

LOCALIZED AREAS OF HIGH ALKALINE PHOSPHATASE ACTIVITY IN THE TERMINAL ARTERIAL TREE

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ABSTRACT

Fresh frozen skeletal muscles of rats, rabbits, and humans were sectioned in a cryostat. Sections 12 to 32 micra thick were incubated in a substrate solution for the histochemical demonstration of non-specific alkaline phosphatase activity. A modified azo dye coupling technique was used at pH 9.5. Localized areas of high enzymatic activity were found in specific and well defined areas along the terminal arterial tree, in addition to the activity which has been previously described in capillary endothelium. Arterial branches with luminal diameters of 25 micra or less showed staining of their endothelium starting abruptly at their origin from the parent vessel and fading distally. Smaller arterial branches showed the same localization of enzymatic activity and stained more intensely. Other organs of rats surveyed showed arterial branches with the same pattern of staining. Identical results were obtained using the Gomori technique for alkaline phosphatase. Extensive saline perfusion of the vascular tree did not affect the observed localization of enzymatic activity. The enzymatic activity described may be part of the mechanism regulating the blood flow.

INTRODUCTION

The presence of non-specific alkaline phosphatase activity in the walls of blood vessels was discovered by Gomori (1, 2) and by Takamatsu (3), who independently developed the first histochemical technique for the demonstration of this group of enzymes. They found activity localized to the endothelium of capillaries, the endothelium of some small blood vessels, and the adventitia of medium sized arteries. Subsequent histochemical studies by other investigators using the same technique with or without modifications (4-7), or using other techniques developed later (8-11), failed to demonstrate any other localized areas of alkaline phosphatase activity in the vascular tree. In these studies a variability of enzymatic activity of blood

vessels was noted in various organs of the same animal or in the same organ in different species of animals.

Quantitative chemical studies of non-specific alkaline phosphatase have been limited to large blood vessels (12-18). The activity of this group of enzymes in such vessels was found to be very low and certainly disproportionate to that of other phosphatases (13-23).

In the course of a histochemical study of enzymatic activity of skeletal muscle fibers, we observed localized areas of high alkaline phosphatase activity at specific and well defined sites along the arterial tree. Since up to the present time there has been no histochemical (24) or quantitative

(25, 26) evidence of any chemically specialized areas along the wall of the arterial tree, our preliminary observations were studied in detail.

MATERIALS AND METHODS

The histochemical studies were carried out on fresh rat and rabbit tissues, as well as on human muscle biopsies. Albino rats weighing 250 to 350 gm and albino rabbits weighing 2 to 3 kg were used. The animals were given ether or Nembutal anesthesia and the tissues were removed while the animals were under deep anesthesia or immediately after the cessation of respiration. The gastrocnemius, plantaris, soleus, and diaphragm were used for most studies. Heart, skin, and spinal cord were surveyed on a few occasions only. These tissues were immediately frozen by being placed on dry ice. They were then mounted on ice, and sections 12 to 32 micra thick were cut in a cryostat. Most frequently the sections were floated directly on the substrate solutions. At times, they were placed on slides, dried in the air for 30 to 60 minutes, and then placed in substrate solutions.

The mesentery, the membranous portion of the diaphragm, and the cremaster muscle were also studied fresh without freezing immediately after their removal from the body. These tissues were gently stretched over a rubber ring, fixed to the ring with pins, and subsequently submerged in substrate solutions.

Human iliopsoas and temporalis muscle biopsies were frozen on dry ice within 5 minutes of their removal from the body. They were mounted on ice and sectioned in the cryostat like the animal tissues.

A modified coupling azo dye technique (27) using alpha naphthyl phosphate and 4-amino-2,5-diethoxybenzaniline (fast blue BBN) in tris buffer at pH 9.5 was used for most experiments. The Gomori technique (1) using beta glycerophosphate at pH 9.0 was used on only a few occasions, to compare the results with those obtained with the azo dye technique. After incubation, the sections were fixed in 10 per cent neutral formalin for 24 hours, counterstained in Mayer's carmalum, and mounted in 50 per cent polyvinyl pyrrolidone.

Three series of perfusion experiments were carried out. The procedure in one series consisted simply of washing blood from the vascular tree as thoroughly as possible. The rat was placed under Nembutal anesthesia, the chest was opened, and, while the heart was still beating, a cannula made from a 15 gauge needle was introduced through the left ventricle into the ascending aorta. A small opening was immediately made into the right atrium and the perfusion was started through the cannula. After introducing 500 ml of warm 0.2 per cent sodium nitrite in normal saline, 900 ml of normal saline were

perfused at a pressure of 1.5 meters of water. As soon as the perfusion was completed, tissue blocks were removed, frozen fresh, and sectioned in the cryostat, and the sections were incubated for alkaline phosphatase.

