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Ladies and Gentlemen, dear Colleagues and Friends,

It is indeed a great honour to receive the Fåhraeus Award, and I would like to thank our European Society very much for this.

I would now like to give you some insight into my experiences in the field of hemorheology and intravital microscopy over the years.

In principal, hemorheology and microcirculation are two different scientific fields of study: with the microscope we examine small objects, especially cells, as well as the construction of tissues, that means more or less stationary structures are examined microscopically. In hemorheology, on the other hand, the dynamic flowing phenomena are investigated. For this reason both fields of science have developed independently for a long time from one another. In my clinical career, however, I encountered a continuous connection between these two areas.

My clinical tutor, Norbert Henning, was a scholar of Paul Morawitz (Leipzig). Both were hematologists and gastroenterologists. Morawitz developed the classical scheme of blood coagulation and described the "agastric anaemia", the relations between blood formation and the stomach. Henning developed the endoscopy and the cytodiagnostics of gastroenterological diseases, whereas I focused on the newer optical methods like phase contrast and fluorescence microscopy.

During my internship I introduced blood coagulation diagnostics in our hospital, so I had to develop my own thromboplastin for the Quick Test. During those days this was only possible when one had good personal connections with a pathologist, as well as a "kitchen nurse". The pathologist was able to supply us with grey matter of the brain, while the "kitchen nurse" had at her disposal the only deep freezer of the hospital, which was necessary for storage. The deep freeze was necessary for maintaining the stability of the thromboplastin preparations. During this time I started to develop an interest for blood coagulation.

I realized later, that even before these early clinical contact with blood coagulation, I already had a certain connection with our teacher and founder Alfred Lewin Copley (Fig. 1). This was at the physiological institute of the University of Würzburg, where I studied medicine. This institute was headed by Edgar Wöhlisch, who in his day was an authority on coagulation physiology. A.L. Copley was a student of his, performing the coagulation tests. For these tests he required large amounts of ox

Fig. 1. Alfred L. Copley, 1910–1992.

blood which he hauled by the bucket from the nearby city abattoir. The blood had to be centrifuged, and this meant that he used the only centrifuge available in this institute for days on end, much to the dismay of his co-workers. A.L. Copley only told me about these and many other episodes, which he experienced during his fulfilled and successful life, years later.

A.L. Copley [2] was an extraordinary person, which most of us are aware of. He was not only fascinated by science but also by abstract art. As a little reminder I would like to show two of his pictures. Figure 2 is in morphological accordance with rheology and Fig. 3 shows his love for detail and colour.

Hemorheology and the vascular wall

My combined path of microscopy and blood coagulation developed after these early years. I would now like to give an overview of my own, mainly experimental work. My aim will be to show the relationships between hemorheological phenomena and vascular walls [3–5,7,10–17,19,21,23,29].

I was mainly concerned with the relationships between plasma proteins and the vessel wall. I looked for the accumulation of individual plasma proteins on the endothelium, especially fibrinogen, fibrinonectin, polypeptide from factor VIII and others; furthermore, the permeation of such proteins through the vessel wall, the influence of the clotting system on the permeability and the phenomena of adhesion of particles on the endothelium.

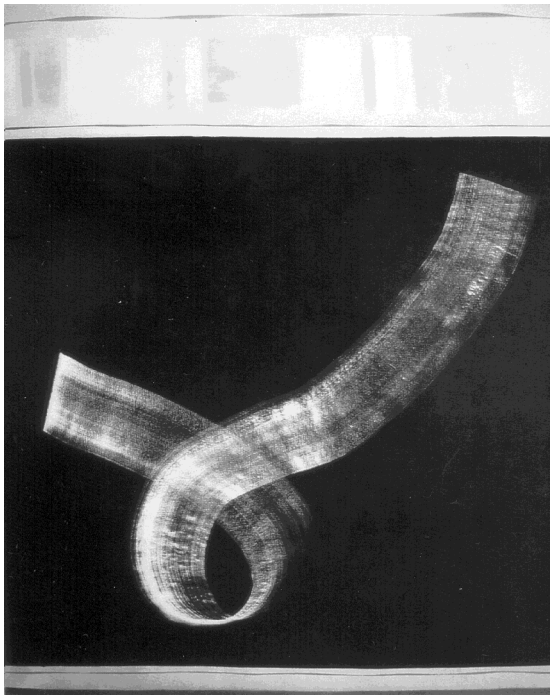


Fig. 2. L. Alcopley, A journey, 1978, 267 × 218 cm, oil on canvas. Published in [1].

Fig. 3. L. Alcopley, City life, 1955, 157 × 193 cm, oil on canvas. Published in [1].

The instrument I used was the intravital microscope and the classical experimental model was the terminal ilial loop of exposed rat mesentery. We developed various microscopic techniques, in particular, fluorescence techniques for labeling anatomical structures and functional processes, observations in transillumination and incident illumination, fluorophotometry, rapid scanning techniques, television

techniques with digital image registration and computer-assisted image processing methods to carry out automatic image analysis [8,9,12,24–26].

Since no blood constituents fluorescence spontaneously, we applied fluorescent dyes which were suitable for our special purposes. They should not be toxic and their binding capacities to plasma proteins or cell components, as well as the spectrum of their physical-optical properties, had to be known.

I do not wish to elaborate more on technical details, although they often brought about decisive advances. This applies especially to the microscopic techniques such as quantitative appraisal with TV in combination with methods of image analysis.

