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On the Presence of a Concanavalin-A Reactive Coat over the Endothelial Aortic Surface and Its Modifications during Early Experimental Cholesterol Atherogenesis in Rabbits

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Summary. In rabbits, guinea pigs, and rats an ultrathin coat containing carbohydrates has been demonstrated in transmission electron microscopy by means of the highly specific Concanavalin-A histochemical reaction over the endothelial aortic surface. In rabbits fed on a hypercholesterolic diet in aortic areas where the Concanavalin-A reactivity of the endothelial surface is lost, an amorphous thick deposit is found.

In earlier papers (Weber *et al.*, 1970; and Tosi, 1971a, b, c, d) we called attention to some morphologic characteristics of the endothelial surface as it looks at "en face" examination in scanning electron microscopy and to some pathologic findings which may be observed on the arterial surface in scorbutic guinea pigs. In guinea pigs and rabbits fed on a hypercholesterolic diet, our findings consisted chiefly in scattered flattenings of the intimal folds and in an amorphous veil-like deposit (in whose thickness blood cells, chiefly erythrocytes and platelets, are recognizable) lying over large intimal areas. This was observed after two months of hypercholesterolic diet in guinea pigs and three weeks in rabbits, i.e. before the first small intimal plaques appeared.

More recently, we have observed after only 15 days of treatment in cholesterol-fed rabbits that the endothelial surface, as seen in the scanning electron microscope, consistently shows fine wrinkles at the origin of the collaterals. This was never observed in the controls (Weber *et al.*, 1972; Weber, in press).

The amorphous veil-like deposit could have some correlation with deposits of the kind demonstrated in experimental material by Parker (1960) and in human material by Sinapius (1968), and chiefly consisting of lipids: but we have not yet been able to obtain convincing results with lipid stains in our experimental material. Nor have convincing results yet been obtained with other staining methods, which have usually given negative results in our hands, except for some positive results with the DMAB method of Adams (1957) for tryptophan (Weber *et al.*, 1972, in press).

A thin amorphous deposition lying on the endothelial surface has very seldom been found by us in histological routine sections obtained by the same vacuum-dried, metal-shadowed aortic blocks used for scanning electron microscope examination and in ultrathin sections of freeze-dried aortic preparations examined in transmission electron microscope (Weber *et al.*, 1972).

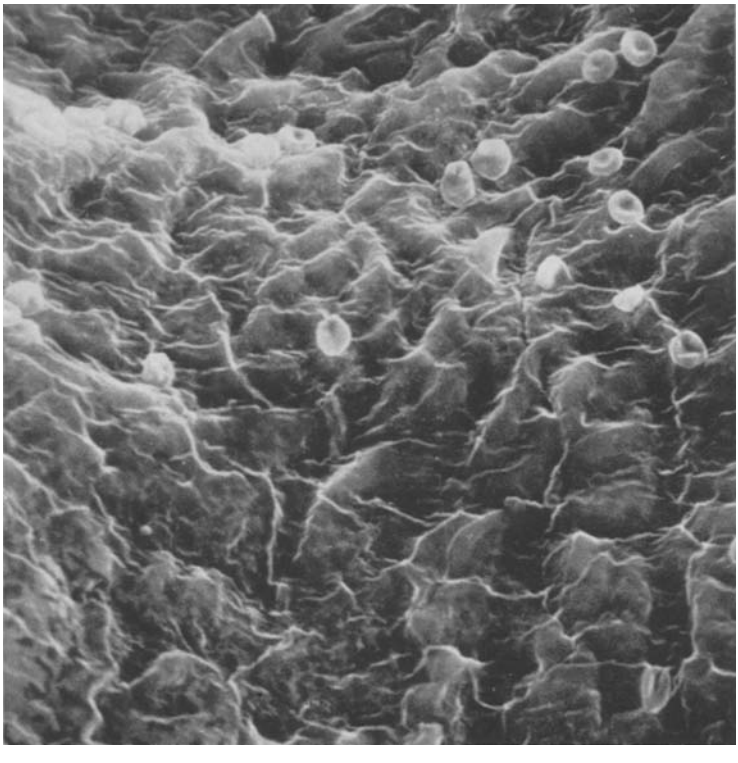

Fig. 1. Aortic intimal surface. Rabbit. Normocholesterolic diet. The intimal folds near the origin of a collateral branch. SEM × 1000

