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INTRODUCTION
ON ERYTHROCYTE AGGREGATION AND DESAGGREGATION

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1. Initial Remarks

As this Conference deals with red blood cell aggregation and its aspects, it is of interest to stress certain observations pertaining to this phenomenon. There are a number of findings and concepts regarding red cell aggregation and their desaggregation which are dealt with by different authors, mainly during the past two decades. Many of these observations and considerations are presented in this Symposium. However, there will be others which may have been observed, but not given adequate thought.

It should be further emphasized that red cell aggregation extra vivum is not necessarily mirrored in vivo. One aspect which appears to me to be significant and could not be sufficiently realized in the past is that red cell aggregation in the living is related not merely to the blood but also to the vessel wall. In 1982, the author proposed the concept of the 'vessel-blood organ', consisting of two portions which comprise the blood vessel wall and the blood (1). This is particularly significant for transcapillary transport, as well as in certain abnormal conditions affecting the vessel wall, as for example in inflammation. The clumping of red cells in the latter condition, although in general occurring as reversible aggregation, involves increased capillary or vascular permeability, leading to the phenomenon of 'compaction stasis'. This and certain other phenomena affecting the vessel-blood organ will be briefly discussed later.

This introduction cannot deal with many aspects, which the topic on erythrocyte aggregation and desaggregation invites. Certain aspects are emphasized, which appear to me to be particularly important. Answers to a number of questions can be given only in part by this Conference. Studies pertaining to these aspects will occupy investigators for many years to come.

2. Van Leeuwenhoek's Discoveries

Anthony van Lewenhoek (or Leeuwenhoek, as his name is usually spelt) gave the first accurate description of the red blood corpuscles, published in 1674 in the Philosophical Transactions of the Royal Society, London (2). However, it was Malpighi who nine years earlier demonstrated their existence, which he mistook for globules of fat (3).

It was Anthony van Leeuwenhoek who first described the aggregation of red blood cells and their desaggregation in a Letter to the Royal Society, dated September 25, 1699 and published

in London in 1702 (4). This communication "Concerning the Circulation and Stagnation of the Blood in Tadpoles" was, to my knowledge, never referred to in the biomedical literature. Van Leeuwenhoeck could not have seen the aggregation of red blood cells with his inadequate microscope. However, he described that blood in the blood vessels "did in a manner stagnate , insomuch that one could discern no separated parts in the blood, for it did appear there to be but one even red colour". He gives a vivid, detailed description of what he saw, which was probably not merely stoppage of blood flow, but red cell aggregation and increased capillary permeability in the affected blood vessels. This phenomenon, already mentioned and named by Copley 'compaction stasis' (5,6), will be dealt with later.

From van Leeuwenhoek's observations of the microcirculation in the tadpole, the following is cited: "So that now it doth plainly appear before our eyes, that the stagnated blood cannot only be made to move again by the motion of the heart, which we call beating of the pulse, nay, even in such a manner, that the coagulated red globules of the blood are uncongealed again, and assume their first figure". It is clear from this description that the red blood cells are not "coagulated", but that they are aggregated. It is further clear from his observations, made 14 days later, that the so-called "coagulated" red blood cells were seen to be desaggregated as single globules. About the size of the red blood cells, he observed the following : "These Particles of the Blood, are, according to my position, so small, that ten hundred thousand of them cannot make up so great a body as the corn of a great Sand". Van Leeuwenhoek's Letter regarding his observations of the microcirculation contains many other interesting descriptions as well as certain comments which cannot be dealt with here.

3. Terminology

It appears necessary to define the term 'aggregation' , as it has been frequently used interchangeably with the term 'agglutination'. In an effort to clarify the terminology, the author proposed in 1958, in a Discussion of a paper by Fåhraeus, presented at the Third International Congress on Rheology at Bad Oyenhausen, West Germany, to define red cell aggregation as reversible clumping, while agglutination as irreversible clumping (5,6). This is illustrated in Fig.1.

