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Ruthenium red staining of blood-bubble interface in acute decompression sickness in rat

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Lehto V-P, Kantola I, Tervo T, Laitinen LA. Ruthenium red staining of blood-bubble interface in acute decompression sickness in rat. Undersea Biomed Res 1981; 8(2):101–111.—The ultrastructure of the blood-bubble interface has been studied in rats decompressed experimentally. Subsequent to staining with ruthenium red there was detected by electron microscopy a continuous envelopelike layer about 20 nm thick at the bubble-facing surface of the interface. The envelopelike structure was visualized also by concanavalin A-ferritin, a glycosyl- and mannosyl-residue–recognizing lectin coupled with an electron-dense probe, but the structure was not at all seen by the use of the conventional stains used in electron microscopy, uranyl acetate and lead citrate. No electron-dense layer was discernible on the application of only osmium tetroxide without further staining. The results indicate that material stainable by ruthenium red, and binding concanavalin A (probably a glycoprotein), is concentrated at the blood-bubble interface upon decompression. It is suggested that it plays a role in stabilization of the bubble and in the hematological alterations that are frequently observable in decompression sickness.

decompression sickness
blood-bubble interface
electron microscopy
ruthenium red
concanavalin A

The blood-bubble interface (1) plays a crucial role in the hematological alterations that are frequently encountered in acute decompression sickness (2). Electron microscopic studies have shown that thrombocytes are attached and aggregated at the interface (1, 3). Moreover, it has been postulated that the interface can trigger hematological alterations, most prominently activation of the coagulation system (2, 4). It has been considered that these derangements contribute essentially to the pathogenesis of acute decompression sickness (2, 4, 5).

In our previous electron microscopy study (6) it was possible to ascertain the gathering of thrombocytes at the blood-bubble interface and to demonstrate by means of scanning electron microscopy that the envelope that borders the bubble is structurally different from the "plasma phase" that surrounds the bubbles. Nevertheless, little has been known about structure, composition, and properties of the interface; these cannot be studied in detail solely by the application of conventional methods of staining for electron microscopy. In this study, staining with ruthenium red and with concanavalin A-ferritin (Con A-ferritin), combined with transmission electron microscopy, has been applied in the investigation of the properties of the

blood-bubble interface in rats that have been decompressed experimentally. Ruthenium red reacts with polyanionic groups (for instance, glycoproteins), and concanavalin A with glycosyl and mannosyl residues of glycoproteins and glycolipids, which are not normally visualized with the uranyl and lead salts applied conventionally for the enhancement of contrast in electron microscopy (7, 8).

MATERIALS AND METHODS

Rats

Six male Sprague-Dawley rats, weighing from 300 to 350 g, were taken from a colony in the Department of Bacteriology and Immunology, University of Helsinki.

Compression-decompression procedure

The rats were exposed for 120 min to compressed air of 5.1 bars in a Siebe Gorman hyperbaric chamber at Helsinki Naval Base. Following this, the pressure was reduced to the ambient level in 105 s (6).

Electron microscopy

Subsequent to decompression, the rats were anesthetized with ether within 2 min of their removal from the chamber and were subjected to a midline abdominal incision. The abdominal cavities of rats 1, 2, and 3 were filled with 3.5% glutaraldehyde (TAAB Laboratories, Emmer Green, Reading, U.K.) buffered with 0.1 *M* sodium cacodylate, pH 7.2, at 37°C, for 60 min. For rats, 4, 5, and 6 the same fixative, made 0.5% for ruthenium red (BDH Chemicals, Ltd, Poole, U.K.), was similarly used (7).

Preliminary fixation and staining were followed by the careful excision of samples from mesenteric veins that contained intravascular bubbles. The samples were immersed in 1% osmium tetroxide in 0.1 *M* sodium cacodylate buffer at pH 7.2 for 60 min. In addition, some specimens from rats 4, 5, and 6 were postfixed in 0.5% ruthenium red in 1% osmium tetroxide, by means of Luft's method (7). For staining with Con A-ferritin, glutaraldehyde-fixed samples were incubated with 1 mg/ml Con A-ferritin (Calbiochem, San Diego, CA) in phosphate-buffered saline at 37°C for 30 min. Thereafter the samples were washed and poststained as above.

After postfixation and dehydration in ethanol and propylene oxide, the specimens were embedded in Epon 812. Ultrathin sections were cut perpendicularly to the long axis of the vessels with an LKB ultratome (LKB Instruments, Bromma, Sweden), and studied in a JEOL 100 B electron microscope at the Department of Electron Microscopy, University of Helsinki, either without further staining, or stained with uranyl acetate and lead citrate (9).

