THE DEMONSTRATION OF
ENZYMATIC ACTIVITY IN PINOCYTIC
VESICLES OF BLOOD CAPILLARIES
WITH THE ELECTRON MICROSCOPE

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ABSTRACT

In aldehyde-fixed myocardium, enzymatic activity to nucleotide substrates was localized to the pinocytic vesicles of blood capillaries with the electron microscope. No other structures of the endothelial cell showed activity under the conditions employed. These findings are discussed in relation to recent concepts of transport across the capillary wall.

A number of histochemical studies have established that the nucleotide substrates, adenosinetriphosphate (ATP), adenosinediphosphate (ADP), and adenosinemonophosphate (AMP) are hydrolyzed in the walls of the small blood vessels (1–5). These observations, made with the light microscope, demonstrate that enzymatic activity is present in or immediately adjacent to the endothelial cells.

In the present work the fine structural localizations of these enzymatic activities were studied in the small blood vessels of the rat myocardium. New techniques of fixation were used and these provided adequate preservation of tissue fine structure as well as retention of enzymatic activity. As a result of these experiments the sites of these activities were localized to pinocytic vesicles. A preliminary report of these results appeared elsewhere (6).

MATERIALS AND METHODS

Freshly excised rat hearts were cut into small pieces (1 to 2 mm³) and fixed in one of the following fixatives: 6.5 per cent glutaraldehyde (7) buffered to pH 7.4 with 0.1 M cacodylate, 12 per cent hydroxyadipaldehyde (7) similarly buffered, 4 per cent formalin

buffered to pH 7.5 with 0.06 M phosphate and containing 7.5 per cent sucrose (8), and 1 per cent buffered osmium tetroxide—containing sucrose (9). Fixation was carried out in the cold (4°C) for varying periods of time. Two-hour fixation gave adequate preservation of structure and enzymatic activity for glutaraldehyde and hydroxyadipaldehyde and 10-hour fixation was used for formalin. It should be noted in regard to these fixatives that glutaraldehyde gave the best structural preservation as compared with the other two aldehyde fixatives and that enzymatic activity was retained by all aldehyde fixatives. Oxmium tetroxide was used as a control fixative which destroyed enzymatic activity in 10 minutes.

After fixation the aldehyde-fixed tissues were washed and stored in cold 0.05 M tris or cacodylate buffer, pH 7.4, containing sucrose for periods ranging from overnight to several days. After storage they were incubated either intact or more finely diced in the standard Wachstein-Meisel adenosinetriphosphatase (ATPase) medium (10) (pH 7.1 to 7.2), containing lead nitrate, $Pb(NO_3)_2$, as the capture reagent, either at room temperature or at 10°C for periods of from 15 to 90 minutes. No significant change in pH was noted at the completion of incubation. Thin (15 μ) and thick (50 μ) frozen sections of the same fixed material were incubated along with the blocks. At various times thin sections

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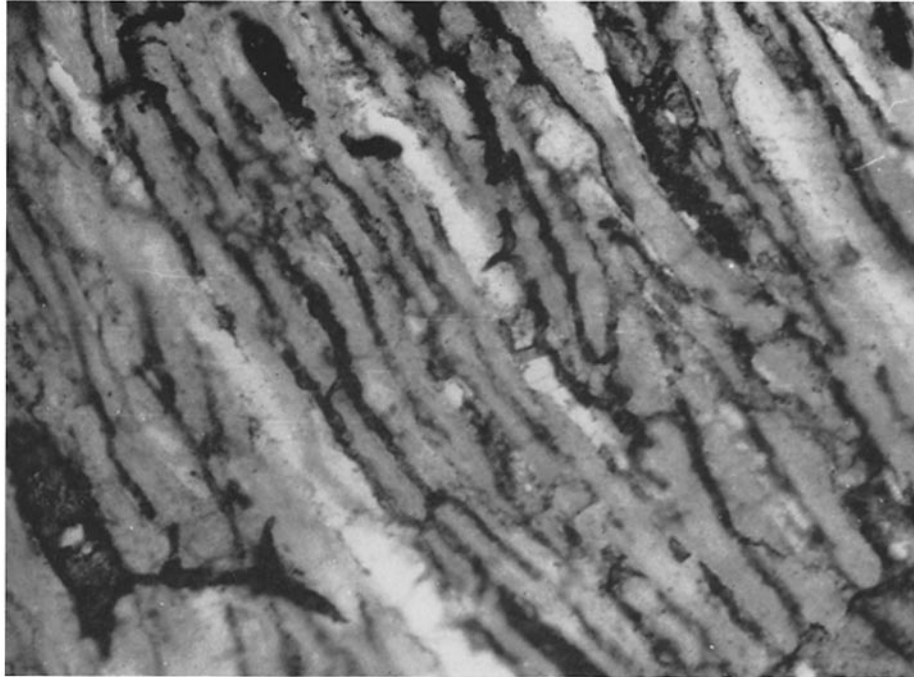
Legends for Figures