Knisely introduced the unhappy term 'sludge' into the literature of red cell clumping, which is clarified in Fig.1 (5,6). Knisely contended that red cell clumping is always a sign of disease, a claim which never could be substantiated and has no basis in fact. In contrast, Fåhraeus could show that red cell aggregation in the form of rouleau formation may facilitate the flow of blood (7). Thus, red cell rouleaux are reversible cellular clumps or aggregates which can occur physiologically. There is a form of irreversible red cell clumping which Knisely observed to occur in several diseases. The differentiation between the agglutination of red cells (which is irreversible) and the aggregation of red cells (which is reversible) is considered by the author to be highly significant. It is, therefore, necessary to look for such irreversible clumps.

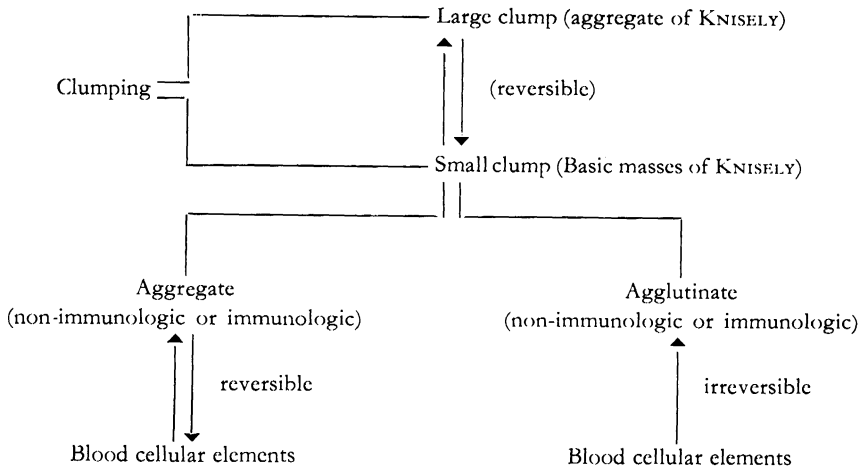

Fig.1

Schema of intravascular blood cellular clumping

There is a kind of large clumps of red cells which do not desaggregate into single red cells, but instead into small irreversible clumps to which Knisely referred as 'basic masses', while the large clumps he named aggregates (Fig. 1).

Clumping is used as a generic term for both reversible aggregation and irreversible agglutination. Furthermore, it should be noted that both aggregates and agglutinates can be formed either non-immunologically or immunologically. The above proposed definitions are meant to avoid confusion as often found in the literature.

4. Erythrocyte Aggregation Extra Vivum and In Vivo

It is not generally appreciated that red cell aggregation extra vivum does not necessarily reflect its significance intravascularly (in vivo). It is, therefore, imperative to differentiate between red cell aggregation formed extra vivum and that formed in the circulation.

Red cell aggregation occurs physiologically in vivo without augmented capillary permeability. According to Fåhraeus, rouleau formation can actually facilitate the flow of blood. In the discussion of this statement by him, given at the Third International Congress on Rheology at Bad Oeynhausen in 1958, I asked the question whether and when he would suggest that therapeutically induced rouleau formation could be used in certain clinical conditions (5,6). Although Fåhraeus responded to some other questions I posed at this meeting, he did not respond to this one. There are nowadays substances known to aggregate red blood cells in vivo, such as some Dextran preparations which may not harm the patient. The question whether, when and how to manage the induction of red cell aggregation needs to be addressed experimentally and clinically.

4.1 Red Cell Aggregation and Rheological Measurements

An instrument, which utilizes the Weissenberg rheogoniometer, developed by Siskovic, King, Huang and Copley (8), permits the visualization of the flow of red cells, suspended in plasma or other physiological fluids, in steady shear varying from 5000 down to 0.005 sec^{-1} and in oscillatory shear from 60 down to 0.00006 Hz. Photomicrographic recordings (9) were reported by Copley, King, Chien, Usami, Skalak and Huang, while cinephotomicrographic recordings by Usami and the above authors (10). Desaggregation occurred if sufficiently high shear rates were applied. At low shear rates the rouleaux remained. A steady state structure was attained when a given shear rate was applied for sufficient length of time. In oscillatory flow, even as low as 0.001 Hz, the motion greatly accelerated aggregation and rouleau formation. Certain relationships between our microscopic and macroscopic findings in oscillatory and steady shear existed and were discussed by Chien et al (11).

