GAS MICROBUBBLES IN FRESH NATIVE BLOOD AND THEIR SIGNIFICANCE FOR ESR MEASUREMENTS

V.L. Voeikov*,**, A.I. Goncharenko*, S.A. Goncharenko*, and I.P. Kaganovskii*

*Lomonosov Moscow State University, Faculty of Biology, Department of Bioorganic Chemistry, Moscow, Russia
**International Institute of Biophysics, Neuss, Hombroich, Germany

vvl@soil.msu.ru

Abstract: Video recordings of the process of erythrocytes sedimentation in whole blood of healthy donors revealed nano- and microbubbles of gas evacuating from blood in course of its settling. At the early stage of sedimentation stormy release of gas is observed at the interface between settling mass of erythrocytes and plasma. Some particles ejected from settling particulate matter were floating up to blood-air interface. These phenomena were observed only if blood was in contact with air. Sedimentation of particulate matter in blood isolated from atmospheric air was much slower and no "boiling" occurred. Thus freshly isolated blood contains gaseous phase besides aqueous and solid phases providing for a higher pressure in blood in comparison to atmospheric pressure. The results suggest that erythrocyte sedimentation rate (ESR) evaluated by standard technique is related to gas evacuation from blood. In particular, high values of ESR result from non-stability of nano- and microbubbles of gas in blood. Revelation that blood represents a complex liquid-solid-gas colloidal system calls for reconsideration of many concepts of haematology, rheology, cardio-vascular physiology.

Introduction

Erythrocyte sedimentation rate (ESR) is a widely used clinical test for inflammatory diseases and in the follow-up of patients. Typically for the performance of the test a pipette filled with stabilized blood is installed vertically and 1 hour later the distance traveled by the boundary between the descended red cell mass and clean plasma is measured. Too fast or too slow sedimentation of red cell mass indicates of abnormal state of health of a patient.

On the other hand this test may potentially provide valuable information about blood as of a complex living tissue. It is plausible that *patterns* of settling of cellular mass in whole blood depend upon blood *behavior* defined by interaction of its components and by metabolic processes taking place in it.

To study the dynamic patterns of erythrocyte sedimentation in whole blood we developed recently an opto-electronic device for monitoring the process of erythrocytes settling in blood. The device provides high-temporal resolution of the changes in the position of the plasma/cellular mass boundary [1]. We named this method ESR-graphy. It turned out that ESR-graphy can give more specific information of a state of health of a patient than usual ESR-test [2]. Besides some new phenomena have been revealed, in particular, essentially non-linear character of the movement of the plasma/cellular mass boundary, apparent upwards movement of the boundary, dependence of its form and fuzziness upon physiological state of blood donors or different manipulations with blood. Several seemingly paradoxical phenomena were observed, for example, slowing down of the boundary movement upon moderate blood dilution with physiological saline and significant acceleration of sedimentation upon addition of even small quantity of own plasma to blood, acceleration of initial rates of sedimentation with shortening of blood column in a pipette, etc. [3].

However in spite of apparent simplicity of the test, the mechanism of sedimentation of red cells and their associates in whole blood is far from being clear [4] making it difficult to interpret data provided by ESR-graphy. To conduct a more deep analysis of processes taking place in blood we performed video recording of settling blood at high magnification. It was revealed that fresh blood of healthy donors contains nano- and microbubles of gas evacuating from blood while it is settling. The results presented here suggest that ESR parameters depend significantly upon gas evacuation from blood whereas the latter depends in its turn upon the conditions providing for the stability of gas phase in blood: the rate of bubbles growth, the ease of their fusion with each other or on the contrary of their collapse.

Materials and Methods

Blood of healthy donors was obtained by finger puncture or from elbow vein using vacutainer. It was stabilized with sodium citrate (1 part of 3,5% citrate to 9 parts of blood). Blood was transferred into a rectangular cuvette (height 50 mm, width 10 mm, thickness 0,8 mm) of in a glass capillary (i.d. 0.6 mm, height 80 mm) as soon as possible. The lower orifice of the capillary was then plugged. In some experiments the capillary

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was completely filled with blood and both its orifices were plugged to avoid blood contact with the air.

