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The localization of alkaline phosphatase activity in cerebral blood vessels[1]

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Since the development of the first histochemical techniques for demonstrating non-specific alkaline phosphatase activity (Gomori, 1939; Takamatsu, 1939) there have been a number of reports of activity in the endothelium of blood vessels of the mammalian nervous system. Differences in the site and intensity of endothelial staining in the nervous systems of different species have been described but there are no reports of attempts to follow the pattern of alkaline phosphatase activity from arteries to veins in the cerebral vascular tree. Recently we have found evidence of localized areas of alkaline phosphatase activity in the vascular system of muscles and other tissues (Romanul and Bannister, 1962), and in view of the distinctive structural and functional properties of cerebral blood vessels it seemed important to examine in detail their alkaline phosphatase activity.

MATERIAL AND METHODS

Male albino rats weighing 250-350 g. were used for all experiments. The animals were anaesthetized with Nembutal and killed by opening the chest. In nearly all experiments the animals were perfused before removal of the brains. A cannula made from a 15 gauge needle was passed through the left ventricle and tied in the ascending aorta. The right atrium was cut and the animal perfused with 1,000 ml. of normal saline at a pressure of 1·5 metres of fluid, until the perfusate was clear. On some occasions, with the purpose of relaxing smooth muscle and making the perfusion more complete, the vessels were washed out with 500 ml. of normal saline containing 0·2% sodium nitrite before perfusion with 500 ml. of normal saline. Perfusion was omitted in some instances so that any effect of the perfusate on the alkaline phosphatase activity could be studied. Some animals were injected with carmine gelatine (Carlton and Drury, 1957) after perfusion with saline in order to fill the whole of the cerebral vascular tree. The brain and upper spinal cord were then removed, frozen with dry ice, mounted on ice, and sections 8 to 60μ thick cut in a cryostat. Sections were dried for 30 to 60 minutes on slides. On a few occasions the brain was fixed in neutral 10% formalin at 4°C. for 24 hours before cutting sections on a freezing microtome.

Staining for alkaline phosphatase was by the Gomori technique (Gomori, 1939) using beta-glycerophosphate at *p*H 9·0 and on a few occasions by means of a modified azo dye technique (Pearse, 1960) using alpha-naphthyl phosphate and 4-amino-2·5 diethoxy benzaniline (Fast Blue BBN) at *p*H 9·5. The alpha-naphthyl phosphate medium consisted of tris buffer (*p*H 9·5) containing 75 mg. % sodium alpha-naphthyl phosphate and 180 mg. % Fast Blue BBN. Sections on slides were placed for 10 minutes in neutral 10% formalin at 4°C., washed in water, and then placed for one to two hours in substrate solution. The sections were then washed in water and placed in neutral 10% formalin for 24 hours. Mayer's carmalum was used as a counterstain in some instances. On most occasions, in order to study the morphology of vessels, alternate serial sections were stained, one with haematoxylin or cresyl violet and the next histochemically for alkaline phosphatase. Sections stained for alkaline phosphatase were mounted in 50% polyvinyl pyrrolidone.

Sections 200 μ thick from formalin-fixed brains, some with and some without previous perfusion of the animal, were stained by Pickworth's method (Carlton and Drury, 1957) in order to demonstrate the distribution of red blood cells in the cerebrovascular tree.

For study of the pial vessels the brains, some with and some without previous perfusion of the animal, were removed and then several different preparations made. 1 Brains were frozen on dry ice and tangential 64 μ sections, from the surface of the brain, including pia, cut in a cryostat, mounted on slides, and stained as already described. 2 Brains were frozen by placing them for a brief period of time on dry ice, so that slices of pia and cortex about 400 μ thick could be cut by hand with a razor. Slices were placed in the alpha-naphthyl phosphate medium for 10 minutes, washed in water, fixed in 10% formalin for 24 hours, washed again in water, and then cleared in glycerine for 48 hours before mounting in 50% polyvinyl pyrrolidone. 3 Whole brains from perfused animals were placed in the alpha-naphthyl phosphate medium for 10 minutes, then washed in water and fixed in neutral 10% formalin for 24 hours. Tangential sections were then cut from the surface of the brain with a razor by hand, cleared in glycerine, and mounted in 50% polyvinyl pyrrolidone.