RESULTS

Macroscopic findings

Intravascular bubbles were observed in the mesenteric and intestinal vessels in all rats. The blood was seen to froth if any major vessel was cut. Bubbling was also observed in fat tissue, particularly in the omentum.

Staining with uranyl acetate and lead citrate (rats 1, 2, 3)

Electron microscopy of mesenteric vein samples stained with uranyl acetate and lead citrate showed a continuous, faintly stained layer at the blood-bubble interface (Fig. 1). The thickness of the layer was about 10 nm (Fig. 2). Thrombocytes were found to be concentrated near the interfaces and were much less frequently found in the area between the bubbles. Aggregation of the thrombocytes was also often discernible (Fig. 1).

Staining with ruthenium red (rats 4, 5, 6)

In sections stained with ruthenium red, in which the stain was present both in the primary fixative and in postfixation solutions, a continuous envelopelike structure, more distinct and thicker than the electron-dense layer in sections stained with uranyl acetate and lead citrate, was observable at the interface (Fig. 3). Moreover, large deposits of intensively stained material could be seen, unevenly distributed, at the bubble-facing side of the envelope (Fig. 3, *arrows*). On occasion, the intensively stained envelope was seen to be partially detached from the interface (Fig. 4). The denser globules were visualized very clearly in Fig. 4 (*arrows*). Staining of a continuous layer at the interface was noted (Fig. 3, *arrowheads*). The thickness of the layer positive to ruthenium red was about 20 nm (Fig. 5). Only the globular deposits were visualized if the ruthenium red was omitted from the secondary fixative (Fig. 6). No material stainable by ruthenium red was seen in plasma, although distinct staining of the membranes of both erythrocytes and thrombocytes was observed (Fig. 3). In both of these cells a faint

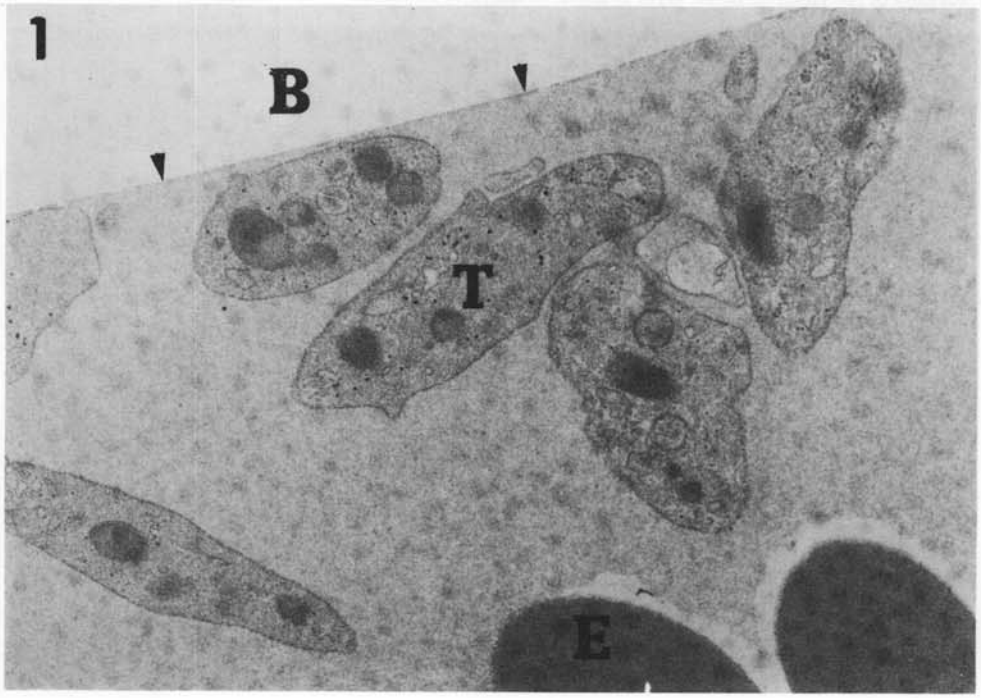

Fig. 1. Staining of the blood-bubble interface with uranyl acetate and lead citrate. A faintly stained continuous layer is discernible at the interface *(arrowheads)*. Thrombocytes are concentrated close to the interface. Aggregation of the thrombocytes has also occurred. T, thrombocytes; E, erythrocytes; B, bubble. × 24,000.