A vessel with blood was fixed vertically on the object table of a horizontal microscope. The table could be moved in two coordinates by micrometric screws. Color TV-camera ACE-S110CHB having image resolution of 470 lines and sensitivity 0,1 lux was fixed on the drawtube. A thick glass bath with water was installed between the lighter and a vessel with blood to prevent heating of the latter.

TV-recording was performed at a rate 25 frames/sec. Objectives allowing to observe fields of vision 800x600 mkm, 340x255 mkm, and 167x125 mkm were used.

Results

Fast movement of cells and their associates in blood in a rectangular cuvette (surface area 10x0,8 mm) starts just after its filling with blood (Figure 1). Erythrocytes and their associates are moving during the first 3-6 minutes along the plasma/cellular mass boundary.

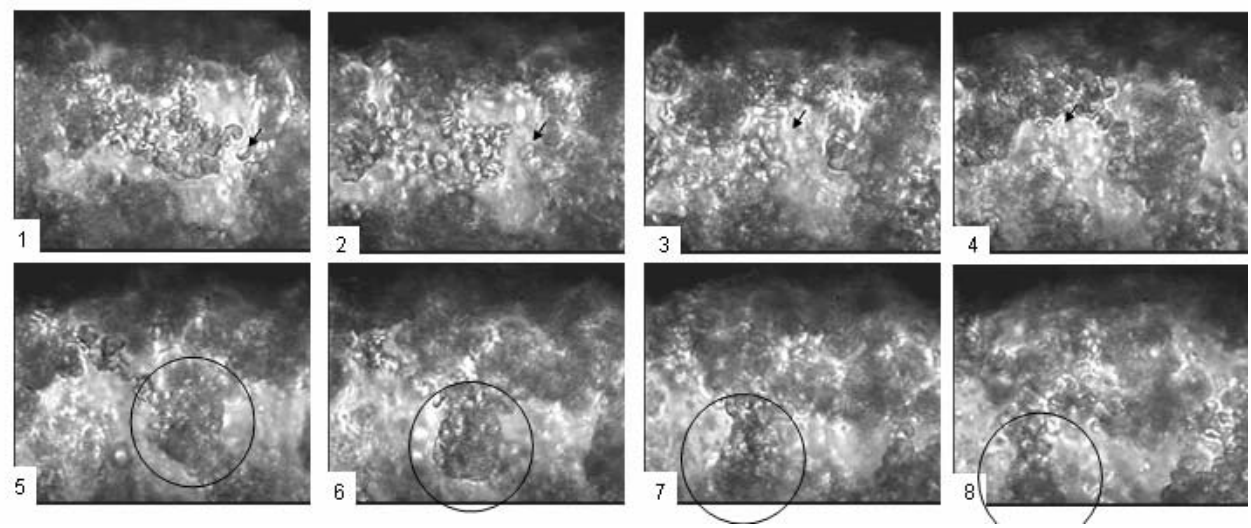

Figure 1: Visualization of cells and their aggregates movement 3 min after filling the rectangular cuvette with blood. Each image was collected for 40 msec, interval between frames 2 sec, field 167x125 mkm. Arrow on frames 1-4 points to a nearly horizontally moving erythrocyte; a group of many erythrocytes encircled at frames 5-8 moves from right to left and somewhat downwards.

The horizontal rate of movement of particles reached 50 mkm/sec or more much exceeding their vertical movement. One may follow a trajectory of a single erythrocyte marked by the arrow at frames 1-4 (Figure 2) and a trajectory of a group of erythrocytes (encircled). Both simultaneously move horizontally but the former has the tendency to an upwards movement and another – to a downwards one. The gap where they move is bounded from below with the settling cellular mass and from above with cells and their aggregates attached to the air/blood interface. Often a thick "plug" of cells at the air/blood interface persists for a long time due to continuous supply of rising cells that stick to it and substitute those that detach from the plug and sink. In general the movement of particles at the stage of plasma/cellular boundary formation is highly turbulent and looks like a snowstorm.

