

# THE ROLE OF MUCOPOLYSACCHARIDES IN VESICLE ARCHITECTURE AND ENDOTHELIAL TRANSPORT

## An Electron Microscope Study of Myocardial Blood Vessels

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### INTRODUCTION

Electron microscope studies have helped to define the fine structure of pinocytic vesicles and have provided information leading to a better understanding of their function (1-3). Although "an endocapillary layer (or sheath)" which would play a role in transendothelial transport was predicted (4), most electron microscope studies did not clearly demonstrate such a structure (1-3) until the ruthenium-red (RR) staining method was introduced (5). Luft described the RR-stained ultrastructure of blood capillary and endocapillary layers (6) and pointed out that RR penetrates pinocytic vesicles under certain conditions and that the dye diffuses into tissue slowly and does not usually penetrate the plasma membrane. Because of the fine granularity and the relatively controllable electron opacity of its end product, the RR stain seemed to us potentially to allow good electron microscope resolution in combination with tracer techniques.

The purpose of the present study was (a) to analyse the fine structure of pinocytic vesicles in relation to mucopolysaccharides in the vesicle architecture, and (b) to obtain a better understanding of the role of the surface coating in endothelial transport.

### MATERIALS AND METHODS

Horse-spleen ferritin (10% solution obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio) was dialysed against 0.1 M ethylenediaminetetraacetate overnight at 4°C and then against 0.1 M phosphate buffer (pH 7.2) at 4°C for 24 hr (7). The resultant 10% ferritin solution in 0.1 M phosphate buffer was slowly injected (in 2 min) into an ear vein of the rabbit, under light pentobarbital

anesthesia, at a rate of 100 mg ferritin/100 g body weight.

Four healthy young-adult female New Zealand white rabbits were given pentobarbital anesthesia, without pretreatment (two rabbits) or 10-15 min after an injection of ferritin (two rabbits). The myocardium was removed and immediately fixed for electron microscopy.

Tissue blocks (smaller than 5 × 5 × 2 mm) excised from the left ventricle were fixed in 2% paraformaldehyde-2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) (8) at 4°C for 4 hr. The blocks were then cut into smaller pieces (smaller than 1 × 1 × 1 mm), rinsed in the cacodylate buffer (0.1 M, pH 7.4), and postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 1 hr. Small pieces of fresh tissue were also fixed directly in the osmium fixative without aldehyde prefixation. After two to three washes in the buffer, they were dehydrated in graded ethanols and embedded in Epon (9). Before dehydration, a sample of the preparations was immersed in 0.5% uranyl acetate in 0.05 M sodium acetate at room temperature for 60 min (3). Thin sections were cut on an LKB Ultratome with glass knives, the *Section Thickness* being set at 400 Å. Sections, which were cut serially, were picked up on bare 300-mesh grids and stained with uranyl acetate (10) and/or lead citrate (11). The sections were examined in a Siemens Elmiskop I equipped with an anticontamination device, a pointed filament, a double condenser system with a 400 μ aperture, and a 50 μ objective aperture. Thinner portions of the sections were selectively photographed at initial magnifications of 40,000 or 80,000, and occasionally for survey at 10,000.

Samples of tissue blocks (smaller than 5 × 5 × 2 mm) were immersed in 2% paraformaldehyde-2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 1000 ppm ruthenium red (12) at 4°C

for 2 hr. The blocks were washed briefly with the cacodylate buffer and then cut into 40  $\mu$  slices on a Sorvall tissue sectioner (13). After brief washing in the buffer, the tissue slices were fixed in the same aldehyde fixative containing ruthenium red for an additional 2 hr. They were then washed in two to three changes of the 0.1 M cacodylate buffer (pH 7.4) containing 1000 ppm ruthenium red, and post-fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) containing 1000 ppm ruthenium red at 4°C for 1 hr. On occasion, the concentration of ruthenium red in the various solutions was lowered to 100, 50, or 20 ppm, and samples of tissue blocks and slices were processed with a series of solutions of the lower concentration of ruthenium red. After rinsing in two to three changes of the cacodylate buffer, the slices were dehydrated in ethanol, embedded in Epon, and prepared for electron microscopy as described above.