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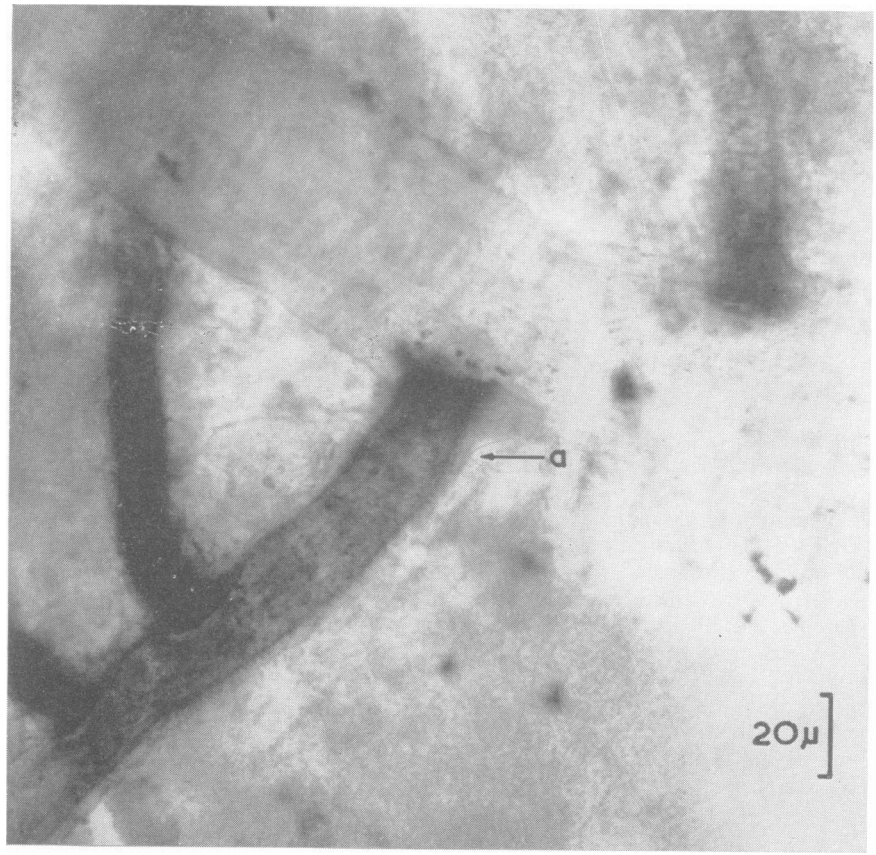

FIG. 1. *Superficial brain slice (approximately 400 μ) from unperfused animal, prepared by method 2, showing pial artery and branches. Incubated with alpha-naphthyl phosphate medium and counter-stained with Mayer's carmalum. The thicker muscular wall and narrower internal calibre can be seen at the origin of the branch artery (a). The apparent super-imposition is due to thickness of the section, the branches originating from the upper portion of the parent vessel.*

RESULTS

The alkaline phosphatase activity of the vessels on the surface and in the substance of the brain was studied in a large number of sections. No alkaline phosphatase activity was found in the walls of arteries larger than 30 μ in luminal diameter. However, on the surface of the brain the largest arteries were some 300 μ in diameter and in these vessels there was alkaline phosphatase activity in the adventitial coat of the vessel. This activity appeared to be continuous with the alkaline phosphatase activity of the pia itself. No alkaline phosphatase activity was seen in the adventitia of veins of similar calibre.

Superficial sections cut in order to demonstrate the pial vessels over the surface of the brain showed that many of the primary branches some 15 to 20 μ in diameter, arising from pial arteries 20 to 40 μ in diameter, had intense alkaline phosphatase staining at their point of origin which decreased gradually distally as shown in Figure 1. Such vessels usually arose at right angles to the parent vessel. In some instances, the staining appeared to extend for a short distance along the wall of the parent vessel from the point of emergence of the branch. On some occasions the luminal diameter of a branch staining intensely for alkaline phosphatase was less at its origin than more distally (Fig. 1) and the muscular wall appeared thicker in the proximal region. Staining was rarely found in the walls of Y-shaped bifurcations even when the side branches originating proximally showed intense staining. Arteries less than approximately 15 μ in luminal diameter also stained abruptly and intensely at their point of origin but in these vessels the staining continued throughout their course. Usually such branches could be followed until they plunged into the brain substance. Using the modified azo dye technique, alkaline phosphatase staining at the point of origin of branches was observed to start earlier during incubation than that of the remainder of the vessel.