The perfused blood vessel

The hemorheologist will look mostly at the perfused blood vessel. The intravascularly flowing blood shows, under normal conditions, a cell-free blood plasma zone near the endothelium. The blood cells move within the central current of the blood. If we inject a suitable fluorescent dye solution intravenously, the blood plasma will be labeled. We can see the cell-free blood plasma flowing near the endothelium, most clearly in venules, and the blood cells occupy the central current under normal undisturbed flow conditions (Fig. 4).

If we apply a rapid scanning technique and put a scanning line traversing an arteriole, we obtain a bell-shaped curve of the fluorescence intensities, corresponding to the circular cross-section of the vessel. In the central flow oscillations of the fluorescence intensity or irregular flattening occurs, since in this region streaming red cells accumulate, which extinguish the plasma fluorescence (Fig. 5) [20].

The boundary region at the vascular wall can be seen clearly. We postulated that the plasma forms an immobile layer between the endothelium and the blood. If we observe this boundary zone closely, slight blurring can be detected. The reason for this is two-fold. Firstly, we have to take into account the spatial and temporal dimensions of the scanning process. Our measuring field in the objective is 1×2 micrometer and the step distance is 0.25 micrometer, the time to collect a scanning line of 200 micrometer, that means 800 fields is 2 s.

There are also physiological reasons for the blurring between blood plasma and the vessel wall, this being the diffusion of the blood plasma through the blood vessel wall. The immobile margin zone of the blood plasma stands functionally in relation to the mobile parts of the streaming blood by the processes known as diffusion and convection.

Finally and most importantly, the processes of blood permeability through the vessel wall into the perivascular tissue preclude a sharp microtopographical demarcation by this intravital fluoromicroscopy.

Before I go into detail about these processes, I would like to mention our results which we obtained regarding the affinity of plasma proteins to the vessel wall.

We fluorochromed individual plasma proteins *in vitro*, then injected them intravenously and observed their intravascular behaviour with regard to the vessel wall. Labeled albumin did not show an accumulation at the inside wall of a vessel. Gamma-globulin showed a corresponding characteristic in this regard, as well as antithrombin III and beta-lactoglobin. For gamma-globulin and alpha-2-macroglobulin, we sometimes found a weak accumulation on the inside wall of venular blood vessels.

As a noteworthy exception we observed a remarkable affinity of fibrinogen for the inside blood vessel wall. Following the intravenous infusion of FITC-labeled fibrinogen (whether from rats or from

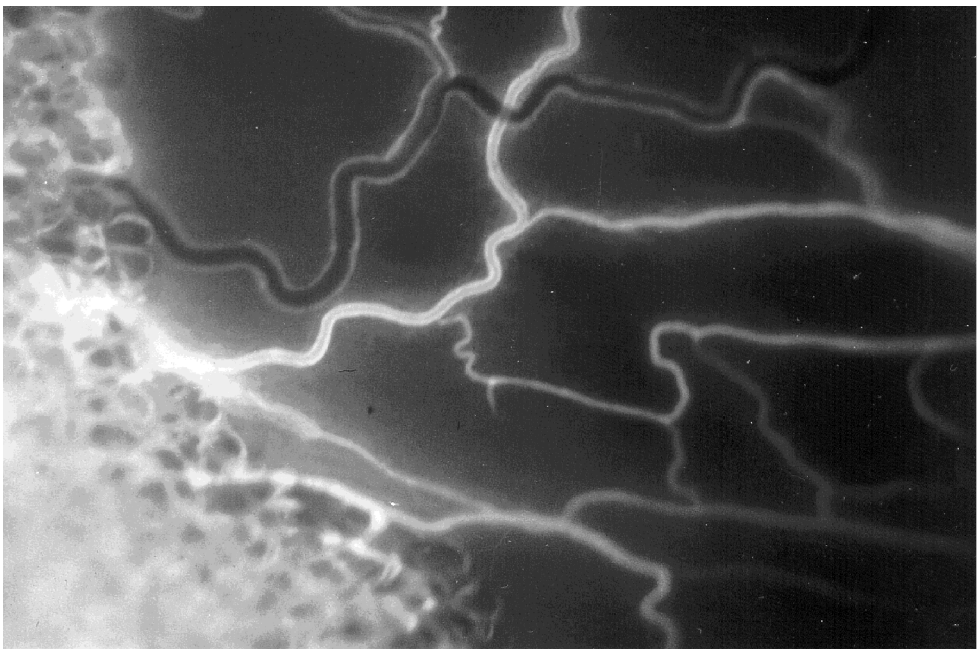


Fig. 4. Exposed rat mesentery, fluorescence microscopy. Terminal bed coming from the mesenteric fat tissue. 2 min after intravenous injection of FITC-labeled plasma proteins. Objective 6.3 : 1.