B, basement membrane   M, muscle cell
E, endothelial cell   R, red blood cell
J, intercellular junction   v, pinocytic vesicle
L, lumen

Although all work was performed on both frozen section and small blocks of tissue, only Fig. 7 is taken from the frozen-sectioned material.

FIGURE 1

Light photomicrograph of a frozen section of myocardium incubated for one hour in ATP-medium. The dense reaction product outlines the small blood vessels. × 300.

were removed from the incubation media, treated with ammonium sulfide, $(NH_4)_2S$, to develop a visible precipitate, lead sulfide (PbS), from the final product lead phosphate, $PbPO_4$, and examined with the light microscope. When a satisfactory reaction was present in the thin sections, the thick sections and small blocks were removed from the incubating media, washed briefly in buffer, and fixed for 2 hours in cold buffered osmium tetroxide—containing sucrose (9). It should be noted that tissue processed for electron microscopy was not treated with $(NH_4)_2S$ since the final product, $PbPO_4$, is electron-opaque.

The same procedures were followed in other experiments in which only the ATP in the incubating medium was substituted for by either ADP or AMP at the same concentrations. The controls consisted of osmium tetroxide–fixed tissue (10 minutes) incubated in the complete medium with ATP, and

aldehyde-fixed material incubated in media from which only the substrate was lacking.

Additional experiments were carried out at the light microscope level with other phosphate substrates in an attempt to evaluate the specificity of the reaction with the above nucleotide substrates. β-glycerophosphate was substituted for ATP and incubated under the same conditions described above and phenyl phosphate was incubated at pH 7.6 (11).

After postincubation fixation with osmium tetroxide, the thick frozen sections and the small blocks of tissue were dehydrated in a graded series of ethanol and embedded in Epon (12). Thin sections, cut on an LKB microtome and mounted on Formvar-coated grids, were examined without any additional staining with an RCA EMU 3F electron microscope.

FIGURE 2

Control preparation. Electron micrograph of part of a capillary wall taken from tissue incubated in medium without substrate. Numerous vesicles (*v*) are present in the cytoplasm of the endothelial cell (*E*). *B* marks the basement membrane and *L* the lumen. X 44,000.

RESULTS

Light Microscopy

When rat myocardium was incubated in the Wachstein-Meisel medium with ATP, ADP, or AMP as substrates, visible reaction product was localized in the small blood vessels. The pattern of the distribution of the final product outlining the blood vessels of myocardium is shown in Fig. 1. The reaction product appears to be precipitated in and/or immediately around the blood vessels. The same localization occurred when ATP, ADP, or AMP was used as substrate and the incubation was carried out in the pH range 7.1 to 7.2. When β-glycerophosphate was used as substrate and incubated in the same pH range no reaction product was seen. Similarly when sections of myocardium were incubated in a medium containing phenyl phosphate and buffered at pH 7.5 to 7.6, no reaction product developed in the small blood vessels. In addition, sections of heart muscle fixed in osmium tetroxide for 10 minutes before incubation in the complete medium also showed no activity.