In another series of perfusion experiments performed in rats the same procedure was used as the one just described, followed by injection with a syringe through the cannula of warm Ranvier's carmine-gelatin (28). A loop of small intestine and mesentery was externalized through an opening made in the abdominal wall. The plunger of the syringe containing the carmine-gelatin was pressed manually while the mesentery and the intestine were being observed. Enough carmine-gelatin was injected to fill the arterial tree as completely as possible without filling the capillaries or veins. Tissues were removed after a waiting period of 5 to 10 minutes, frozen fresh, sectioned in the cryostat, and incubated for alkaline phosphatase.

In the third series of perfusion experiments the vascular tree was washed of blood as outlined in the first series of perfusion experiments. After introducing 500 ml of 0.2 per cent sodium nitrite in normal saline and 1000 ml of normal saline, 900 ml of filtered azo dye alkaline phosphatase incubating medium at pH 9.5 were perfused. After 1 hour, the vascular tree was rinsed with 300 ml of normal saline. Tissues were then removed, frozen fresh, and sectioned in the cryostat, and the sections were floated on buffered formalin.

RESULTS

Serial sections of skeletal muscles of rat incubated in alpha naphthyl phosphate substrate solution permitted a detailed study of the enzymatic activity of blood vessels. The large arteries showed no staining in their walls. Primary branches of such arteries, however, with luminal diameters of 25 micra or less, displayed moderately intense staining in the endothelium starting abruptly at their point of origin from the larger vessel and gradually decreasing distally (Figs. 1, 2, and 3). Secondary branches originating from an unstained part of the primary branch showed the same pattern of enzymatic activity of endothelium starting abruptly at their point of origin and fading somewhat distally (Fig. 1). In these branches, however, the staining was more intense, and extended throughout the length of the vessel. Capillaries originating from these branches stained with the same intensity as the distal portion of such a secondary branch. The capillaries had rather uniform enzymatic activity even at their points of