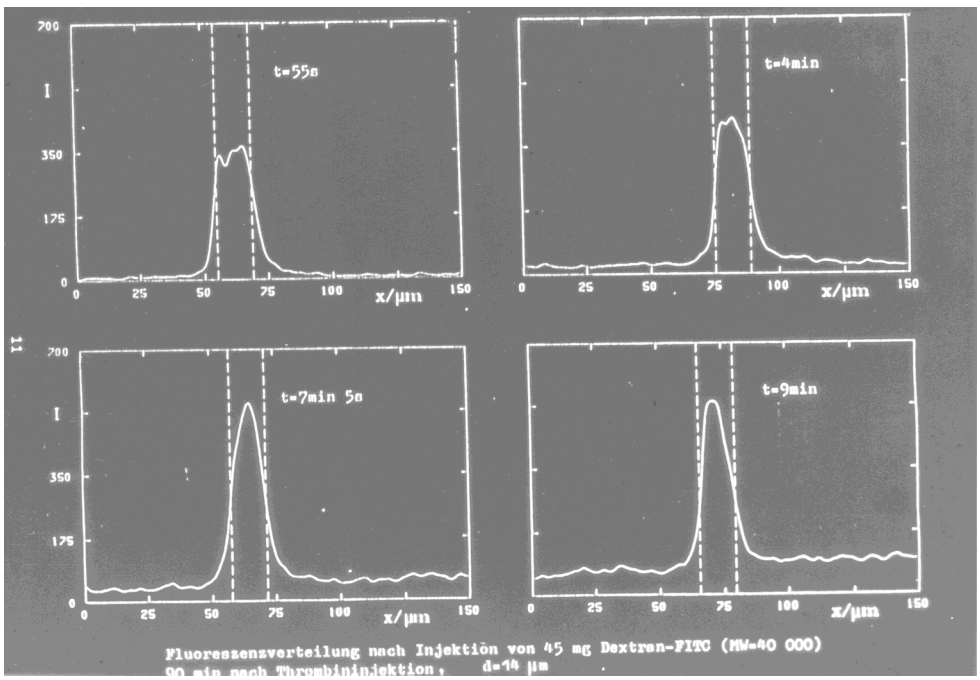


Fig. 5. Rapid line scanning technique traversing an arteriole, diameter 14 μm. Various times after intravenous injection of 45 mg Dextran-FTIC (MW 40.000)/100 g body weight. Animal was pretreated for 90 min with Thrombin-infusion; increase of microvascular permeability.

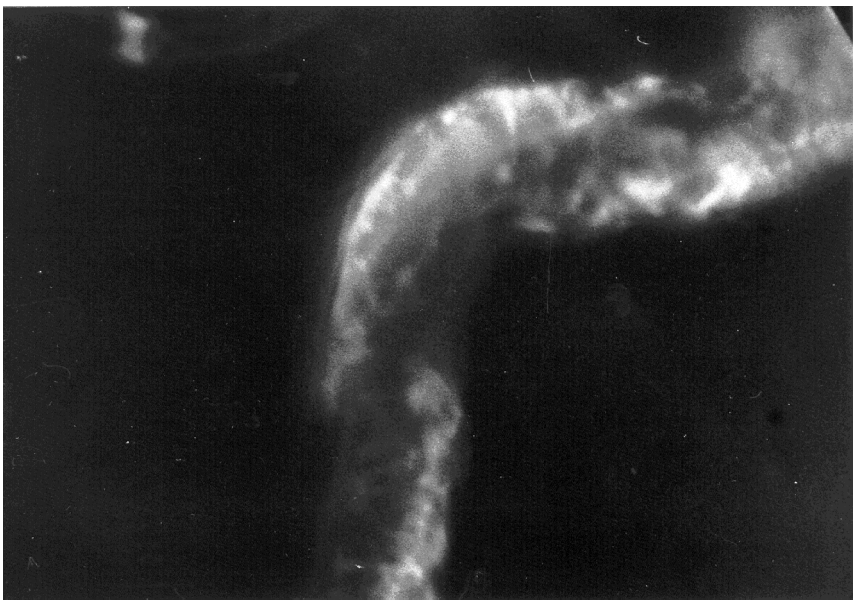


Fig. 6. Similar object as in Fig. 4. Venule 65 min after intravenous injection of FITC-labeled human fibrinogen solution. Objective 40 : 1.

humans) we can see fluorescent accumulations in the boundary zone between blood and the inside wall of blood vessels in form of granules, fine stripes and faint networks, preferentially (Fig. 6) on venules. The blood flow, however, does not change. The accumulation of the labeled proteins arises at about 5–15 min beside a diffuse fluorescence of the circulating blood plasma.

On arterioles or capillaries no, or only very occasional, granular deposits occur.

At high microscopic magnification and with a combined illumination of bright field and fluorescence, we could identify the fibrinogen accumulation on the inside wall of the venules. Reticular shapes of the labeled fibrinogen indicated, in my opinion, a preferential deposition at the interendothelial cell boundaries.

These phenomena remained constant during approximately one hour, or even increased in size, but a complete wall-paper-like lining of the marked fibrinogen did not develop.

We measured the amount of locally adherent, labeled fibrinogen by scanning microfluorometry, and found that it amounts to eight times that of the circulatory blood plasma (Fig. 7).

We attempted to modify the affinity of fibrinogen to the endothelium–plasma interface. Pre-treatment with Heparin, even in high doses, did not change the fibrinogen accumulation. When we blocked the fibrinolytic activity of the blood with tranexamic acid we found an enhanced deposition of fibrinogen. Pre-treatment with the bioflavonoids Diosmin or Hesperidin, which activate the tissue plasminogen activator, significantly reduces the fibrinogen accumulation on the venular endothelial surface.

From this, we concluded that the affinity of plasma fibrinogen to the endo-endothelial surface of venular vessels is not associated with thrombin induced clotting activity, but that the fibrinolytic system may play a part in this [22].

