CLINICAL HEMORHEOLOGY, Vol. 1, pp. 9-72, 1981
0271-5198/81/010009-63$02.00/0 Printed in the USA.

HEMORHEOLOGICAL ASPECTS OF THE ENDOENDOTHELIAL FIBRIN LINING AND OF FIBRINOGEN GEL CLOTTING. THEIR IMPORTANCE IN PHYSIOLOGY AND PATHOLOGICAL CONDITIONS

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CONTENTS

I. Introduction

The overview deals with the author's concepts of the endoendothelial fibrin lining (EEFL) and of thrombogenesis due to fibrinogen gel clotting without thrombin participation. They are based in great part on in vivo and extra vivum studies by Copley and associates during the past thirty years. Hemorheological and other biological aspects concerning these concepts play important roles in health and disease.

The concept of the EEFL of the blood vessel wall was first proposed by the author in 1953 (1,2) and developed by him ever since (3-10). He related the EEFL to homeostasis between steady fibrin formation and continuous fibrinolysis. These homeostatic processes have been known for many years to occur in the circulating blood. However, their localization in close proximity to the vessel wall is essential in the concept of the EEFL. As was postulated by the author, both homeostatic processes of fibrin formation or so-called 'fibrination' and of fibrinolysis occur in the more or less immobile portion of the plasmatic zone, undisturbed by the flow of blood.

In 1971, the author published the concept of in vivo intravascular fibrinogen gel clotting (10-15), which is related to the concept of the EEFL. This concept of fibrinogen clotting without thrombin activation concerns the initiation of thrombus formation, both for the genesis of thrombosis and for the initiation of hemostasis, i.e., the arrest of hemorrhage in minute or capillary blood vessels.

This overview is particularly long, as it provides detailed information to a number of ramifications, which grew out from the postulation of the author's two concepts. They stimulated hemorheological and other biomedical research, resulting in findings of new phenomena which are discussed in some detail. These concepts led also to the postulation of other concepts of unexpected diversity pertaining to physiology and to pathological conditions, such as, among others, vascular patency, conformational changes of fibrinogen due to external forces (Blombäck-Copley hypothesis), ecchymosis, pathogenesis of decompression sickness, cancer therapy, and atherogenesis.

The following remarks were made in the introduction of a plenary lecture, given by the author, at the European Symposium 'Hemorheology and Diseases', held in October 1979 at Nancy, France (16). "Many years ago, Sir Howard Walter Florey, in a discussion with the author, was highly sceptical about the existence of the proposed EEFL. A few years later, however, he changed his mind entirely on the physiological presence of the EEFL. This was in 1963 in his Shapey-Schafer Memorial Lecture (17) which possibly was his last presentation. Florey made the following statement in this lecture about the 'theory that there may be a layer of protein molecules on the luminal surface of the small vessels. Some investigators go further and suggest that fibrin is being formed continuously at this site and equally continuously being removed by a plasma enzyme which is a fibrinolysin. This proposition is not so fantas-

tic as at first sight it may seem, for it can be shown that endothelial cells do , in fact, liberate something which is capable of activating plasmin in plasma to form fibrinolysin'. Eight years ago Florey was no longer alive, as otherwise he might also have found the author's theory of the initiation of thrombosis by the gel clotting of fibrinogen, a process of adsorption, layer upon layer, without the action of thrombin, (to put it in Florey's words) 'not so fantastic as at first sight it may seem'."

The predictions, suggested by the above cited remarks, have been superseded by the publication of direct evidence provided by Witte (18,19) for the validity of the author's concepts of the EEFL and of thrombogenesis.

II. The Endoendothelial Fibrin Lining (EEFL)

1. The blood capillary

The term "blood capillary" is defined similar to Landis (20) as any minute blood vessel from 5 to 20 μ or more in diameter, because no morphologic criteria exist for establishing precise boundaries between the capillaries and the smallest arterioles and venules (21). A number of authors are not aware of the above definition of the term "capillary", which they consider to be synonymous with the so-called "true capillary" of Chambers and Zweifach (22). However, Chambers (23) never committed this error and in conversations with the author thought that such interchangeable usage will lead to confusion. Since the word 'blood capillary' was always used as a generic term for minute blood vessels, there is no need to use the word 'microvessels' instead of capillaries. Only after a particular capillary blood vessel can be well characterized, the author suggested (13) that it should no longer be referred to as capillary or microvessel, but given the proper term in accordance with the nomenclature introduced by Chambers and Zweifach in 1944 (22).

2. Hemorheological studies of the plasmatic zone

Copley and Staple (24), who made in vivo hemorheological studies of the plasmatic zone in the microcirculation of Chinese and Syrian hamsters, found in 1958 suggestive evidence for the existence of an immobile layer of plasma (Fig. 1) next to the vessel wall (24,25) first postulated by Poiseuille in 1839 (26). **We suggested that in this relatively immobile layer physiological processes could take place undisturbed by the flow of blood, such as the exchanges between the blood and the tissues across the vessel wall.**

The localization of the EEFL in close proximity to the endothelial cells, shown in Fig. 1, is based on the more or less immobile portion of the plasmatic zone. This portion would permit the production and maintenance of the EEFL.

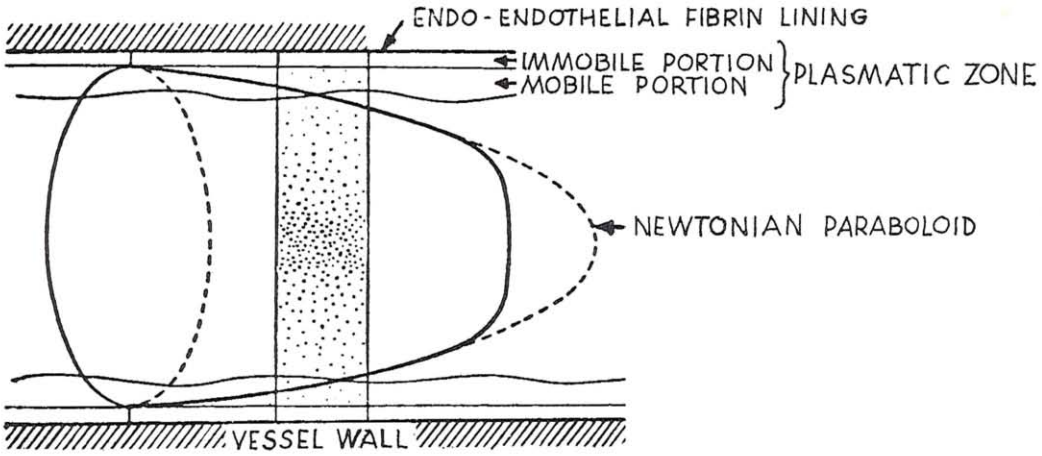

Fig. 1. Diagrammatic figure of extrusion of blood.

A drawing of the plasmatic zone by Poiseuille, published in 1839 (26), pertaining to his studies on the plasmatic zone is shown in Fig. 2.

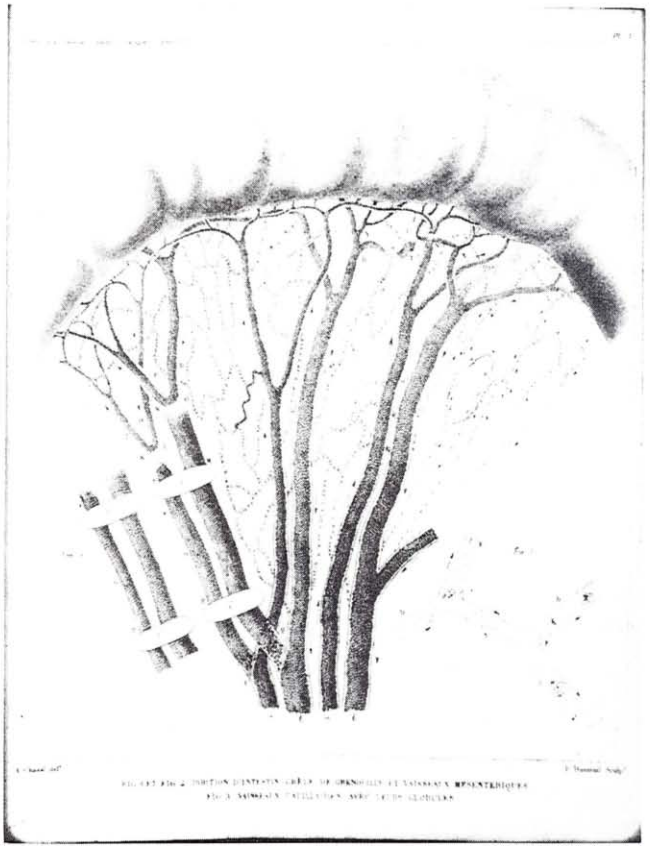

Fig. 2 Reproduction of figures in the treatise of Poiseuille, published in 1839 (26).

3. Homeostasis of endoendothelial fibrination and fibrinolysis.

Originally, in 1953, the author postulated that fibrin in submicroscopic dimensions lines the inner aspect of the vessel wall in direct contact with the endothelium (1, 2). He considered this fibrin layer to occur close to the vessel wall and to be controlled by homeostasis of continuous fibrinolysis and of steady fibrin formation at this site. This homeostatic equilibrium is pictured diagrammatically in Fig. 3.

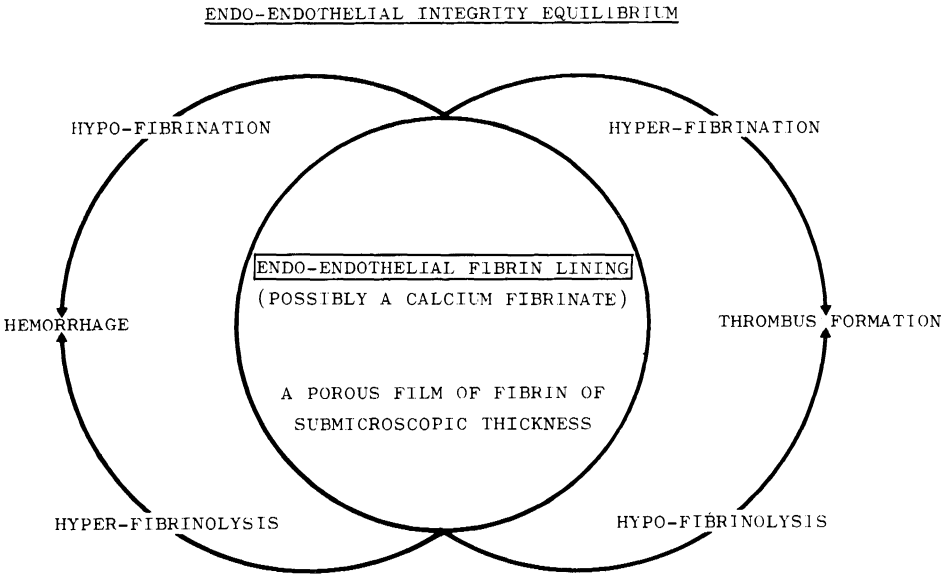

Fig. 3. Homeostasis of endoendothelial fibrination (formation and deposition of fibrin) and fibrinolysis continuously occurring close to the endothelial cells, in the immobile portion of the plasmatic zone in all blood vessels.

4. The endoendothelial fibrin lining as an integral part of the microstructure of the blood capillary wall

The structures associated with the EEFL are the diaphragms of plasmalemmal vesicles and the basement membrane. According to the author's concept both the endoendothelial fibrin lining and the basement membrane contain fibrin in different forms which he named "cement fibrin".

A diagram illustrating the layers of the capillary wall is presented in Fig. 4.

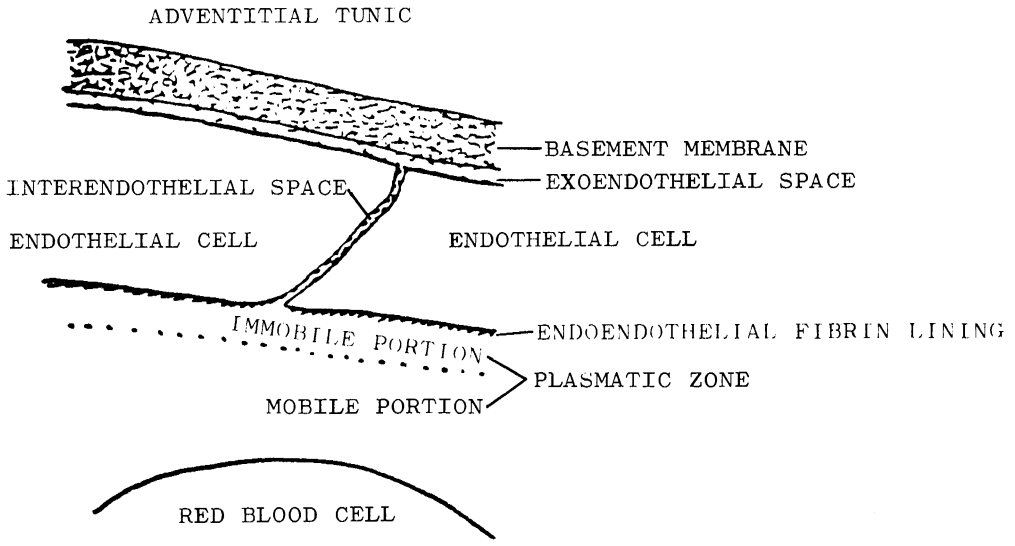

Fig. 4. Diagram illustrating the layers of the blood capillary wall and the location of the cement fibrin in the basement membrane and surrounding the endothelial cells (including the endoendothelial fibrin lining) in accordance with Copley's concept. The drawing is meant to convey a general idea without giving scale or dimensions and without simulating the appearance of elements in the microstructure of the vessel wall.

Until 1964 the existence of a layer, lining the interior of blood vessels, has been denied by most electron microscopists. However, Luft, using the new technique of ruthenium red staining, reported in 1964, that in fenestrated endothelium the fenestrae are closed by a thin, delicate layer or diaphragm and showed that the endothelial cells are covered by a thin, irregular layer of material that can be stained with ruthenium red (27). He found that ruthenium red stain binds and thus precipitates a wide variety of acid polymers (e.g. heparin and chondroitin sulfate) and it fails to react with some proteins (e.g. insulin and fibrinogen), but he was able to show later that ruthenium red will interact with fibrin clots (28). Thus, Luft could not exclude fibrin as the principal component of the coating of the endothelial cells. Nevertheless, he concluded that, since ruthenium red is thought to be specific for anionic grouping, it must have reacted only with the anionic polysaccharide in the endocapillary layer. Luft's findings led Copley and Scheinthal (29) to investigate the ability of ruthenium red to react with various proteins and anionic polysaccharides, known to exist in the blood, the vessel wall and its surrounding connective tissue matrix. Commercial ruthenium red was further purified to free it from ruthenium violet.

From the data presented by us in 1970 (29), it is evident that ruthenium red will react equally well with fibrinogen and with fibrin. These findings demonstrate that Luft's conclusion can no longer be sustained, that ruthenium red must have reacted in the endo-capillary layer solely with polysaccharides. These findings strengthen Copley's suggestion that ruthenium red may well react in great measure with the fibrin component, contained in the endoendothelial layer. We also found that ruthenium red reacts with proteins not containing carbohydrates (29). Thus, the carbohydrate moiety of fibrinogen or fibrin may not necessarily be responsible for this reaction. However, since Copley found that fibrin constitutes a highly reactive surface, and that fibrinogen, fibrinolysin and other proteins can readily be adsorbed or "anchored" to the cement fibrin, which he proposed, carbohydrates may likewise be adsorbed (29).

With uranyl acetate staining, Palade and Bruns found in 1964 similar diaphragms closing the fenestrae of the endothelium of visceral capillaries and the opening of endothelial vesicles in visceral as well as in muscle capillaries (30). Palade postulated in 1968 that these diaphragms are either part of a continuous, independent layer, supposedly made up of protein which covers the cell membrane of the endothelium on both the lumenal and tissue sites, or layers of cell membrane itself, left behind by a process of cell membrane-vesicle membrane fusion (31).