## RESULTS

### *General Remarks*

In the preparations without ruthenium red (RR) treatment, capillaries and small blood vessels (the present study was limited to vessels of less than 20  $\mu$  diameter) displayed ultrastructural features comparable to those described previously (1-3). Pinocytic vesicles showed a variety of interactions with each other, with plasma membrane, and with cytoplasmic vacuoles. The unit-membrane structure of the vesicular membrane, with all detail comparable to that described by Palade and Bruns (3), was found after considerable effort.

In the RR-treated preparations, finely granular electron-opaque precipitates (absent from the preparations without RR treatment), which according to Luft (5, 6, 12) represent ruthenium red-osmium complexes that localize acid mucopolysaccharides, were found in the blood vessel structure. The precipitates were mainly on the luminal surface of the endothelium, in the cytoplasmic vesicles and vacuoles, and in the basement membrane (Fig. 1). On many occasions they had a very high electron opacity and appeared as fine granules of about 20 A diameter which often formed larger aggregates when heavily deposited. The highly electron-opaque granular precipitates were common in specimens treated with a higher RR concentration (1000 ppm) (Figs. 1 and 7), and the less granular lower electron opacity precipitates were more common with a lower RR concentration (20, 50, or 100

ppm) (Figs. 5 and 6). In all instances, the electron opacity of the precipitates was enhanced by electron staining with uranyl or lead (Figs. 2-4).

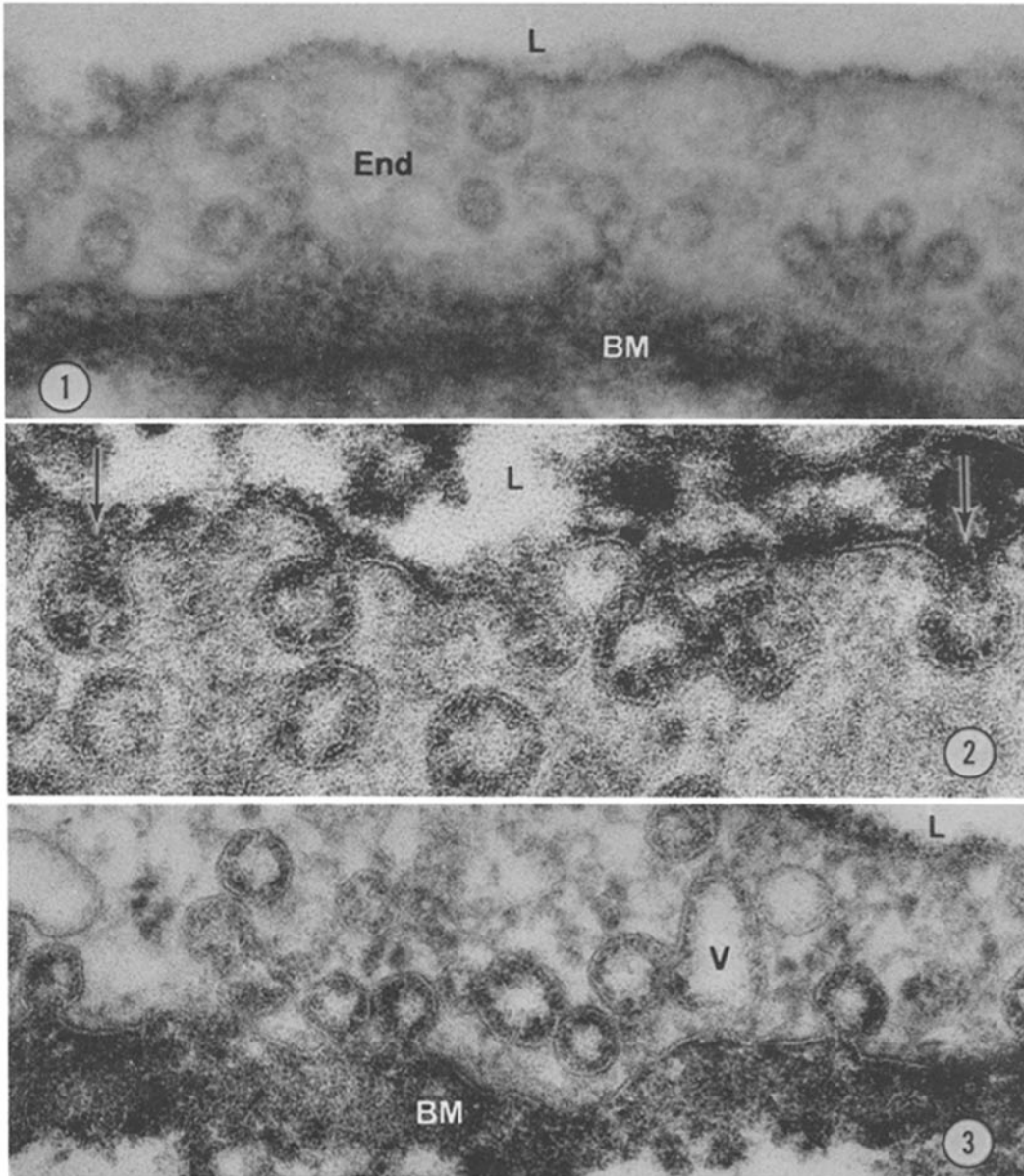
The layer of the RR-stained substance on the luminal endothelial surface was relatively thick (up to 1000 A) and covered the entire surface in some vessels, was thinner and interrupted in others, and was nonexistent in some. Such variations of the coating were found among capillaries as well as small arterioles and venules. The coating was observed to adhere closely to the plasma membrane so that the outer leaflet of the unit-membrane structure of the plasma membrane often could not be distinguished from the coating (Fig. 2).