Wondering if our scanning electron microscope pictures could therefore have something to do with the presence of soluble surface substances such as a "polysaccharide" or "carbohydrate coat" (cf. Bennett, 1968; Parsons and Subjeck, 1972), we started research on ultrathin sections of adjacent portions of the same aortas by transmission electron microscopy, though the existence of a layer lining the blood vessel intima has been denied by workers using conventional ultrathin section methods. The ultra-histochemical methods used until now have given rise to conflicting interpretations: we shall mention here only the papers by Luft (1964, 1966), Groniowski *et al.* (1969), Fuchs (1971), Cossel *et al.* (1971), who considered ruthenium red was capable of reacting specifically with carbohydrates, and those by Copley and Scheinthal (1970), who meanwhile have demonstrated that ruthenium red is capable of reacting not only with polysaccharides, but also with fibrinogen and fibrin, proteins not containing carbohydrates.

We have therefore tried the new highly specific ultrahistochemical staining method recently proposed by Bernhard and Avrameas (1971); this makes use of Concanavalin-A, the agglutinin of *Canavalia ensiformis* (jack bean).

Concanavalin-A has two active sites, both of which can react with sugars or glycoproteins containing branched terminal non-reducing α-D-glucopyranosyl, α-D-mannopyranosyl, or β-D-fructofuranosyl residues. Concanavalin-A is fixed

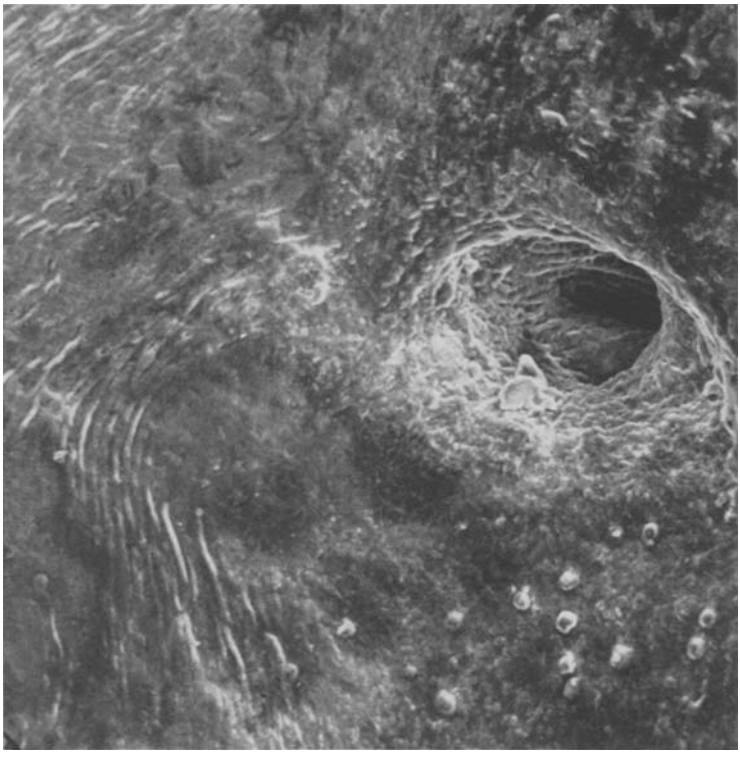

Fig. 2. Aortic intimal surface. Rabbit. After 15 days of hypercholesterolic diet. Near the origin of a collateral branch, in some areas, scattered flattenings of intimal folds; in others, the endothelial surface is covered by an amorphous coating in whose thickness blood corpuscles are incorporated. SEM × 100

to sugar only at one of its active sites. The other active site remains free and operates as a receptor for another sugar secondarily added to the system, such as horse-radish peroxidase (which is a glycoprotein containing 18% carbohydrates). The catalytic activity of the peroxidase molecule can finally be revealed by the diaminobenzidine (DAB) method of Graham and Karnovsky.

Material and Methods

Twelve rabbits weighing 1500 g were fed a standard rabbit diet with 1% added cholesterol dissolved in ether. A second group of 8 rabbits (control group) was fed the same standard diet without added cholesterol. Four guinea pigs and four rats were also used as controls.

The hypercholesterolemic rabbits were killed by bleeding after 15, 20, 22, or 35 days, as were the controls.