The importance of calcium ions in the regulation of capillary permeability has been recognized for a very long time. Chambers and Zweifach proposed in 1944 that the cement substance which binds the endothelial cells together is a calcium proteinate, i.e., a calcium protein complex (22). Copley considered the endoendothelial fibrin lining to be a calcium fibrin complex which he designated, as shown in Fig. 4, as a calcium fibrinate (4). In 1969 Clementi and Palade reported their studies on structural effects of EDTA (the sodium salt of ethylenediamine tetraacetate) on intestinal mucosa in the rat, and found removal of fenestral apertures (32). Bruns and Palade stated that this "implies that Ca^{++} are necessary either for binding together the components of the aperture, presumably proteins and polysaccharides, into a thin film or for anchoring the latter in the fenestral frame or both" (33).

On the basis of extra vivum findings, the author pictures the EEFL to contain a more or less contiguous porous film of fibrin, while other parts have fibrin deposits, interspersed with other substances (5). He concluded that even patchy fibrin deposits would exert some anticoagulant action.

5. Cement fibrin

The author ascribed an entirely new significance to the physiological role of fibrin in submicroscopic dimensions, which he proposed to contribute to the integrity of the wall of the capillary blood vessels. Thus, the presence of typical and atypical fibrins, comprising what he named "cement fibrin", being formed

steadily in close proximity to the endothelial cell and in the basement membrane, is considered as a physiological manifestation (5,13).

Typical fibrins include: 1 - fibrin monomers and 2 - fibrin in different forms of polymerization, the filaments of which can be recognized by the characteristic transverse striation with a periodicity of 22 Å. When fibrinogen is converted to fibrin monomer by the enzymatic action of thrombin, there are two fibrinopeptides split off. The A peptide (M.W. 1600) is released rapidly and the B peptide (M.W. 1400) is released slowly. Atypical fibrins include breakdown particles of fibrin due to fibrinolysis. A diagram illustrating the layers of the blood capillary wall is presented in Fig. 4. The pools of granular material, which Majno and Palade observed in the exoendothelial space, between the endothelial cells and the basement membrane, in their electron microscopic studies of capillary permeability (34), might well represent such fragmented fibrin. It may be also, as Majno and Palade suggested in 1961, fibrin at an earlier state of polymerization. Other atypical fibrin can be derived from fibrinogen molecules which have been altered by fibrinolysis and still retain the capacity to be transformed by thrombin into 'fibrin'.

The fibrin monomer, formed at low concentrations of thrombin which are expected at the vessel site, or with a coagulant enzyme isolated from the venom of any of the two Brazilian snakes 'Bothrops jararaca' and 'Bothrops atrox', concomitant with a very small rate of release of fibrinopeptide B, as compared with fibrinopeptide A, can lead to the formation of fibrinogen-fibrin complexes (35). These complexes, which Copley et al described in 1964 and 1965 are formed as a product of the inhibition of the polymerization of this form of fibrin monomer by fibrinogen and are dissociable (35,36).

Numerous observations have been reported by Copley et al which provide indirect evidence for the presence of 'cement fibrin' in closed proximity to the endothelial cell and in the basement membrane. They concern the anticoagulant (37-39) and antithrombotic (5) actions of fibrin, its wettability (40), its lowering of apparent viscosity (13,41-44) which constitute major factors in aiding the microcirculation by reducing the resistance to the flow of blood. They concern, as well, capillary permeability and capillary fragility (8,13,21,45).

6. Evidence for the physiological presence of the EEFL

The experimental production of petechiae in the capillaries of the nictitating membrane of the rabbit's eye led the author in 1953 to postulate the existence of the EEFL (1,2).

Of significance is the experimental production of vascular purpura with guinea-pig antifibrin rabbit serum in guinea-pigs and hamsters, which Copley et al reported in 1956 and 1957 (46-48). These findings support strongly the physiological presence of the EEFL (1,2).

The detection of fibrin by a modification of the Coons method in heart and kidneys of patients soon after death and from freshly slaughtered cattle was reported by Copley in 1964 (5). A fibrin lining in minute blood vessels could be shown by the apple green hyperfluorescence (Fig. 5). However, the method is inadequate to localize the fibrin lining directly on the endothelial cells and in the basement membrane, because of the scattering of the fluorescence to adjacent vessel wall structures. In their electron microscopic studies on capillary permeability as effected by histamine and serotonin, Majno and Palade observed in the wall of capillary blood vessels filaments of fibrin, which were not seen outside the basement membrane in the connective tissue (34).

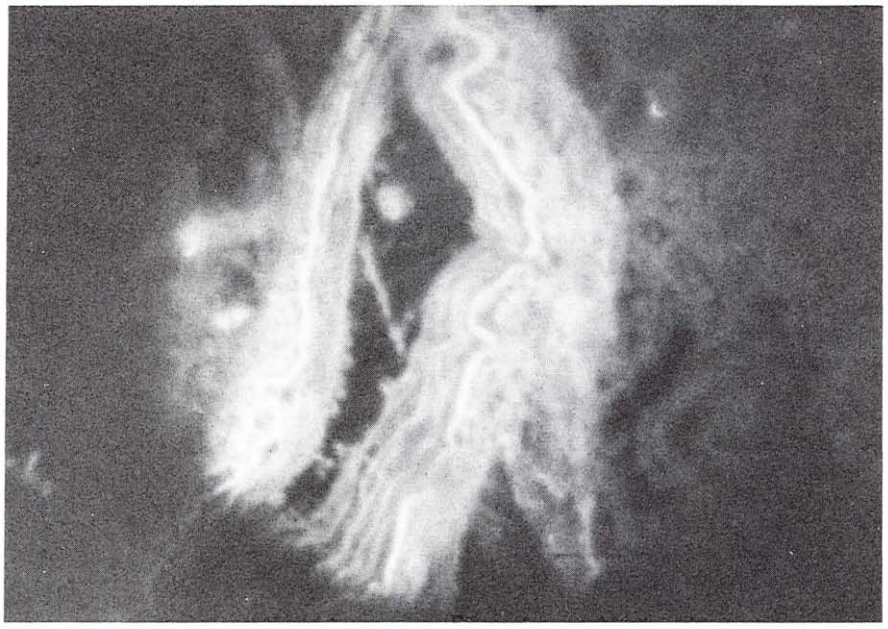

Fig. 5. Fibrin lining in a human renal artery at 450 x magnification. Note: As this Figure is not reproduced in color, the part of the wall in proximity to the vascular lumen is in green color which signifies the fibrin lining, while the adjoining lateral aspects of the wall are in blue colors, signifying autofluorescence (5).

Lipowsky and Zweifach reported in 1977 their in vivo hemorheological findings of simultaneous measurements of pressure differentials of flow of single unbranched microvessels of the intestinal mesentery of the cat (49). They claimed that their in vivo studies provided a means of examining the nature of the blood-endothelial wall contact surface, which, they believe, cannot be simulated by in vitro techniques. These authors reported low asymptotic apparent viscosities, which they found for microvessels, and considered these values to support the presence of the EEFL by its action in lowering the apparent viscosity of circulating blood.

It should be reemphasized that a number of proteins, as well as other substances, have a strong affinity to fibrin (5,37-39). Thus, the adsorption of different substances to the EEFL can be postulated.

Witte could show that following i.v. injection of fluorescent fibrinogen it can be detected adherent to the inner lining of the vessel wall, in particular on the venous site of the microcirculation in the animals he used. These in vivo findings on the affinity of fibrinogen to the vessel wall were shown with purified fibrinogen, which was labeled with fluorescein isothiocyanate and injected intravenously into the rat, having an intestinal loop exposed into a transparent chamber for fluorescence vital microscopy (50).

In an additional report, Witte (18) stated that he could actually demonstrate the presence of the postulated EEFL. He found accumulations of the FITC labeled fibrinogen, shown in Fig. 6. He observed a granular net-like accumulation at the inner aspect of the vessel wall, preferably at the interendothelial spaces. He considered this accumulation at these sites of the endothelial lining as an especially rapid turnover of fibrinogen with increased capillary permeability. This suggested to him fibrin formation with the release of fibrinopeptides, which we found to augment capillary permeability (67).

Certain cell surface proteins like fibronectin are known to interact with fibrinogen under certain conditions. The plasma fibronectin is considered by Mosesson and Amrani (51) as human plasma cold-insoluble globulin.

Recently Witte (52) communicated to the author his findings that the affinity of fibrinogen to the lumenal lining of the vessel wall is shared by cold-insoluble globulin as well as by factor VIII derivatives. He found also that other proteins do not exhibit this affinity which occurs predominantly in the venules. He described "phenomena of thrombogenesis" which are manifest as "plastic viscous layers of labeled fibrinogen at venular vessels and as blood plasma sections with an accumulation of both platelets and fibrinogen within stagnant blood columns".

It was found by Bloom and Giddings (53b) that "when blood vessels were examined by immunohistology using rabbit antiserum to factor V or fibrinogen, these proteins could be seen lining the intimal surface. However, after washing in saline, the immunofluorescent staining was greatly abolished, suggesting that factor V and fibrinogen were adsorbed to the surface of the endothelium from which it could be removed by washing in saline". They "could also observe fibrinogen related antigen and material which reacted with antisera to fragments D and E situated throughout the blood vessel even when these are obtained from neonates". From their thus far unpublished observations pertaining to fibrinogen, Bloom asserts that Copley's concept of the EEFL "thus does, in fact, have a basis in experimental observation" (53a).

There are recent findings by investigators who offer other experimental evidence which is in support of the existence of the

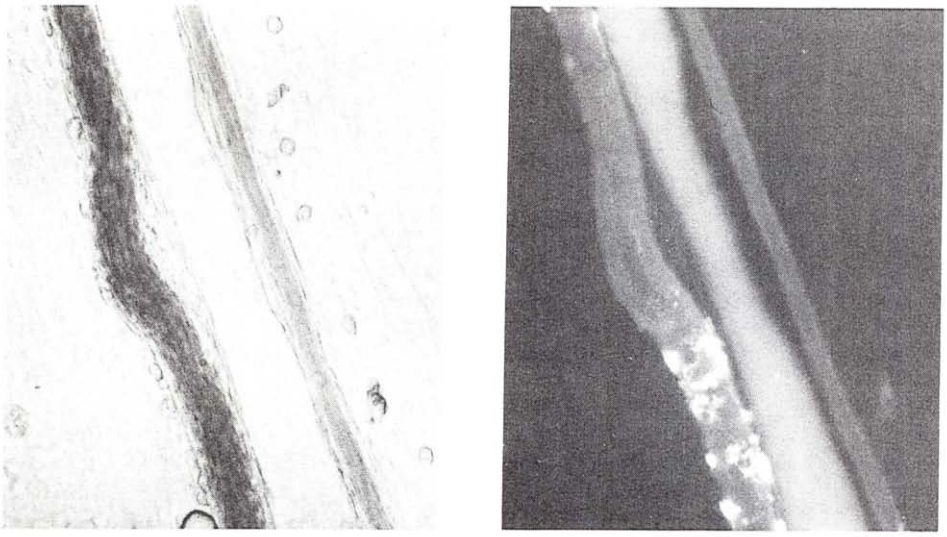

Fig.6 Part of rat mesentery with arteriole, lymph vessel and venule, 35 min after i.v. injection of fluorescein-isothiocyanate (FITC) labeled human fibrinogen (0.2 ml/100 g body weight). Objective 10:1, ocular 8x. (a) Light microscopy; (b) Fluorescence microscopy. Note the homogenous intravascular fluorescence and granular net-like accumulation of the labeled fibrinogen at the venous wall.

EEFL. In experimentally produced afibrinogenemia, Schröer reported in 1980 that capillary permeability was markedly decreased. He considered this finding to support the concept of the EEFL (54).

The above mentioned new findings by different authors provide additional direct and indirect evidence to that already provided earlier by Copley et al for the physiological presence of the EEFL.

7. On the antagonism between capillary permeability and capillary fragility

Capillary permeability (CP) and capillary fragility (CF) are physical properties, which were found by the author (21,55-62) to be antagonistic. CP, which is a physiological phenomenon, denotes leakiness of the vessel wall, without which there could be no exchange of fluid between the circulating blood and the tissues. Only if CP ia augmented, it can become pathological, such as in inflammation.

An attempt has been made by the author (6,21,45) to correlate his findings on the antagonism between CP and CF with those known in physics and rheology. Several points are reemphasized here.

In structure mechanics it is necessary to design a structure with as many compensatory mechanisms as possible. According to Freudenthal (63), the integrity of the whole structure is not ne-

cessarily identical with the local failure but depends on the interaction with the various parts. Local failure may therefore relieve local forces and mobilize a resistance of different parts. Quantitatively expressed chance to withstand certain stresses is so-called "structural reliability". The strength of the vessel divided by the maximum force under which it operates would be the safety factors. (Tensile strength of the vessel divided by the force in the vessel due to pressure and other effects.) This ratio will always be larger than 1. If it is smaller than 1, the vessel will break. If the ratio refers to average values of higher than 1, there is still the possibility that for a particular vessel in a given subject the vessel may break, as in that instance the actual values may differ widely from the average values.

The permeability should increase with extension due to pressure in a leaky system such as in an elastic tube or blood capillary. In increased CP the immediate surroundings of the capillary wall will be saturated by fluid so that any further extension by pressure in the capillary, which could lead to breakage, will have to proceed against the viscous resistance of the surrounding fluid. Fracture could then require a higher pressure than that required in a vessel loosely embedded in its surroundings. A blood vessel, when embedded in rather loose or less dense tissues, will, under pressure, deform elastically. In a viscous surrounding the blood vessel can only deform together with the surrounding medium. In this case, the response is visco-elastic, i.e., a combination of its own elasticity with the viscosity of its own surroundings. In vascular fragility, the energy which is required to deform the vessel and its surroundings to conditions of fracture, i.e., to a certain extension, is larger than that required for breaking the vessel in loose surroundings. The cushioning effect around the more or less permeable capillary would produce a lowered CF.

There might as well be two directions of flow through the permeable wall, one from within and the other from without the blood vessel. These directions are determined by the sign of the pressure difference, long established in physiology (20), particularly since Starling's hypothesis proposed in 1896 (64), i.e., an outward flow would have a higher pressure in the vessel and an inward flow a higher pressure outside the vessel. In physics, permeability is defined as coefficient which characterizes the resistance of the membrane to permit the passage of flow across it (or the capacity of the membrane to permit passage of flow). Permeability is therefore a property of the membrane and not, as often referred to by biologists, a movement of material across a barrier.

In fracture mechanics, the largest defect causes failure according to the weakest link concept. In a capillary blood vessel, the largest pore opening or gap represents the largest defect and the chance of failure is thus determined by the chance of having a large opening. An index for CP would be the distribution of pore sizes in different blood vessels, because the frequency could be estimated upon extrapolation to large pore sizes. Therefore, those vessels which have a stronger tendency to large pores would have a predilection for CF. The fact that the permeability characteristics are maintained in vascular purpura, a condition with no ede-

ma, is an indication that the surface of the defect is negligible as compared to the surface of the entire vascular system. In CF the defect is gross, widely spaced and of very small total surface. In CP, the significant structural failure, manifested by the size of pores or channels, is minute and closely spaced from a few to hundreds of Ångstrom units in dimension, while in the case of CF the dimension of the openings are of the order of 0.1 micron and more. The antagonism between CP and CF was shown by us in cases which decreased CP and increased CF, as in scorbutic guinea pigs or in animals in which we produced increased fibrinolysis. Particles due to lysis of fibrin molecules could block the pores or channels in the basement membrane, resulting in decreased CP (21).