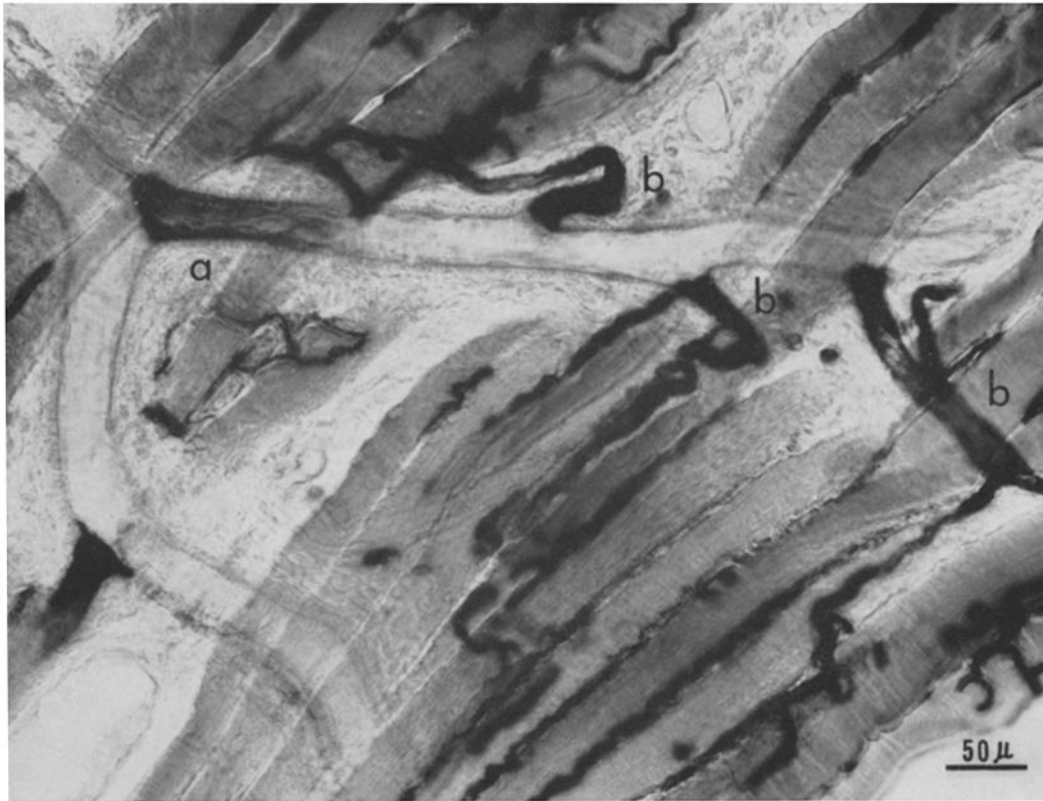


FIGURE 1

Arteries in rat soleus incubated in alpha naphthyl phosphate substrate solution for alkaline phosphatase. An artery with a lumen diameter of 45 micra is unstained and is visible only by its high refringence. Its primary branch (*a*) stains abruptly at its origin and the staining fades gradually distally. The secondary branches (*b*) also stain abruptly at their origin. Their staining continues throughout their length but decreases to some extent distally. Capillaries are seen running parallel to muscle fibers. $\times 220$.

branching. There was slight decrease in their intensity of staining near the venous end.

The staining of the proximal portion of small arterial and arteriolar branches was more intense than that of the capillaries. When the floating tissue sections were observed under the microscope during incubation, the staining was first seen to appear at these sites of branching and only later in capillaries.

No enzymatic activity was demonstrated at Y-shaped bifurcations of arteries, even when side branches originating proximally showed intense staining at their origin. The small collecting venous channels and the venules and veins, including their junctions, as well as the lymphatics, were entirely free of stain.

The study of many arterial branchings sectioned longitudinally and across permitted clear localization of the enzymatic activity to the endothelium, the inactive muscular layer of the vessel wall being distinctly visible around it (Figs. 2 and 3). In some proximal portions of larger arterial branches, where the staining was not excessive, a more detailed localization of enzymatic activity was possible. The nuclei of the endothelial cells were unstained. The cytoplasm around these nuclei stained intensely. The cytoplasm at greater distance from the nuclei, as well as the intercellular substance, was free of stain (Fig. 2).

The staining of the endothelium of the arterial branch started abruptly inside the parent vessel at the point of junction of the endothelium of the