On the abluminal side, the basement membrane was usually heavily loaded with the RR-stained substance, and, again, the image of the outer leaflet of the plasma membrane often blended with that of the densely-stained basement membrane (Fig. 3). At the junction of endothelial cells, the RR-stained substance filled the gap between two apposing membranes and usually could be followed continuously from both the luminal surface and the basement membrane to the area of tight junction (zonula occludens) which usually was free of the stain.

### *Pinocytic Vesicles and RR-Stained Substance*

The majority of the pinocytic vesicles in preparations treated with ruthenium red contained the RR-positive substance. The substance usually formed a thin (usually <300 A) layer along the surrounding membrane and did not fill the entire vesicular lumen, so that the central portion of the vesicle appeared empty. The image of the coating layer often fused with that of the inner leaflet of the vesicular unit membrane (Figs. 2-4). The RR-laden vesicles, while abundant in the luminal and the abluminal surface cytoplasm, were scattered throughout the endothelial cytoplasm.

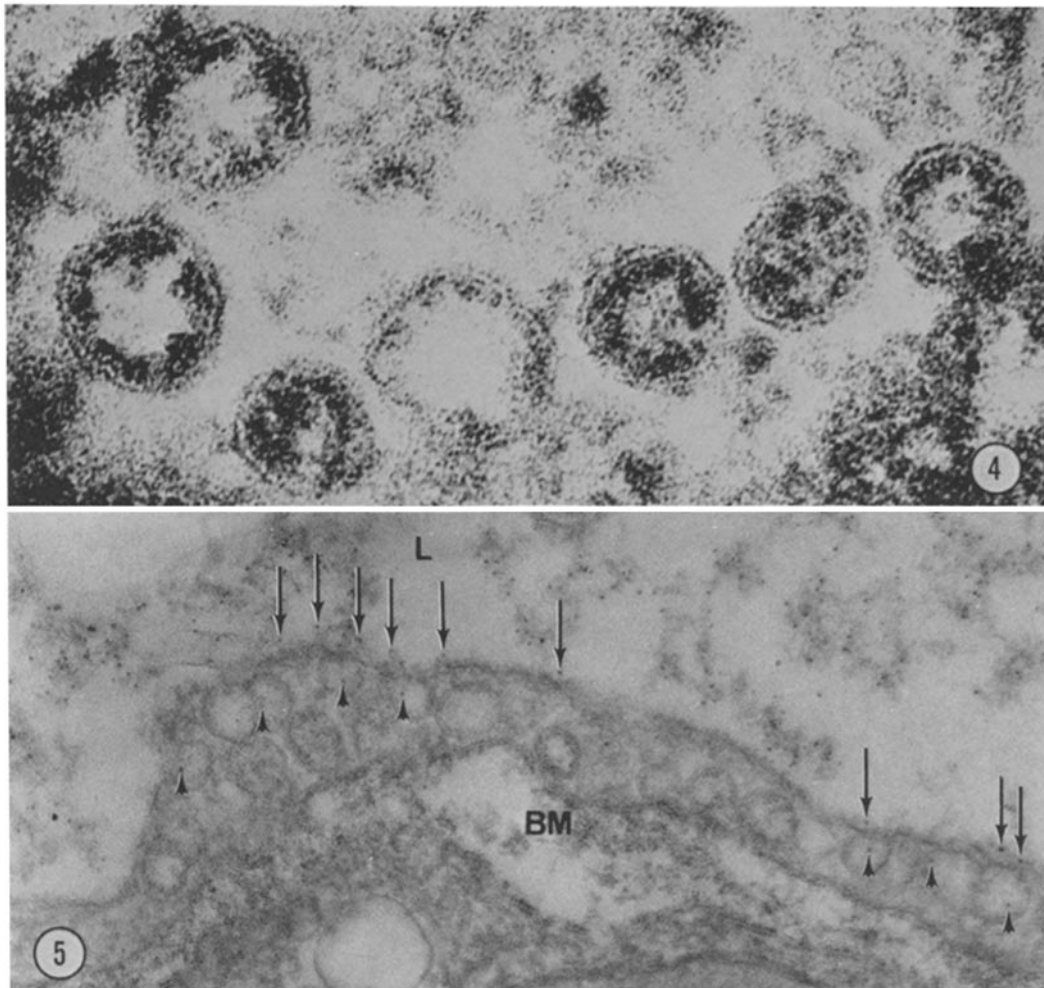
At the luminal surface of the endothelium, the vesicles showed a variety of interactions with the plasma membrane. The RR-coated plasma membrane often formed an indentation or a pit of 500-1000 A diameter. When the pit opening was narrower than 500 A, it was often filled with RR-stained substance which separated its lumen from the vascular lumen (Fig. 2). In addition, a variety of views representing the intermediate stages between the plasmalemmal pit and the cytoplasmic vesicle (3) were revealed by means