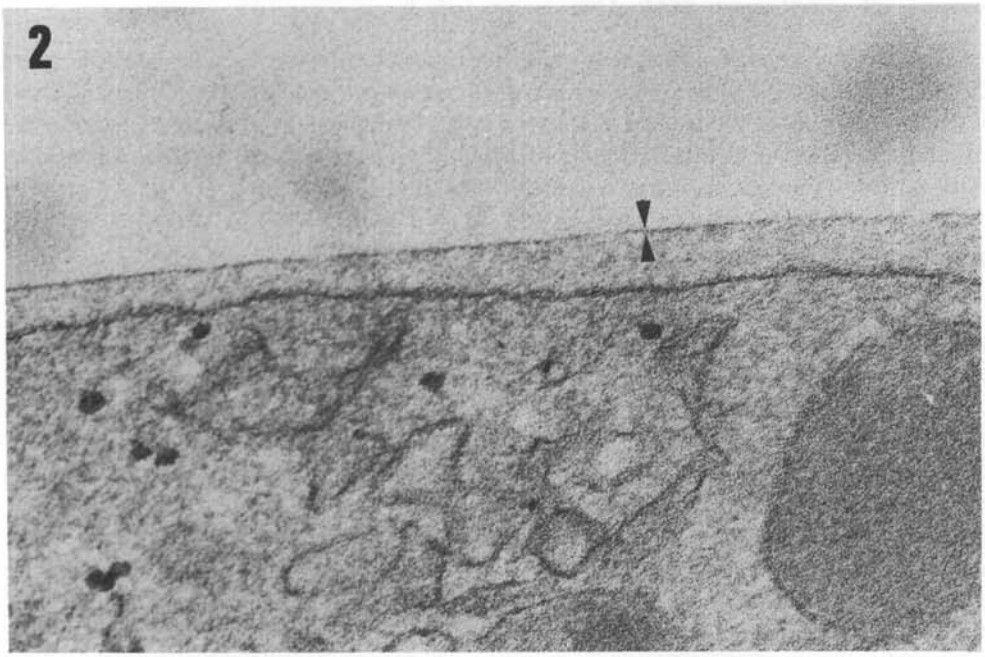

Fig. 2. The interface at a higher magnification, after staining with uranyl acetate and lead citrate. Thickness of the electron-dense layer is about 10 nm *(arrowheads)*. × 140,000.

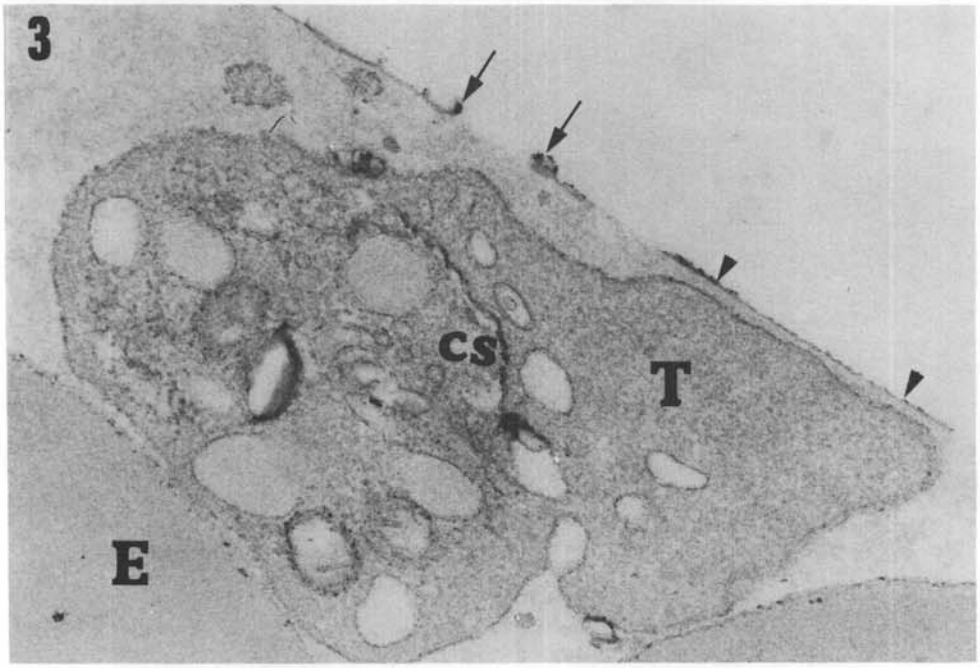

Fig. 3. Staining of the interface with ruthenium red. The stain was present in both the primary and secondary fixative; no counterstain. Note the staining of a continuous layer at the interface *(arrowhead)*. Globulelike structures are also discernible *(arrows)*. A continuous coat positive to ruthenium red is observable on thrombocytes (T) and erythrocytes (E). The staining is particularly distinct in the surface-connected canalicular system (CS). B, bubble. × 46,000.