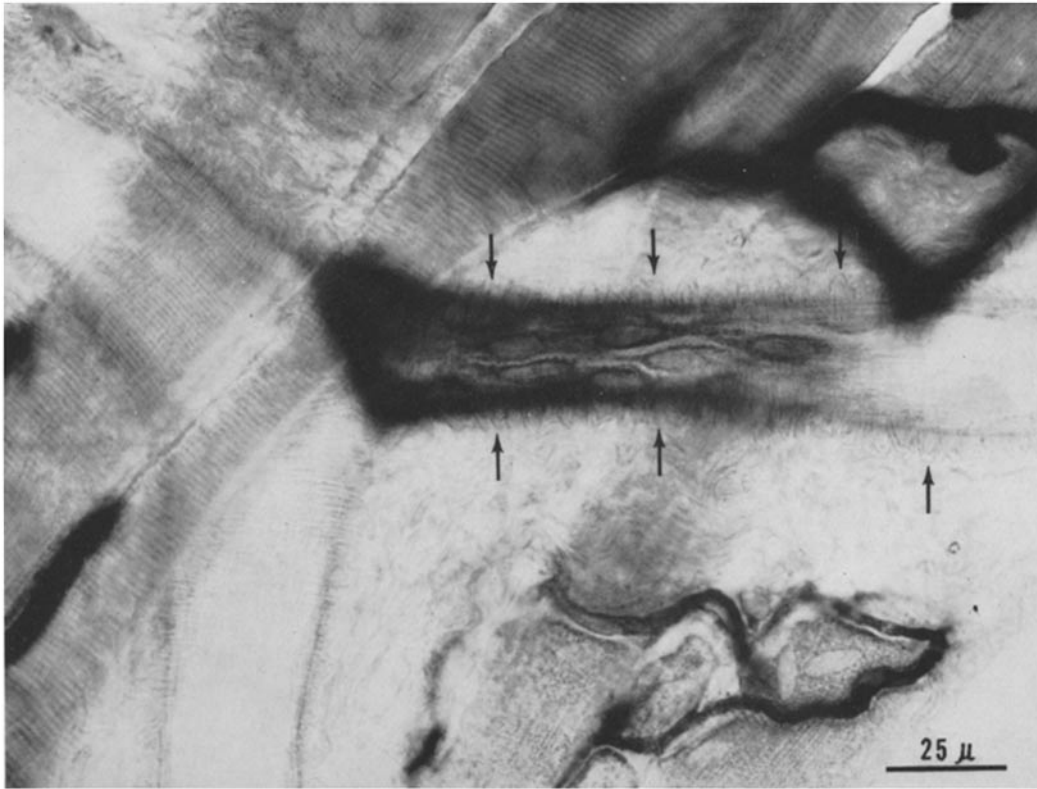


FIGURE 2

Higher magnification of the primary arterial branch *a* from Fig. 1. The staining of the wall is clearly in the endothelial cells. The cytoplasm of the endothelial cells is outlined by the stain while the junctions between the endothelial cells, as well as their nuclei, are free of stain. The circular muscular layer is faintly visible by its high refringence (arrows). The entire stained proximal portion of the branch is somewhat constricted. $\times 640$.

parent vessel and that of the branch (Figs. 1 to 6). The stained endothelium of the branch could be seen therefore coursing through the entire thickness of the wall of the parent vessel (Figs. 5 and 6). At some arterial branching sites the endothelial cells of the branch were seen to project into the lumen of the parent vessel (Fig. 6). Sometimes at the point of origin of a small arteriole, the stained most proximal endothelial cells were bulging inside the lumen of the arteriole, producing a moderate to marked narrowing of its lumen. The entire stained proximal portion of an arterial branch was at times slightly dilated, but more often somewhat constricted (Fig. 2).

The pattern of enzymatic activity described above was found in the arterial tree of all the rat, rabbit, and human skeletal muscle studies. In

addition to muscle, several other tissues in the rat were surveyed, namely, heart, mesentery, skin, and spinal cord. Arterial junctions with the described distribution of staining were seen in all these tissues and could be studied particularly well in the mesentery. The results obtained with the azo dye technique and with the Gomori technique (Fig. 5) were identical. A detailed account of the alkaline phosphatase activity of the central nervous system will be the object of a separate communication (29).

Extensive washing of the blood from the vascular tree by perfusion with saline did not modify the pattern or intensity of enzymatic activity of the blood vessels (Fig. 6). The study of tissue sections of animals in which carmine-gelatin was injected into the arterial tree, after extensive washing of

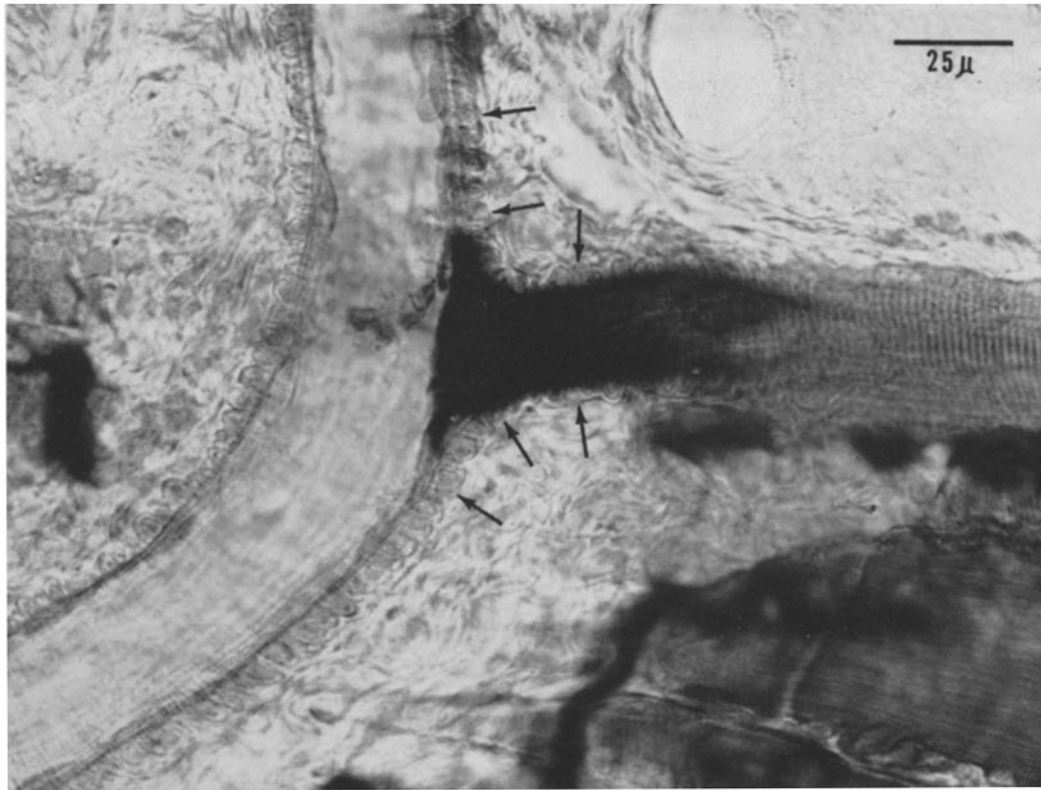


FIGURE 3

Artery in rat soleus incubated in alpha naphthyl phosphate substrate solution for alkaline phosphatase. The primary branch of the artery stains abruptly at its origin. The unstained muscular layer of the branch is clearly visible by its high refringence (arrows). $\times 640$.