Small portions of aorta were fixed for two hours in 2.5% glutaraldehyde in 0.2 M phosphate buffer at pH 7.2, coated with platinum-gold after vacuum-dehydration at room temperature and observed with a scanning electron microscope JSM 2 at 25 Kv. Adjacent aortic portions were subjected to the Concanavalin-A reaction (Bernhard and Avrameas, 1971) or prepared for transmission electron microscopy examination without previous Concanavalin-A treatment. The tissue was fixed in buffered glutaraldehyde, washed in buffer, postfixed in osmium tetroxide and embedded in Epon.

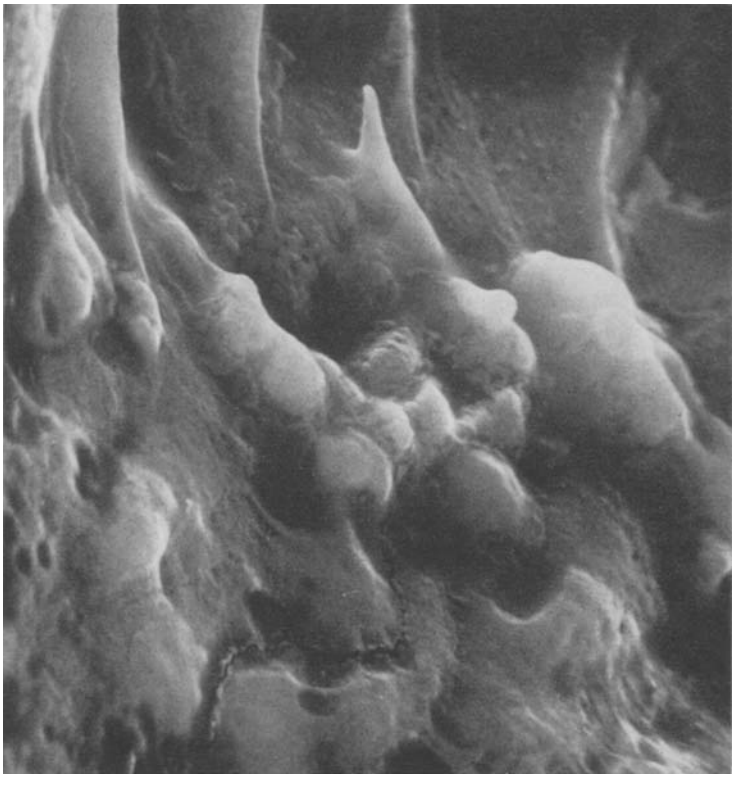

Fig. 3. Aortic intimal surface. Rabbit. Already after 15 days of hypercholesterolic diet, a heavy coat is to be seen in some areas. Erythrocytes and platelets are incorporated in it. SEM × 3000

Micrographs of ultrathin sections have been obtained with Philips EM 300 and Siemens Elmiskop 1A, operated at 60 and 75 Kv respectively.

Results

1. Ultrathin sections obtained from aortic blocks not subjected to previous Concanavalin-A treatment do not show any "coat" on the endothelial surface either in the normo- or in the hypercholesterolemic rabbits. In these animals, the endothelial cells appear swollen as by an increase in their intracellular fluid accumulating chiefly beneath their luminal surface. In the fluid, vesicles and an amorphous material are to be seen; no "endo-endothelial film" is found with conventional methods of transmission electron microscopy.

2. In ultrathin section obtained from aortic blocks subjected to Concanavalin-A treatment the findings are quite different.

a) An ultrathin (about 200 Å thick) Concanavalin-A reacting coat is evident on the endothelial aortic surface and in the pinocytotic vesicles near it in all the control guinea pigs, rats and rabbits examined.

