentration (arrows *pc*) of enyzmes at the stage when the nucleolus (arrow *Nu*) touches the nuclear wall in the Purkinje cell. ×1360.

Fig. 10. Distinguish the intracytoplasmic acid phosphatase positive granules in the perinuclear area (with nucleolus, *nu*, touching the nuclear membrane) of the Purkinje cell. Note also the extracellular positive elements ( = synapses) as seen at unlabelled arrows in the field around the cell. ×1360.

Fig. 11. Note the broad zone of enzymic activity (arrow) and the centrally situated nucleolus. ×1360.

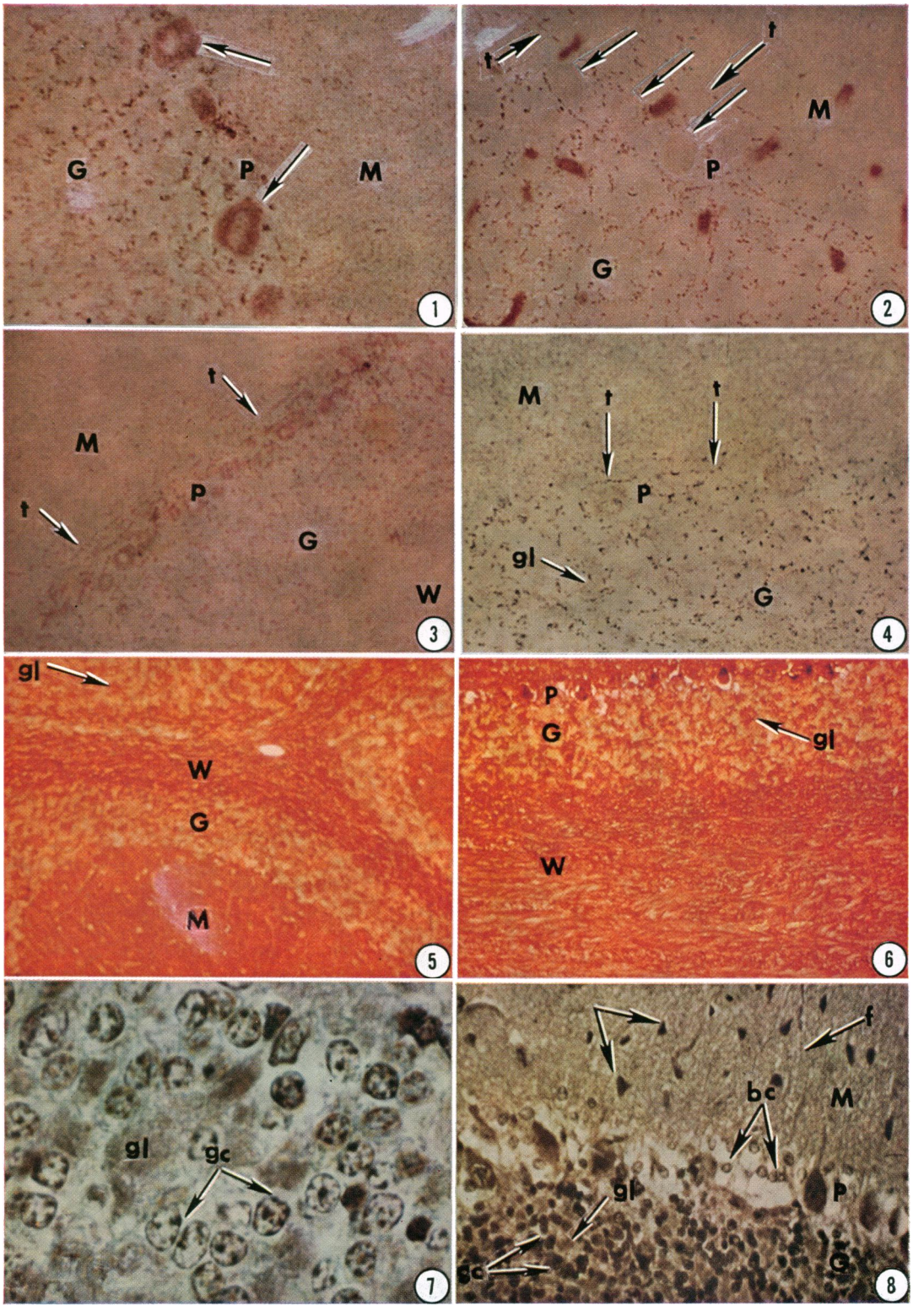
Fig. 12. Note enzymic activity in the entire cytoplasm (compare this stage with the perinuclear concentration, *pc*, and middle zone of activity, arrow, of figs. 9 and 11). Further compare the central location of the nucleolus (*Nu*), of fig. 12 with the eccentric portion of the nucleolus (*Nu*) of fig. 9; also the positive elements (unlabelled arrows) of figs. 10 and 12. ×1360.

Fig. 13. Note the general distribution and increase in intensity of acid phosphatase reaction in the cytoplasm of the Purkinje cell (compare with less intense granular reaction of figs. 9–12). Positive elements, as seen at arrows, occupy the neuropil around the cell. ×1360.