blood by saline perfusion, showed again the above described distribution of enzymatic activity in the arterial and capillary bed (Fig. 4). Sections of tissue from these animals in successfully performed experiments permitted easy and certain identification of the arteries since the carmine-gelatin had not reached the lumen of the capillaries or veins. The staining of the proximal portions of blood vessel branches was confirmed to be present only in arteries. The good filling of the lumen of arteries with carmine-gelatin at such branching sites excluded the possibility that blood was retained in the lumen of the arteries. Tissue sections from animals perfused with alpha naphthyl phosphate substrate solution for alkaline phosphatase, after the vascular tree was washed of blood, showed the same localization of enzymatic activity in the vascular tree as that obtained by the incubation of tissue sections.

DISCUSSION

When the staining of the proximal portion of small arterial branches was first seen, several questions arose with respect to the validity of this histochemical localization of enzymatic activity. The subsequent experimental procedures were designed so as to make possible a critical evaluation of this finding.

The first question which posed itself was whether the enzyme is actually located in the endothelium of the arterial branch or whether the endothelium stained as a result of enzymatic activity of blood caught in the lumen of the artery at such points. The results presented here indicate that the enzyme is localized in the endothelium. This was demonstrated by identical staining with unchanged intensity in longitudinal and cross-sections of arterial branchings incubated by floating, where