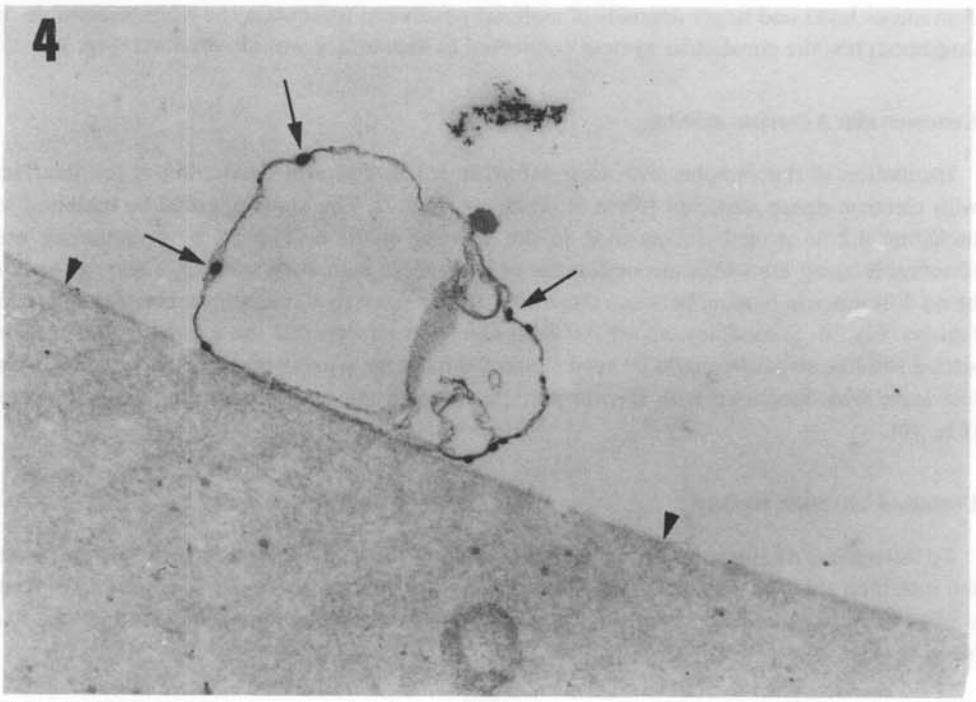

Fig. 4. Same staining as in Fig. 3. The envelopelike layer positive to ruthenium red is partially detached from the interface. Note dense globules *(arrows)* and the lack of staining in the adjacent region *(arrowheads)*. B, bubble. × 34,000.

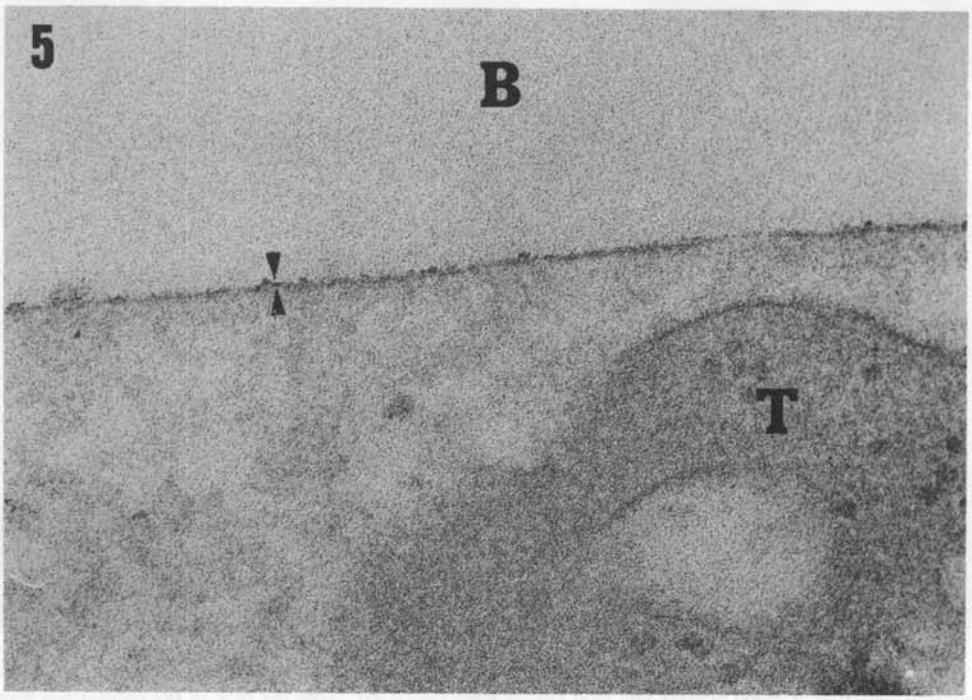

Fig. 5. Same staining as in Figs. 3 and 4. The thickness of the layer positive to ruthenium red is about 20 nm. B, bubble; T, thrombocyte. × 140,000.

continuous layer and larger deposits of material positive to ruthenium red were discernible. In thrombocytes, the canalicular system connected to the surface was also stained (Fig. 3).