**FIGURE 1** A portion of vascular wall, treated with ruthenium red (1000 ppm), but no uranyl or lead stain. Finely granular, electron-opaque precipitates form a thin layer on the luminal (*L*) surface of the endothelium (*End*) and in the periphery of the vesicular lumen. The coating is 100–200 Å thick, with a few exceptions. The basement membrane (*BM*) is filled with the precipitates. The structure of the membrane system and of other cell organelles is not clearly visible.  $\times 100,000$ .

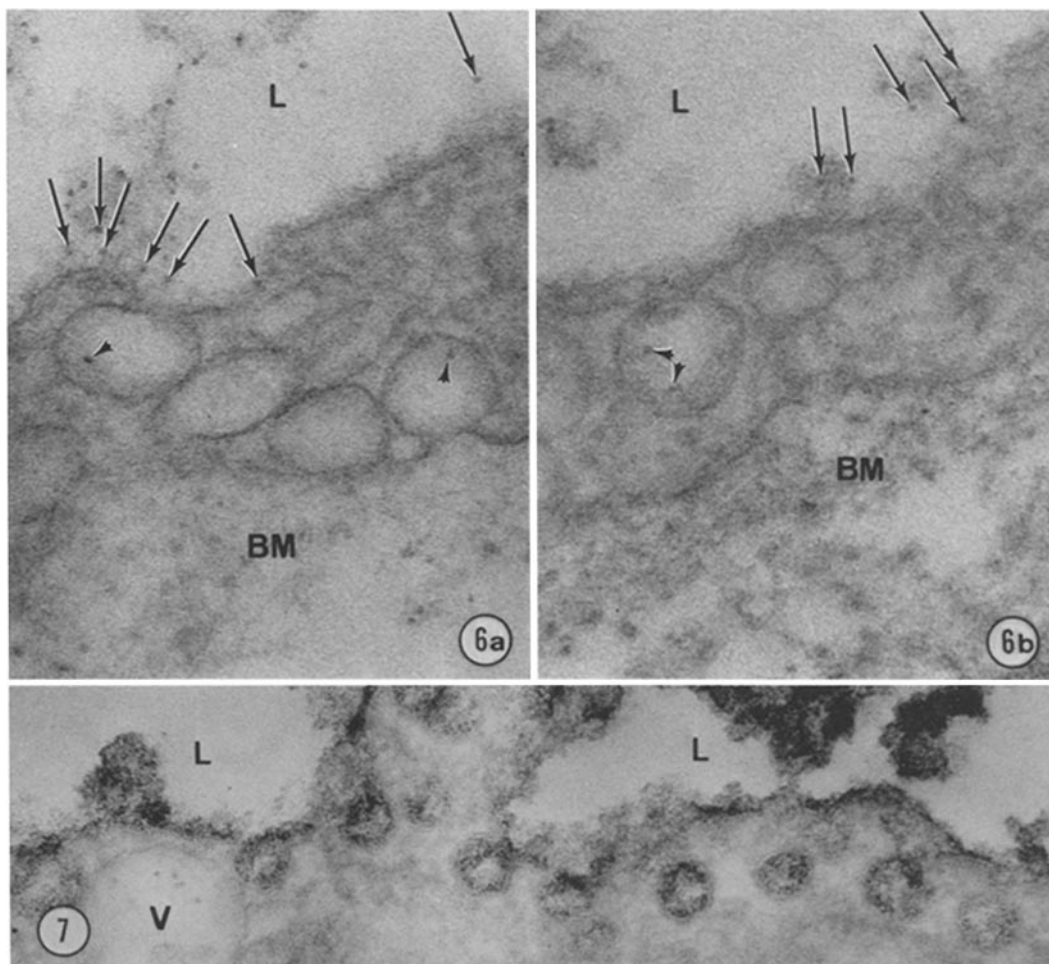
**FIGURE 2** In this luminal portion of the endothelium, treated with ruthenium red (1000 ppm) and stained with uranyl and lead, the electron opacity of the RR-positive substance is considerably enhanced by the electron staining, and the membrane structures are now readily visible. The RR-stained substance covers the endothelial surface and lines the vesicles. The outer leaflet of the unit membrane structure of the plasma membrane as well as the inner leaflet of the vesicular membrane is often indistinct from the layer of the RR-stained substance. The plasma membrane often forms plasmalemmal pits with the RR-positive coating. The opening of the pit, when it is narrower than 500 Å, is often filled by the substance (arrows). Features representing the intermediate stages between the plasmalemmal pit and the cytoplasmic vesicle are also seen in association with the RR-positive coating. *L*, vascular lumen.  $\times 160,000$ .