We performed similar experiments with fibronectin and obtained identical results. If both proteins, fibrinogen and fibronectin, are given to the same animal in 30-minute intervals, both proteins were found on the same side of the venular endothelium in the same configuration. Fibrinogen and fibronectin

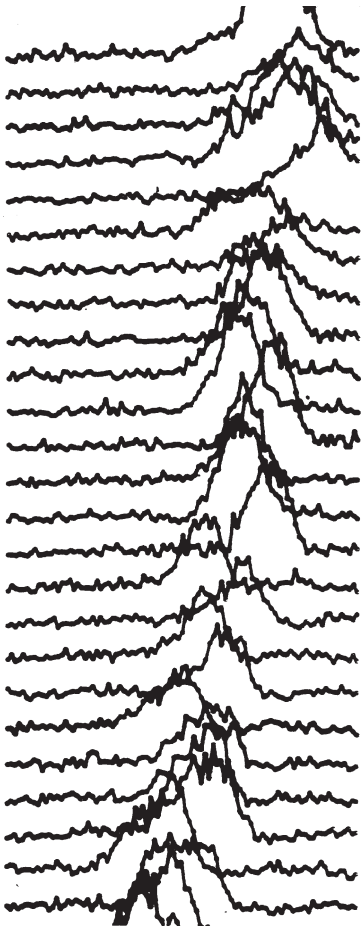


Fig. 7. Rapid scanning microfluorometry, similar object as in Fig. 6. Objective 10 : 1. Venule with great amounts of adherent labeled fibrinogen.

were labeled with different fluorescent dyes, so that each substance could be identified by its different fluorescent colour.

Another factor derived from the clotting system, a polypeptide fraction of the antihaemophilic globulin which has haemostypic effects, also accumulates on the venular endothelium, it does so more slowly and in a more continuous band-like manner than fibrinogen or fibronectin.

How should we interpret these findings?

The concept of a turnover of fibrinogen in the periphery located at receptors on the endo-endothelial surface may explain this. Such receptors may be located closer together on venular blood vessels or the turnover of fibrinogen at such venular receptors may be more rapid than in other parts of the periphery.

Protein permeability

When discussing the relation between hemorheology and the vascular system we should not forget that the plasma proteins also spread in the extravascular space as part of the interstitial fluid and the lymph. Our fluorescent microscopic studies revealed the following findings on this topic:

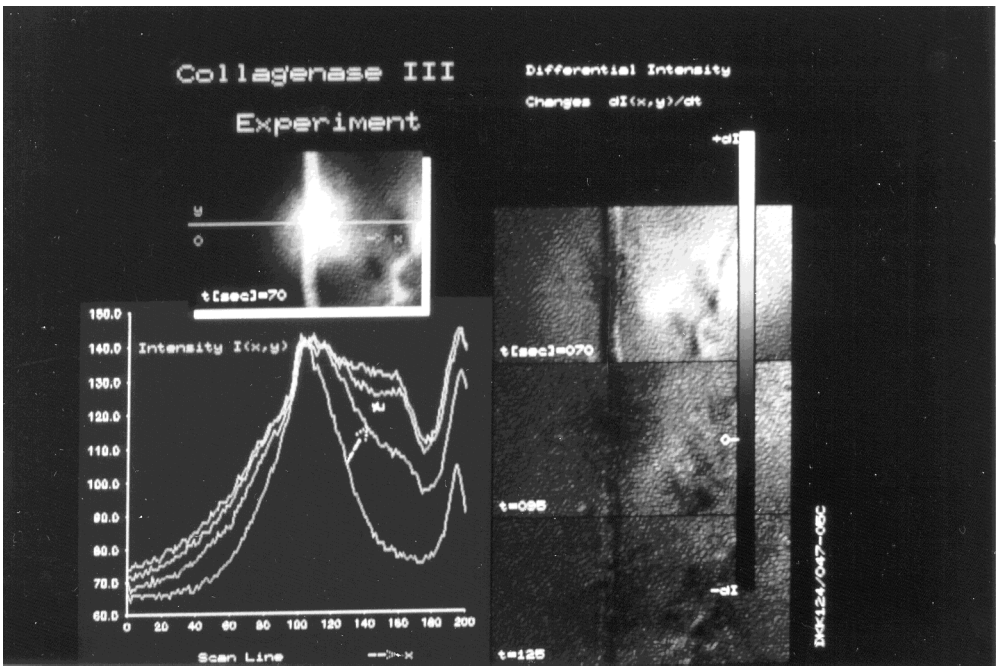


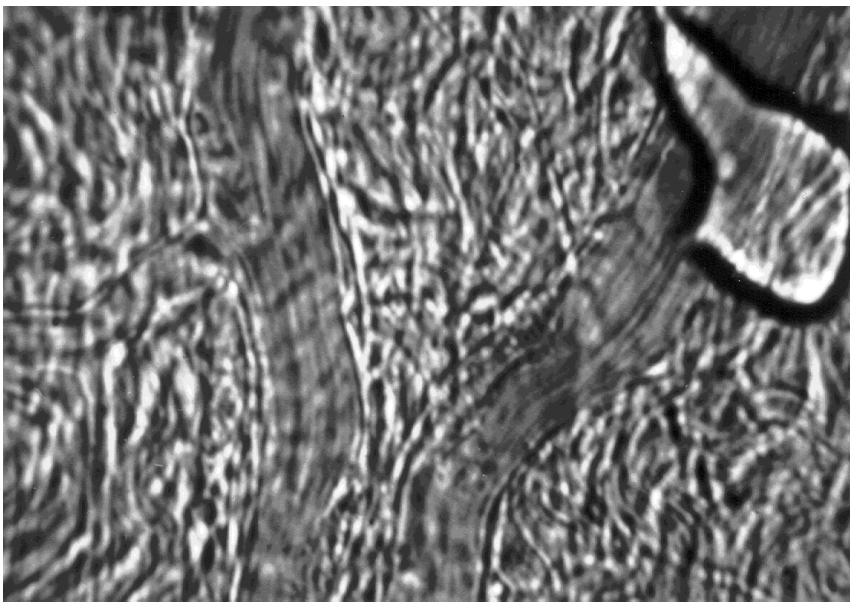
Fig. 8. The increase of plasma protein permeability after local injection of Collagenase III. Exposed rat mesentery, perivascular injection of Collagenase III at the right side of an arteriole. Scanning lines of fluorescence intensities after intravenous injection of FITC-labeled human albumin, starting with a time of 70 s up to 125 s after intravenous injection of the labeled protein. Upper left picture – scanning line at 70 s. Right picture – differential intensity changes $\mathrm{d}I(x,y)/\mathrm{d}t$ at 3 times. Note the increased protein permeability at the right side of the arteriole.