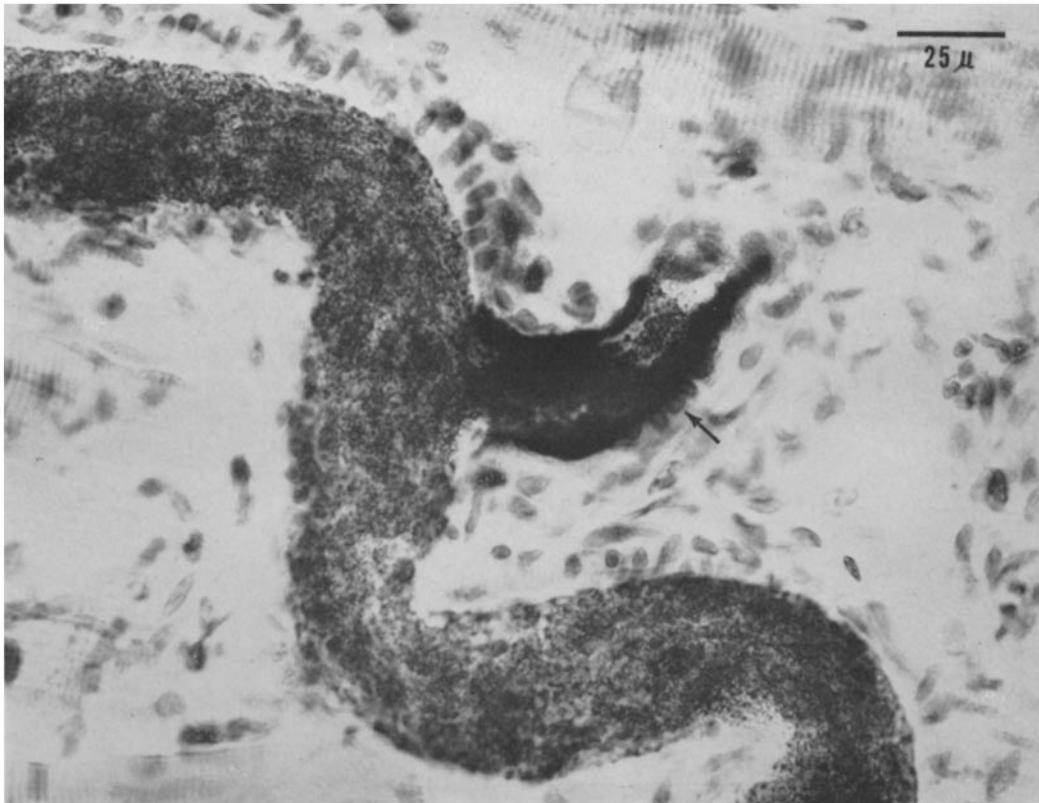


FIGURE 4

Artery in rat soleus incubated in alpha naphthyl phosphate substrate solution for alkaline phosphatase. Prior to the removal of the muscle, the vascular tree was washed by perfusion with saline and the arterial tree was injected with Ranvier's carmine-gelatin. After incubation for alkaline phosphatase, the section was counterstained with Mayer's carmalum. The artery and its branch are filled with carmine-gelatin. The endothelium of the proximal portion of the arterial branch stains intensely for alkaline phosphatase. The muscular layer of the branch shows no enzymatic activity. The nuclei of the muscular layer of the branch are shown by counterstain (arrow). $\times 580$.

no blood was retained in the lumen. It was also shown in tissue sections of animals with washed vascular tree with or without subsequent carmine-gelatin injection.

The second question that should be considered is whether this localization could be falsely produced by a differential penetration of substrate. In animals perfused with substrate solution, all the endothelium of the vascular system was exposed to substrate. In sections from non-perfused animals incubated by floating, the substrate could easily reach the endothelium of the vessels through their lumen. There is no doubt, therefore, that the localized enzymatic activity of the endothelium of the proximal portion of arterial branches cannot

be accounted for by differential penetration of substrate through tissue. Neither can it be attributed to greater permeability of these endothelial cells to the substrate solution. This was clearly demonstrated by the lack of staining of the endothelial cells which were cut through at the point where the parent vessels and the more distal portion of the arterial branches reached the surface of the section.

Thirdly, one should question whether the localization of enzymatic activity demonstrated in this communication is not secondary to physical and chemical alterations of the tissue produced by freezing. Such a possibility is excluded by the fact that the same localization was obtained in

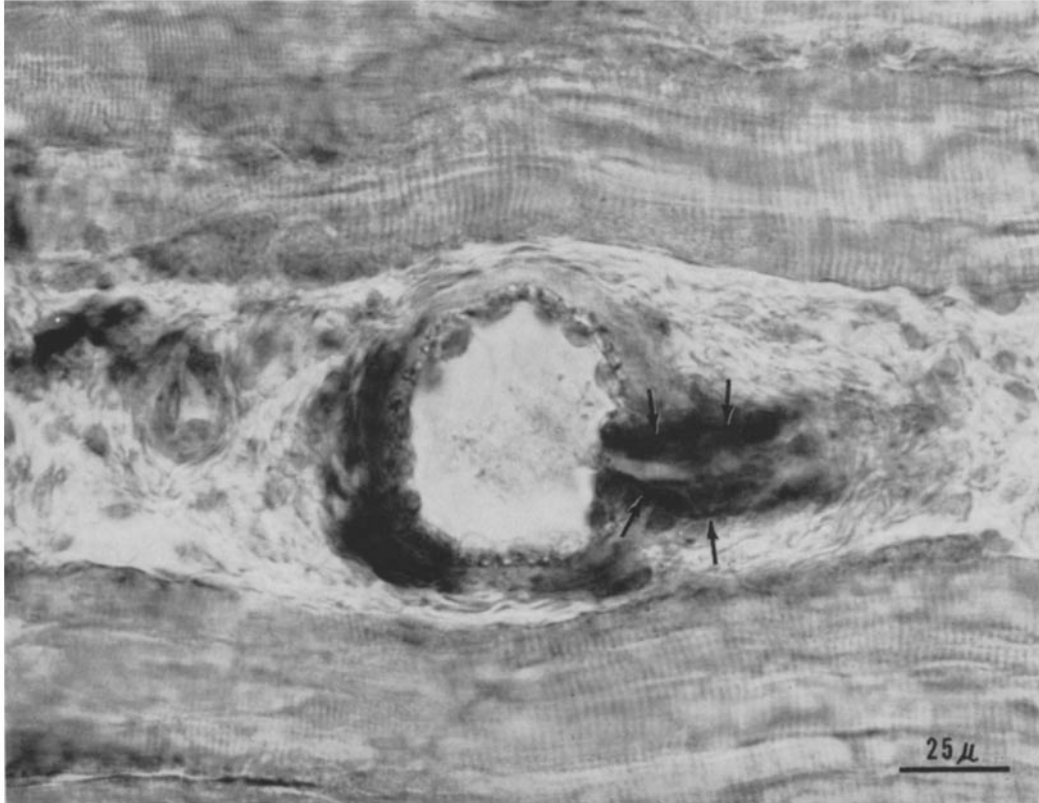


FIGURE 5

Small artery in rat soleus incubated for alkaline phosphatase according to Gomori's technique and subsequently counterstained with Mayer's carmalum. The small artery seen in cross-section shows only some staining in its adventitia. Its branch shows intense activity in the endothelium. The branch was sectioned somewhat obliquely and the resulting contour of its endothelial lining tends to be elliptical (arrows). $\times 580$.