**FIGURE 3** In this abluminal portion of vascular wall, pinocytotic vesicles with internal coating demonstrate the varying interrelationships with the abluminal endothelial plasma membrane, with each other, and with a small cytoplasmic vacuole (*V*) which is also internally coated. The basement membrane (*BM*) is heavily loaded with the RR-stained substance, and abuts closely the abluminal endothelial plasma membrane. A small portion of vascular lumen (*L*) is seen at right upper corner. Treated with 1000 ppm ruthenium red and stained with uranyl and lead.  $\times 120,000$ .



**FIGURE 4** Close-up picture showing relationship of the RR-stained substance and the ultrastructural framework of the vesicle. The substance forms a thin layer internally along the vesicular membrane. The image of the inner leaflet of the unit membrane structure of the vesicular membrane fuses, in many places, with that of the coating substance, although the intermediate electron-lucent space and the outer leaflet of the membrane are distinct. The thickness of the coating usually measures less than 300 Å, and varies from vesicle to vesicle and even from place to place within a vesicle. The central portion of the vesicle usually appears empty. Treated with 1000 ppm ruthenium red and stained with uranyl and lead.  $\times 240,000$ .

**FIGURE 5** 10 min after a ferritin injection. By treatment with 50 ppm ruthenium red and without uranyl or lead stain, the RR-positive substance has a much lower electron opacity (compare with Figs. 1 and 7). The coating is now recognized as fuzzy material with low electron opacity on the luminal endothelial and the internal vesicular surface (this is not seen in specimens untreated with RR). Basement membrane (BM) generally shows a higher electron opacity than that in untreated controls, and also is sparsely populated with finely granular, very electron-opaque precipitates. Ferritin particles are identified within the coating substance on the luminal endothelial surface (arrows) as well as on the internal vesicular surface (arrow heads). Vascular lumen (L) contains many ferritin particles and some plasma protein structures, although the content seems to be partially "extracted."  $\times 90,000$ .



FIGURES 6 *a* and *b* Higher power electron micrographs for clearer demonstration of the relationship of ferritin particles and the surface coating, prepared as in Fig. 5. The RR-positive substance, which shows low electron opacity, can be traced on the endothelial surface, on the internal vesicular surface, and in the basement membrane (*BM*). Ferritin particles are located within the internal coating of the vesicles (arrow heads) and also in the endothelial surface coat (arrows). *L*, vascular lumen.  $\times 160,000$ .