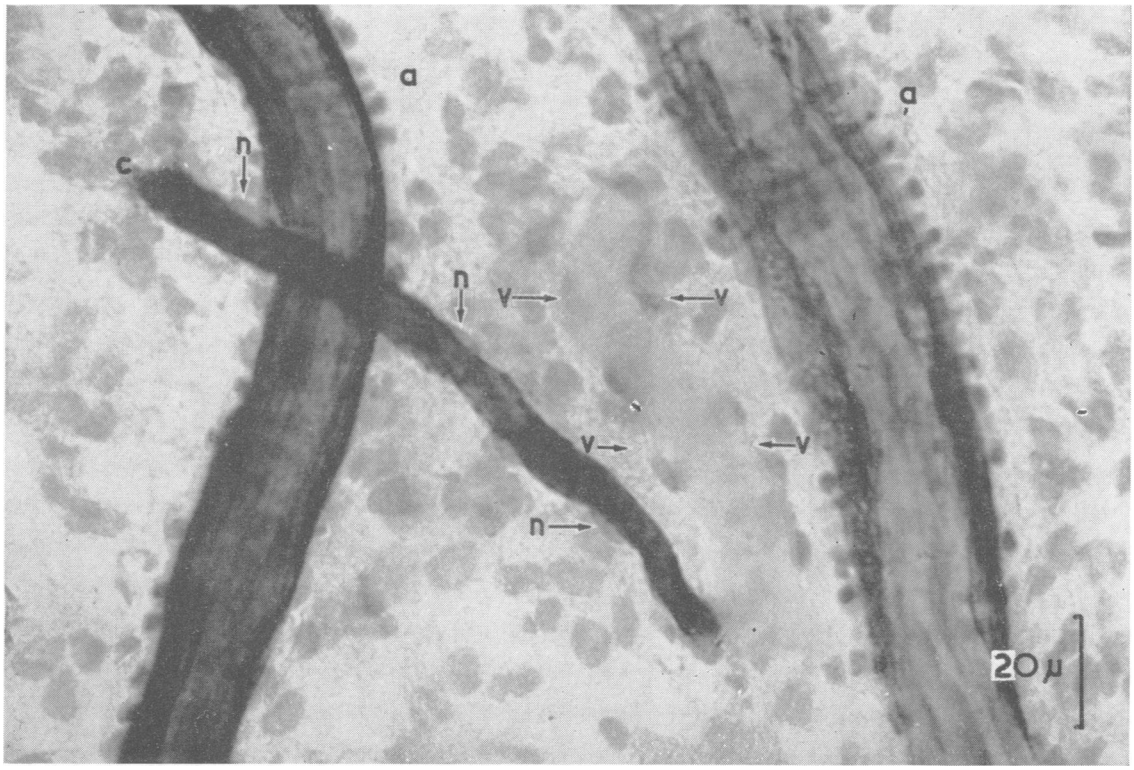

FIG. 2. *Cross section of brain* (32 μ) *showing vessels penetrating the inferior surface of the hippocampus. Animal perfused with saline, injected with carmine gelatine, and sections stained for alkaline phosphatase and counterstained with Mayer's carmalum. The circular muscle layer of the two small ateries* (*a, a*) *can be seen outside the darkly staining endothelium. A capillary* (*c*) *crosses the artery on the left and the nuclei* (*n*) *of its endothelial cells can be seen outside the alkaline phosphatase staining. A small vein* (*v*) *with its lumen filled with carmine gelatine, marked by arrows, lies parallel to the artery on the right, between the artery and the capillary. There is no alkaline phosphatase staining of the vein.*

Sections were incubated for only 10 minutes since with longer incubation the staining of the vessels was obscured by staining of the pia.

In the substance of the brain, in cortical grey matter, white matter, and the basal ganglia, arteries 20 to 30 μ in luminal diameter showed little or no alkaline phosphatase activity. When alkaline phosphatase staining was found in such arteries (Fig. 2), it was most marked in the perinuclear cytoplasm of endothelial cells. The alkaline phosphatase activity appeared to be confined to the endothelial cell cytoplasm, though it was not possible to exclude additional staining of the basement membrane and of any substance adhering to the luminal surface of the endothelial cell. The nuclei of smooth muscle cells were clearly visible outside the alkaline phosphatase staining (Fig. 2). Veins of similar calibre showed no alkaline phosphatase activity (Fig. 2). In general, branches 8 to 15 μ in diameter arising from larger arteries in the substance of the brain were found to stain suddenly at their point of origin, the staining then continuing as far as the capillary network (Fig. 3). In some instances the parent vessel was also stained lightly.