(1) Plasma proteins pass mostly through small venous vessels.
(2) The passage depends on the molecular size of the proteins.
(3) The permeation occurs at some particular location, for instance, at vessel bends and is thus probably affected by local hemorheological conditions.
(4) The blood clotting system affects the vascular permeability. A reduced blood coagulability leads to an increased passage of plasma proteins between blood and the interstitial space. The pathophysiological concepts of these correlations are greatly stimulated up to today by the idea of an endo-endothelial lining of fibrinogen at the immobile plasma layer on the vessel wall, which was developed by A.L. Copley.
(5) During the passage of proteins through the vessel wall, there is a certain accumulation of the permeating proteins in the vessel wall.
(6) Collagen appears to affect protein permeability. We found that after local application of collagenase a greatly increased albumin permeability occurred even in arterioles, which normally do not have any measurable permeation of plasma albumin (Fig. 8) [27].

Cells and the vessel wall

Finally, I would like to briefly discuss the hemorheological findings regarding the relationship of cells to the vessel wall. The endothelial cells cannot be identified even at high magnification *in situ* because of their 3-dimensional situation. We can, however, stain them *in situ* by the fluorescent dye

(a)



(b)

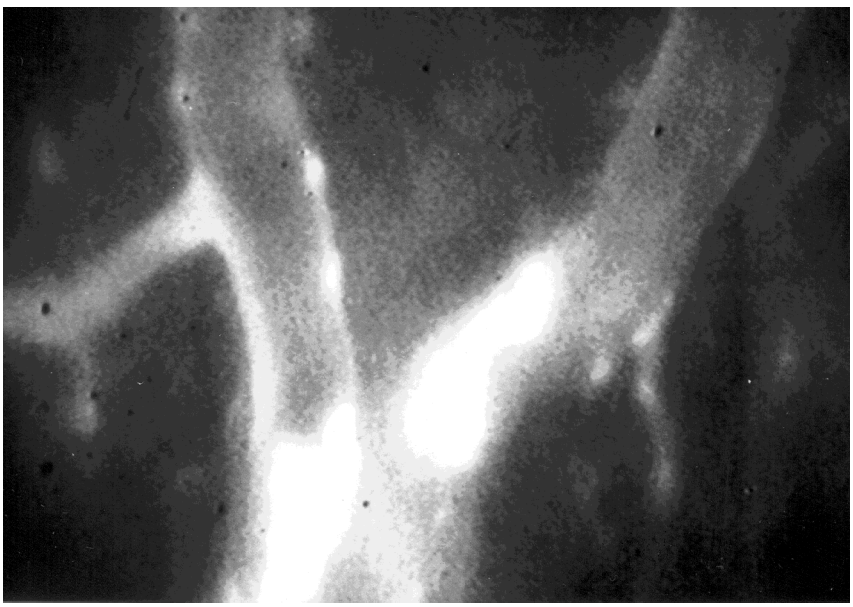


Fig. 9. Similar object as in Fig. 6. (a) Two venules, phase contrast, objective 40 : 1. (b) Same field after intravenous injection of acridine orange, see text.

acridine orange which binds to the nucleic acids of the cell nuclei (Figs 9(a) and (b)). When the dye diffuses into the tissue, it also stains cells in the perivascular tissue.

As a second method to mark cell nuclei we can use an ultraviolet microscope. Here nucleic acids are identified by their monochromatic absorption at 263 nm and staining is not necessary (Fig. 10). Proteins have a monochromatic absorbence at 280 nm, as can be seen in a lymph vessel (Fig. 11).