Concanavalin A-ferritin staining

Incubation of the samples with Con A-ferritin led to a typical decoration of the interface with electron-dense particles 10 nm in diameter (Fig. 7). The staining could be inhibited by including 0.2 *M* α-methylmannoside in the staining solution (Fig. 8). Con A-binding was observable along the whole circumference of the bubble with even spacing. There seemed to be no difference in binding between those sites that seemed to contact thrombocytes and other regions (Fig. 9). Sometimes, an artifactual folding was observed at the interface, and at these sites a skinlike structure could be seen separated from the plasma phase. The detached, skin-like layer was decorated with ferritin particles at both the plasma- and bubble-facing sides (Fig. 10).

Unstained ultrathin sections

To determine whether the postfixation in osmium tetroxide alone was sufficient to render the interface electron positive, unstained sections (postfixed in 1% osmium tetroxide) were studied. Nevertheless, no structures were visible that resembled that indicated in Figs. 3–6 (see Fig. 11).

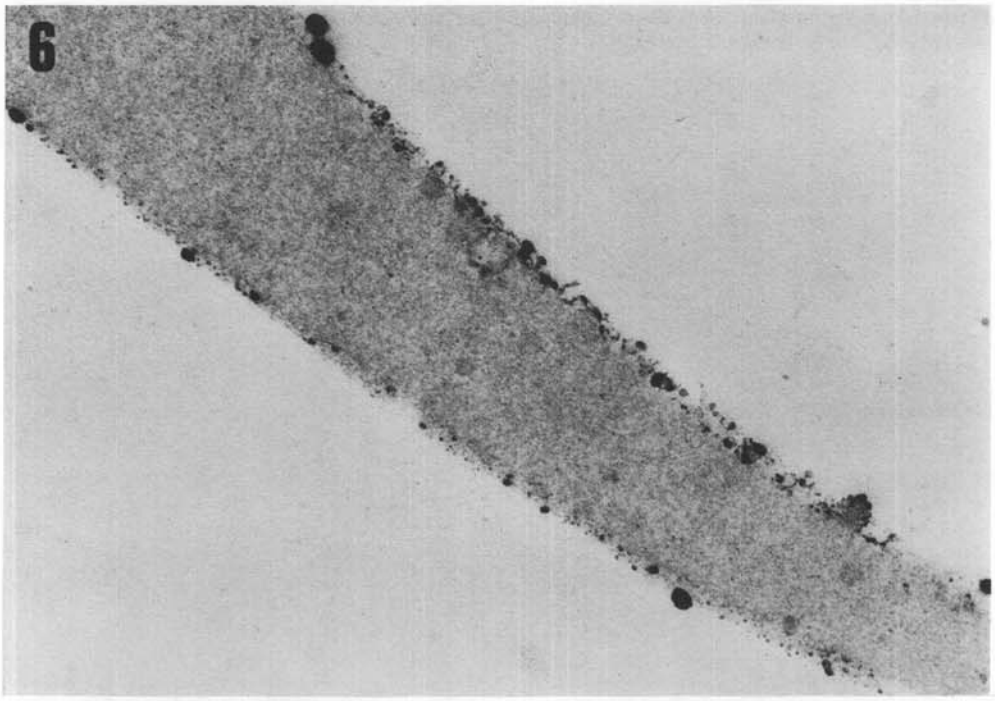

Fig. 6. The area between two bubbles. Ruthenium red was present in primary fixative but not in osmium tetroxide. Uranyl acetate and lead citrate were also used. Note the lack of the continuous layer positive to ruthenium red, but the presence of a very distinct visualization of the globular structure. × 63,000.