Electron Microscopy

Fig. 2 is an electron micrograph of a part of a capillary wall which is representative of the appearance of the vessels in myocardium fixed in glutaraldehyde, incubated in medium without substrate, and refixed in osmium tetroxide. The fine structures of the endothelial cells prepared and treated in this manner are similar to those found in tissue fixed in osmium tetroxide alone. The pinocytic vesicles were of particular interest in this study and in control preparations (incubation without substrate or inhibition of enzymes by osmium tetroxide fixation), the vesicles were numerous and irregularly distributed. As indicated by other authors (13–18), some of these vesicles were directly continuous with or abutting on the plasma membranes of both the luminal and extra-

vascular surfaces. The membrane-bounded vesicles within the cytoplasm measured 500 to 800 A in diameter and occurred singly or occasionally agminated. In the latter case either two vesicles were joined together or many vesicles formed a rosette pattern. *No lead precipitate occurred in any of the formed elements of endothelial cells in any of the control preparations.*

In the glutaraldehyde-fixed material the final product of the enzymatic reaction was clearly localized to the pinocytic vesicles of the endothelium (Fig. 3). In some instances vesicles were filled with final product and in other cases the reaction product was deposited on or adjacent to the inner surface of the vesicle membrane (Figs. 4, 5). The plasma membranes of the endothelial cell (both luminal and extravascular) showed no activity when present as a linear structure. However, when vesicle formation was occurring as invaginations or as indentations of the plasma membranes, these sites showed activity (Figs. 4, 5). No reaction product occurred in the cytoplasm of endothelial cells, in other cell organelles, at the sites of intercellular junctions (Fig. 5) or in the basement membrane. It should also be noted that no activity was seen on the surface of muscle cells surrounding the capillaries (Fig. 3), but reaction product was regularly seen distributed in a spotty fashion on the surface of erythrocytes.

This distribution of reaction product was also obtained when hydroxyadipaldehyde or formalin was used as the preincubation fixative (Figs. 6, 7). In addition the same distribution occurred when the aldehyde-fixed tissue was incubated either as a thick frozen section (Fig. 7) or a small block.

DISCUSSION

The electron micrographs clearly demonstrate that the reaction product produced by the hydrolysis of nucleoside phosphate is localized within vesicles in the cytoplasm of the endothelial cells. The same localization was found when the tissue