8. Capillary permeability, capillary pseudo-hemorrhage and capillary fragility

There is great complexity in association with capillary-tissue fluid exchange. In the tissue compartments there is division of space between the rather immobile supporting phase and the fluid capable of movement. This problem was treated by Apelblat, Aharon Katzir-Katchalsky and Silberberg (65). These authors considered the Darcy law as a good approximation for flow in tissue which is a region of low permeability.

Indirect evidence for the possible occurrence of the formation of fibrin and the process of fibrinolysis at the site of the vessel wall was reported by Copley and associates from 1964-1967. We reported that plasminogen (66) and plasminopeptides (21), which are freed in the conversion of plasminogen to plasmin, as well as fibrinopeptides A and B, liberated in the transition of fibrinogen to fibrin monomer, enhance capillary permeability (8,67). Thus, these peptides, if liberated in contact with the capillary wall in vivo, may contribute directly to the physiological capillary permeability dealt with above. (See also II-6).

The physical property of capillary or vascular fragility manifests itself by the occurrence of petechial hemorrhages which have to be differentiated from capillary pseudo-hemorrhages (45). The latter can be found in inflammation or subsequent to treatment with histamine or serotonin (34,45), in which there is an escape of red blood cells, white blood cells and platelets through a gap between the endothelial cells. It should be noted that this phenomenon of blood cell captivity in the exoendothelial space between the endothelial cells and the basement membrane is not a true extravasation of blood, since the basement membrane remains intact.

9. The differentiation between petechial hemorrhage and ecchymosis

There exists a qualitative difference, which we observed, between petechial hemorrhage and ecchymosis (68-70). The latter is considered generally as indicating a quantitative increase in capillary fragility (70). In the author's appraisal the occurrence or production of petechiae indicates increased capillary fragility without the presence of a hemostatic defect. Conversely, ecchymosis is a manifestation of augmented vascular fragility combined with impaired hemostasis (70). For the differentiation of pete-

chial hemorrhage and ecchymosis we developed the ecchymosis test (68,69), which promises to be useful clinically and is therefore recommended for investigations in conditions with hemorrhagic diathesis.

Hyperfibrinolysis at a given site can affect the cement fibrin contained in the basement membrane, resulting there in true hemorrhage, as we reported in 1963 (6,70).

Earlier in this discussion, it was pointed out that, in accordance with the author's contention, processes of fibrin formation and fibrinolysis occur simultaneously and steadily in the proximity of the endothelial cell and in the basement membrane. It is reemphasized that the release of fibrinopeptides and plasminopeptides at these vascular sites can be related to their capillary permeability augmenting actions (67).

10. The Copley-Scott Blair phenomenon

Oka (13, 41) referred to the original observations by Copley (42) and the subsequent ones by Copley and Scott Blair (43,44) that there is a marked decrease in apparent viscosity when blood systems were in contact with fibrin-coated or "fibrinized" surfaces as compared with glass and other surfaces. Oka named this phenomenon the "Copley-Scott Blair phenomenon" (13,41).

The existence of a plasmatic zone, as pointed out by Oka (41), will result in a decrease of the apparent viscosity, since the apparent viscosity of the plasmatic zone which he considered as "a kind of lubricant" is less than that of the central core.

According to Oka (41) "the characteristics of the blood flow in living vessels as compared with glass capillaries lie in the electrostatic or electrokinetic forces upon red blood cells due to either the fibrin or the proteins of the membranes of the endothelial cells." Oka, therefore, anticipates that the apparent viscosity will always show a decrease, when blood is in contact with substances that are negatively charged. He concluded that his theoretical approach to the effect of the wall surface condition in hemorheology concurred with the concept of Copley. If the endoendothelial lining of all blood vessels is composed of a film of substances such as fibrin, the circulation will be aided by decreasing the apparent viscosity of blood. Oka added that the marginal zone may be markedly influenced by the negative charge on the fibrin film.

Tamamushi (71) as well as Müller (72) dealt with the Copley-Scott Blair phenomenon and Gross and Aroesty (73) emphasized that a marked reduction in the viscosity of the plasma layer could exert a significant effect on the fluid mechanical behavior of the red blood cells in the blood capillaries.

Oka (74), who discussed further the Copley-Scott Blair phenomenon, referred to several publications in the Japanese literature by Koyama, Wada et al, who investigated this phenomenon. According to Oka (74) these authors found that fibrin surface "lowers the apparent viscosity of blood and that the effect is more marked

for blood than plasma in agreement with the findings of Copley and Scott Blair". Oka stated in 1975 that there is as yet no established theory of the Copley-Scott Blair phenomenon and he discussed several explanations given thus far for it.

The following example gives an idea of the vastness of the endothelial surface area facing the vascular lumen and the corresponding immense surface area of the EEFL. In the lungs alone, there is a capillary surface area of 5 to 7 million cm^2 at 3/4 of lung capacity, which can vary by closing and opening of the capillary blood vessels. In our studies of fibrin-coated or fibrinized capillary viscometers, we found about 20 percent decrease in apparent viscosity for blood plasma when flowing through a fibrin coated or so-called fibrinized glass capillary of 980 μ. The decrease in apparent viscosity should be even more important in tubes below 100 μ in diameter than in capillary tubes of the rather large diameter, which we employed (42-44). The physiological importance of the EEFL has been also dealt with by Copley in an article on the endothelium-plasma interface (13).

The importance of the EEFL for the integrity of the capillary vessel wall has been emphasized in many of Copley's publications. This is also dealt with in the above remarks on capillary permeability and capillary fragility. Such a fibrin surface would aid the action of the heart, because the immense surface area, which it would cover as the inner aspect of minute and capillary blood vessels, would be of considerable aid in enhancing the circulation of the blood. This, of course, is based on our earlier findings that fibrin surface decreases markedly the apparent viscosity of blood and plasma (13).

11. On the plasmin neutralizing action of fibrin surface and of the EEFL

In 1962 the author reported his findings of inhibition of fibrinolytic activity by fibrin surface. Highly purified urokinase activated fibrinolysin (plasmin) was added to plasma or to serum and then exposed to fibrin powder (5). Such fibrin contact plasma or serum had lost its own fibrinolytic activity as well as that of the added highly potent plasmin. On the basis of these findings he postulated (5) that the EEFL may act as an antifibrinolysin (antiplasmin). He thought that antiplasmin activity in the circulating blood might be mainly brought about by the immense surface of the EEFL with which the circulating blood comes into direct contact.

The above mentioned findings may offer an explanation for the observations by Copley and Stefko on the effect of plasmin in blood vessel chambers, experimentally produced in dogs (4). In spite of the large amount of plasmin employed, no hemorrhages occurred. Our in vivo and extra vivum findings at that time may, in part, explain the inactivation of plasmin following its intravenous administration, for which antiplasmin in the circulating blood has been held responsible by many investigators.

We cannot enter into a discussion on antiplasmin, but should like to refer to the recent studies on α_2-plasmin inhibitor by Mül-

lertz (75), Collen (76), Aoki et al (77), and Harpel (78). During the past decade several investigators reported studies on the interaction of plasminogen and its tissue activator with fibrin (79 , 80 , 81).

Thorsen, Glas-Greenwalt and Astrup (79) found that tissue activator is strongly adsorbed to fibrin in contrast to urokinase. Wiman and Wallén (80) concluded from their studies that the lysine binding site in plasminogen is responsible for its interaction with fibrin. However, it will need to be established to which extent the EEFL contributes to the antiplasmin activity in the cardiovascular system.

12. The anticoagulant and antithrombotic actions of fibrin surface

a. The anticoagulant action of fibrin surface extra vivum

Several communications were published by Copley et al on the anticoagulant and antithrombotic properties of fibrin surface, which is responsible in great part for the fluidity of blood in the circulation (5,37-39). When the author first discovered this phenomenon in his laboratory in London in 1957 (38), he was quite astonished. When he discussed these findings with Professor R.G. Macfarlane in Oxford in 1958, he also would have expected the opposite to occur. In repeating the experiments, Macfarlane could fully confirm the author's findings (82). This anticoagulant property of fibrin surface is of great significance for the development of prosthetic devices pertaining to the heart and blood vessels. Perhaps, biomaterials in prostheses, which would come into contact with circulating blood, might preferably have a surface which would behave like that of fibrin. (See II-12d).

The way in which fibrinogen is deposited onto artificial heart valves, vascular prostheses, dialyzers and oxygenators may determine whether or not the deposit will be beneficial or harmful. As fibrin it may be beneficial because of its antithrombotic action, and as fibrinogen it may be the nucleus for thrombus formation.

The initial event at the blood-biomaterial border or at non-physiological boundaries is not the adhesion of platelets but the spontaneous adsorption of proteinaceous matter, which seems to be now generally considered to be fibrinogen. However, it may very well be fibrin or a fibrin-like material. The latter could be identical to fibrinogen which, due to the high shear forces at the wall of the prosthesis, may have undergone conformational changes, similar to those which Blombäck and Copley hypothesized to occur in the successive aggregation of fibrinogen molecules (14). (See also IX).

b. Joseph Lister's experiments in sheep (1863)

It came as a surprise, when in 1959 the author noted in reading the Croonian Lecture, given by Lister (83) in 1863, that it was

Lister who originally observed the antithrombotic action of fibrin surface without recognizing it as such. Lister's experiments offer the first evidence that in the living animal, fibrin acts as an in vivo anticoagulant. He made a prosthesis, using a long vulcanized India-rubber tube between the severed jugular vein of a sheep. He then ascertained that the blood was circulating freely through the tube and that it remained partially fluid in the prosthesis after the lapse of three hours. He gave the following account: "The portion of the blood that first came in contact with the walls of the tube had coagulated. And it is to be observed that in these experiments I never found the blood altogether fluid, even after a comparatively short time: there has always been a certain amount of coagulation, and only a certain amount of fluidity. A layer of blood having thus coagulated upon the internal surface of the tube, the fresh blood which continued to flow through it was not brought into contact with the walls of the tube at all, but with their lining of coagulated blood". Lister has been "aware of the great influence exerted upon the blood by exposure for a very short time to a foreign solid". He continued to state: "I feel many of my own experiments, and many performed by others, have been vitiated for want of this knowledge".

Although Lister was mainly concerned with disproving the theory of ammonia influence on coagulation, accepted at that time, he made the above-mentioned as well as other observations, which have a direct bearing on our studies of the anticoagulant action of fibrin lining.

c. The author's observations of antithrombotic action of fibrin surface in dogs (1962)

The following is the account given by the author (5) in his presentation at the IX Congress of the International Society of Hematology in Mexico City in 1962. It is cited in toto. Although printed in the proceedings of the congress, it is not easily available and probably therefore not known as its importance warrants it:

"Because of the significance of Lister's experiments, Stefko and I have repeated some of his studies, and used dogs instead of sheep.

These experiments were carried out in healthy mongrel dogs of about 10 kg in weight. (Through a short incision directly over the jugular vein, the latter was exposed for approximately 3 cm. Two ligatures were placed around the jugular vein, one proximally and one distally. Bulldog clamps were then applied proximally and distally. An incision was made into the jugular vein extending for a distance of 3 cm). A special rubber tube (a so-called cigarette drain), of 1 mm thickness measuring 16 cm in length and 7 mm in diameter with two pieces of polyethylene at each end, was inserted into the longitudinally sectioned jugular vein. Both ends of the tube were connected with the vessel so that the current of blood could flow through the tube and then continue its natural course. The blood was permitted to circulate freely through this tube until it was ascertained that no air existed in the rubber tubing. Thereafter, seven ligatures were placed approximately 2.5 cm apart on the rubber tubing which was segmented into six air-tight chambers con-

taining blood. The entire tube was then removed and allowed to stay in this condition for approximately three hours. Upon opening each segment, it was found that the blood in each chamber remained mainly fluid. After the fluid blood was placed in contact with porcelain plate surface, it coagulated from 6 to 10 minutes.

There was on the inner wall of the tube a very thin, red-colored coating, probably less than 1 mm in thickness, which was coagulated whole blood and appeared like the surface of the intima. It was even, smooth, and well attached to the inner wall of the tube. While it was well adherent, it could be peeled off in the form of a membrane.

In the course of numerous cannulations of the dog's carotid artery to record blood pressure, one could observe readily, at the conclusion of the experiment that a fine layer of blood existed on the polyethylene catheter. The layer was found to be coagulated blood which adhered strongly to the tube and could be peeled off as parts of a membrane. The blood in contact with this layer did not coagulate during the experiments, which was not due to the anticoagulant employed. These experiments are mentioned, as they demonstrate in the dog's artery similar findings as in the vein.

Our experiments demonstrate the in vivo anticoagulant effect of a lining of coagulated blood in tubes used as prostheses between severed large blood vessels in dogs. These observations furnish experimental evidence that fibrin as an anticoagulant acts physiologically in the prevention of thrombosis in the living animal."

d. Fibrin surface at cardiovascular prostheses

The importance of Lister's observation of the antithrombotic action of fibrin surface was, however, not realized until the author could confirm in 1964 Lister's experiment (see above II-12-b and c). The findings by Lister as well as by Copley and Stefko, which appear to be not known to investigators of biomaterials to be employed in cardiovascular prosthetic devices, are of great significance in future studies on blood compatible surfaces. (See II-12-a).

Macpherson (84) emphasized that the body performs automatically the maneuver of fibrinization, i.e., the coating with fibrin of a surface foreign to blood, of the wall of vascular prostheses. Fibrinization of glass capillaries was employed by Copley et al for their studies of apparent viscosity of blood and plasma in capillary viscometers (see above under II-10).

Of interest are also the findings by Macpherson (85). He found that when a homologous graft, which has been preserved by freezing or freezing and drying, and consequently is dead, or a plastic prosthesis is used to replace the portion of a disused human artery, the inner surface of such graft or prosthesis is coated by a microscopically structureless substance which appears to be fibrin. He concluded from this observation in patients that this occurrence of fibrin in dead tubes carrying blood suggests that the fibrinolytic activity is a function of the living endothelium rather

than of the plasmatic layer. Subsequent to the deposition of the fibrin lining the latter becomes organized to form a "pseudo-intima". He found that the appearance of this fibrin layer is much more common in the human than in the experimental animal. The layer of fibrin was not found in fresh living autografts and Macpherson contended that, if it exists, it is presumably very thin because the endothelial cells in a fresh autograft are living and functioning.

The problem of producing satisfactorily blood compatible surfaces continues to present a critical obstacle to the production of safe and useful artificial organs. For the use of synthetic vascular grafts to repair defects in blood vessels, the effectiveness of porous fabrics as substrates for the ingrowth of blood compatible so-called "pseudointimal" or "neo-intimal" linings seems to be favored by several vascular surgeons. However, the antithrombotic effect of porous neo-intimal vascular prosthesis is only poorly understood.

It has been found that a blood vessel prosthesis should be of a material which would provide a porous network and serve as a blood compatible lining for the device in order to prevent thrombosis, hemolysis and other damaging effects that foreign materials can have on blood. What prevents the clogging or obstruction of the lumen of the prosthesis, in case the latter is made of porous material, can probably be explained on the basis of the author's concept of the EEFL. Fibrinolysis could only go on steadily at the inner wall site of the vascular prosthesis, if it can be activated there. Activating agents will have to be supplied by the tissues surrounding the vascular prosthesis to activate the plasminogen, contained in the circulating blood. For the conversion to plasmin, the prosthetic material would need to be porous to allow the activation. Otherwise, fibrinolysis could not take place to control the thickness of the fibrin lining on the inner aspects of the vascular prosthetic device.

The EEFL is part of the normal vascular endothelium and thus is the most blood compatible material. It is, therefore, necessary that a biomaterial will need to be developed for prosthetic devices of the heart and blood vessels which simulates as closely as possible the nature of the EEFL.

13. On the antithrombotic action of the EEFL and on prostacyclin

Both extra vivum and in vivo findings by Copley et al in studies made from 1958-1964 and referred to above (under II-12a,c) established the anticoagulant action of fibrin surface (5, 37-39) and the antithrombotic action of fibrin layers on vascular prostheses in the living circulation of dogs (5). These important experimental findings explain the antithrombotic action of the endothelium and provide additional evidence for the physiologic presence of the EEFL.