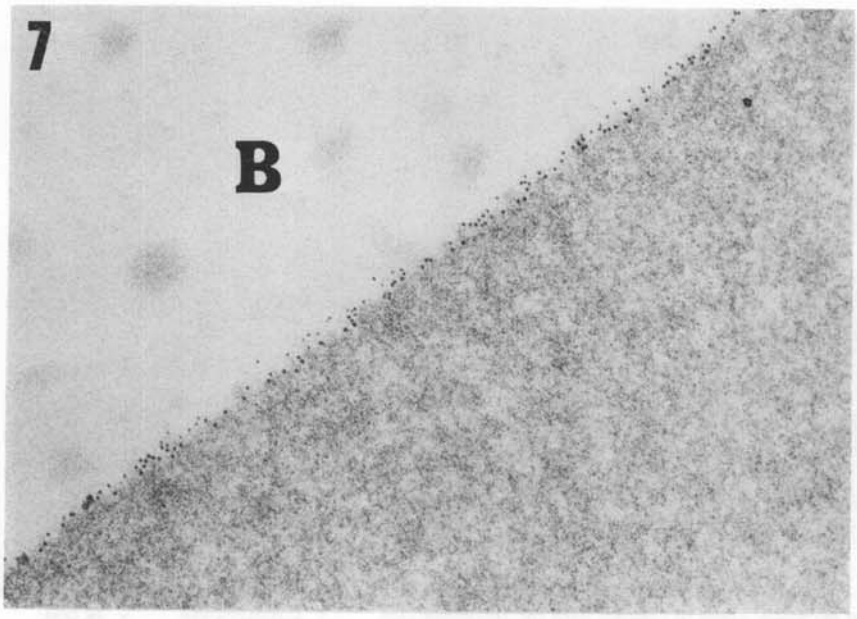

Fig. 7. Con A-ferritin staining of the interface. Note the even distribution of the ferritin particles along the interface. B, bubble. × 60,000.

DISCUSSION

A continuous layer positive to ruthenium red was observable in this study at the blood-bubble interface. Apparently, the continuous layer corresponds to the "osmiophilic" or "electron-dense" layer seen in other studies (1, 6, 10, 11) and also seen, in this study, in sections stained with uranyl acetate and lead citrate. Subsequent to ruthenium red staining, however, it was thicker and was visualized more clearly. The structural integrity of the layer positive to

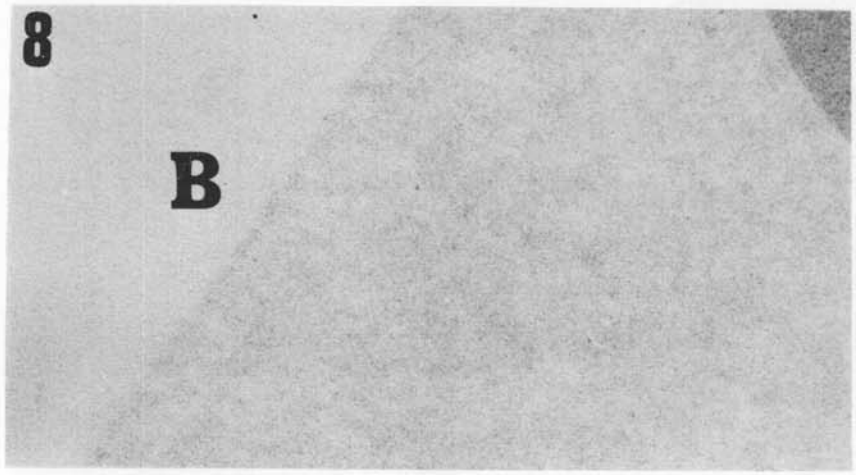

Fig. 8. Inhibition of the Con A-ferritin staining of the interface by incubating the tissue sample with Con A-ferritin in the presence of 0.2 *M* α-methylmannoside. B, bubble. × 55,000.

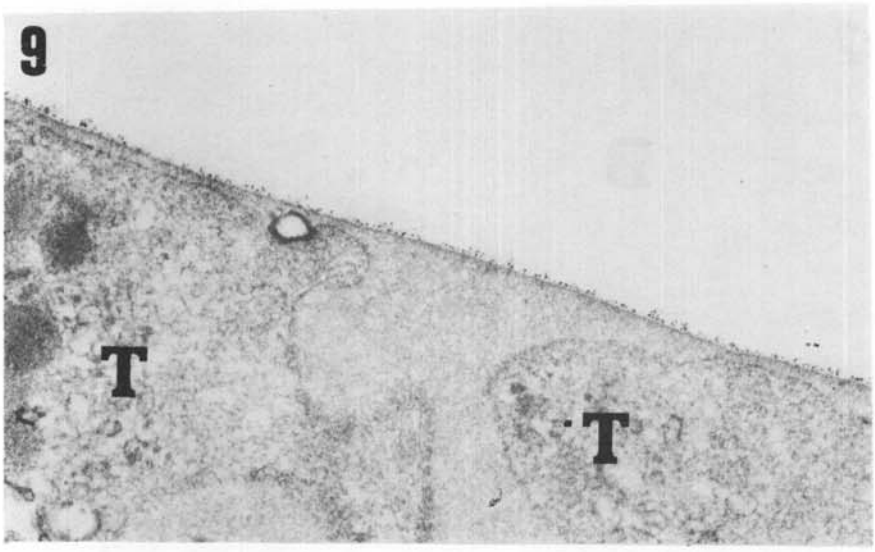

Fig. 9. Con A-ferritin staining of the interface. No clustering of the ferritin particles can be seen at areas that probably come in contact with the thrombocytes. T, thrombocyte. × 38,000.