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FIGURE 3

Electron micrograph of part of a capillary wall taken from glutaraldehyde-fixed material incubated with ATPase medium for 1 hour. The dense reaction product is confined entirely to small localized sites in the cytoplasm of the endothelial cell (*E*). Part of a muscle cell is seen at the right (*M*). X 39,000. The sites of deposition (outlined by brackets) are seen at higher magnification in inset. Final product of histochemical reaction is entirely confined within the membrane lined vesicles. X 75,000.
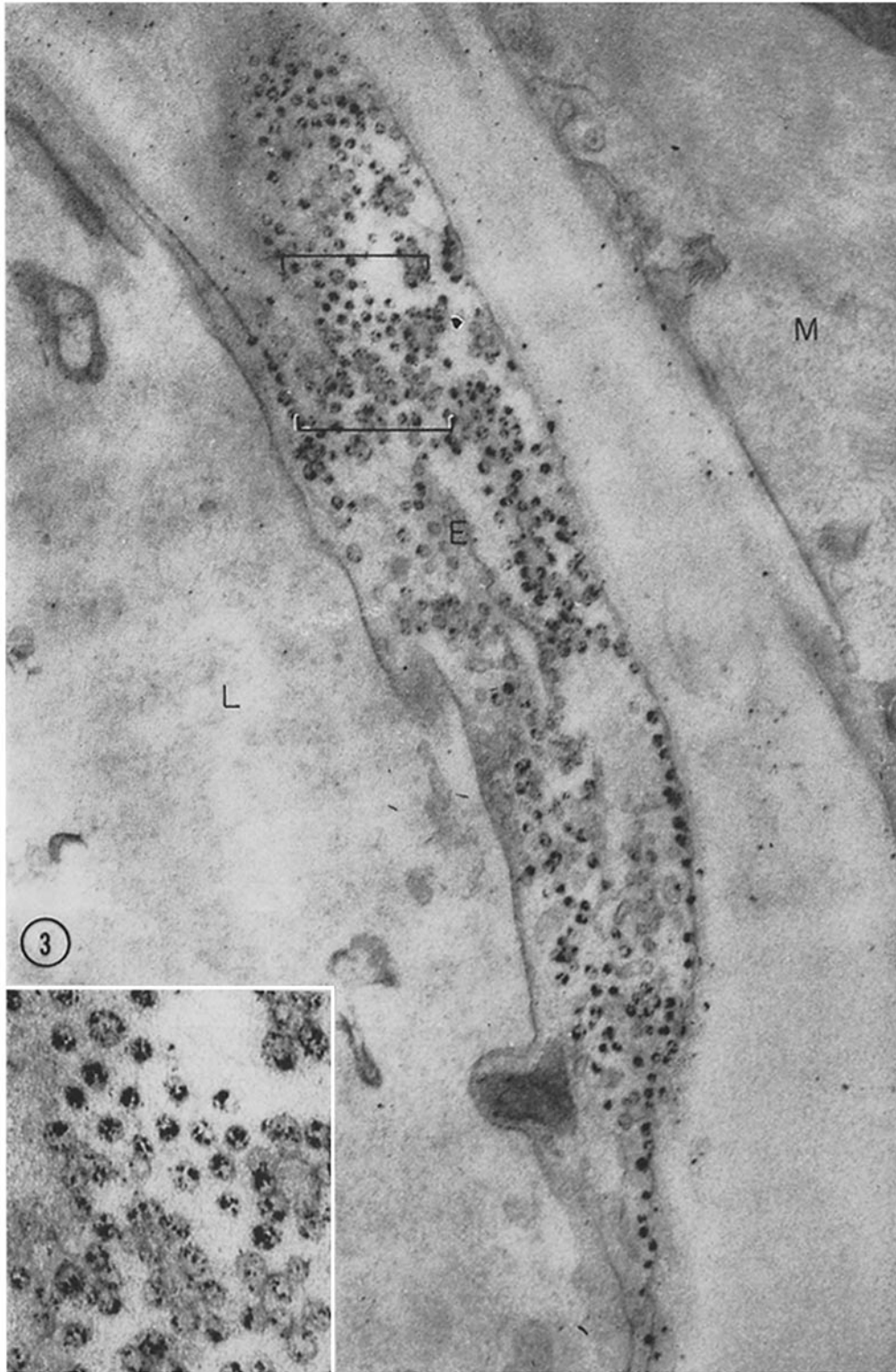
was fixed in either glutaraldehyde, hydroxy-adipaldehyde, or formol-phosphate-sucrose.

Since there are sound theoretical objections concerning the localization of enzymatic activity in tissue incubated in block form (19, 20), the reactions were also carried out on fixed frozen sections. Under these conditions the reaction product was localized to the same sites found within the tissue blocks. It should be emphasized that the use of frozen sections resulted in both poorer structural preservation of the tissue and apparently greater enzymatic activity. In this study the frozen sections served the useful purpose of corroborating the localization of activity in the better preserved blocks of tissue.

The evidence presented suggests that the vesicles of endothelial cells contain an enzyme(s) capable of hydrolyzing the adenine nucleotides, ATP, ADP, and AMP. In the present study we have not yet determined whether this activity is due to the presence of "specific" nucleotidases, e.g. ATPase or 5'-nucleotidase, or to a non-specific phosphatase.[1] However, no activity was found when the phosphatase substrates, β-glycerophosphate or phenyl phosphate, were incubated under the same conditions for incubation of the nucleotides. This suggests that the hydrolysis of the nucleotides is probably not due to the action of the non-specific alkaline phosphatase, although the possibility cannot be excluded. In this regard Novikoff, Hausman, and Podber (21) suggested

[1] It is well known that when β-glycerophosphate or phenyl phosphate are used as substrates for the demonstration of alkaline phosphatase (pH 9.1), activity can be localized to the blood capillaries with the light microscope. We confirmed this localization for myocardial capillaries.

that the enzyme activity demonstrated by the Wachstein-Meisel technique with ATP as substrate is most likely a specific ATPase rather than a non-specific phosphatase. Further characterization of the enzymes associated with myocardial capillaries is in progress.