The alkaline phosphatase activity in the vascular tree was followed from artery to vein in many cross sections of brain of 60 μ thickness and compared in the grey and white matter of various regions, with special attention to the basal ganglia. Small arteries identified histologically as arteries on adjacent sections by the large, elongated endothelial cell nuclei and narrow transversely placed smooth muscle fibre nuclei, were followed as they branched into progressively smaller vessels. The smallest vessels of the capillary network were less than 5 μ in luminal diameter and their endothelial cell nuclei were clearly seen outside alkaline phosphatase staining (Fig. 2). These vessels then joined larger vessels

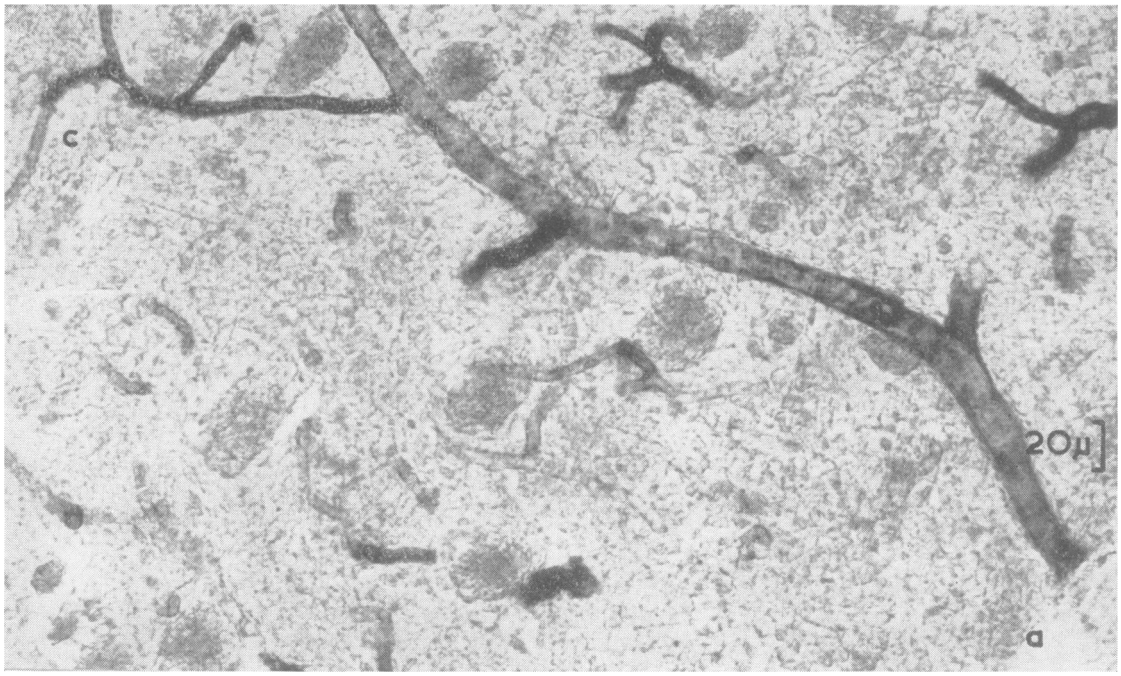

FIG. 3. *Cross section of brain (60 μ) showing an artery (a) in the caudate nucleus with a branch extending to the white matter of the corpus callosum. Animal perfused with saline, injected with carmine gelatine, and sections stained for alkaline phosphatase. The fading of the alkaline phosphatase activity is seen as the capillary (c) enters the white matter. The apparent background staining is due to the high refringence.*

which in turn joined vessels which could be identified as veins on histological grounds by the rounded appearance of their endothelial cell nuclei and by the absence of the regular pattern of muscle of the arterial vessels of corresponding size. On all occasions when such vascular networks were traced a gradual decrease in the alkaline phosphatase activity of the vessel was found towards the venous end of each vascular network (Fig. 4). The lengths of the small vessels staining and the intensity of staining were both greater with longer incubation periods in the medium. Also, in all small vessels the intensity of staining was slightly less in the part more deeply placed in the section, due presumably to the effect of lack of penetration of the substrate. However, with different methods of fixation and staining a gradual fading of alkaline phosphatase activity invariably occurred before the vessel increased in calibre before joining a vein. No alkaline phosphatase activity was found in the walls of veins. The gradual fading of activity at the venous end of the capillary network was in marked contrast to the appearance of alkaline phosphatase activity on the arterial side of the vascular tree which was commonly found to begin abruptly.