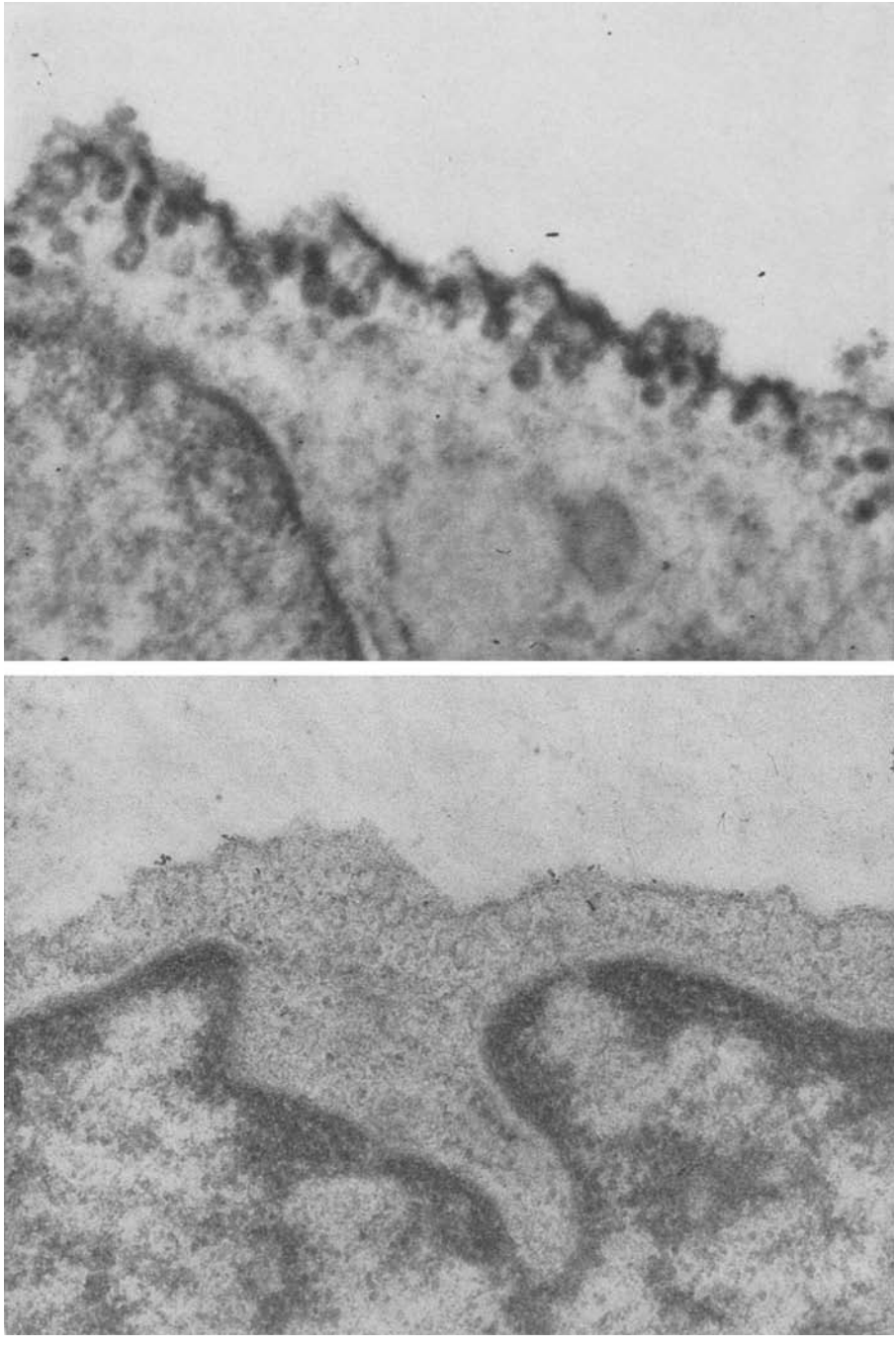

Fig. 4a. Aorta. Rabbit. Normocholesterolic diet. A carbohydrate containing coat is evident on the endothelial surface and in the pinocytotic vesicles near it (Concanavalin-A method). TEM × 61500. b Aorta. Rabbit. Normocholesterolic diet. The endothelial surface as it looks without previous Concanavalin-A treatment. TEM × 61500