The absence of reaction product in other organelles of the endothelial cells should not be taken as sufficient evidence that similar enzymatic activity is absent from these sites. The possibility is strong that some enzymes (e.g. mitochondrial ATPase) were selectively inhibited by fixation. In addition, capillaries in different tissues may show different enzymatic activities and/or different fine structural localization of activity (22, 23). It has been found that vesicles in cerebral capillaries do not show nucleoside phosphatase activity but this activity was localized to the basement membrane of these capillaries and in the plasma membrane of glial end-feet abutting on these vessels (23). It is also probable that the vesicles of heart capillaries have other enzymes in addition to those demonstrated here.

The findings of enzymatic activity in the vesicles of endothelial cells offers interesting correlations with recent studies on the physiological function of these structures. Palade (13) was the first to describe the existence of vesicles in the cytoplasm of endothelial cells of capillaries as seen with the electron microscope, and he suggested that the vesicles might play a role in the transport of materials across the capillary wall (13, 14, 17). This suggestion was augmented by Bennett and his coworkers (16, 24) and others (15, 25).

A number of attempts to demonstrate experimentally that the vesicles of endothelial cells were active in the transport of colloidal particles were

FIGURE 4

Part of the endothelial cell seen in Fig. 3. Numerous vesicles (v) with reaction product are open to the extravascular side of the endothelial cell but no reaction product is seen on the plasma membranes where vesicular indentations do not occur. The reaction product is localized on the inner surface of the membrane and inside the vesicles (arrows). No reaction product is seen in the basement membrane. (B). X 83,000.