There is no unanimity about the criteria for distinguishing histologically between arterioles, venules, and capillaries (Majno, Palade, and Schoefl, 1961) but since the distinction cannot be made on grounds of size alone a study was made of the structure of the vessel walls and an attempt made to correlate this with the alkaline phosphatase activity. On the arterial side of the vascular tree the most intense alkaline phosphatase activity occurred in branches 8 to 15 μ in diameter, arising from arteries 20 to 30 μ in diameter which had an organized layer of circular muscle fibres in their walls. These branches had smooth muscle nuclei in their walls at the point of origin but then progressively fewer nuclei at progressively longer intervals could be definitely identified as smooth muscle nuclei. This pattern occurred in arteries of grey matter, white matter, and basal ganglia, despite the differing arrangement of the vascular tree in these three regions. From observations made in the course of the study, no consistent differences in the appearances of the endothelial cells and perivascular cells were found that made distinction between arteriole, capillary, and venule easy in the case of any particular cerebral vessel less than 6 to 8 μ in diameter. In general, as

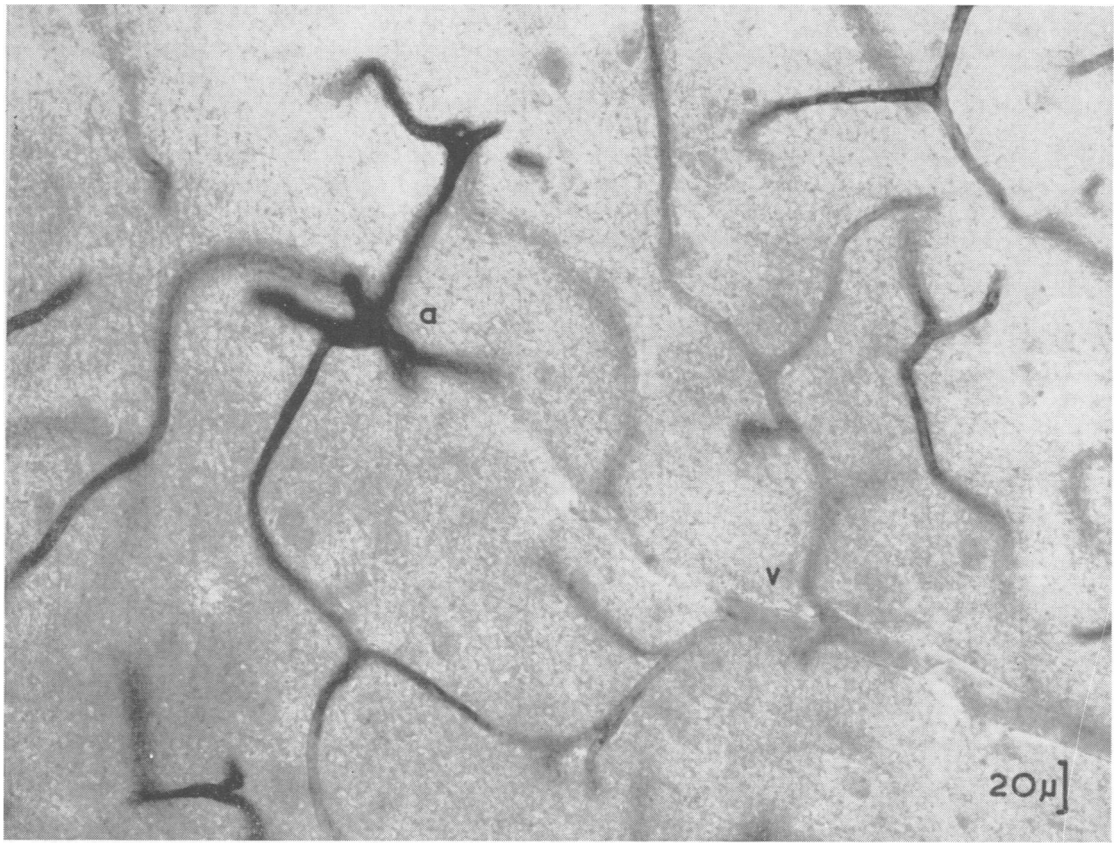

FIG. 4. *Cross section of brain (60 μ) from the interior frontal region, lateral to the lateral olfactory tract showing the capillary network between a small arteriole (a) and venule (v). Animal perfused with saline, injected with carmine gelatine, and sections stained for alkaline phosphatase. The venule (v) shows no alkaline phosphatase staining, the apparent grey colour being due to the carmine gelatine cast of the vessel.*

has been found in the vascular tree of other organs, the endothelial cell nuclei of vessels on the arterial side tended to be larger, more elongated in the direction of the vessel, and more closely spaced. Nuclei winding around the vessel or astride its junctions, with the appearance of the nuclei of muscle cells, were more frequent in vessels on the arterial side than in vessels of the same size on the venous side of the capillary network. Alkaline phosphatase activity was found to be strikingly different in small vessels of similar calibre and structure and its presence or absence was seemingly only related to the proximity of the vessel to the arterial or venous side of the vascular tree.