Figs. 2 and 3 demonstrate the effect of red cell aggregation on rheological measurements (R.G. King, unpublished data).

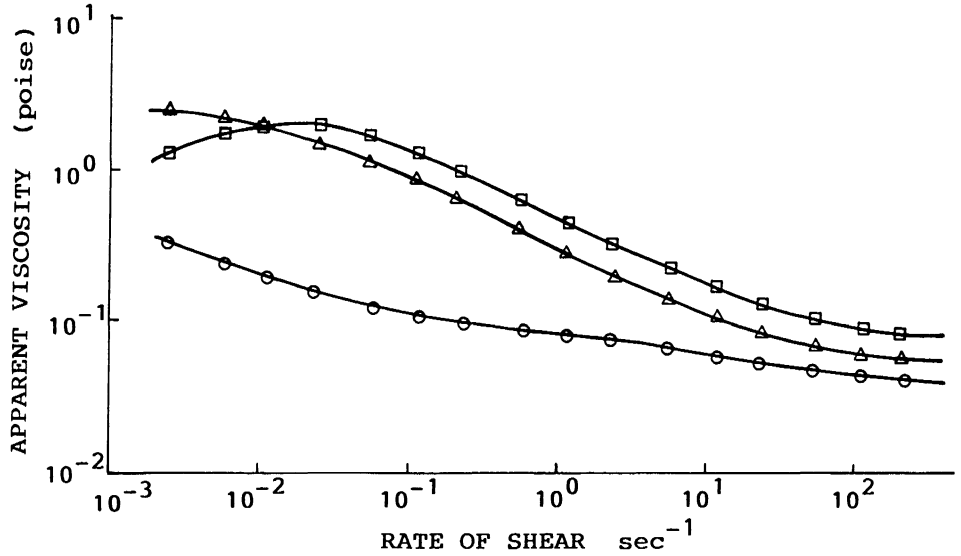

Fig. 2
Comparison between flow curves of preparations of 50% red cells suspended in plasma - △ -, in plasma together with Dextran 150 (4 grams percent) - □ -, and in Ringer-albumin solution - ○ -.

Figure 2 shows a comparison between flow curves of preparations of 50 percent red cells suspended in Ringer-albumin solution and in plasma, secured with EDTA. The red cell-plasma preparation aggregates, whereas the red cell-Ringer-albumin preparation does not. The addition of Dextran, 150,000 M.W., enhances aggregation. At low shear rates, this enhanced aggregation, coupled with sedimentation of the aggregates, causes a marked reduction

in torque measurements at shear rates below 0.05 sec^{-1}. Below this shear rate large cell-free areas develop on the cone and plate geometry and the system of packed red cell aggregates slips at the geometry wall. This is demonstrated by the change in slope at very low shear rates.