FIGURE 7 10 min after a ferritin injection. Treated with 1000 ppm ruthenium red but no uranyl or lead stain. Ferritin particles are not found in the central "empty" areas of pinocytic vesicles, suggesting that they are localized within the internal coating which displays higher electron opacity than ferritin particles. Several ferritin particles are seen in a cytoplasmic vacuole (*V*). *L*, vascular lumen.  $\times 120,000$ .

of the associated RR-positive coating (Fig. 2).

Comparable interactions were observed among the vesicles, between the vesicle(s) and the cytoplasmic vacuole, and between the vesicles and the abluminal plasma membrane (Fig. 3).

#### *Coating and Injected Ferritin Molecules*

In the animals injected with ferritin, the vascular lumen of small blood vessels and capillaries

were usually loaded with many ferritin particles. However, the lumens of some vessels whose surface coating was well stained with ruthenium red contained only few ferritin particles and structures representing plasma protein (as discussed by Luft (6) and considered to be a result of extraction of the vessel content during preparation). Pinocytic vesicles occasionally contained a few ferritin particles.

At selected areas where the coating had lower electron opacity (especially in the specimens treated with 20, 50, or 100 ppm ruthenium red), ferritin molecules were identifiable in the coating as electron-opaque, 60–80 Å diameter particles (Figs. 5 and 6). Some ferritin particles were found on and within the endothelial surface coating (Figs. 5 and 6), but their distribution was perhaps only one fifth of that of the particles in the free vascular lumen. About one in 20–30 pinocytic vesicles contained one to three ferritin particles. In these vesicles virtually all (more than 90%) ferritin particles were located on or in the internal coating of the vesicle (Figs. 5 and 6). This localization of ferritin particles within the coating was verified by a negative finding, i.e. practically no ferritin particles were present in the central “empty” areas of the pinocytic vesicles when the internal coating of the vesicles displayed an electron opacity higher than that of the ferritin particles (Fig. 7). The distribution of ferritin in the vesicular coating was low and comparable to that within the endothelial surface coating.

Most cytoplasmic vacuoles contained ferritin. The distribution of ferritin was relatively high in the vacuolar space compared to the free vascular lumen, and lower in the internal RR-stained coating of the vacuoles. The latter coating was similar to that of the endothelial surface and the vesicles.

#### DISCUSSION

The character of the end product of ruthenium red staining, for example, fine granularity and a partly controllable electron opacity, made this method more useful for the present study than other methods that have been used to demonstrate mucopolysaccharides at the ultrastructural level, i.e. colloidal iron (14–17), colloidal thorium (15, 16, 18, 19), periodic acid–silver methenamine (18, 20), phosphotungstic acid (21), Alcian blue (22), and probably lanthanum staining (14, 23–25). As pointed out by Luft (5, 6, 12), however, there are some problems with the ruthenium-red staining method. Several trials in our pilot experiment also revealed that (a) infiltration of ruthenium red into the tissue is very slow (5, 6, 12), and that (b) the mixture of ruthenium red and osmium tetroxide in solution forms very fine black precipitates in a relatively short period of time, although the presence of ruthenium red in the osmium fixative (5, 6, 12) is helpful or even

essential for the production of better staining (especially the highly electron-opaque precipitates). To minimize these undesirable effects, we employed 40  $\mu$  tissue slices rather than tissue blocks for the ruthenium-red staining preparation. Nevertheless, unevenness of the staining condition in a specimen (6) was not fully avoided. Therefore, although the present results support the concept that the condition of the mucopolysaccharide coating varies somewhat from vessel to vessel as well as from vesicle to vesicle, other possible interpretations (6) should be considered; for example, the dye did not penetrate well in the unstained or less-stained site, or the mucopolysaccharides had been extracted before the stain reached to the area.

The present study has demonstrated morphologically the probable sequential interaction of mucopolysaccharide-coated plasma membrane and pinocytic vesicles. This plasma membrane-vesicle interaction corresponds well with that previously described by Palade and Bruns (3). However, the demonstration of a plasmalemmal pit whose opening is closed by the coating substance alone is a new observation, and may be inserted between the two stages previously proposed: a plasmalemmal pit which has a free opening to the vascular lumen and a pit which is closed by a “diaphragm” (3). It is uncertain, however, on this purely morphologic basis, whether the sequence represents the formation or the discharge of the vesicles. However, one finding in the present experiments with ferritin as a tracer may be informative. The participation of the ferritin molecules in these structural complexes at the luminal endothelial surface shortly (10–15 min) after the injection, when no significant number of ferritin particles is counted in the perivascular elements, lends credence to the idea that the sequential interactions represent the events that accompany formation of the vesicles.