FIGURE 5

Part of the wall of a small glutaraldehyde-fixed capillary incubated for ATPase activity. No reaction product is seen at the site of the intercellular junction (J). Final product occurs in relation to only pinocytic vesicles or indentations. R is part of a red blood cell in the lumen showing spotty localization of final product on the surface. X 92,000.
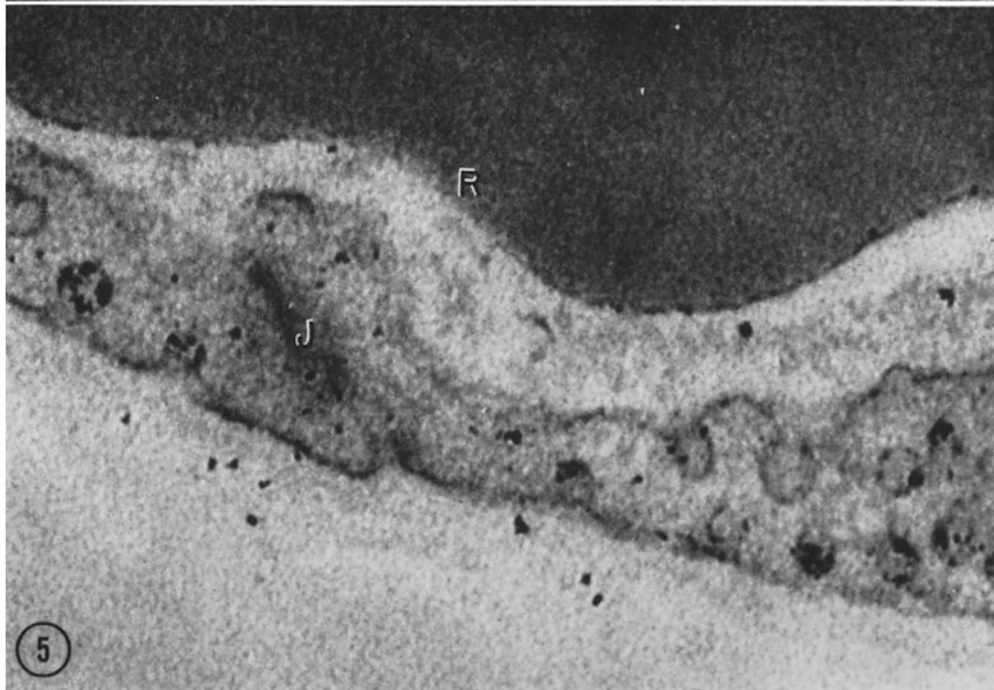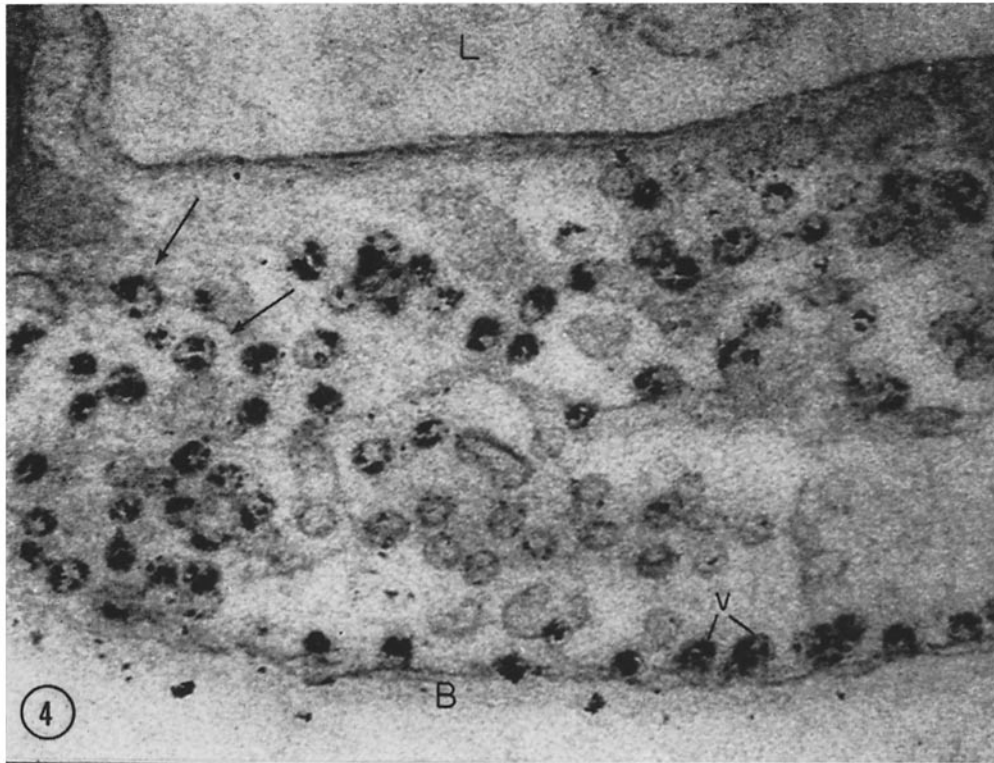
FIGURE 6
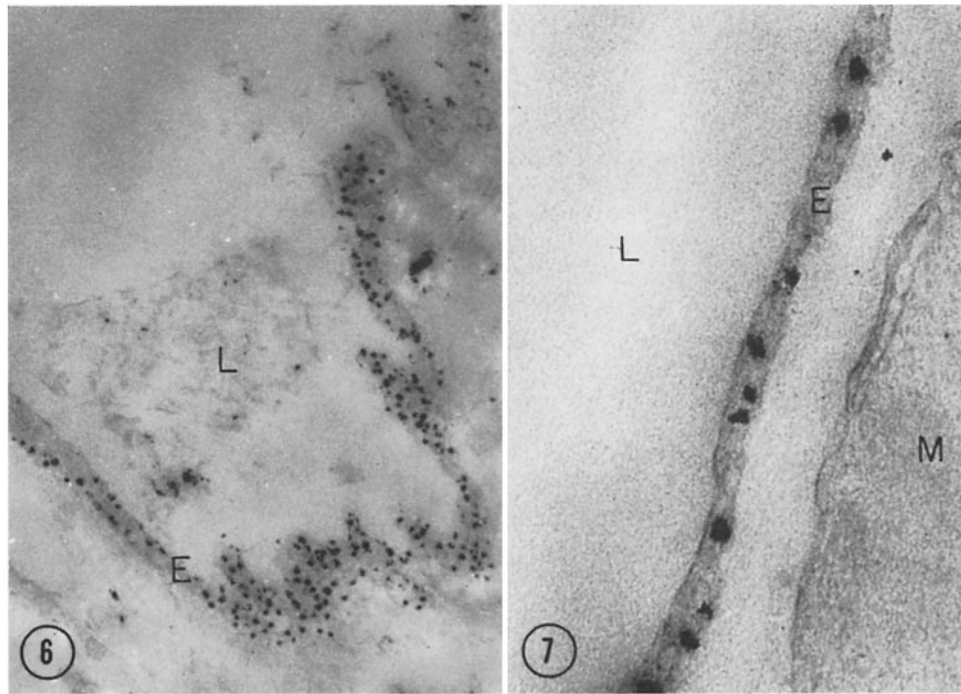
Low magnification electron micrograph taken from tissue fixed in hydroxyadipaldehyde and incubated with ATP for 1½ hours. The same punctate pattern of reaction product is confined to the endothelial cell (E) of a small blood vessel. × 18,000.