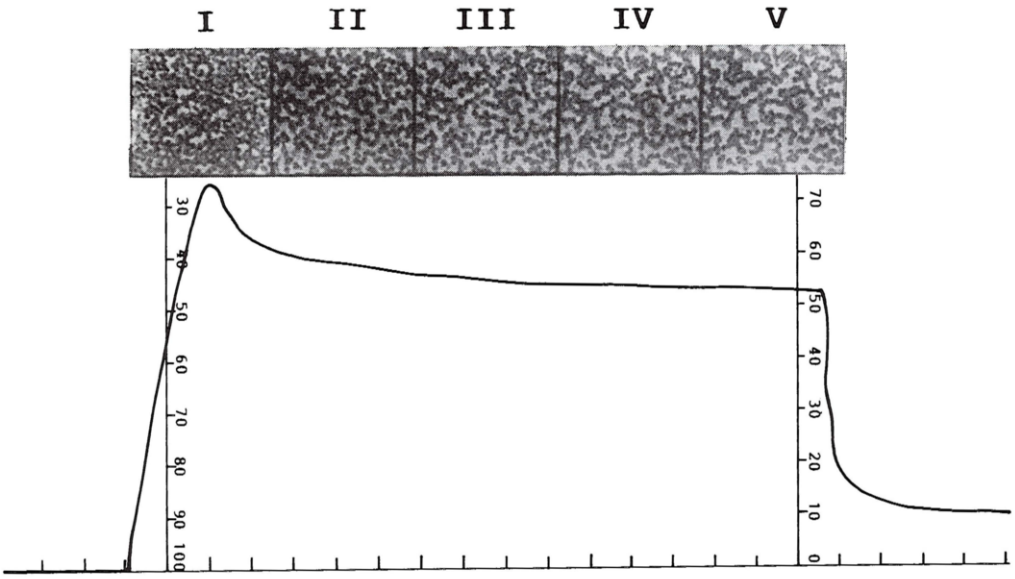

Fig.3

A recorder trace of tangential stress in steady shear of red cells in plasma at 40% hematocrit at 0.05 sec^{-1}. Photomicrographs are taken at the start of shearing, and at one minute intervals thereafter.

Figure 3 shows a recorder trace together with a series of 5 photomicrographs. The first frame was taken when the torque reaches a peak. At this time the red cells are still in the process of aggregation. Thereafter the frames are taken at 1 min. intervals. As aggregation continues, the torque value falls and reaches a plateau after approximately 3 min. At this time the aggregation is complete. This can be seen in frames III, IV, and V.

4.2 Recent Studies on Erythrocyte Aggregation

At the conference on surface hemorheology, organized by Copley and Seaman (12) and held by The New York Academy of Sciences in 1983, several investigators presented studies on cell aggregation, including red cell rouleaux, and desaggregation. It is advisable to consult in particular in this volume the contributions by Evans and Parsegian (13), Silberberg (14), Skalak and Chien (15), Seaman (16), Chien et al (17), Goldsmith et al (18), Copley (19), Seno et al (20) and Dintenfass (21).

4.3 Fibrinogen-Fibrin Complexes, Fibrinogenin Aggregation and

Red Cell Aggregation

Fåhraeus emphasized the role of fibrinogen in red cell aggregation (7). There are quite a number of substances known to initiate red cell aggregation. However, I should like to deal here with fibrinogen in its different physiological manifestations. In 1966 we reported at the First International Congress on Hemorheology our findings on fibrinogen-fibrin complexes which bring about red cell aggregation (22). To my knowledge, no new work pertaining to fibrinogen-fibrin complexes and rouleau formation has been presented since that time. We emphasized that fibrinogen-fibrin complexes are reversible and separate into fibrinogen and des-A fibrin monomer, thus facilitating red blood cell desaggregation, with the splitting off of fibrinopeptide A.

There is another reversible phenomenon in which fibrinogen is involved which is the clotting of fibrinogen without thrombin participation. The occurrence of this phenomenon has been thoroughly investigated by different researchers and is well founded on experimental grounds (23). The term 'fibrinogenin' was introduced by me for this form of clotting, which occurs in two steps, viz., aggregation followed by gelation (23). It may be surmised that the fibrinogenin gelation may not be reversible, although this can only be decided in future studies.

Fibrinogenin aggregation has hitherto not been considered in red cell aggregation. As both are reversible processes, they can act synergetically or cumulatively. As fibrinogen may be adsorbed to the surface of the red cell membranes, the aggregation of red cells may be brought about by fibrinogenin aggregation, not merely in pathological conditions, such as polycythemia, but physiologically as well. No studies have thus far been made on red cell aggregation pertaining to fibrinogenin aggregation. Such proposed investigations appear to me necessary to throw new light on the aggregation of red cells.

4.4 Red Cell Aggregation Under Zero Gravity

Recently, Dintenfass (24,25) reported his experiment on red cell aggregation under zero gravity made during a NASA space shuttle. The observations were recorded during one space flight from the Kennedy Space Center, and Dintenfass emphasized that they need to be confirmed in future experiments.

Dintenfass and his team developed automatic instrumentation for conditions of zero gravity and placed it on the space shuttle "Discovery" for the flight in January 1985. A parallel and simultaneous experiment was performed in ground laboratories provided by NASA at the Kennedy Space Center.

Photomicrographs of red cells were obtained under zero gravity and under conditions of vibration. Dintenfass summarized his findings as follows: (I) under zero gravity, the red cells do not change their shape; (II) they aggregate in rouleaux, although the size of aggregates is smaller than on the ground; (III) the red cell clumps in blood samples obtained from patients with histories of myocardial infarction, diabetes, cancer, among others, show rouleaux under zero gravity in contrast to "patterns of compact aggregation under ground conditions"; (IV) in "normal" blood, which exhibited rouleaux on the ground, the red cells are mono-

disperse and in doublets.