Counts of the number of small vessels with alkaline phosphatase activity were made in various regions of the grey matter, white matter, and basal ganglia. The extent and intensity of the alkaline phosphatase activity of the vessels in the grey matter and basal ganglia were found to be similar despite the different patterns of branching in these regions. However, the intensity and extent of alkaline phosphatase activity was less in the capillaries of the white matter. On several occasions sudden cessation of staining was seen as a capillary passed from the surface of the section, and for this reason it was concluded that the reduction of alkaline phosphatase activity of vessels in the white matter was probably due more to reduction of penetration of the substrate into the white matter than to any metabolic difference between capillaries of white matter and grey matter.

Greater alkaline phosphatase activity was found in the vessels of the hypothalamic nuclei, as has already been described (Eränkö, 1951; Leduc and Wislocki, 1952), though in these regions the distribution of the

activity in the vascular tree was similar to that found elsewhere in the brain.

The extent and intensity of the alkaline phosphatase activity of the vessels of the grey and white matter of the spinal cord was similar to that of vessels in the grey and white matter of the brain, respectively.

No difference was found in the alkaline phosphatase activity of blood vessels in brain previously perfused with sodium nitrite solution when compared with those perfused only with saline. Sections from brains of animals not perfused with sodium nitrite or saline showed alkaline phosphatase activity similar to that of perfused animals except that there was more staining of the walls of arteries 15 to 30 μ in calibre, so that the more intense staining of branches of these arteries, though present, was by contrast less marked. There was no alkaline phosphatase activity found on the intimal surface of veins or large arteries even when red cells could be seen lying in the lumen of the vessel.

Thick sections from brains of animals not perfused showed, with Pickworth's stain, apparent continuous filling with blood of the arteries and veins of the cerebral vascular tree and segmentation of the red cells into clumps of various sizes in vessels less than 8 μ in calibre approximately. The sites of branching of arteries showed no difference in distribution of red cells by comparison with the remainder of the vascular tree. No staining with Pickworth's stain was found in sections from perfused brains.

A similar intensity and distribution of alkaline phosphatase activity was found with the Gomori and the modified azo dye technique. There was less background staining and better localization with the Gomori technique, and results which have been described were with the Gomori technique unless specifically stated.