Moncada et al (86,87) proposed that the generation of prostacyclin in layers of the aortic wall, which they found highest in

the intima, offers an explanation for the antithrombotic properties of the vascular endothelium. They based this interpretation of their findings on the inhibition of the aggregation of platelets by prostacyclin.

In recent articles by the author (88,89), in which he discussed these findings by Moncada et al, he demonstrated that there are several primary roles of platelets, none of which, however, partake in the initiation of thrombosis or of hemostasis (88-90). He emphasized that these primary roles mainly concern physiological mechanisms of defense and transportation in the circulating blood.

The possibility exists that prostacyclin synthesis in the vessel wall may contribute to the lack of thrombus formation on normal vascular endothelium. But it must be pointed out that the antithrombotic action of the EEFL cannot be ignored. Moreover, according to Copley's theory of thrombogenesis, which is discussed below (under X-2), thrombus formation is initiated by the aggregation of fibrinogen and subsequent gelation without thrombin participation. Whether prostacyclin prevents fibrinogen gel clotting and thus acts as an antithrombogenic agent will need to be studied.

The antithrombotic action dealt with above is more aptly designated as 'antithrombogenic', a new term introduced under X-4.

The general usage of platelet aggregation as a criterion for thrombogenesis is considered as a fallacy by the author. He stated that he "cannot emphasize enough, probably to the consternation of many workers on platelets, and on blood clotting in general, that such a primary role assigned to the platelets is still a belief and not a fact" (88,89).

III. Studies of Rigidity and Viscoelasticity of Surface Layers of Solutions of Highly Purified Fibrinogen

Surface rheological studies of highly purified fibrinogen were made, employing a method, which King and Copley developed in modifying the Weissenberg rheogoniometer (11,12,14,91,92). The thromboid surface layers of the tested sample transmit, when the lower platen is rotated, a torque to the measuring upper platen (Fig. 7).

A detailed account of the measuring systems is given by Copley, King, Kudryk and Blombäck (93). It contains descriptions of the modified Weissenberg rheogoniometer, rigidity measurements in steady shear, measurements of viscoelasticity in oscillatory shear, and the formulae for calculations of measurements of the gel-like structures of the surface layers of the fibrinogen systems.

In all experiments, reported here, we have used highly purified fibrinogen of 98 to 100% clottability (IMCO, Stockholm, Sweden) prepared by the method of Blombäck and Blombäck (94).

There has been some question with regard to the air-fibrinogen layer interface. We have not found any difference in values

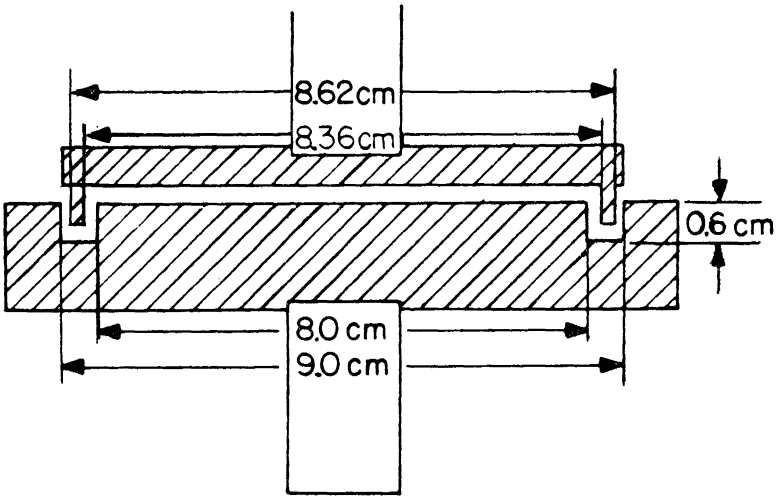

Fig. 7. Plan view of special geometry for direct measurements of rheological properties in steady or oscillatory shear of surface layers.

if the air was replaced by oil. This is shown in Fig. 8 with a 0.4 per cent human fibrinogen solution. The oil used was a light mineral oil. In a number of other experiments, we always found that there were no changes whether or not we used oil to cover the protein system (14).

Fig. 9 shows surface viscosity findings which we obtained with fibrinogen from sheep, bovine and human species. The rigidity of the surface layers of all these fibrinogen solutions was at least as marked as would be with steel or cement of the same thickness. Smith and Morrissey found the thickness of fibrinogen surface layers to vary from 200 to 600 Å (95). We did not measure the thickness of these fibrinogen surface layers and made our calculations on

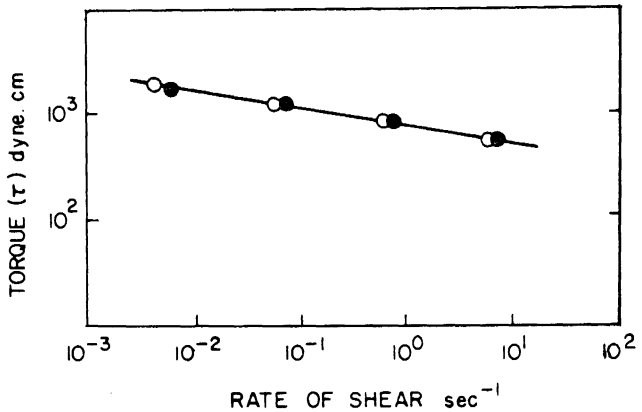

Fig. 8. A comparison of torque values, secured from surface layers of a 0.4 per cent human fibrinogen solution, formed at (-•-) oil-fibrinogen and (-o-) air-fibrinogen interfaces.

the basis of an assumed thickness of 100 Å. If the thickness of these layers were, for instance, 10 times more we would need to divide our calculations by a factor of 10 and still have an extremely high rigidity of all the various fibrinogen concentrations employed.

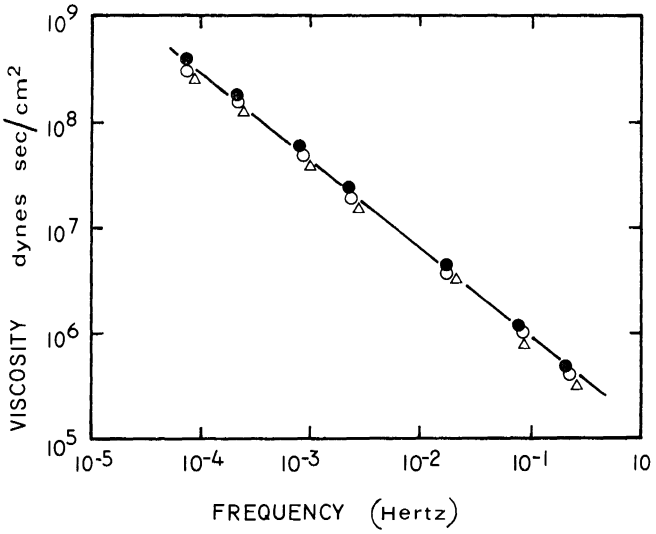

Fig. 9. Plot of viscosity (dynes. sec/cm^2) versus frequency of 0.4 per cent solutions of fibrinogen from sheep (-●-), bovine (-o-), and human (-△-) origins.

In the literature, values for surface viscosity are reported in so-called 'surface poise'. However, this does not take into account the thickness of the layers. They are treated in two dimensions and not as they actually are. This approach in two dimen-

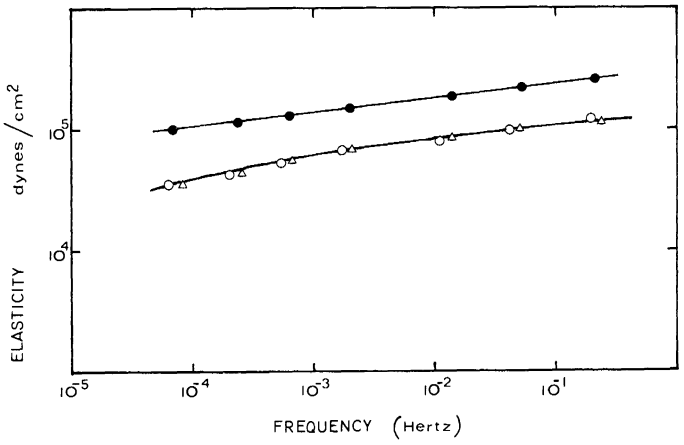

Fig. 10. Comparative elastic modulus data of surface layers of 0.4 per cent solution of fibrinogen from sheep (-●-), bovine (-o-), and human (-△-) origins.

sions gives only a qualitative measure and the values given in surface poise are reduced by about 10^6 as compared to the three-dimensional approach. This is mentioned to acknowledge that we are aware of this mode of presentation, which, however, we do not consider to be adequate.

In Fig. 10., values of elastic moduli of surface layers of sheep, bovine and human fibrinogen solutions are given, which show high degrees of elasticity, which are highest with sheep fibrinogen.

Studies of viscosity in poises and of elasticity of surface layers of human fibrinogen in various concentrations from 0.4% down to 0.00004% have been made. As shown in Fig. 11., the values of viscosity or, in this case, of the rigidity of these layers are surprisingly extremely high (14,92,93) even at such low concentrations as 0.00004% (92,93).

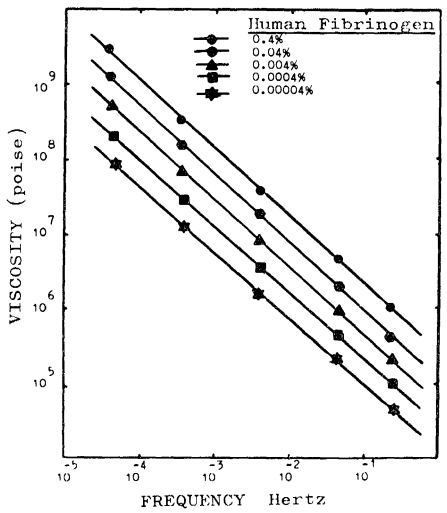

Fig. 11. Viscosity of surface layers of varying concentrations of highly purified human fibrinogen versus frequency.

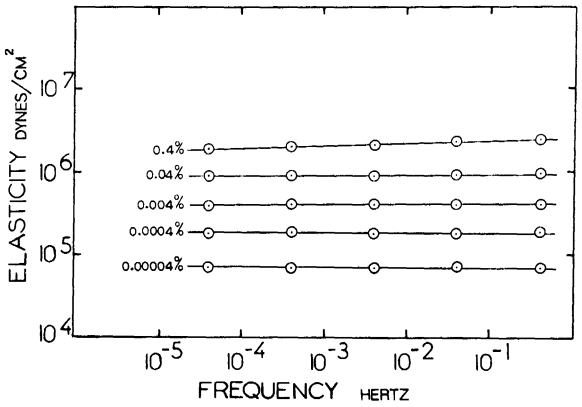

Fig. 12. Elastic moduli of surface layers of varying concentrations of highly purified human fibrinogen versus frequency.

In Fig. 12. the elastic moduli of the fibrinogen surface layers are also unexpectedly high (14,92,93) also at the very low concentration of 0.00004% fibrinogen (92,93).

IV. On the Patency of the Vascular Lumen

From measurements of pressure in the capillaries of the rat mesoappendix, Baez et al (96) found at pressure changes from 15 to 80 mm Hg that these vessels are relatively non-distensible.

Fung et al (97) claimed that the rigidity of blood capillaries was due to the gel-like material surrounding the blood vessel wall. Fung (98) commented that a mesenteric capillary or a muscle capillary will not collapse when the entire tissue is subjected to a hydrostatic pressure. The elastic behavior of blood capillaries was considered by him to depend also on the mechanical properties of the surrounding medium. The patency of blood capillaries was discussed by Fung on the basis that mechanically they are like "tunnels in gels".

A different explanation for the elastic rigidity of blood capillaries and their patency was advanced in a new concept by the author, although the elastic rigidity of the different surrounding media appears to be an important factor in capillary patency. The author's explanation, based on findings of extremely high values of viscosity and elastic modulus of surface layers of fibrinogen systems, was proposed in 1974 (13,99). Thus, the patency of blood vessels would be aided by the endoendothelial fibrin lining, not merely because of the antithrombotic and anticoagulant properties of fibrin surface (5, 37-39).

The polymers of fibrinogen and fibrin molecules may behave similarly with regard to their mechanical properties. There is therefore a need for comparative studies of the elastic rigidity of surface layers of fibrin and of fibrinogen. There is also the possibility that highly sheared fibrinogen may exhibit similar mechanical behavior as may be the case with fibrin. (See also IX).

As the EEFL is continuously exposed to the very high shearing forces at the walls of all blood capillary vessels (see IX), their walls would thus become highly rigid. In our viscoelasticity studies of surface layers of fibrinogen solutions, we found that these layers have an elastic component (92,93). The author considers this component to contribute to the elasticity of the blood capillary wall.

V. The Pathogenesis of Decompression Sickness Based on the Viscoelasticity of Fibrinogen Surface Layers

The application of information secured from our surface rheological studies of fibrinogen systems is also of interest to blood bubble generation in decompression sickness (DS). Most decompressions from any significant depth are accompanied according to Philp (100) by bubble formation and the occurrence of DS is probably dependent upon the size, frequency and location of the bubbles. Vroman et al (101) and Philp (100) noted that a layer of protein, probably fibrinogen, is deposited at the blood-bubble interface.

The extent of reactions in DS depends upon the total surface area of the gas emboli, independent of the actual number and sizes (100,101). Thus, the total area of gas which is in contact with blood becomes sufficient to interfere with the physiological blood-tissue exchanges in the affected microcirculations of different organs, leading to clinical manifestations of DS.

Our measurements on the rigidity of surface layers of fibrinogen systems in contact with air (13,14,92,93) appear to be of great significance in considerations of the formation and physical importance of these embolizing air bubbles in DS. The rigidity which they add to the air bubbles, coated by fibrinogen and other plasma proteins, makes them behave like highly rigid foreign bodies. Thus, our surface rheological studies, in which we found viscosities of several million poises of fibrinogen surface layers, results of which are given in Fig. 13, provide a new basis for the understanding of the above cited findings in the recent literature on DS. These surface rheological studies constitute an approach which hitherto has not been thought of in studies of decompression sickness.

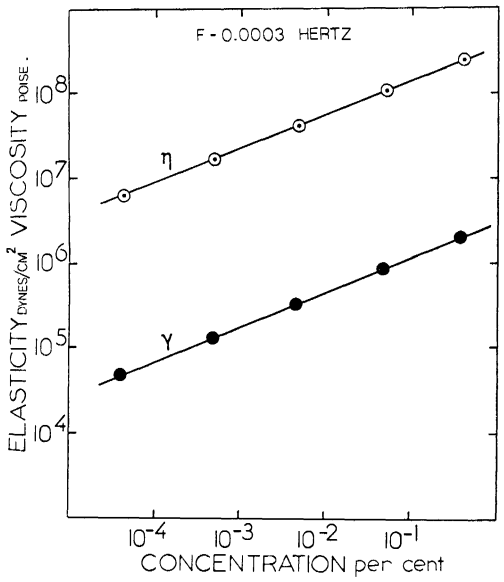

Fig. 13. Comparisons of viscosities (η) and elasticities (γ) plotted against different concentrations of human fibrinogen at frequency F = 0.0003 Hertz.

VI. Adsorption and Aggregation of Fibrinogen Observed by Different Techniques

Scarborough, Mason, Dalldorf and Brinkhous (102) found, in transmission electron microscopic studies of the organization of adsorbed plasmatic and blood cellular materials in cross section, the following initial event. On both uncoated and silicone coated glass surfaces, exposed to blood without anticoagulant, the almost instantaneous adsorption of a thin, irregular film of electron dense material, approximately 200 Å thick, became manifest. These authors

observed a trilaminar cross-sectional structure developed on glass, but not on the silicone coat. They found that only in subsequent events fibrin formation occurred, as did the adherence of platelets and their alterations in shape. Mason (103) stated in a communication to the author that no artifacts in their protein adsorption experiments were ever observed or reported. He concluded that he did not consider "that the presence of an air-liquid interface invalidates the model in any way. Indeed, we have seen similar ultrastructural features at the blood-solid interface in test systems with or without an air-liquid interface."