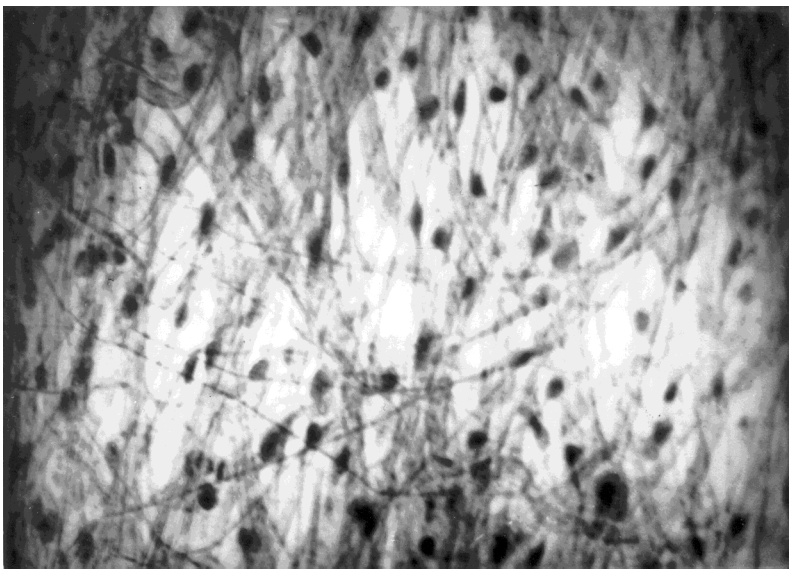


Fig. 10. Similar object as in Fig. 6. Mesentery without fat and vessels. Monochromatic ultraviolet illumination at 263 nm. Specific absorbance of nucleic acids in tissue cells. Objective ultrafluar 40×.

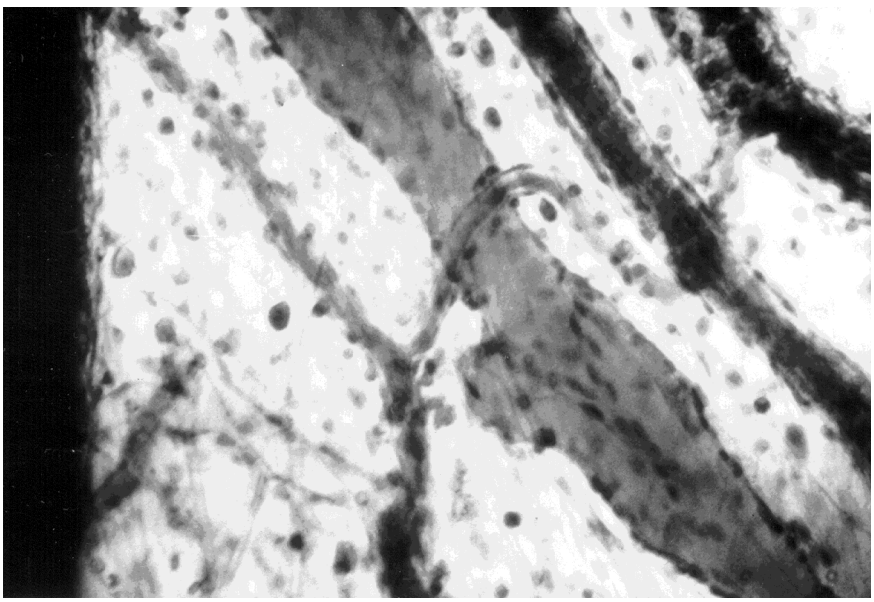


Fig. 11. Similar object as in Fig. 10. Monochromatic ultraviolet illumination at 280 nm. Specific absorbance of protein having aromatic amino acids. Objective 40 : 1. The lymph vessel in the centre of the field shows high absorbance due to its plasma protein content of the lymph fluid. Also shown are the cells of the vessel walls and of the mesenterial plate.

A circulating leukocyte in a blood vessel is of great interest to hemorheologists. The relationship of leukocytes and microcirculation is two-fold [6]. They have a tendency to flow more slowly in venules than the red blood cells do, thus coming more into contact with the vessel walls, a phenomenon which is termed “rolling” when they remain in circulation (Fig. 12). This is in contrast to the “sticking”,

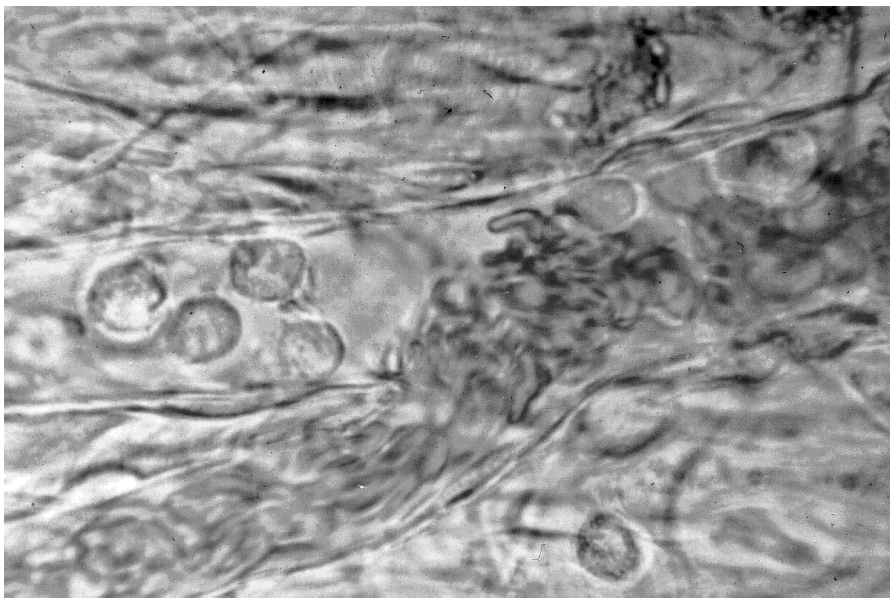


Fig. 12. Similar object as in Fig. 11. Branching of a venule with slowing of the blood stream and an accumulation of slowly rolling leukocytes. Bright field, flash illumination, objective 100 : 1.

which is the adherence to the vessel wall and likewise on venules, which remains constant over time and at the same position. These phenomena of rolling and sticking are fundamentally different from one another and are currently being researched (Figs 13 and 14).