DISCUSSION

Alkaline phosphatase staining of the endothelium of capillaries and the adventitia of arteries of medium size in various organs and in different species was first reported by Gomori (1939) and independently by Takamatsu (1939). Gomori (1941) described staining for alkaline phosphatase in the endothelium of the capillaries of the nervous system of the rat and guinea-pig and 'less regularly' in the dog. Landow, Kabat, and Newman (1942) made a special study of the alkaline phosphatase activity of the nervous system of chicken, mouse, cat, and man, and concluded that vessels of all sizes except large arteries showed staining of the endothelium, though in their paper there was no indication that they were able to identify endothelial staining of veins. Alkaline phosphatase staining was found by Shimizu (1950) to be variable in the brain capillaries of different species and he was unable to find any staining of the brain capillaries in the rabbit. Leduc and Wislocki (1952) found intense staining only in arterioles of the rat brain, the vessels being identified as arterioles by their method of branching. More recently Bourne (1958) and Becker, Goldfischer, Shin, and Novikoff (1960) have confirmed the alkaline phosphatase activity of the endothelium of capillaries of rat brain. One factor which may have contributed to the variations in the extent and intensity of capillary staining noted is the use of various modifications of the Gomori technique by different authors. However, other techniques of staining for alkaline phosphatase (Maengwyn-Davies and Friedenwald, 1950; Burstone, 1958, 1961), though not used with special reference to the nervous system, have shown in general a similar distribution of this group of enzymes to that obtained by the Gomori technique. A second factor which may have contributed to the apparent variability of results is the lack of agreement over the definition of the terms 'arteriole', 'capillary', and 'venule' (Majno *et al.*, 1961).

Thus there have been many reports of the alkaline phosphatase activity of cerebral vessels but reports have not suggested a consistently greater activity in the endothelium of particular regions of the cerebral vascular tree. This study provides evidence that such differences in activity do exist. Alkaline phosphatase activity was found to be intense in the endothelium of small arteries at their origin from larger arteries. In pial arteries the activity was frequently confined to this region of the vessel whereas in the substance of the brain the activity then continued into the capillary network. This finding was strikingly similar, though less frequently encountered with such prominence as in blood vessels elsewhere, in particular those of muscle (Romanul and Bannister, 1962). It was at first thought that the localization of alkaline phosphatase activity to the endothelium of small arteries at their junctions with larger arteries might be artifactual, due either to enzymatic activity of blood retained at these points or to differential penetration of the substrate or due to physical or chemical alterations in the vessel occurring at or after death. Consideration of the results of perfusion experiments (Romanul and Bannister, 1962) led to the conclusion that greater enzymatic activity probably occurs at the junctional region in intact vessels *in vivo*.

In the present study similar perfusion experiments were undertaken with similar results. There was more intense alkaline phosphatase activity in the walls of unperfused small cerebral arteries than in

perfused arteries, which suggested either that there was greater diffusion of phosphatase from small arteries during perfusion or that some of the activity found here may have been due to retained blood. However, perfusion did not appear to reduce significantly the intensity of the staining of the proximal region of arterial branches. Moreover, though some plasma may have remained in the vessels, the Pickworth stain failed to show any red cells in the sections from perfused brains. Since studies of the circulation of other organs have shown that intravenously injected carbon particles adhere to the capillary wall at the venous end of the capillary (Chambers and Zweifach, 1940), which is also more permeable to dyes (Rous, Gilding, and Smith, 1930), there is no reason to believe that plasma is selectively retained at arterial junctions. It seems likely therefore that, as in skeletal muscle, the alkaline phosphatase activity found at arterial junctions is not due to retention of plasma in the vessel. On some occasions a reduction in luminal diameter of the proximal part of small cerebral arteries was found which corresponded to the regions of intense endothelial alkaline phosphatase activity. It might be argued that, if this reduction in diameter was due to constriction of the vessel at the time of death, it might have caused an apparent local increase in the intensity of alkaline phosphatase activity which would have appeared more uniform in the absence of such constriction. However, the inconsistency of the finding of constriction in relation to intense alkaline phosphatase activity made it unlikely that this was more than a partial explanation of the localization of alkaline phosphatase activity in the endothelium of small arteries at their origin from larger vessels.

Though the functional significance of this observation is not known, some evidence of specialized function of the proximal region of small arteries at their junction with larger arteries may be deduced from anatomical and physiological observations. In the present study it was found that the circular muscle coat often appeared thicker, sometimes with relative reduction of the luminal diameter of the vessel, in the region where intense alkaline phosphatase activity occurred. It is of interest that in the blood vessels of some other parts of the circulation, the proximal regions of the smallest arteriolar branches, at the point where smooth muscle round the endothelium ends, have been described as 'precapillary sphincters' because of their observed contractile properties (Zweifach, 1950). However, electron microscopic studies of small cerebral arteries (Maynard, Schultz, and Pease, 1957; Bennett, Luft, and Hampton, 1959) as well as studies on the vessels of other tissues (Moore and Ruska, 1957; Fawcett, 1959) have so far failed to demonstrate any distinctive morphological features of the endothelial cells of the proximal region of small arteries.