Vroman et al (104,105) found that, onto glass and similar surfaces, normal plasma or blood deposits predominantly fibrinogen within 2 seconds, and that the plasma "converts" (perhaps replaces, distorts or masks) the adsorbed fibrinogen within 20 sec at room temperature.

In his newly proposed method of rheo-simulation, Hartert (106, 107) considers Copley's concept of fibrinogen polymerization to furnish an explanation for the plateau formation in his rheo-thrombelastogram (107,108).

Hartert (108) considers the unidirectional precession movement of his method of rheosimulation extra vivum to resemble natural flow of blood in a living blood vessel. In the gap between an outer and inner cylinder it acts upon the clotting substrate at a shear rate of 10 sec^{-1}. According to Hartert the quality of transmission depends on the impedance of the clotting substrate. He finds "gel formation during the clotting of blood a long time prior to the crosslinking and the complex structure forming process of fibrin production. The clotting process seems to proceed layerwise from the moving to the resting cylinder." He also stated that "the understanding of the layerwise clotting in vivo partly depends on the knowledge of the mutual influence of gelation and rheopexy, over the course of time, on the mode of deposit of clotted material in the vessel." On the basis of electron micrographs he could conclude that the involvement of platelets is only secondary. Hartert reported that his findings "confirm Copley's theory" of thrombogenesis.

In a recent communication Hartert (108a) could differentiate three phases in the development of a clot structure in his newly manufactured 'resonance thrombograph'. He stated that "the first phase can have a comparatively long duration, while the last two phases occur rapidly. In the first phase there is a marked increase in viscosity which I consider to correspond to fibrinogen aggregation, since thrombin may not yet have been activated. However, this activation becomes manifest by fibrin formation and coagulation which occur in the second phase, while the platelets, if present, produce an extremely high rigidity of the clot in the third phase."

VII. Extra Vivum Fibrinogen Gel Clotting Without Thrombin Participation

1. Processes of blood clotting

Many years ago the author recommended that the word "clotting" (3) be used as a generic term for fibrin coagulation and blood cellular clumping (Fig. 14). Recently, he extended this usage of the noncommital generic term "clotting" to include the process of time-dependent progressive adsorption of plasma proteins including fibrinogen (13,14). This process is considered as a hitherto unrecognized form of clotting. All three processes are rheological in nature.

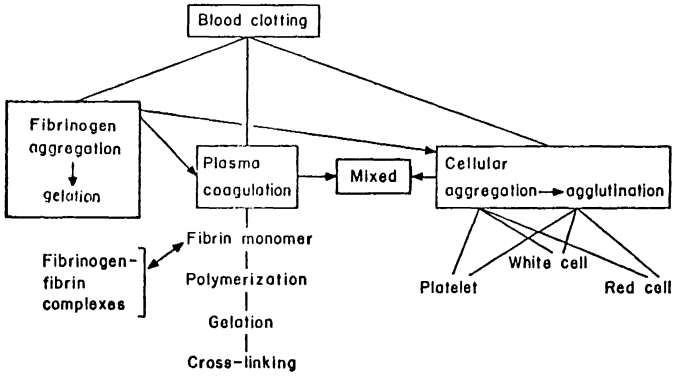

Fig. 14. The three major processes of blood clotting.

2. Clotting of fibrinogen without thrombin action

Carolyn Cohen and Nancy Tooney found that the structure of bovine fibrinogen aggregates depends critically on the ionic conditions of precipitation (109,110). These authors showed that at high ionic strengths (greater than 0.2), where charges are shielded, native fibrinogen precipitates as fibers, which appear indistinguishable from fibrin in the electron microscope. Native fibrinogen was precipitated with divalent cations or fluoride at an ionic strength of 0.2 or greater and formed fibers that look like fibrin. However, at low ionic strengths (less than 0.05), where charges are not shielded, native fibrinogen forms fibers which appear aperiodic in the electron microscope.

"The small angle X-ray results", described by Tooney and Cohen," indicated that gels of native fibrinogen, with fibrinopeptides intact, can also form an aggregate similar to fibrin." These investigators stated, "the critical factor was that the ionic strength in the gels be high enough to 'swamp out' the charge on the fibrinopeptides."

From their recently published paper on crystalline states of a fibrinogen, modified proteolytically by a crude bacterial enzyme to produce highly ordered crystalline forms, it appears that fibrinogen molecules can aggregate without thrombin interaction (110).

In view of these recent findings by Tooney and Cohen and earlier findings with protamine sulfate by Stewart and Niewiarowski (111), it no longer appears to be doubtful that fibrinogen mole-

cules can aggregate without thrombin action and without cleavage of fibrinopeptide A.

Of great interest are the findings by Witte and Dirnberger (111a) on the action of protamine sulphate on fibrinogen extra vivum and in vivo, reported in 1952. These authors found clotting of fibrinogen by minute amounts of protamine sulphate as well as by clupeine methyl ester extra vivum, while in vivo the intravenous injection of protamine sulphate led to a marked decrease of fibrinogen in the circulating blood. Witte and Dirnberger cited also findings of plasma clotting by protamine sulphate without thrombin participation reported by different investigators as early as 1929.

3. The antagonistic actions of red blood cells and platelets on the rigidity of surface layers of plasma

Copley and King (112) found that red blood cells lower the rigidity or viscous resistance of surface layers of plasma, as measured in torque values, while platelets exert the opposite action and increase markedly the rigidity.

Torque ($\mathcal{T}$) values of surface layers of 10 and 43 percent red blood cells, suspended in 0.4 percent fibrinogen solution, were compared with those of the fibrinogen solution as control. The decrease in $\mathcal{T}$ values occurred with both red cell concentrations, but is more marked with the 43 percent blood cell suspension (Fig. 15).

The rigidity of surface layers of a 10 percent blood cell suspension in platelet rich plasma was compared to that in platelet-free plasma. The $\mathcal{T}$ values of the surface layers of the red cell suspension in the platelet-rich plasma were markely increased, as compared to the red cell suspension in platelet-free plasma (112).

A comparison of $\mathcal{T}$ values was made between the surface layers of a platelet-rich plasma containing 2 percent red cells and of the same plasma in the absence of red cells. The concentration as small as 2 percent red cells counteracted strongly the increase of $\mathcal{T}$ values in platelet-rich plasma, as shown in Fig. 16. Copley and King stated that this experiment may mirror to some extent the situation as it can exist in the microcirculation, for instance in the true capillaries. The number of red cells, which circulate there in an apparently platelet-rich plasma, is markedly reduced (112)

Red blood cells and platelets are known to adsorb to their surfaces fibrinogen and other plasma proteins. Studies on the adsorption of plasma proteins to red cells and platelets should therefore be made, together with rigidity and viscoelasticity measurements, of the corresponding blood systems as well as of the surface layers of these systems.

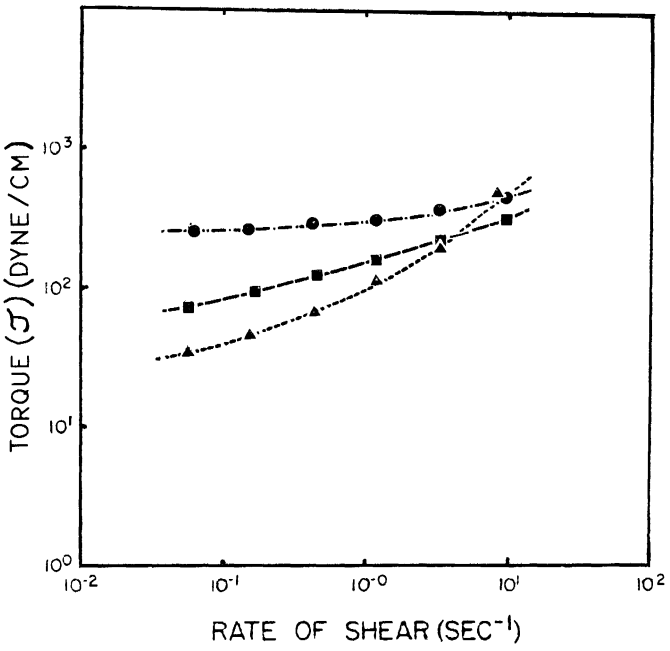

Fig. 15. Torque ($\mathcal{T}$) measurements of —●— 0.4% fibrinogen solution; —■—10% red blood cell suspension in 0.4% fibrinogen solution;—▲— 43% red blood cell suspension in 0.4% fibrinogen solution.

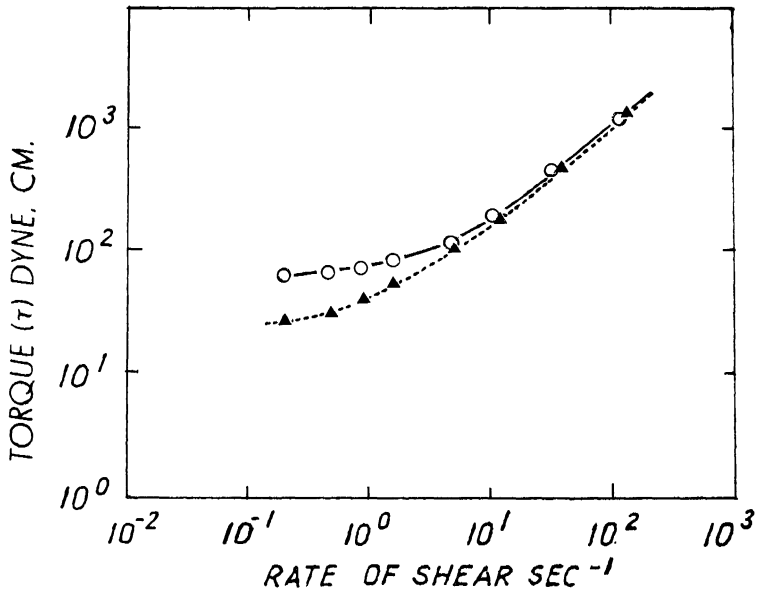

Fig. 16. A comparison of viscous resistance or torque values between the surface layers of a platelet-rich human plasma containing 2% red blood cells and the surface layers of the same plasma without the red blood cells. —o—Platelet-rich plasma; -▲- 2% red blood cells in platelet-rich plasma.

4. The rigidity and viscoelasticity of surface layers of systems of plasma proteins

Copley and King (11,12) reported measurements of rigidity or viscous resistance, viz., torque values in response to shear, derived from surface layers of systems of bovine and human fibrinogen and other plasma proteins as well as of systems of human plasma and serum. The measurements were made at shear rates from 10^3 to less than 10^{-1} sec^{-1}, and the torque values ($\mathcal{T}$) of these polymolecular layers were determined.

Fibrinogen solutions gave the highest $\mathcal{T}$ values when compared with γ-globulin, while albumin exhibited the least. The higher the concentration of each of the tested proteins, the higher was the $\mathcal{T}$ value. This was also the case with 5 to 90 percent concentrations of platelet-free oxalate plasma and serum obtained from apparently healthy human subjects.

A critical concentration of plasma or serum was noted, which ultimately may provide an indicator in the recognition of proneness towards thrombosis and may serve in the management of thrombotic conditions (12).

Vroman et al (104,105) found that albumin in concentrations like those in normal plasma compete rather successfully for certain surfaces against fibrinogen and globulins. However, albumin fails to be adsorbed out of whole plasma on almost all surfaces tested. These complex interactions may well be related to our measurements of viscous resistance of surface layers of these proteins, with fibrinogen always showing the highest values and albumin the lowest.

In another study, Copley and King (113) found that highly purified γ-globulin (99%) and β-lipoprotein have a reducing action on the viscous resistance of surface layers of highly purified (98 - 100%) fibrinogen.

Fig. 17 shows $\mathcal{T}$ values of surface layers of fibrinogen-β-lipoprotein systems and, as controls, of β-lipoprotein and fibrinogen. It can be seen that 0.025% β-lipoprotein, added to the fibrinogen solutions, decreases markedly the $\mathcal{T}$ values of the surface layers of the β-lipoprotein-fibrinogen system, while the 0.25% β-lipoprotein added to fibrinogen solution, exhibits a 45° slope, indicating no viscous resistance from the surface layers. These findings are interpreted that the β-lipoprotein interferes with the bonding of the fibrinogen molecules, resulting in very weak structures of fibrinogen layers, if formed. Any fibrinogen gels will thus be very soft and highly fragile.

Our studies of viscoelasticity of surface layers of plasma proteins were mainly limited to fibrinogen systems. There is, however, the requirement to study the viscoelasticity and rigidity of surface layers of different plasma proteins per se and in combinations with fibrinogen without and with blood cellular elements, as emphasized above. Surface rheological studies are also indicated of plasma and serum from healthy subjects and from patients with different vascular disturbances and other pathological conditions.

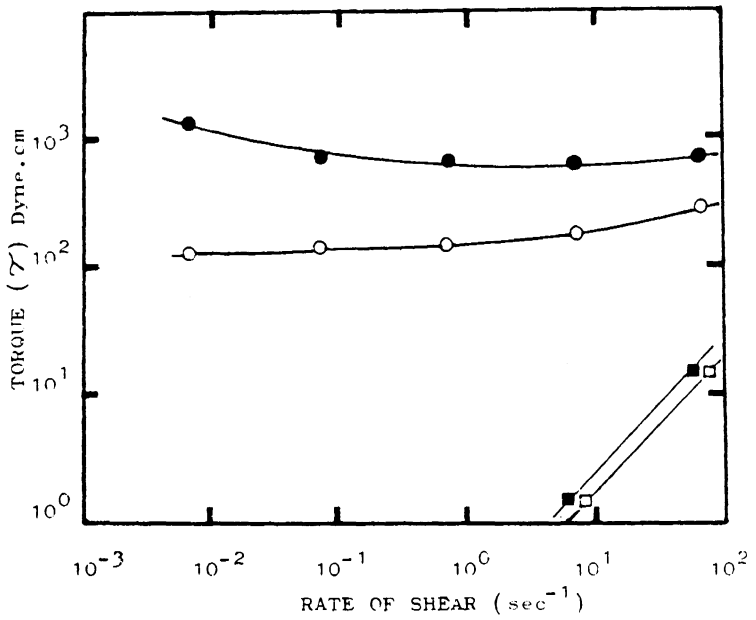

Fig. 17. Comparisons of torque values from surface layers of -●- 0.4% bovine fibrinogen and -□- 0.25% β-lipoprotein as controls. -○- 0.4% fibrinogen plus 0.025% β-lipoprotein; -■- 0.4% fibrinogen plus 0.25% β-lipoprotein.

VIII. The Physiological Occurrence of Thrombi and the Pathological Condition of Thrombosis

There is a need to clarify certain terms as they are being used carelessly by many investigators and thus have contributed to confusion which unnecessarily complicates the understanding of processes which by their nature remain rather complex. Thrombus formation is always an in vivo occurrence regardless of whether it manifests itself in hemostasis, in thrombosis, or as particle-platelet clumping in vivo. The latter becomes manifest as the result of the police or defense function of platelets. According to Copley and Witte (88 - 90) this constitutes one of the primary roles of platelets. It rids the circulating blood of any invaders foreign to it, surrounding them by adhering to them and by further clumping with other platelets. This is a special formation of platelet thrombi which in no way is associated with hemostasis and not necessarily with the development of processes leading to thrombosis.

It is well known that, as proposed by Rudolf Virchow, a thrombus is defined as an intravital, intravascular clot (114). This definition is still valid after more than 100 years.