The disclosure of the internal coating of the pinocytic vesicles in the previous (5, 6) and present studies may somewhat change the interpretation of vesicle ultrastructure in relation to vesicle function. Although quantitative measurements cannot be made, since it is difficult to assess precisely what proportion of the capacity of the vesicle is occupied by the coating material and what type of macromolecular configuration the coating substance displays, the unit capacity of the vesicles for fluid transport, which is considered

to be the prime function of pinocytosis (1-3), should be considerably reduced from that previously estimated from conventional electron micrographs (1, 2).

With regard to macromolecule transport by the pinocytic vesicles (1, 2, 7), the presence of a stage with a plasmalemmal pit closed by the coating material alone may have some significance. If one accepts this structure as a step in vesicle formation, the finding suggests that the entrance of the plasmalemmal pit becomes closed for certain macromolecules much earlier than expected from conventional electron micrographs. Moreover, the demonstration of the inside coating in the majority of the pinocytic vesicles and the sequential aspects suggesting transfer between the endothelial surface (luminal and abluminal) coating and the vesicular internal coating suggests a significant role for the pinocytic vesicles in the trans-endothelial transport of mucopolysaccharides, at least those which constitute the coating and the basement membrane.

Virtually all published electron micrographs of tissues stained with ruthenium red have shown finely granular, highly electron-opaque precipitates as the end product of the staining (6, 12, 14, 22, 23, 26, 29). According to Luft (5, 6, 12), however, the electron opacity of the stain is not essentially due to the density of the dye itself but is produced by osmification of the dye which binds the specific sites in the tissue. On the basis of this concept, it has seemed reasonable to anticipate that (a) the precipitates, as the reaction product, should be very fine when the reaction is carried out under ideal conditions because of the low molecular weight of ruthenium red as well as of osmium tetroxide, and that (b) the electron opacity of the end product should be amenable to control by varying the dye concentration and/or the degree of osmification (30, 31). Indeed, the RR-stained endothelial coating in the micrographs of an early publication by Luft (1) showed lower electron opacity, and the results of our pilot experiment generally support this concept.

For this reason, the experiment was undertaken with ferritin molecules as tracers for the transport of macromolecules. Ferritin molecules within the coating were indeed recognizable as the particles with higher electron opacity and larger dimensions than the staining end product, in selected areas where the coating demonstrated light or moderate electron opacity. The present results

support the idea that adhering to or being trapped in the coating is probably an important step for the molecules that are transported by pinocytosis, and may be the first morphological demonstration of such a mechanism in the blood vessels. This further suggests that the selectivity of endothelial transport with respect to various different molecules (1, 2, 4) may in part depend on this coating. It would seem reasonable to speculate that molecules which are attracted to the coating because of their physical and chemical nature may be selectively taken into and transported with the pinocytic vesicles.

#### SUMMARY

Rabbit myocardial blood vessels (<20  $\mu$  in diameter) have been studied with ruthenium-red (RR) staining and ferritin tracer techniques in order to delineate the role of mucopolysaccharides in the ultrastructural organization of pinocytic vesicles and their significance in pinocytic transport of macromolecules. The interactions of the plasma membrane and the vesicles suggest a sequence of events that result in the thin (<300 A) RR-stained endothelial coating being transposed into the pinocytic vesicles to compose a thin inside coating as the vesicles are formed. By careful selection of areas in which the electron opacity of the RR end product was reduced (by lowering the dye concentration or the grade of osmification, or both), it was possible to identify the ferritin particles within the RR-stained substance. Far fewer ferritin particles were found in the pinocytic vesicles than in the free vascular lumen, and these particles appeared to be localized almost exclusively within the internal coating. Moreover, the distribution of ferritin particles within the vesicular coating was comparable with that in the endothelial surface coating, suggesting an important role of the coating in the vesicular transport of macromolecules. The fine structure and function of pinocytic vesicles have been discussed further on the basis of these results.

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