FIGURE 7

Electron micrograph of part of a capillary wall from tissue fixed in formol-phosphate-sucrose and incubated as a 50μ frozen section in ATP medium for 40 minutes. The reaction product is localized in areas that correspond to the vesicles of endothelial cells (E) though no limiting membrane is apparent surrounding the final product. × 56,000.

reported (26–28). These results suggested that the vesicles were involved in the transport process, but the observations related primarily to particle uptake by the vesicles and not to transport across the cell (29).

Recently this problem was investigated using the perfused heart preparation (30), and under these conditions colloidal particles were transported across the capillary walls within the cytoplasmic vesicles.

By analogy to the phenomenon of pinocytosis described first by Lewis (31), these vesicles in the endothelial cells (and those in other cell types as well) have been called pinocytic vesicles although it appears that their principal function may be to transport materials across the endothelial cell

rather than to incorporate substances within the cell.

Now it is clear that the pinocytic vesicles of endothelial cells contain enzymes which are active towards nucleotide substrates including ATP and the evidence is suggestive that this enzymatic activity is present in or on the membranes that make up the vesicles. Other investigators have described the localization of an ATPase on the cell membranes of hepatic cells (22) and kidney tubules (2) using histochemical techniques, and more recently it has been shown that ATPase activity is present in cell membrane fractions as assayed biochemically (32–34). There is also a considerable body of evidence which suggests that an ATPase may play a role in the transport of

Na$^+$ and K$^+$ across the cell membranes of a variety of tissues (35–44).

It seems likely that the enzymatic activity in the vesicles of endothelial cells plays a specific role in the transport function of these structures. Whether this occurs through the activation or synthesis of vesicle membrane (39, 40) with the participation of an ATP/ATPase energy-producing system or through some other parameter remains to be determined. However, it may be significant that the surface membrane of endo-thelial cells did not show activity; this was manifest only where pinocytic invaginations or indentations occurred.