Though the main regulation of cerebral blood flow is likely to be through the effect of cerebral tissue activity on flow in small vessels (Roy and Sherrington, 1890; Cobb and Talbott, 1927; Schmidt and Hendrix, 1938; Sokoloff and Kety, 1960), some direct observations of the pial vessels support the view that the proximal region of arteries at their junction with larger arteries may have special contractile properties. Florey (1925), who studied directly the pial vessels of the anaesthetized cat, noted that some but not all arterial branches arising from a large artery presented at their origin an annular constriction. He suggested that the constriction was of the nature of a sphincter but commented that this region did not exhibit an increased irritability to any form of stimulus. Using a skull window to observe the pial vessels in anaesthetized cats, Forbes (1928) observed cessation of flow on two occasions in small anastomotic arterioles but was impressed that pial vessels showed none of the periodic opening and closing of small vessels that has been observed in other parts of the circulation, for example, in the ear vessels of the rabbit (Clark and Clark, 1932).

There are a few direct observations of the responses of pial vessels to humoral, chemical, and nervous stimuli, though most recent studies on the effect of these stimuli have been directed to measurement of changes of cerebral blood flow rather than alterations in the lumen of pial vessels. Early observations (Florey, 1925) failed to show any effect of intravenous epinephrine on pial vessels of the cat, but Forbes, Finley, and Nason (1933) have shown that epinephrine does have a weak vasoconstrictive action, though as with other humoral factors causing alterations in the calibre of pial vessels (Forbes and Wolff, 1928; Forbes, Wolff, and Cobb, 1929), the precise sites of the change in calibre have not been described. It is of interest that Sandison (1932) observed that the constriction of arterioles in the rabbit's ear after an intravenous injection of adrenalin was more marked at the point where they took origin from the parent artery. Wolff and Lennox (1930) studied in anaesthetized cats the effect on pial vessels of variations in the oxygen and carbon dioxide content of the blood. Hyperventilation was followed by a constriction of some arteries approximately 200 μ in diameter at their origin from larger arteries. They demonstrated that dilatation of these regions occurred after hypoventilation, when the blood pCO_2 was increased to the normal range. It seems likely that such alterations in the calibre of these arteries are the result of changes in the chemical content of incoming

blood, in contrast to alterations in the calibre of capillaries which result from changes in the activity of the surrounding brain.

The function of alkaline phosphatase in the endothelium of blood vessels is unknown but the presence of this group of enzymes in intestine and kidney epithelium at sites of active transport has bed to the suggestion (Landow *et al.*, 1942) that in the vascular endothelium of cerebral capillaries too it may be associated with active transport. Active transport at the proximal region of small arteries would be unlikely to serve the purpose of supply to the surrounding tissues. The direct observations of the alterations in calibre of pial arteries at their origin from larger vessels in response to chemical agents raise the possibility that active transport at these sites might be related to some system of continuous sampling of the blood for the purpose of regulating the lumen of the artery. However, since within the substance of the brain alkaline phosphatase activity of the endothelium is usually continuous from the origin of a small artery to the capillary, it is difficult to postulate different functions for alkaline phosphatase in these two regions, though the structure and function of these two regions of the vessel clearly differ. In conclusion, the purpose of this communication is to describe a consistent localization in the pattern of alkaline phosphatase activity of the endothelium of the cerebral vascular tree, though for the present one can do no more than speculate about its possible functional significance.

SUMMARY

The alkaline phosphatase activity of blood vessels was studied in sections of fresh frozen rat brain and spinal cord which were stained for alkaline phosphatase by the Gomori method and on some occasions by means of a modified azo dye technique. Within the vascular tree the alkaline phosphatase activity of the endothelium was not uniform. Intense activity was in general found in the endothelium of the proximal part of small cerebral arteries less than 20 μ in diameter, at their point of origin from larger vessels. In pial arteries this was usually found to decrease distally whereas in the substance of the brain this activity continued as far as the capillary network. In the capillary network intense endothelial activity was found at the arterial end of the capillary but it decreased gradually towards the venous end. The possible functional significance of these differences in endothelial alkaline phosphatase activity in different parts of the cerebral vascular tree is discussed.

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