Even though further experiments under the same conditions may confirm the findings of Dintenfass, there may be differences in blood samples from human subjects secured in orbit and on the ground. Moreover, comparisons should likewise be made in biomicroscopic observations of the conjunctiva or other capillary beds in human subjects in orbit and on the ground, as well as in experimental animals. Such comparative observations, to be made under well controlled conditions, may show whether gravity or its absence affect red cell aggregation in vivo and extra vivum.

It appears probable that what Dintenfass called "patterns of impact aggregation" are irreversible red cell clumps or agglutinates. In obstructing the circulation, such irreversible red cell clumps would contribute in producing severe disturbances which may ensue in necrosis of the affected tissues. This goes to show that we must differentiate diagnostically between different forms of clumps.

Kimzey (26) reported significant changes in the distribution of red cell shapes during flight and exposure to weightlessness. These findings are supported by Russian reports of an alteration in red cell shape for crewmen following extended Soviet Salyut missions. No red cell clumps were mentioned by Kimzey in his review. As no cell shape changes were observed in the Dintenfass experiment under zero gravity, there appear to be marked differences between blood samples from astronauts in orbit and those secured on the ground and later exposed to zero gravity.

4.5 Red Cell Sedimentation At Varying Shear Rates

Copley et al (30) subjected the erythrocyte sedimentation of blood from healthy human subjects to varying shear rates. Whole blood, anticoagulated with EDTA, was exposed to shear rates from 0.0001 to 10 sec^{-1}. They considered their findings to mirror flow properties in certain parts of the microcirculation, since erythrocyte sedimentation changes with varying shear rates. It is pointed out that erythrocyte agglutination (which is an irreversible process of clumping) may be an important factor in increased sedimentation rates and mainly responsible in pathological conditions.

5. Compaction Stasis and Erythrocyte Desaggregation

In certain conditions of inflammation, the red cells are compacted, resulting in compaction stasis, as mentioned under 1. This phenomenon occurs with restriction of the fluid phase of blood due to increased capillary or vascular permeability. Referring back to van Leeuwenhoek's observations of the circulation in the tadpole's tail, the initial stoppage of blood flow ceased, the circulation was restored in the affected blood vessel and the red cells could be seen as single entities. The initial observation in a blood vessel of the tadpole's tail which van Leeuwenhoek described as "coagulated" might have occurred in an inflamed vessel where the flow of blood stagnated. If this should have been the case, van Leeuwenhoek could be credited also for discovering inflammation as it affects the vessel-blood organ. However, it was Julius Cohnheim who gave detailed description of his biomicroscopic ob-

servations of the microcirculation in the web and tongue of the frog during inflammation. He found increased capillary permeability and stoppage of blood flow, which he called 'stasis'. In 1957 I introduced the term 'compaction stasis' for this phenomenon, since the red cells are compacted in the affected blood vessels. Last year, at the Conference in Siena, I proposed to name this hemorheological discovery the "Cohnheim compaction phenomenon" (3).

6. Erythrocyte Desaggregation Extra Vivum and In Vivo

Red cell desaggregation (also spelled disaggregation) is a physiological process and can occur in inflammatory conditions in restoring blood flow, as mentioned above. In case such restoration does not occur, reversible aggregation leading to desaggregation is no longer possible in the affected vessel segment of the vessel-blood organ.

Chien et al (27) quantitated the shear stress needed to desaggregate red cell rouleaux in a flow channel under microscopic observations. They found stepwise increases in shear stress to cause a progressive peeling of the top cell of the rouleau. The desaggregating energy was found by Chien (28) to result from electrostatic repulsion between the sialic acids on cell surfaces and mechanical shearing stress.