The general usage of the term 'hemostasis' as synonym for extra vivum processes of fibrin formation and of platelet clumping can obviously be misleading, as its present usage does not necessarily mirror in vivo processes. The living blood vessel and its

wall, as well as the flow properties of blood in vivo, cannot be ignored in considerations of hemostasis, since the term 'hemostasis' means arrest of hemorrhage. The term 'hemostaseology', which is widely being used, would not be objectionable, if its usage would be limited to the field pertaining to the arrest of hemorrhage which remains an in vivo process. Unfortunately, this term is frequently employed in extra vivum processes, not associated with hemostasis.

It should be kept in mind that a wound thrombus may grow intravascularly and propagate excessively, leading to a thrombotic condition. Such a development, if it takes place, may well be a rare occurrence.

In other words, a thrombus per se is not necessarily a clot which leads to the pathologic condition of thrombosis. As mentioned above, a thrombus can very well be an entirely physiologic occurrence and as such can have a special physiological function (70,88-90). This physiological function is, of course, obvious in sealing of a traumatized blood vessel and, as a "wound thrombus", of numerous severed blood vessels in the process of hemostasis (115). However, a physiologic role is not obvious in other situations, in which the production of thrombi occurs.

We must differentiate between a thrombus which is an intravascular clot, with partial or complete obstruction of the lumen affecting a particular vascular segment and a thrombus which grows contiguously leading to thrombosis. The latter is a pathological condition affecting a large part of the circulation and involving one or more organs.

IX. Shear Rates at Vessel Walls and the Blombäck-Copley Hypothesis

The shear rates are known to be very high at the wall of the different blood vessels particularly in the small or minute vessels, as can be seen in Fig. 18.

In discussion of these wall shear forces Blombäck and Copley developed a working hypothesis that the high shear forces present at the blood vessel wall may bring about conformational changes of fibrinogen molecules at certain sites in the circulation (14). Recently, it has been demonstrated by Blombäck and associates that fibrinogen contains polymerization sites in its fragment D and N-DSK portion (116,117). The sites residing in N-DSK become activated upon treatment with thrombin or thrombin-like enzymes. This appears to be due to unfolding of the polymerization sites or their exposure after release of the fibrinopeptides. It is likely that polymerization processes, whether enzymatic (116, 117) or nonenzymatic, always involve conformational change in N-DSK, whereby polymerization sites are exposed. It is the Blombäck-Copley working hypothesis that these conformational changes can also be induced by shearing forces with subsequent exposure to polymerization. These shearing forces, as was demonstrated, are especially high at the vessel wall (14).

It is not known to us whether shearing forces can open up polymerization sites and thus simulate an enzymic action, as we proposed in the Blombäck-Copley hypothesis to simulate the action of thrombin in the initiation of fibrinogen polymerization. Of interest are, therefore, the comments by Ephraim Katchalski-Katzir, who in a recent letter to the author communicated the following: "As a result of my own studies on the conformational fluctuations occurring in biopolymers I am very much interested in the effect of external nonhomogeneous fields on the conformation of synthetic and native polymers. Your hypothesis has thus stimulated me to reconsider the matter more seriously. In due course I will do my homework and analyse the possible conformational changes that might be expected in biopolymers exposed to an external nonhomogeneous field of forces from the theoretical and experimental point of view" (118).

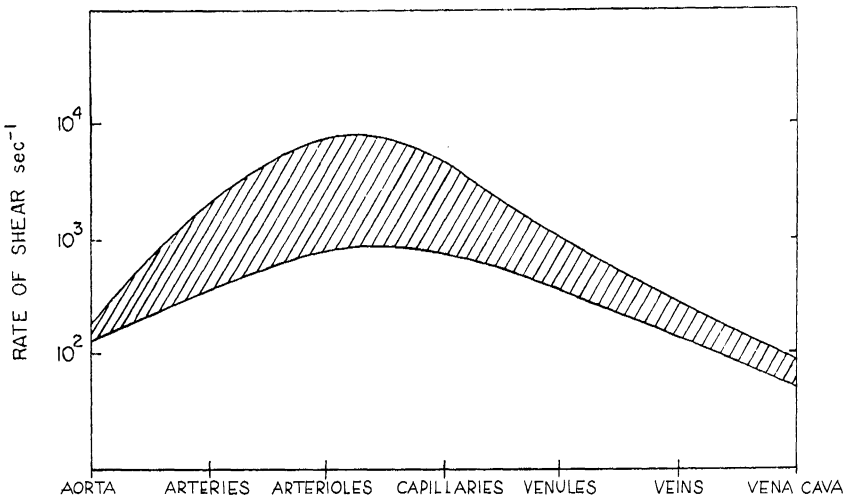

Fig. 18. Compilation of values of wall shear rates in different blood vessels from data by Whitmore (1968), Chien (1972) and Charm and Kurland (1974). See: Copley and King (14).

X. In Vivo Fibrinogen Gel Clotting as the Initial Phase of the Genesis of Thrombosis

1. Surface hemorheological and other considerations pertaining to fibrinogen gel clotting in vivo

The in vivo hemorheological observations in arterioles and venules by Copley and Staple on more or less immobile layers next to the endothelium, first proposed by Poiseuille in 1839 (24 - 26), and the phenomenon of wall adherence, described by Copley, Scott Blair, Glover and Thorley (42 - 44), may also have a bearing on the formation of polymolecular layers of fibrinogen and other plasma proteins in flowing blood.

Investigations of wall adherence of blood and blood systems have not yet been made in clinical conditions. Several studies of

wall adherence were reported by Copley et al. They were made in comparison with studies of apparent viscosity, measured in the capillary frame viscometer (43,44). The blood systems were studied contacting glass as well as fibrin in fibrin coated or fibrinized glass capillaries.

The wall adherence test, as implied by its name, measures a surface hemorheological phenomenon, viz., the width of a layer of blood or blood system left behind adherent to the surface of the artificial capillary, through which an index or column of such a system is allowed to travel. It is a rather simple procedure, developed by the author in 1957 (42,44). This test, when employed together with other hemorheological tests and in association with clinical laboratory methods, may well provide useful information of aid in the practice of medicine.

The rigidity as well as the viscoelastic properties of thrombus-like or thromboid surface layers of fibrinogen and other plasma proteins reflect the organization of the thromboid structure.

Surface layers, which form in systems of solutions of fibrinogen and other plasma proteins, have non-Newtonian behavior. The author considers this behavior to be caused in part by the non-homogeneous distribution of these adsorbed proteins and a decrease or imbalance of protective colloids which act as antiadsorbents. By non-homogeneous distribution is meant the time-dependent, progressive adsorption of fibrinogen and other plasma proteins from the solution at the interfaces with the vessel wall and the free surface of the adsorbed proteins. Such a possibility may be related to considerations of Copley and Staple (24) as to whether a suspension of macromolecules would show radial distribution when flowing through a capillary tube, in which the velocity gradient from the axis to the wall of the tube was steep. The findings on electrical field-flow fractionation of proteins by Caldwell, Kesner, Myers and Giddings (119), published in 1972, support the earlier considerations by Copley and Staple (24). Field flow fractionation is a separation method in which various applied fields, working in conjunction with cross-sectional flow nonuniformities in a narrow tube, cause the differential migration of molecules and ions. There may well be a higher transport rate for larger molecules than for smaller ones. The findings by Caldwell et al (119) apparently substantiate our contention of a nonuniform distribution of particles across a flow channel.

There is an alternative possibility for the formation of surface layers of plasma proteins. A decrease in solubility of plasma proteins including fibrinogen may lead to the deposition of these molecules on the inner surface of the vessel wall with subsequent polymolecular layer formation.

According to Silberberg (120) macromolecules tend to adsorb well on most surfaces. This he considers to be due to "the fact that macromolecular solutions are only stable because of repulsive interaction between the solute macromolecules. Hence if an interface, even if it is only mildly attractive, is presented to a macromolecule in solution, adsorption ('precipitation') of the dissolved

material occurs over the surface. Generally speaking only one layer of adsorbed macromolecules is to be expected since the surface, now coated with the macromolecules, would normally be repulsive and oppose further adsorption in much the same way as the macromolecules in the solution space repel each other. Should, however, the interaction with the surface have produced a conformational change in the material, such that attractive segments, which were internally compensated, now move to the outside, such a macromolecular coat could bind fresh material and the effect could conceivably propagate.

The idea that the endothelial lining has a fibrinogen monolayer attached to it is thus well within physico-chemical expectation. It is most reasonable, moreover, that as a result of an altered endothelial wall which, say, renders the surface more hydrophobic, adsorbed fibrinogen undergoes a conformational change, induces further fibrinogen binding and induces the formation of an extensive, layered surface phase. This is in keeping with the observation that at the air/water interface (a typically hydrophobic interface) extensive surface films of fibrinogen do arise and can be characterized rheologically and by other means.

It is clearly of the utmost significance to investigate this problem at the vessel wall, and, if it can be shown to occur, to relate it to thrombogenesis and other aspects of vessel wall malfunction" (120).

2. The Copley concept of thrombogenesis

In 1971 a new concept of the initiation of thrombosis was advanced by the author on the basis of his rheological findings of the non-Newtonian behavior of surface layers of fibrinogen and other plasma proteins (11).

Concepts on the genesis of thrombosis have hitherto been based mainly on two major processes of in vivo clotting, found to occur either separately or mixed, viz., the clumping of blood cellular elements and the coagulation of plasma by the formation of fibrin with subsequent gelation and cross-linking.

Any in vivo clotting involving the endothelium and progressing to vascular obstruction may lead to manifestations in various organs comprising the different conditions of thrombosis, which is a purely hemorheological disease or disorder.

The initiation of thrombus formation will depend on the formation of the fibrinogen aggregate which will need to adhere to some surface stationary as compared to the flowing blood. Such an aggregate will grow by additions to it of other molecules of fibrinogen or other plasma proteins as well as of single or clumped platelets, red and white blood cells, or thrombin-induced fibrin coagula which would ultimately form what is long known as the so-called "mixed thrombus". (See also Fig. 14).

The external surfaces of the adsorbed fibrinogen molecules could adsorb at their sites additional fibrinogen molecules, layer upon layer. Although Copley has pictured the fibrinogen as being

present as layers on the endothelial surface in the initiation of thrombus formation, this should conceivably be viewed as a transition from the layered state to a surface gel state with consequent large increase in volume.

The thrombus nucleus, which would be the initial fibrinogen aggregate or gel, must satisfy the requirements that it be attached to the stationary surface. In the author's view the endoendothelial fibrin lining would present to the contacting blood a surface which can incorporate blood constituents for growth, because of the great affinity of fibrin surface to other proteins including fibrinogen.

Any of the three major processes of blood clotting can go on separately or mixed. However, it is re-emphasized that the initial thrombus formation, both for the development of thrombosis and the arrest of hemorrhage in minute blood vessels, depends chiefly upon the proposed primary process of the aggregation of fibrinogen molecules. This concept is based mainly on our rheological findings obtained with surface layers formed on solutions of plasma proteins.

According to the author's concept, the course of events in the occurrence of what constitutes the "mixed thrombus" is as follows: The process of thrombus formation begins with the adsorption of fibrinogen, followed by a growth process of the adsorption of fibrinogen and other plasma proteins, layer upon layer, until the lumen of the blood vessel involved is partially or possibly completely obstructed. It is only after this process is initiated that other clotting processes occur, such as the aggregation of platelets and/or red blood cells, as well as the coagulation of fibrin. Fibrin coagulation means the polymerization of fibrin, followed by its subsequent network formation or gelation and ending in cross-linking.

The in vivo, intravascular fibrinogen clotting without thrombin action may occur within a very short time (70), i.e., possibly even within one second.

Several observations reported in the literature (106-108,121), supporting this new concept of the initiation of thrombosis, have a bearing on our findings of thromboid layers of fibrinogen and other proteins. Hartert's observations using his new method of rheosimulation, reported over the past several years, appear to provide evidence in support of this concept (107,108). (See also VI.)

3. Witte's observations of "thrombogenesis in statu nascendi". Direct evidence for the validity of the author's concept of thrombogenesis

Witte (19) reported new biomicroscopic findings of the behavior of fibrinogen, labeled with fluorescein isothioyanate (FITC), as observed mainly in the venous part of the microcirculation of the rat's mesentery. The fluorescence microscope was equipped with a scanning fluorometry device for quantitative measurements of the

labeled fibrinogen in situ. He described "phenomena of thrombogenesis" which became manifest as "plastic viscous layers of labeled fibrinogen at venular vessels and as blood plasma sections with an accumulation of both platelets and fibrinogen within stagnant blood columns."

Witte found an up to eight-fold accumulation of the labeled fibrinogen at the vessel wall. Of particular interest is the accumulation of platelets without their aggregation, as the platelets were seen as single entities in a plasmatic segment free of red blood cells. However, at this site a strong accumulation of the labeled fibrinogen occurred (Fig. 19), which Witte emphasizes as follows: "Obviously we observe here a process of thrombogenesis in statu nascendi." In summarizing his observations, Witte can distinguish two modes of intravascular fibrinogen accumulation which he considers as pathogenic processes of thrombogenesis, viz., fibrinogen aggregation without and with the accumulation of platelets.

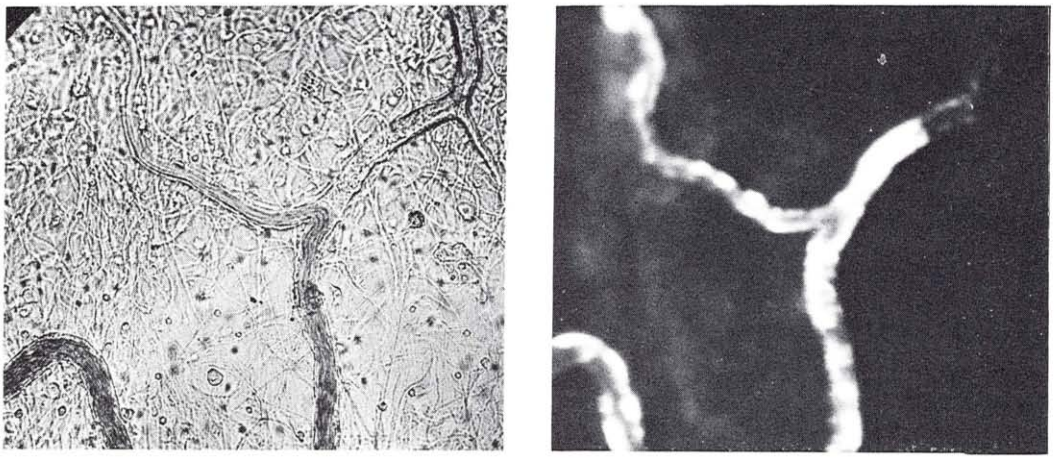

Fig. 19. Part of rat mesentery. Note the branch of a stagnant venule from a capillary with good blood flow. In the stagnant blood vessel segment there is an accumulation of platelets in plasma and, distal from it, a compaction of erythrocytes. 20 min after i.v. injection of FITC labeled human fibrinogen (0.2 ml/100 g body weight). (a) Light microscopy; (b) Fluorescence microscopy. Strong accumulation of fluorescence in the platelet rich stagnant plasma. Intensive accumulation of the labeled fibrinogen in the blood vessels in which there is good blood flow.

Apparently, Witte's observations provide the first direct evidence, thus far obtained, for fibrinogen gel thrombogenesis and support Copley's concept that the initial phase of thrombogenesis for the development of thrombosis is not the aggregation or clumping of platelets, as is generally believed, but the aggregation of fibrinogen.

4. On the differentiation between antithrombogenic and antithrombotic action

The term 'antithrombogenic' is introduced for the inhibition of thrombogenesis. Such inhibition in the initiation of thrombus formation can be brought about by a property of a surface, such as that of the EEFL, or by an agent.

An antithrombogenic agent would be primarily one which would be used prophylactically for the prevention of fibrinogen gel thrombus formation.