BIBLIOGRAPHY

1. PADYKULA, H., and HERMAN, E., *J. Histochem. and Cytochem.*, 1955, **3**, 170.
2. SPATER, H. W., NOVIKOFF, A. B., and MASEK, B., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 765.
3. NOVIKOFF, A. B., DRUCKER, J., SHIN, W-Y., and GOLDFISCHER, S., *J. Histochem. and Cytochem.*, 1961, **9**, 434.
4. WACHSTEIN, M., and MEISEL, E., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 119.
5. FREIMAN, D. G., and KAPLAN, N., *J. Histochem. and Cytochem.*, 1960, **8**, 159.
6. MARCHESI, V. T., and BARRNETT, R. J., *Anat. Rec.*, 1962, **142**, 317.
7. SABATINI, D. D., BENSCH, K. G., and BARRNETT, R. J., *J. Cell Biol.*, 1963, **17**, 19.
8. HOLT, S. J., and HICKS, R. M., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 31.
9. CAULFIELD, J. B., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 827.
10. WACHSTEIN, M., and MEISEL, E., *Am. J. Clin. Path.*, 1957, **27**, 13.
11. MOLBERT, E. R. G., DUPSIVA, F., and VON DEIMLING, O. H., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 387.
12. LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
13. PALADE, G. E., *J. Appl. Physics*, 1953, **24**, 1424.
14. PALADE, G. E., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 85.
15. MOORE, D. H., and RUSKA, H., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 457.
16. BENNETT, H. S., LUFT, J. H., and HAMPTON, J. C., *Am. J. Physiol.*, 1959, **196**, 381.
17. PALADE, G. E., *Circulation*, 1961, **24**, 368.
18. FLOREY, H. W., *Quart. J. Exp. Physiol.*, 1961, **46**, 119.
19. NOVIKOFF, A. B., BURNETT, F., and GLICKMAN, M., *J. Histochem. and Cytochem.*, 1956, **4**, 416.
20. HOLT, S. J., and HICKS, R. M., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 47.
21. NOVIKOFF, A. B., HAUSMAN, D. H., and PODBER. E., *J. Histochem. and Cytochem.*, 1958, **6**, 61.
22. ESSNER, E., NOVIKOFF, A. B., and MASEK, B., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 711.
23. TORACK, R. L., and BARRNETT, R. J., *J. Neuropath. and Exp. Neurol.*, in press.
24. BENNETT, H. S., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 99.
25. YAMADA, E., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 445.
26. WISSIG, S. L., *Anat. Rec.*, 1958, **130**, 467.
27. ALKSNE, J. F., *Quart. J. Exp. Physiol.*, 1959, **44**, 51.
28. PALADE, G. E., *Anat. Rec.*, 1960, **136**, 254.
29. FAWCETT, D. W., in The Microcirculation, (S. R. M. Reynolds and B. W. Zweifach, editors), Urbana, University of Illinois Press, 1959, 1.
30. JENNINGS, M. A., MARCHESI, V. T., and FLOREY, H. W., *Proc. Roy. Soc. London, Series B*, 1962, **156**, 14.
31. LEWIS, W. H., *Johns Hopkins Hosp. Bull.*, 1931, **49**, 17.
32. ACS, G., GARZO, T., GROSZ, G., MOLNAR, J., STEPHANECK, O., and STRAUB, F. B., *Acta Physiol. Acad. Sc. Hung.*, 1955, **8**, 269.
33. EMMELOT, P., and BOS, C. J., *Biochim. et Biophysica Acta*, 1962, **58**, 374.
34. CUMMINS, J., and HYDÉN, H., *Biochim. et Biophysica Acta*, 1962, **60**, 271.
35. SKOU, J. C., *Biochim. et Biophysica Acta*, 1957, **23**, 394.
36. SKOU, J. C., *Biochim. et Biophysica Acta*, 1962, **58**, 314.
37. POST, R. L., MERRITT, C. R., KINSOLVING, C. R., and ALBRIGHT, C. D., *J. Biol. Chem.*, 1960, **235**, 1796.

38. Dunham, E. T., and Glynn, I. M., *J. Physiol.*, 1961, **156**, 274.

39. Hokin, L. E., and Hokin, M. R., *J. Gen. Physiol.*, 1960, **44**, 61.

40. Hokin, L. E., and Hokin, M. R., *Biochem. J.*, 1961, **81**, 32p.

41. Bonting, S. L., Simon, K. A., and Hawkins, N. M., *Arch. Biochem. and Biophysics*, 1961, **95**, 416.

42. Bonting, S. L., Caravaggio, L. L., and Hawkins, N. M., Arch. *Biochem. and Biophysics*, 1962, **98**, 413.

43. Hoffman, J. F., *J. Gen. Physiol.*, 1962, **45**, 837.

44. Auditore, J. V., *Proc. Soc. Exp. Biol. and Med.*, 1962, **110**, 595.