animals with the vascular tree perfused by substrate solution. The localization of enzymatic activity cannot be ascribed to an artifact produced by the histochemical technique, since two entirely different techniques were used. It seems justified therefore to conclude that this localization of enzymatic activity in the vascular tree is present in intact fresh vessels and therefore *in vivo*.

The meaning of this localization of high alkaline phosphatase activity at specific sites in the vascular tree is not immediately obvious. This finding should be evaluated, therefore, in the light of available anatomical, physiological, and chemical information on blood vessels.

The endothelium of capillaries and that of arteries including their branching sites have not been found to have morphological differences by

light microscopy (24, 30, 31). In the electron microscope, with the exception of structural differences of specialized capillaries in some organs (32), the fine structure of the capillary endothelium (33-36) was not found to be different from that of arteries (34-37). Observations of blood vessels in living mammals have disclosed that the terminal arterial tree shows the greatest "spontaneous" change in caliber, called vasomotion (38-45), and the greatest response to vasoconstrictor agents such as adrenalin (41-49). These are the parts of the vascular tree which contain smooth muscle in their wall (40-45). The areas of high alkaline phosphatase activity of endothelium demonstrated in this study do not outline entirely the terminal arterial vascular bed containing smooth muscle in its wall, but only the proximal portions of these

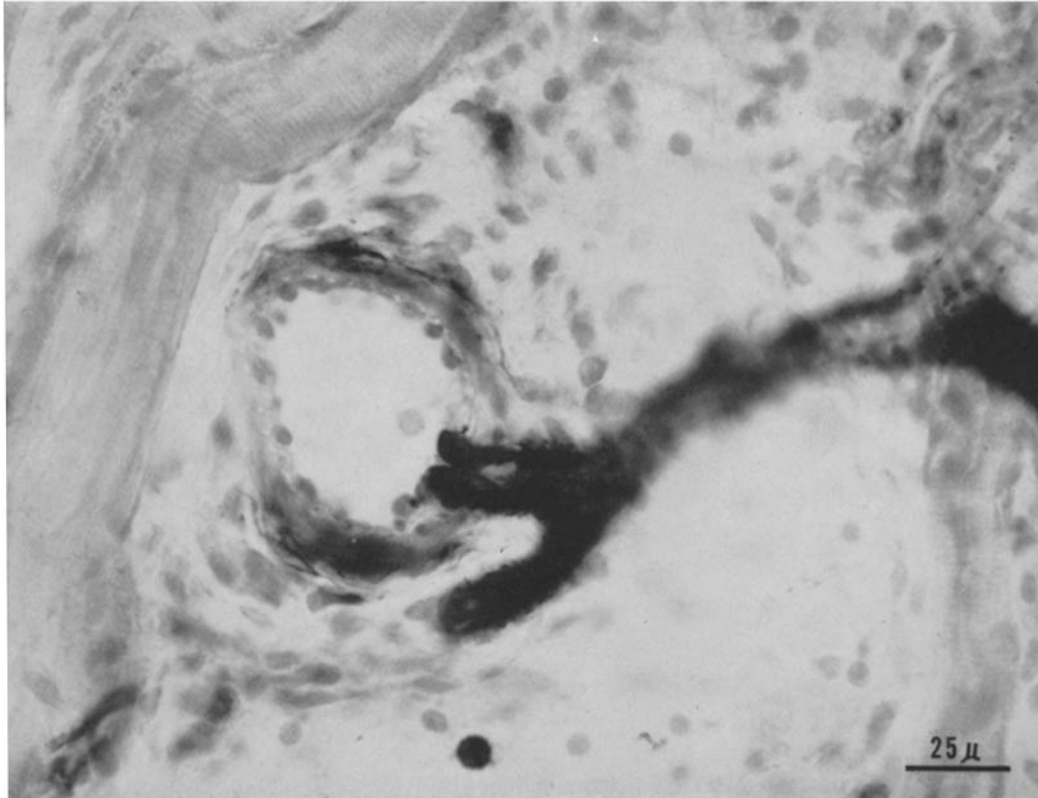


FIGURE 6

Small artery in rat soleus incubated in alpha naphthyl phosphate substrate solution for alkaline phosphatase after extensive washing of the vascular tree by perfusion with saline. The section is counter-stained with Mayer's carmalum. The staining of the small artery and its branch is essentially the same as shown with the Gomori technique (Fig. 5). $\times 560$.

vessels. In this respect the observation of Sandison (48) is of interest. He studied an artery 125 micra in diameter and its branches in a rabbit's ear after intravenous injection of adrenalin. He observed the large artery to constrict more in its proximal half and the arterioles to constrict most at the point where they took origin from the large artery.