The term 'antithrombotic' is recommended as a generic term for the inhibition of any of the processes in the formation of a mixed thrombus, similar to that of mixed clotting, with further development of the pathologic condition of thrombosis. The term 'antithrombotic' should therefore be used for any of the three clotting processes in vivo leading to thrombosis. It is applicable as well to counteract any hemorheological and hemodynamic disturbances which would augment the propagation of the mixed thrombus and lead to the pathological condition of thrombosis. (See also II-12.)

For the past decade Copley and King (122) made numerous studies on the effect of many preparations of heparin on the rigidity and viscoelasticity of fibrinogen layers. These studies were prompted by our finding of marked decrease in rigidity (or viscous resistance) of surface layers of heparinized human plasma as compared to citrate plasma, secured from the same blood withdrawal (12). In this extensive study many non-commercial and commercial preparations of heparin were used. We found only some heparin preparations with either high or low antithrombin activity which inhibited the rigidity of the fibrinogen surface layers and may be considered antithrombogenic. Nevertheless, the usefulness of certain preparations of heparin as inhibitors of fibrinogen gel clotting will need to be studied comparatively on surface layers of fibrinogen-heparin systems, to which different plasma proteins are added, as well as on surface layers of plasma with the heparin preparation under investigation.

Since purified preparations of β-lipoprotein and γ- globulin (113) were found by us to inhibit the rigidity of surface layers of fibrinogen, they may have antithrombogenic action. (See also under VII-4).

There is no antithrombotic agent known which could counteract all three clotting processes, shown diagrammatically in Fig. 14. Heparin, which can be a very powerful antithrombotic agent, inhibits fibrin formation and its subsequent gelation both extra vivum and in vivo. However, it does not prevent the agglutination of platelets either extra vivum or in vivo, as Copley and Robb origi-

nally discovered in 1940 (88-90,123-125). Although no "ideal" antithrombotic agent exists, heparin can be highly effective in counteracting vascular occlusion and the growth of the thrombus. This antithrombotic action of heparin is brought about by its antithrombin and antigeloplastic actions, affecting the larger vessels of the microcirculation and, in particular, the vessels of the macrocirculation.

XI. In Vivo Fibrinogen Gel Clotting in the Initiation of Hemostasis

Thrombus formation in hemostasis is a physiological process. The "wound thrombus", a term introduced by Lalich and Copley (115) in 1943, differs from other thrombi. It occurs both intravascularly and extravascularly. The arrest of hemorrhage or of blood extravasation involves the sealing of the wound of a severed vessel wall, but usually of numerous traumatized vessels. Such intravascular clots are, in general, contiguous with the extravascular clot, formed by the clotting of the extravasated blood in the affected surrounding tissues (70, 126).

The author discussed recently the possible role of fibrinogen layers on the vessel wall in connection with the production of wound thrombi in the mechanism of hemostasis (70). It is possible that with the onset of bleeding an altered hemorheological situation will alter the even distribution of plasma proteins at the extravasation site. Consequently, a dynamic process occurs, viz., the initial adsorption of fibrinogen and other plasma proteins, followed by a growth process of the adsorption, layer upon layer, of these proteins, constituting thrombus formation. This is then followed by platelet deposition and clumping and thrombin induced fibrin coagulation. The sequence of these processes needs to be investigated both in vivo and extra vivum. However, the fibrinogen gel clotting in hemostasis is considered as an initial intravascular process and not involved in the clotting of the extravasated blood. The extravascular clotting is considered as a later phase in hemostasis. (See also VIII).

XII. The Role of pH on the Rigidity of Surface Layers of Fibrinogen

1. Varying pH on the rigidity of surface layers of fibrinogen solution

Copley, King, Kudryk and Blombäck, who first reported their findings in 1976 (93,127-129), employed for their rigidity studies of fibrinogen surface layers two different buffers, viz., tris-maleic buffer and tris-imidazole buffer. The rigidity measurements in steady shear were made at varying pH from 5.4 to 8.6 (92,93,127, 130). Our findings with the two buffers are shown in Figs. 20-22.

From **Figs.** 20 - 22 it can be seen that at a pH below about 6.4, there is a marked increase in the rigidity of the surface layers, while at a pH of about 7.8, there is a marked decrease in the rigidity of the surface layers.

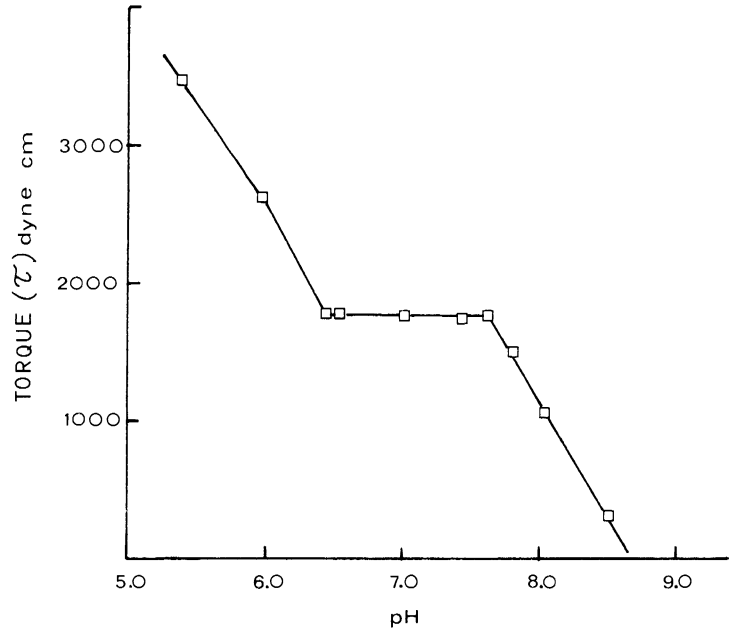

Fig. 20. The effect of pH on the rigidity of surface layers of highly purified human fibrinogen solutions employing tris-maleate buffers.

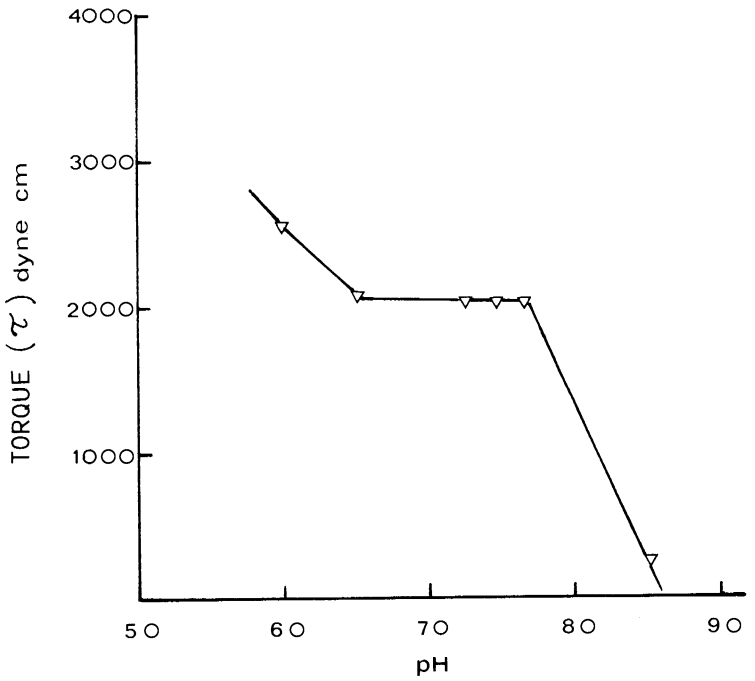

Fig. 21. The effect of pH on the rigidity of surface layers of highly purified human fibrinogen solutions employing tris-imidazole buffers.

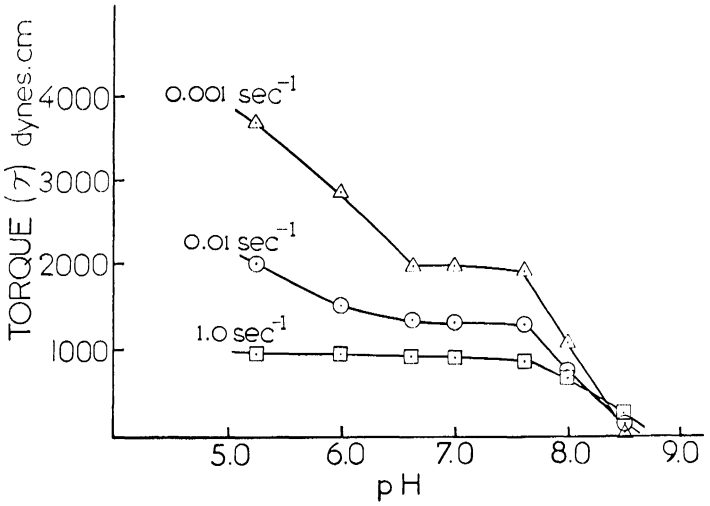

Fig. 22. Comparison of rigidity values of surface layers of 0.4% fibrinogen at varying pH and at shear rates varying from 0.001 to 1.0 sec^{-1} employing tris-maleate buffers.

2. Focal intravascular acidosis as trigger in the genesis of thrombosis

The buffering capacity of blood is such that the pH differs only slightly between 7.35 and 7.40. Only in severe cases of acidosis the pH was found to fall as low as 7.0, and, conversely, in severe alkalosis to rise beyond 7.70.

The results (92,93,127-130) given above (under XII-1) indicate, when applied to situations in vivo, that at a low pH, which may occur at certain sites of the vessel wall in the circulation, in particular in the microcirculation, there may be the initiation of thrombus formation at a pH below 6.4. Conversely, at a pH above 7.8, inhibition in the formation of surface layers may occur. These results, which we reported and discussed in more detail (93) may contribute to an understanding why at an apparently undamaged site of the vascular wall the initiation of thrombosis will take place.

The lowering in pH values at certain sites in proximity to the endothelium may well be one major cause of thrombus formation due to clotting of fibrinogen without thrombin participation, and thus may initiate thrombosis (93).

In the presentation of their findings, Copley et al (93) have discussed the question how a fall of pH below 5.4 could occur intravascularly and thus initiate thrombogenesis. Of particular significance in this discussion are the findings by McLaren and Seaman, to which they refer (131-133). Seaman, who discussed bulk and surface pH, found that the value of the pH will be lower for a negatively charged surface than the bulk value, whereas for a posi-

tively charged surface the pH value will be higher than this value (133).

Most biological cells and surfaces such as the endothelial cells are negatively charged and as a consequence the pH at the surface, or in the region where the charges are located, will be lower than the bulk pH. According to Seaman (133) charges occur often in patches, arrays or clusters and in such microregions the pH could be 2 or 3 or more pH units lower than in the bulk.

For further elucidation Seaman communicated to the author the following account: "Hartley and Roe (134) proposed that the local concentration of ions close to a surface is determined by the zeta potential of that surface. Thus the "surface" hydrogen ion concentration will be $(H^+)surface = (H^+)bulk \cdot e^{-\varepsilon\zeta/kT}$ where ε is the electronic charge, k is the Boltzmann constant and T is the absolute temperature or $pH_{surface} = pH_{bulk} + \varepsilon\zeta/2.303kT$. For a negatively charged surface $pH_{surface} < pH_{bulk}$. At 25°C, substituting values for ε, k and T, one obtains $pH_{surface} = pH_{bulk} + \varepsilon\zeta/59$. Thus for every 59 millivolts of zeta potential there is a difference between bulk and surface pH of one unit. Now charges can occur in patches on a cell surface and one could have local potentials as high as 200 mV. This means that a region of the cell membrane that is + 200 mV could differ in pH from another part of the cell membrane, that is - 200 mV by almost 8 pH units, i.e. if the bulk pH were 7 the positive region would be at about pH 11 and the negative region at pH 3. Virtually all cell surfaces carry a net negative charge under physiological conditions, so, in general, surface pH values at biological surfaces will be lower than the bulk values.

A discussion of the underlying theory relevant to the concept of surface pH value has been given in a review by McLaren and Babcock (135). Surface pH and surface and bulk dissociation constants have been discussed by Seaman (136). The distribution of charge at a biological surface is obviously an effective means of controlling enzyme activities and ensuring that enzymes with very different pH optima are able to function at the same time in the same biological system" (137).

Several authors, including this author, claimed that thrombogenesis can occur without an injury to the vascular wall (14). According to the Blombäck-Copley hypothesis, dealt with above under IX, high wall shear forces may bring about conformational changes of fibrinogen molecules at certain sites of the circulation. Copley et al (93) suggested that these sites may well be where there is a lowering of the pH.

3. On the adsorption of fibrinogen to the membrane of red blood cells and their increase of rigidity at low pH

Dintenfass and Burnard (139-141) found that the blood pH affects the viscosity of blood in particular at high hematocrit. A 30-fold increase of viscosity was found at a pH of 6.0. Dintenfass made the increase of rigidity of red blood cells at a low pH responsible for the increase in viscosity which was also described by Teitel and Nicolau (142), Murphy (143), and recently, by Schmid-Schönbein et al (144,145).

Lee et al (146) pointed out the findings by other investigators that red blood cells, secured from human subjects in normal states of health, possess a protein coating mainly of molecules of fibrinogen, α and β globulins. It may be surmised that fibrinogen molecules, when not merely attached to the endothelium but also to red cells, may clot by gelation upon lowering of the pH in the affected vessel segment and thus cause its obstruction. This phenomenon of increased viscosity of the blood due to lowering of the pH may, therefore, not solely depend upon the rigidity of the membrane of the red cell directly affected by the action of a decreased pH on it, but also on its membrane coating of fibrinogen which will be altered by the decreased blood pH. This irreversible form of red blood cell clumping must be differentiated from rouleau formation, viz., the reversible clumping or aggregation of red blood cells, for which fibrinogen-fibrin complexes have been considered to be responsible by Copley et al (35).

XIII. Fibrinogen, pH, Vascular Obstruction and Cancer Therapy

During the past 120 years numerous authors reported findings of beneficial treatment of cancer by hyperthermia and during the past half century by the induction of low pH in cancerous tissues (147).

As dealt with above (under XII), Copley, King, Kudryk and Blombäck reported in 1976 that the rigidity of fibrinogen surface layers markedly increased below pH 6.5 (127). We considered these findings as a contribution in the understanding of what may trigger the initial phase of thrombogenesis, particularly in the microcirculation, brought about by the aggregation of fibrinogen on the lumenal aspect of the vessel wall. Recently we published a detailed report on these and other findings (93) which we discussed above.

In several publications Ardenne et al claimed that decreases in pH at and below 6.5 were responsible for the destruction of cancerous tissues in patients (145,147). Ardenne et al introduced their so-called Cancer Multistep Therapy (CMT) and claimed that the blood microcirculation is interrupted irreversibly and selectively in the cancer tissue, initiating its destruction. These authors employed a combination therapy of selective hyperacidification, by increasing the blood glucose concentration up to 500 mg/100 ml for a period of about 4 hours, combined with local hyperthermia.

It is noted that hyperthermia is being explored by many investigators often in many-fold combinations in the treatment of cancer (147).

Ardenne et al (145,147,148) reported that the microcirculation in tumor tissue comes almost completely to a standstill as soon as the pH drops to 6.5. In their publications since 1977 these authors referred to the observations by Schmid-Schönbein et al (144) who noted at low pH an increase in the rigidity of the membrane of red blood cells, resulting in their diminished flexibility. However, as discussed under XII, the increase in rigidity of the red cell membrane may not be solely due to the lowering of pH below 6.5 on the membrane proper. The low pH will also increase the rigidity of

any fibrinogen adsorbed to the red cell membrane, since adsorption of fibrinogen to the surface of red blood cells, to which we referred (93,147), has been claimed to occur by several authors.