From a hemorheological point of view two questions arise. What are the consequences of the "sticking" mechanism on blood flow and secondly what conclusions can be drawn from the sticking to an altered endothelium?

Regarding the first question – the sticking seems for me, only to draw hemorheological consequences when it occurs on a large scale. Of course, when this occurs, it is not possible to determine whether the adhesive leukocytes mechanically retard the blood flow or whether the sticking occurs due to localized damage to the vascular wall and increased vascular permeability for blood plasma and leading to localized hemoconcentration, which may also retard blood flow.

To answer the second question – is the sticking due to a changed characteristic of the leukocyte surface? – we can use "microbeads" which are carboxylated latex beads with diameters down to a minimum of 50 nm and which are marked with a fluorescent dye. They can be detected by high magnification immersion objectives and image amplifier techniques even down to a diameter of 50 nm. These beads do not show any affinity to the vessel wall under normal conditions. If they adhere to the endothelium, they label activated endothelial cells, more precisely, the endo-endothelial boundary zone of the endothelium (Fig. 15).

The microbeads may also mark gaps in the endothelium in cases where they spread sub-endothelially in the vessel wall (Fig. 16).

By using these new techniques we are able to study the relationships between blood flow and the vessel wall in a differentiated manner in pathophysiology, and even more importantly, in pharmacology.

The hemorheological phenomena experienced a marked expansion through the much researched and described area of *Perihemorheology* by A.L. Copley ten years ago. Some facts and perspectives

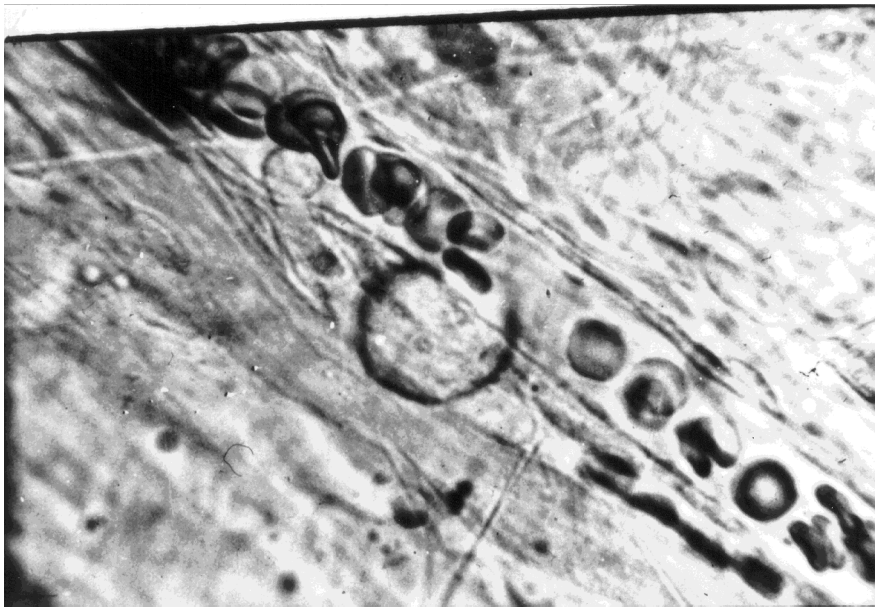


Fig. 13. Similar object as in Fig. 12. A leukocyte is sticking at an entrance of a capillary. Bright field, flash illumination, objective 100 : 1.

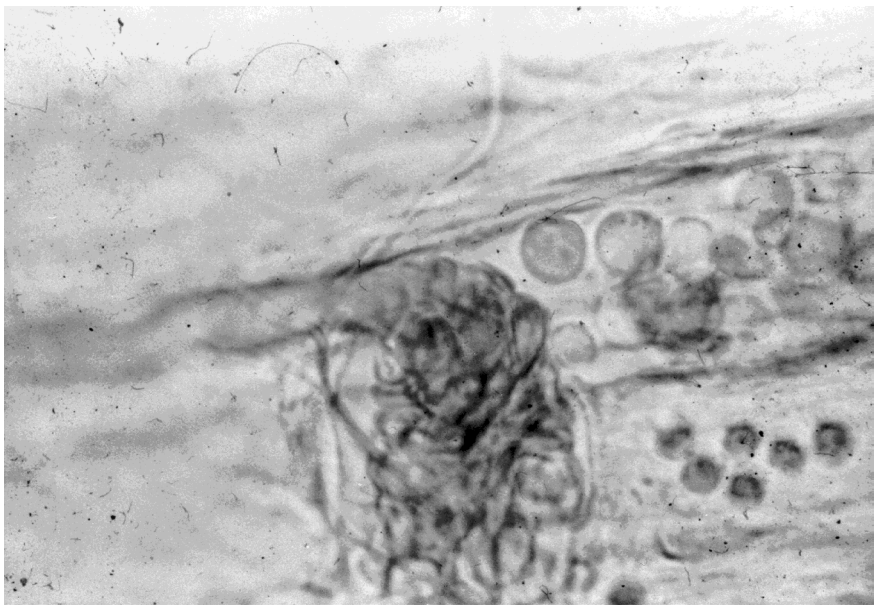


Fig. 14. Similar object as in Fig. 12. Branching of a venule. Standstill of the right branch with leukocytes, causing deviation of the streaming red cells into the left branch. Bright field, flash illumination, objective 100 : 1.