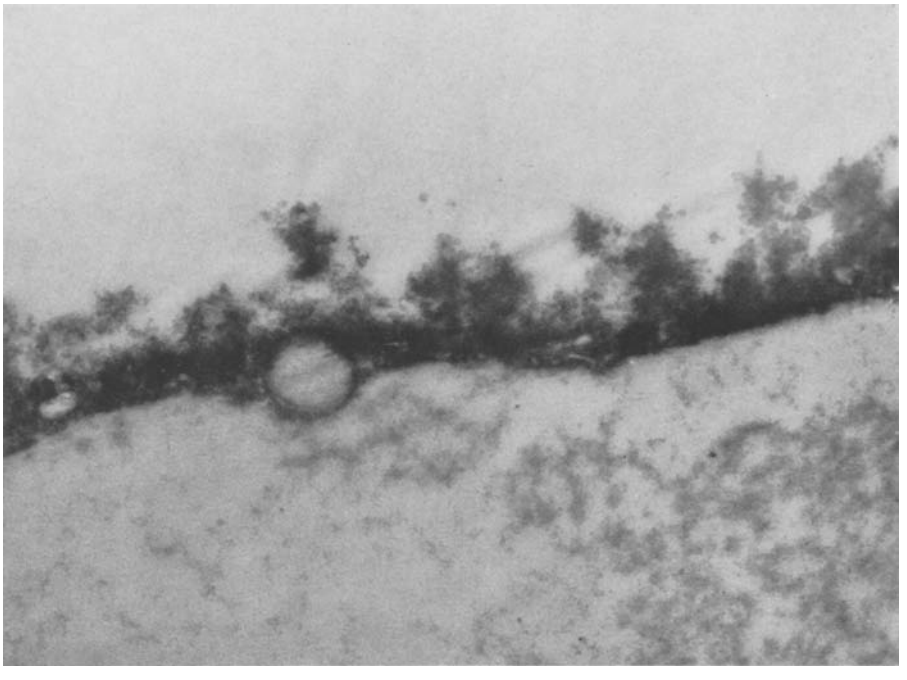

Fig. 5. Aorta. Rabbit. After 15 days of hypercholesterolic diet, in some areas the Concanavalin-A reactivity looks strongly increased. TEM × 30000

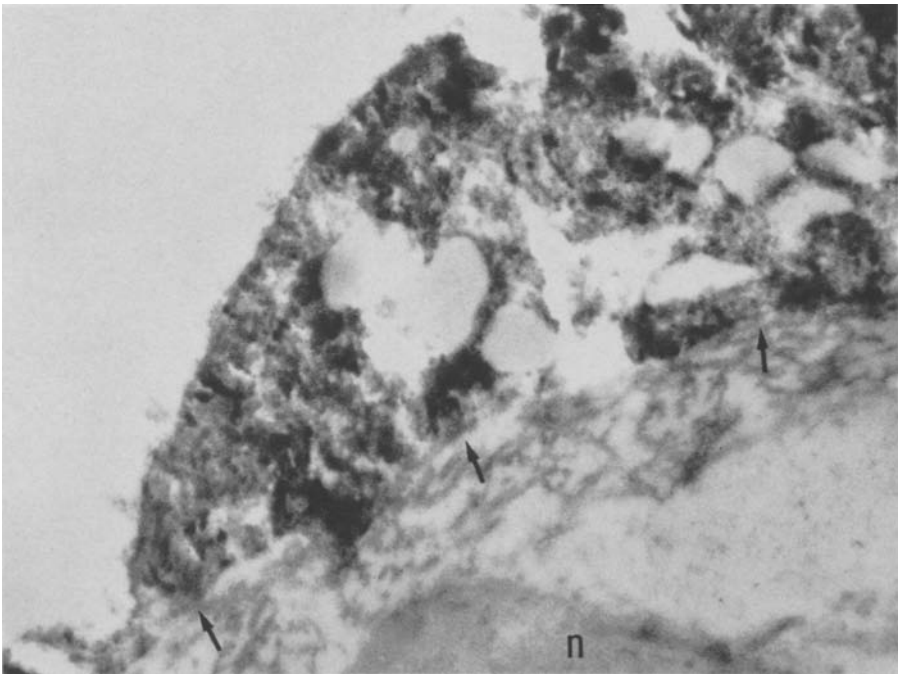

Fig. 6. Aorta. Rabbit. After 22 days of hypercholesterolic diet, on the endothelial surface, in areas where the Concanavalin-A reactivity has disappeared, a heavy amorphous deposition may be seen adhering to the endothelial surface (n = endothelial cell nucleus; the arrows show the cellular membrane, where the Concanavalin-A reactivity is lost). TEM × 30000