Recently, Vayo, Skalak, Brunn and Chien (29) presented a model for the desaggregation (which they spell disaggregation) of two-cell rouleaux, formed with Dextran, by fluid shear stress. This model incorporates several features of rouleau desaggregation observed experimentally. They computed the equilibrium position of a two-cell rouleau by the use of an energy balance in which the elastic, adhesive and mechanical energies are included and the total potential energy is minimized. Their findings suggest that "shear disaggregation may induce a translocation of the intercellular adhesive bonds which become concentrated near the edge of separation, thereby effectively increasing the surface adhesive energy in that region with progressive disaggregation" (29).

Skalak and Chien (15) consider the formation of rouleaux as an interaction of the surface adhesive energy and the elastic strain energy of the red cell membrane. They developed a dynamic energy equation which they claim is applicable to both processes of aggregation and desaggregation by external forces. Desaggregation is studied in a model in which the red cell rouleaux are idealized as cylindrical forms. During desaggregation they consider that the strain energy stored in the cell contributes to the work done in overcoming the adhesive energy.

7. Erythrocyte Aggregation and Desaggregation, Fibrinogen and the Vessel-Blood Organ

Silberberg (14) discusses macromolecules in cell-cell encounters and includes in his considerations the vascular endothelium together with the surface of the circulating cells, both of which "bear upon them a stubby coat of glycoproteins". These surface held glycoproteins fulfill a number of specific functions. According to Silberberg, macromolecular diffuse surface layers

will at times prevent and at other times permit the sticking of the cells circulating in blood to each other or to the walls of the vessel. He analyzes the forces that arise between the cells and discusses how forces induced by the flow match up to forces of repulsion that can be generated.

Recently it has been shown by the author that fibrin and/or fibrinogenin are probably more significant than glycoproteins as the endoendothelial lining, the substance in the interendothelial junctions, one of the major constituents of the basement membrane and possibly also contained in endothelial vesicles (31,32).

As far as the red cell membrane coating is concerned, fibrin-fibrinogen complexes may be significant in red cell aggregation as dealt above under 4.3. As red cell aggregation is a reversible process, there is likewise the problem how desaggregation involving fibrinogen may come about. Erythrocyte desaggregation may be favored by substances found to desaggregate fibrinogenin. Such desaggregation was recently found by Copley, King, and Chien (33) to occur with chondroitins A,B, and C, as well as with certain low molecular heparins, as also found in an experimental survey by Copley and King (34). Although our findings with these substances are considered to be important in the prevention of thrombogenesis, they may likewise be active in preventing the formation of red cell rouleaux.

The divalent nature of fibrinogen was first proposed by Blombäck (35) and now is well established. In a recent discussion with Blombäck (36), he suggested that this divalency of the fibrinogen would have it act as a glue between red cells, provided that there are binding sites for the red cell on the molecule. In the case of desaggregation the non-covalent bond could be split by different substances competing for the binding sites on either the fibrinogen or the red cell surface. Finally, there may well be physiological regulatory mechanisms at different sites of the vessel-blood organ, which bring about erythrocyte aggregation and desaggregation, thus controlling the flow of blood. Such mechanisms may involve the interplay of chemical and physical actions. Disturbances of such homeostatic processes could lead to pathological conditions in the affected parts of the vessel-blood organ.

8. Concluding Remarks

I have taken you on a path which emphasizes the difference between red cell aggregation extra vivum and in vivo. It further points out the occurrence of reversibility with regard to fibrinogen-fibrin complexes and fibrinogenin aggregation. It touches upon compaction stasis as well as irreversible red cell clumping, which probably only occurs in pathological conditions which may lead to disease processes. I have further emphasized the entity of the vessel-blood organ as a way of considering the difference between extra vivum erythrocyte aggregation and that occurring in vivo. It is a path entirely open to exploration and may lead to discovery.

It is no longer possible for a scientist to express in a scientific paper the delight experienced in having made a discovery. It gives me great pleasure to cite again Anthony van Leeuwenhoek

from the same Letter to the Royal Society, from which I quoted in the beginning of this presentation. He expressed his feeling about his discovery on erythrocyte aggregation and desaggregation as follows: "The motion of the Blood in these Tadpoles exceeds all the rest of small Animals, and Fish, I have seen; nay, this pleasure has oftentimes been so recreating to me, that I do not believe that all the pleasure of Fountains or Water-works, either natural or made by Art, could have pleased my sight so well, as the view of these Creatures have given me".

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