Fig. 14. Simple esterase reaction (Burstone technique; AS-D acetate and Fast Blue RR salt). Positive cytoplasmic granules and extra-cellular elements (=synapses). Compare these (unlabelled arrows) with similar structure in acid phosphatase preparations (unlabelled arrows of figs. 10 and 12). ×1360.

Fig. 15. Alkaline phosphatase reaction (Burstone technique, AS-MX phosphate, red violet LB salt). The Purkinje cell shows light positive reaction (compare with Purkinje cells at arrow, fig. 2). Identify the positive structures (arrows) around the cell (compare with structures at unlabelled arrows of figs. 10, 12, 13 and 14). Note the intense reaction in the blood vessel at the right-hand corner. ×1360.

Fig. 16. 5-Nucleotidase reaction (Wachstein and Meisel technique). Note the positive elements (= Nissl bodies? arrows *Nb*) in the Purkinje cell (also absence of positive elements around cell as seen in figs. 10–15) in this preparation. ×1360.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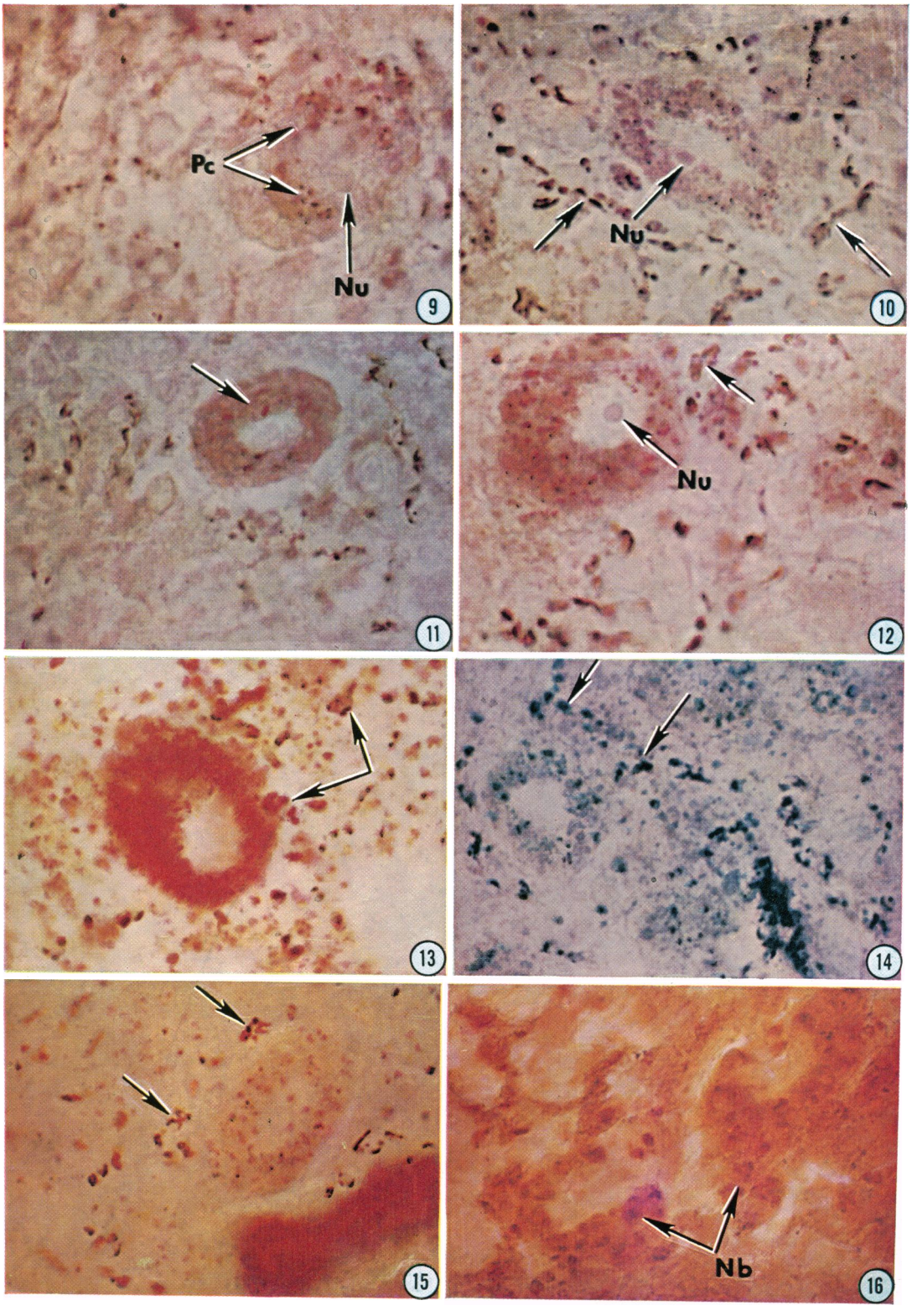
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