ruthenium red was indicated when we found that it could be separated from its surroundings as a thin film. Ruthenium red staining was observed also on erythrocytes and thrombocytes in which the staining pattern was similar to that described earlier (12, 13). The nature of the globular deposits, seen when the stain was present only in the primary fixative, is not clear. It may, however, represent a nonspecific reaction, since a mixture of ruthenium red and osmium

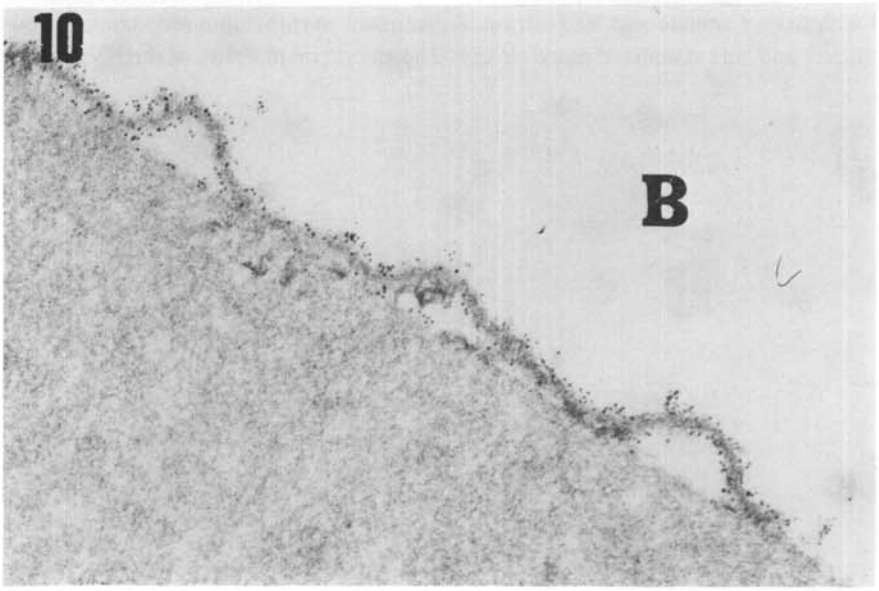

Fig. 10. Con A-ferritin staining of the interface. An artifactually folded skinlike layer is decorated with the ferritin particles of plasma and bubble-facing sides. B, bubble. × 55,000.

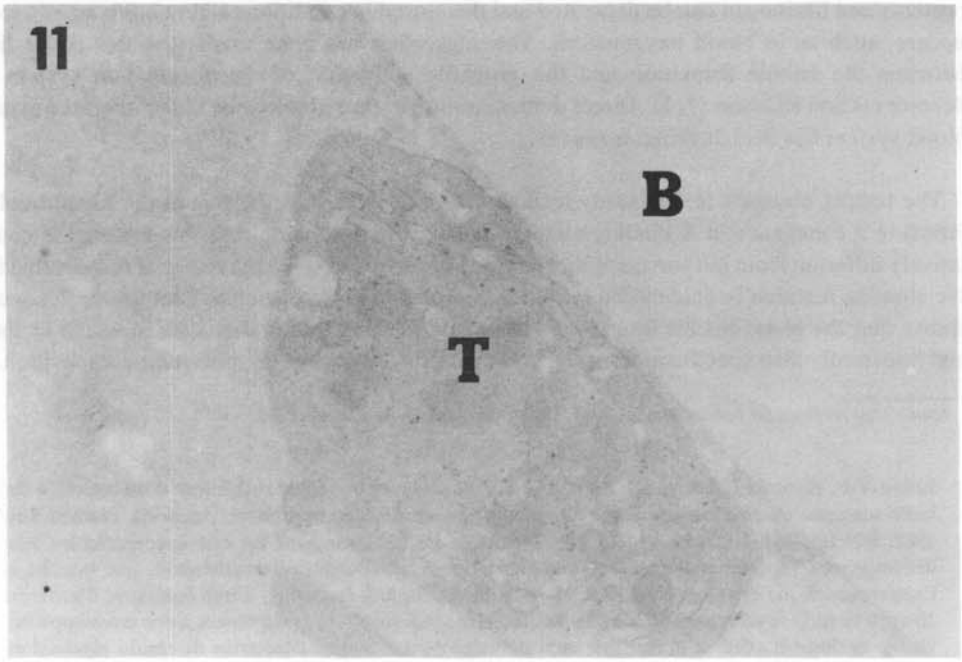

Fig. 11. Unstained section. The contrast is very low. No envelopelike or globular structures are visualized at the interface. B, bubble; T, thrombocyte. × 35,000.