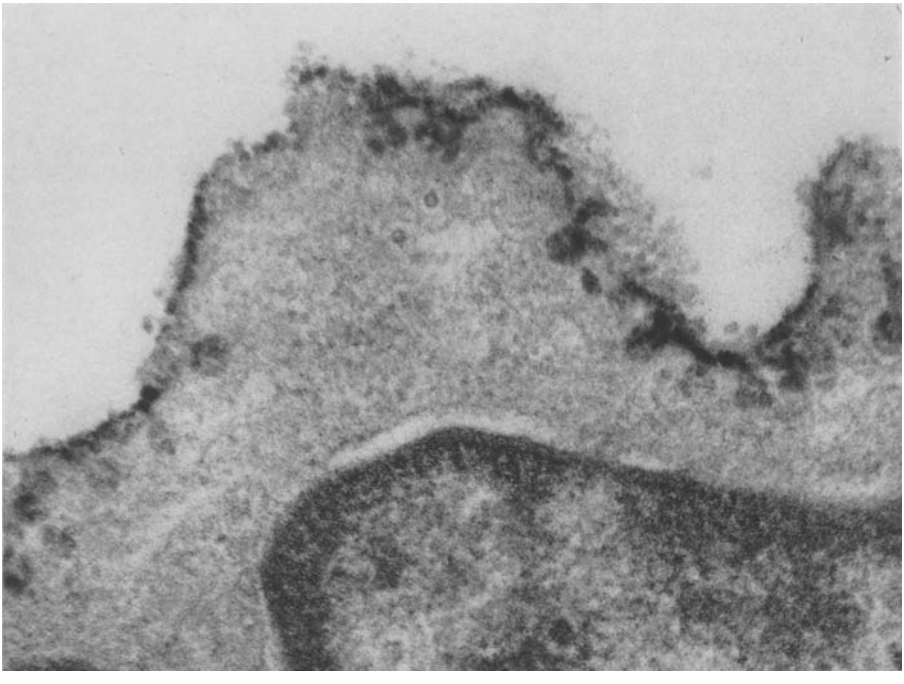

Fig. 7. Aorta. Rabbit. Even after 35 days of hypercholesterolic diet thick depositions are not evident where Concanavalin-A reactivity, though diminished, still persists. TEM × 75000

b) The Concanavalin-A reacting coat thickness is considerably increased (up to 0,2–0,6 μ) in some areas of the rabbit aortas after fifteen days of hypercholesterolic treatment. From then on, the Concanavalin-A reactivity on the free surface of the endothelial cells decreases.

c) In some areas where the endothelial-surface reactivity to Concanavalin-A disappears (on day 22nd of hypercholesterolic diet or even before) rather widely an amorphous deposition 1–2 μ thick, appears, adhering to the denuded surface of the endothelial cells. It is composed of an irregular mixture of roundish clear "globules" and of a coarse granular material, scarcely reacting with Concanavalin-A. Small monocyte-like cells are sometimes contained in it.

Comment

1. Earlier observations published by other Authors (Luft, 1966; Groniowski *et al.*, 1969; Fuchs, 1971; Cossel *et al.*, 1971) who used ruthenium red, revealed the presence of a "glycocalyx" on the capillary endothelial surface, but Copley and Scheinthal (1970) questioned the specificity of the ruthenium red reaction. An ultrathin coat containing carbohydrate has been demonstrated in our study by means of a highly specific method, the Concanavalin-A reaction, in aortas of different animal species (rabbits, guinea pigs and rats).

2. The increasing thickness of this ultrathin coat in rabbit aortas after 15 days of hypercholesterolic diet could well explain our scanning electron microscope

findings near the origin of the collateral branches, where the endothelial surface looks wrinkled.

3. The amorphous thick coat, scarcely reacting with Concanavalin-A, and lying over the endothelial surface in the areas where the Concanavalin-A reactivity has disappeared, may represent an equivalent of the veil-like deposit observed in large intimal areas by means of scanning electron microscopy. The nature of this material (as well as the real nature of the small monocyte-like cells which are sometimes found in it) must be further investigated: it looks as if it results from substances derived from the blood, possibly mixed with the ones belonging to the carbohydrate-containing coat.

The presence of a carbohydrate-containing coat on the endothelial surface in aortas of different animal species, now demonstrated by means of a highly specific ultra-histochemical reaction, could well represent the basis, as French (1971) suspected, of some functional properties of the endothelial surface, for instance of its normally being "unwettable".

The thick amorphous deposit (seen both by scanning electron microscopy and by transmission electron microscopy) which occurs in areas where the Concanavalin-A reactivity has disappeared could indirectly confirm the protective function of the carbohydrate-containing coat: the thick deposit is in fact found in areas where it has disappeared.

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