Song (149,150) reviewed the recent literature on the changes in blood flow and pH due to hyperthermia in normal tissues and in tumors. The clinical trials and experimental data suggest that hyperthermia may cause greater damage in tumors than in normal tissues. The blood flow in normal tissues, i.e., in skin and muscle, increases markedly when heated at temperature from 42-46°C. Blood flow in tumors increases slightly when heated at temperatures below 41-42°C, but decreases drastically above 42°C. The heated tumors demonstrated histologically vascular occlusion and hemorrhage. Song concluded that upon heating the pH in tumors is significantly decreased, and that there appears to be a close relationship between the decrease in blood flow and in pH in the vasculature of rat sarcoma.

Of interest are the studies by Song et al (151) who found a severe vascular occlusion occurring in the SCK tumor of mice, a fast growing mammary adenocarcinoma, after heating at 41.5-45°C. The pH in the tumors significantly decreased while that in the muscle increased upon heating. They found the clonogenic cell number to decrease continuously when the SCK tumors were left in situ after hyperthermia. These authors considered the vascular occlusion and the increase in acidity to account for the progressive death of tumor cells after heating.

Harguindey et al (152) studied the effects of dietary-induced acidosis on the growth and rates of complete regression of sarcoma 180 in mice. They could demonstrate in these experiments that mineral acidification of laboratory food products produces a late decrease in tumor growth and that there was a significant increase in the rate of complete tumor regression. However, it should be stressed that this method of acidification may induce thrombogenesis in the healthy tissues as well.

Ardenne et al (148) gave a description of their new radiofrequency (rf) procedure, i.e., their "CMT Selectotherm" technique. Recently, Ardenne (153) reported that "since the rf applicator executes a scanning motion, a widely homogeneous temperature distribution in the order of 42.5°C is attained. By stepwise shifting the rf field from one body section to the other, all parts can be overheated without exceeding the tolerable core temperature of 40.5°C. Hyperthermia of 42.5°C combined with tumor acidification, which is caused by the excess glucose supply, triggers irreversible hemostasis in tumors and metastases followed by dramatic regressions within 3 weeks, but without overloading the circulation with toxic degradation products, as was documented in several clinical trials." He concluded that this combined treatment can be repeated in case of partial regression or recurrence without endangering the life of the patient.

The author suggested recently (147) that the effect of triggering thrombus formation by fibrinogen gel clotting without thrombin participation due to low pH values, induced by Ardenne's Cancer Multistep Therapy, may be augmented in modifying this therapy as

follows. The glucose infusion could be followed by the infusion of fibrinogen solution or substituted by the infusion of fibrinogen added to glucose in solution, since more fibrinogen would thus become available for clotting without thrombin participation in securing vascular obstruction. Such a fibrinogen-glucose infusion could be further modified by the added infusion of platelet suspensions, because platelets increase the rigidity of surface layers of plasma (112, 147). The infusions of fibrinogen and of platelet suspensions would as well prevent the risk of hemorrhages in different parts of the body. These infusions would compensate for the depletion of fibrinogen and platelets in the circulating blood as a result of the therapeutic vascular occlusions of the cancer tissues.

As promising as Ardenne's Cancer Multistep Therapy may be, whether or not modified by the author's above suggestions, it should always be kept in mind that the decrease of pH in cancerous tissues by the induced glucose infusion and hyperthermia will, if possible, have to be limited to the vasculature of these tissues. Strict safeguards will therefore have to be developed for cancer patients receiving Ardenne's CMT, without or with the additional infusion(s) suggested above. In other words, the induced intravascular acidosis should not affect non-cancerous areas of the patient's body, because there could also be an imminent danger of thrombogenesis in these areas.

Endrich, Zweifach, Reinhold and Intaglietta (154) observed that leukocytes stuck to the vascular wall in postcapillaries and collecting venules upon heating of rat sarcoma, grown in transparent chambers. On the basis of Witte's observations, discussed above under X-3, it would be advisable to extend the observations of Endrich et al by the use of Witte's fluorescence microscopic methods. It appears that the thrombogenesis in statu nascendi, viz. fibrinogen aggregation, which Witte observed in the normal rat, may well be found as the primary event in acidosis-induced cancer therapy.

XIV. The Uptake of β-Lipoprotein by the EEFL and the Fibrinogen Gel Thrombus as Alternate Pathways in the Author's Theory of Atherogenesis

In 1957, Copley wrote two letters which were published in the Lancet with regard to the development of atherosclerosis (155,156). At that time, he thought that, since many different substances have an affinity to fibrin, the possibility of interactions between the endoendothelial fibrin lining with cholesterol and lipoproteins could explain processes which initiate atherosclerosis. It has been shown, as mentioned above, that the growth of atherosclerotic plaques occurs commonly at certain favored sites which have been correlated with local flow conditions. The transport of matter between the blood and the wall influences the development of atheroma. It was shown by other investigators that the transport and diffusion of cholesterol and proteins augment by increasing the wall shear stress.

Caro et al (157-159) have made observations with regard to the genesis of atherosclerosis which appear to relate to our findings

on viscous resistance of fibrinogen surface layers following exposure to high shearing forces. They observed that the distribution of fatty streaking and early plaques may be strongly influenced by high shear rates at the arterial wall and also at the site of bifurcations of arteries when there is turbulence and the shear rate is high.

The author's new theory of atherogenesis, first presented in 1978, (138,160-163),is based on the adsorption of β-lipoproteins or low density lipoproteins (LDL) via two pathways. They are: (A) on the endoendothelial fibrin lining and (B) on fibrinogen gels, loosely structured due to a hitherto unknown action of β-lipoprotein which we discovered (113,138,161). The theory is furthermore based on the recently postulated theoretical approaches by Oka (164,165) involving hemorheology, polymer physics and other physical factors, and on recent surface chemical findings by Miller et al (166).

The theory, diagrammatically shown in Fig. 23, concerns the early development of atheroma and proposes two pathways of uptake of low density lipoprotein.

Pathway 1 concerns the adsorption of β-lipoprotein or low density lipoprotein to the endoendothelial fibrin lining (EEFL), as shown in Fig. 23.

The affinity of cholesterol carrying lipoprotein to fibrin was suggested by the author in 1957 (155,156) as stated above. He proposed the uptake of lipoprotein and cholesterol by the EEFL in contrast to Duguid's modification (167) of the Rokitansky theory and against the thesis, that an injury to the vessel wall would be necessary for initial thrombus formation in the process of atherogenesis.

Pathway 2 (Fig. 23) concerns the uptake of low density lipoprotein on a thrombus, initially formed by the aggregation or clotting of fibrinogen without thrombin participation. This form of clotting is, as dealt with above, a growth process, layer upon layer, of fibrinogen, and the formation of a gel.

The proposed second pathway of early atheroma formation is based on our rheological findings on surface layers of fibrinogen-β-lipoprotein systems (113,138,161).

We employed a highly purified β-lipoprotein (168), prepared and kindly supplied by Dr. M. Burstein of the Centre National de Transfusion Sanguine, Paris, France, as we reported in 1974 (113).

We presented torque values ($\mathcal{T}$) of surface layers of fibrinogen-β-lipoprotein systems and, as controls, of β-lipoprotein and of fibrinogen (Fig. 17). We showed that 0.025 percent β-lipoprotein, added to the fibrinogen solution, decreased markedly the torque values of the surface layers of the β-lipoprotein-fibrinogen system, while the 0.25 percent β-liprotein added to the fibrinogen solution exhibited a 45° slope, indicating no viscous resistance from the surface layers (113), Fig. 17. This experiment is

interpreted that the β-lipoprotein interferes with the bonding of the fibrinogen molecules, resulting in very weak structures of fibrinogen layers, if formed. Such fibrinogen gels will thus be very soft and highly fragile (113).

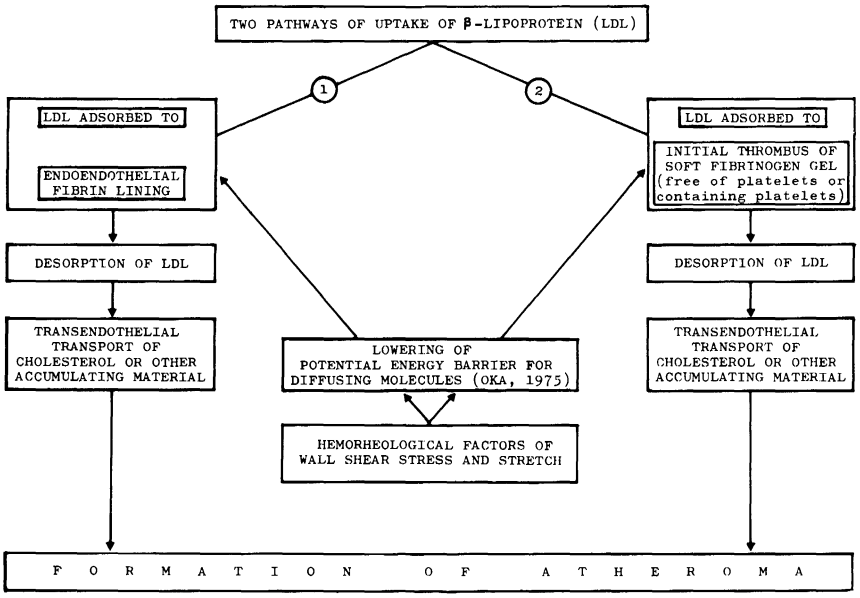

Fig. 23. Schematic diagram of the author's theory of the genesis of atheroma formation.

We found later in numerous experiments that preshearing at 1000 sec^{-1} increased markedly the τ values of surface layers of fibrinogen systems (14). Because of these findings, we presheared a solution of 0.25 percent highly purified β-lipoprotein and 0.4 percent highly purified bovine fibrinogen (Fig. 24). The preshearing of the fibrinogen solution, used as control, increased markedly the τ values of its surface layers. However, the preshearing of the β-lipoprotein-fibrinogen system gave the same results as those obtained without preshear, viz., a slope of 45°, i.e., as mentioned above, no viscous resistance from the surface layers (138,161).

Our findings that the β-lipoprotein-fibrinogen surface layers, which, if formed, are extremely weak or fragile structures, are important for the consideration of the newly proposed second pathway of uptake of β-lipoprotein or LDL (low density lipoprotein). On the basis of our experimental findings on surface layers of fibrinogen-β-lipoprotein systems, the following tentative explanation of the role of β-lipoproteins in the early development of atheroma is as follows. The LDL containing fibrinogen gel, which, on the basis of our surface rheological findings, is considered to be poorly structured, would permit increased uptake of β-lipoprotein carrying cholesterol or/and other accumulating material.

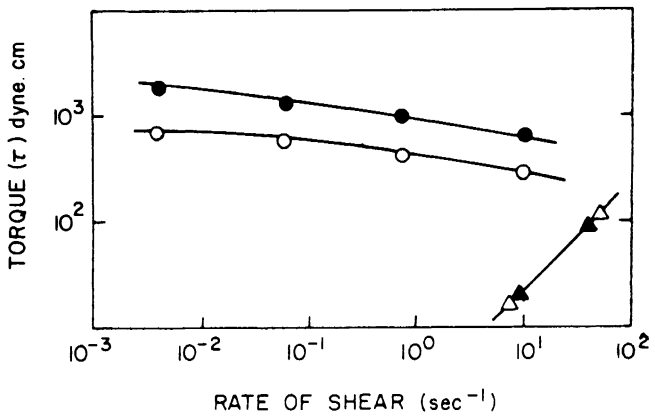

Fig. 24. Comparisons of torque values from surface layers between 0.4% fibrinogen solution and 0.25% highly purified β-lipoprotein added to the 0.4% fibrinogen solution with and without preshearing at 1000 sec^{-1}. —●— Fibrinogen after pre-shear, —○— fibrinogen without pre-shear, —▲— fibrinogen plus β-lipoprotein after pre-shear, —△— fibrinogen plus β-lipoprotein without pre-shear.

Of particular significance are the recent adsorption experiments by Miller et al (166), who studied cholesterol exchange between surface layers and plasma proteins in bulk. Their findings suggest that lipoproteins, which serve, as is well known, as vehicles for transport of cholesterol, can adsorb reversibly on proteinaceous surface. According to these authors, lipoprotein molecules can be desorbed readily and may maintain transient contact with the proteinaceous blood vessel surface, which the author considers to be mainly fibrin or/and fibrinogen. Metabolic digestion of the proteins (138,161) or of fibrin or/and fibrinogen, leaving the lipids behind, may then result in the formation of fatty streaks, lesions and atheromas.

Prior to or simultaneous with the processes of adsorption and desorption of β-lipoprotein and other accumulating material, the hemorheological and other physical factors of wall shear stress and stretch, proposed by Oka, come into play. In accordance with Oka's theoretical approaches (164,165) applying, among other factors, polymer physics and the theory of rate processes, which have been referred to earlier, the lowering of the potential energy barrier for diffusing molecules, included in the schematic diagram of Fig. 23, contributes to the formation of atheroma.

In a discussion of the author's theory of atherogenesis Farrell (169) cited a number of findings by different authors in the current literature. He concluded that their findings are not incompatible with the theory.

XV. Conclusions

As a student of phenomena affecting the circulation of blood, the author has been preoccupied since 1932, i.e., for nearly half

a century, with the flow properties of blood, with the obstruction of the lumina of blood vessels due to clotting processes, with other processes affecting the blood vessel wall and with those which keep the vascular lumina patent.

On the basis of the findings discussed in the overview, hemorheological and related processes pertaining to the circulating blood and the blood vessel wall can now be seen in a new light and studied accordingly. There appears to be sufficient experimental direct evidence for fibrin to constitute the inner lining of blood vessels or for the existence of the endoendothelial fibrin lining (EEFL). It affects such physiological processes as they exist in the exchanges between the blood and the tissues. It also involves the permeability and the fragility of capillary blood vessels.

There are the other processes which either promote or inhibit the various forms of clotting or, after clotting has already set in, lead to the lysis or disintegration of clots of different origins. These occurrences will, of course, continue to be a main concern of studies related to thrombosis subsequent to its initiation as well as to hemostasis. The phenomena of the aggregation of fibrinogen molecules extra vivum and intravascularly, layer upon layer, and subsequent gelation, will need to be studied widely at many levels in a variety of scientific fields and disciplines. In the case of cellular clumping and of blood coagulation, such studies have been going on for more than one hundred years, an enterprise in which many investigators continue to be active.

The various findings dealt with in the overview provide new tools for further experimental scrutiny of the EEFL, the Copley-Scott Blair phenomenon, vascular patency, the initiation of hemostasis, the pathogenesis of decompression sickness, the genesis of thrombosis and the initial phase of atherogenesis. Of particular significance are the author's findings of anticoagulant and antiplasmin activities both of fibrin surface and of the endothelium. They relate the phenomenon of antithrombotic action of the endothelium to the EEFL. Moreover, they recommend the usage of fibrin-like surfaces as biomaterials in cardiovascular prostheses. Such biomaterials would mimic the compatibility of the endoendothelial lining, since they would have surface characteristics and behave like fibrin surface.

Certain findings offer a rationale for cancer therapy due to induced hyperthermia and acidosis. Infusions of fibrinogen and platelet suspensions are recommended as modifications of such therapy for the prevention of hemorrhages and as compensation for the depletion of these constituents in the circulating blood as a result of the induced vascular occlusions.

As far as the pathophysiology in other diseases or pathological conditions is concerned, it involves, among others, augmented capillary permeability in inflammation (where fibrinogen gel clotting may also play an important role), certain hemorrhagic disorders affecting the fragility of capillary blood vessels, edema formation in different pathological conditions.

It is anticipated that the information provided in this over-

view will stimulate research both in experimental and clinical hemorheology.

There can be little doubt that more and more evidence will be accrued in future studies, supporting the validity of both the physiological occurrence of the EEFL and the fibrinogen gel clotting in thrombogenesis. The application of knowledge pertaining to these two hemorheological manifestations will become of paramount importance in the practice of medicine and surgery for the management of many different disease processes and for the maintenance of health.

Acknowledgement

The author thanks Professor Dr. Siegfried Witte of Karlsruhe, Federal Republic of Germany, for permission to use the photomicrographs (Figs. 6 and 19) in this presentation.

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