The electron microscopic studies of endothelium of capillaries (32-36) have not solved the problems of capillary permeability (24, 36). The pores thought to exist in the endothelial membrane on physiological grounds (50-52) have not been found. In muscle capillaries, intravenously injected ferritin was found in endothelial cells both free in the cytoplasm and within vesicles (53), whereas intravenously injected colloidal gold was found only in membrane-bounded vesicles within the

endothelial cells (54). It is still not clear (24, 36) whether the endothelial cell (55) or the intercellular cement substance (56, 57) is responsible for the normal capillary permeability. Concerning the location of areas of highest permeability along the vascular tree, the small venules revealed the greatest permeability to intravenously injected dyes of various molecular sizes, followed by the venous end of the capillaries (58). Colloidal carbon particles injected into the blood stream have been found to adhere to and penetrate the "intercellular cement" between the endothelial cells at the venous end of the capillaries and in small venules (56, 57). The same distribution was found in the vascular tree after local injection of histamine, with much greater accumulation of carbon particles in the capillaries and what was interpreted to represent venules in the region of

histamine injection (59). The areas of high alkaline phosphatase activity in the proximal portion of the arterial branches reported in the present study outline the cytoplasm of the endothelial cells, and therefore give a negative image of the picture obtained by the carbon accumulation at the intercellular junctions of the branching of veins (56, 59, 60). After intravenous injection of colloidal carbon, heavy carbon accumulation was described in the endothelium of the lymphatic vessels in the mesentery (57). In the present study, no alkaline phosphatase activity was found in the endothelium of the lymphatic vessels in the mesentery.

Surveys of quantitative studies of various enzymes in blood vessel walls (25, 26) reveal that the overwhelming majority of studies were limited to the aorta or very large arteries and that no attempt was made to study various segments of small arteries or arterioles. Separate determinations on individual layers of blood vessel walls were seldom carried out. With respect to dephosphorylating enzymes, the large arteries were found to have very low alkaline phosphatase activity (12-18) and high acid phosphatase (13, 18), 5-nucleotidase (14-18, 23), and adenosinetriphosphatase (16-22) activities. Of these, only adenosinetriphosphatase was determined in veins and arteries of various sizes (18, 20, 22), the highest activity being found in muscular arteries (18, 20) or in the aorta (22). No activity was found in veins (22). These quantitative results are concordant with the histochemical demonstration of high acid phosphatase (61, 62), 5-nucleotidase (16, 63-69), and adenosinetriphosphatase (16, 67-70) activities in the media of arteries.

In view of the fact that the small arteries and arterioles are the segments of the vascular tree most responsive to epinephrine, it is of interest to consider the localization of monoamine oxidase in the vascular tree. This enzyme was studied quantitatively in veins and arteries of various sizes (71)

and its activity was found to be highest in the largest arteries, lower in smaller arteries, and lowest in veins. Histochemically, monoamine oxidase activity was seen to be localized in the media of blood vessels (72, 73). From the above review, it is apparent that monoamine oxidase and dephosphorylating enzymes other than alkaline phosphatase do not show any parallelism in the distribution of their activities along the vascular tree with that of non-specific alkaline phosphatase.

In summary, the histochemical localization of alkaline phosphatase in the endothelium of the proximal portion of arterial branches does not correspond to areas of the vascular tree with special morphologic differentiation or to areas of highest permeability. The enzyme activity is present in the small branches of the arterial tree which show the highest "spontaneous" contractions and the greatest response to hematogenous humoral agents. It is possible that the function of alkaline phosphatase at these sites in the vascular tree is that of active transport as in the proximal tubules of the kidney and in the mucosa of the small intestine. Active transport through the endothelium of the proximal portion of arterial branches could represent a basic mechanism whereby the blood vessel continuously samples the chemical content of the blood for the purpose of regulating the size of its lumen and therefore the blood flow. Such an interpretation, however, is entirely speculative.

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