tetroxide has been regarded as a prerequisite for occurrence of the specific staining reaction (7).

It has been found in both theoretical and experimental work that ruthenium red, by ionic interaction, marks a number of anionic groups such as carboxyl groups of sialic acids and amino acid residues, phosphate groups of phospholipids, and sulfate groups of glycolipids and glycosaminoglycans (7, 14, 15). That the material positive to ruthenium red is a glycoprotein is suggested by the finding that concanavalin A also is bound at the blood-bubble interface. Concanavalin A is a seed lectin that has been widely used to study the distribution of glycoproteins in both cultured cells and tissues (8). Complete inhibition of the staining reaction with α-methylmannoside (8) indicates that the lectin binds to a specific oligosaccharide at the interface and excludes the possibility of unspecific binding. Moreover, the results with Con A support the idea that there is a separate layer facing the bubble, since decoration of a skinlike structure from both sides could be seen in some bubbles.

It has been shown also in earlier electron-microscopic studies that an electron-dense layer about 20 nm thick exists at the blood-bubble interface (1, 3, 6, 11). On purely theoretical grounds, moreover, it has been predicted that stabilizing, impermeable skin exists at the gas-liquid interface (16). For the most part, however, the information concerning the composition of the interface has been obtained from experiments relating to interactions between blood components and foreign substances other than gas, such as glass, artificial organ graft material, and other plasma-solid interfaces. These studies have indicated that an electron-dense layer deposited on the foreign surface is, from a morphological aspect, similar to that observable in decompression sickness and is necessary for the subsequent deposition of thrombocytes at the surface (17). It has been suggested that fibrinogen and gamma globulins are the main components of this coating layer (10, 18). Furthermore, Lee and Hairston (19) have shown that plasma

proteins and fibrinogen can be deposited and denatured in conditions where bubbling of blood occurs, such as in blood oxygenators. The suggestion has been made that this is the link between the bubble formation and the probable activation of the coagulation system in decompression sickness (1, 2). Direct demonstration of these molecules at the interface in gas-blood system has been lacking, however.

The results obtained in this study lead to the conclusion that there is at the blood-bubble interface a concanavalin A-binding material positive to ruthenium red; this material is qualitatively different from the surrounding plasma. For the time being, the material responsible for the staining reaction is unidentifiable, but it is probably a glycoprotein. Moreover, this work shows that the blood-bubble interface is amenable to ultrastructural studies in which lectins, and apparently also specific antibodies, can be used to elucidate its molecular composition.

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Lehto V-P, Kantola I, Tervo T, Laitinen LA. Coloration en rouge de ruthénium d'un interface de bulle sanguine chez le rat lors d'une attaque chronique de décompression. Undersea Biomed Res 1981; 8(2):101–111.—L'ultra-structure de l'interface de bulle sanguine fut examinée parmi les rats décomprimés expérimentalement. Aprés coloration avec du rouge de ruthénium, une couche à l'apparence d'une enveloppe fut détectée par microscopie à électrons, d'une épaisseur d'environ 20 nm à la surface en regard de la bulle de l'interface. La structure ressemblant à une enveloppe fut visible également avec de la ferritine concanavalin-A, une lectine détectrice de résidu glycosyl et mannosyl couplée à une sonde à électrons profonde, mais la structure ne fut pas visualisée avec les tâches classiques de la microscopie à électrons, l'acétate d'uranyl et le citrate de plomb. Aucune couche épaisse en électrons fut discernable lors de la simple application de tétroxyde d'osmium sans coloration. Ces conclusions démontrent que le matériau pouvant être coloré par le rouge de ruthénium, et la liaison de concanavalin A (sans doute une glycoprotéine), se concentre dans l'interface de bulle sanguine aprés décompression. Il est proposé que ceci joue un rôle de stabilisation de la bulle et lors des changements hématologiques qui sont observés fréquemment dans la maladie de décompression.

la maladie de décompression
interface de bulle sanguine
microscopie à électrons
rouge de ruthénium
concanavalin A

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