of perihemorheology were elucidated by myself five years ago [18]. These I divided into three categories: permeation of the blood vessel wall, transport within the interstitial space, and lymph formation and transport. These areas have over the last 5–8 years become more accessible to experimental and clinical research. This has lead to some very interesting new findings. Unfortunately, due to time

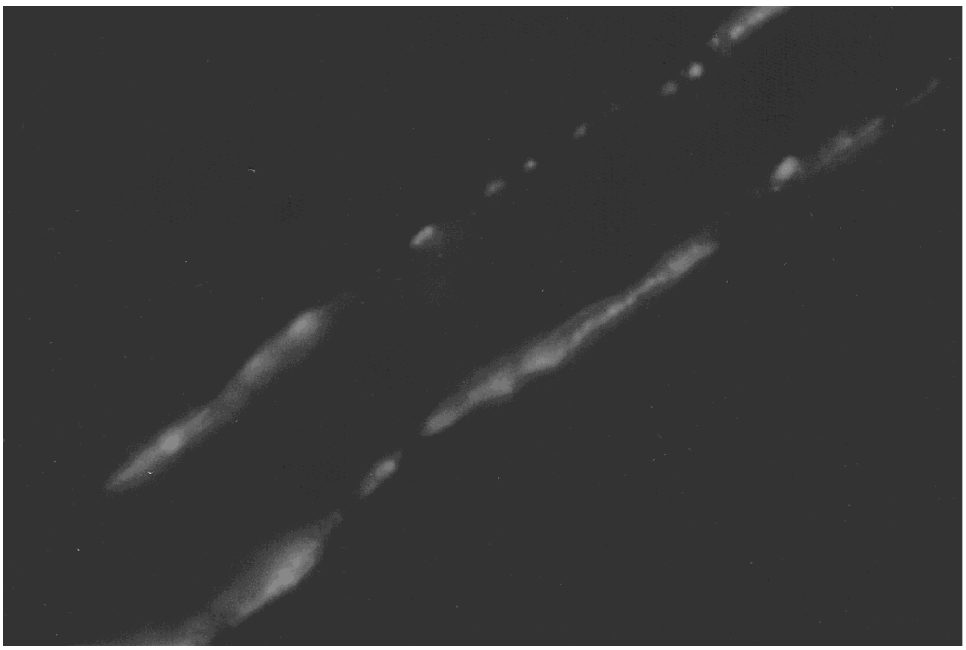


Fig. 15. Exposed rat mesentery, venule after intravenous injection of FITC-labeled microbeads (mean diameter 60 nm). Local application of fMLP (N-formyl-methionyl-leucyl-phenyl-alanin) 10^{-5} mol/ml 1 min. Blue violet fluorescence combined with a dampened bright field illumination. Objective 40 : 1, n.A. 1.2. TV intensifier technique, digitalized images obtained by an exposure time of 50 ms.

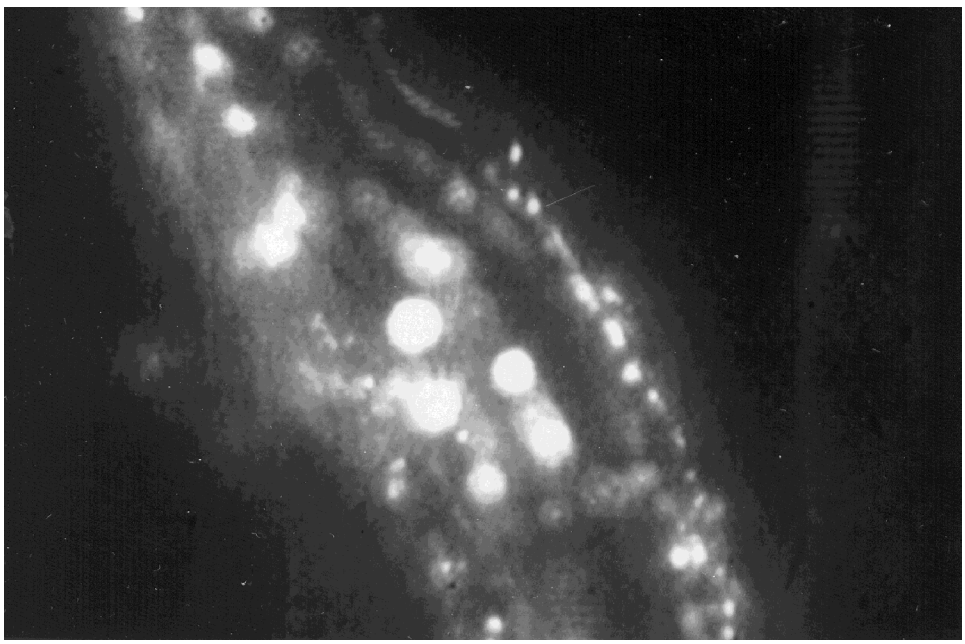


Fig. 16. Same technique as in Fig. 15. Local application of nfMLP and Thrombin 13.5 NIHE/ml. Blue violet fluorescence, objective 63 : 1. Increased adherence of the microbeads at the endo-endothelial boundary zone and spreading sub-endothelially in the vessel wall.

constraints, I will not be able to elaborate on these further. However, I hope that our journal with its new expanded title *Clinical Hemorheology and Microcirculation* will soon be receiving relevant articles, thus validating our hitherto existing subtitle "Blood flow, vessel wall, interstitium, transport and interactions".

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