As the plasma/cellular mass boundary descends it becomes more distinct, but at the same time it is permanently broken due to the appearance of "craters" at different sites of it. Emergence of such a "crater" is illustrated in Figure 2. Single cells and even big associations of cells are ejected from a cellular mass through these "craters" back to plasma. Typical rouleaux composed of erythrocytes are indicated by numbers 1 and 2. Whereas mass of cells generally settles, ejected particles float; some of them quickly sink back, other stay for some time suspended, while some other continue to float to the surface. Often one may observe in one and the same field of vision that one group of particles sediments under the action of gravity while another particle in its neighborhood is either suspended or moves upwards.

Movement of particles in opposite direction is well seen in blood plasma at a distance of several millimeters above the boundary (Figure 3). Here a group of erythrocytes (encircled) and a single erythrocyte (indicated by the downwardly directed arrow in the upper right corner) fall down. An upward movement of a group of particles looking as bright small spots in the transmitted light occurs between them in the same field of vision.

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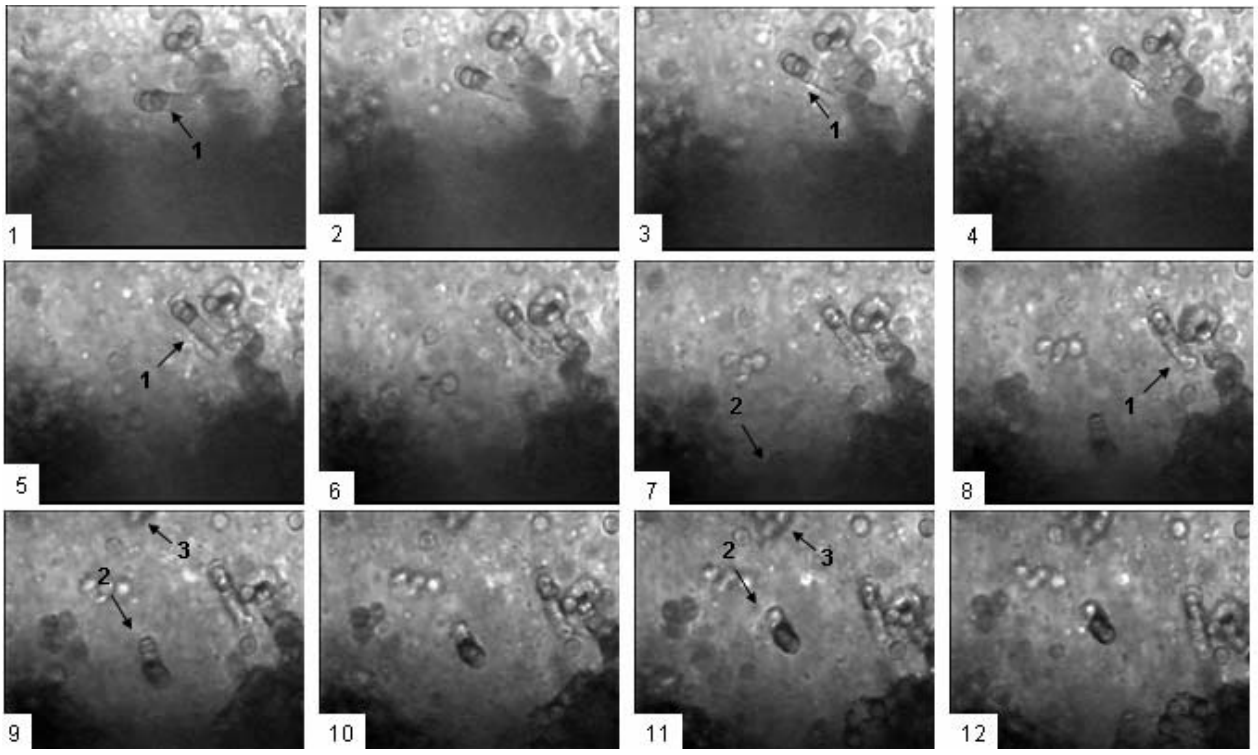

Figure 2: "Boiling" observed in blood 15 minutes after filling a cuvette. Each image was collected for 40 msec, interval between frames 2 sec, field 167x125 mkm. Numbered arrows Nos 1 and 2 point to particular erythrocytes and their aggregates ejected from the "crater" while arrow No 3 points to an aggregate falling from above.

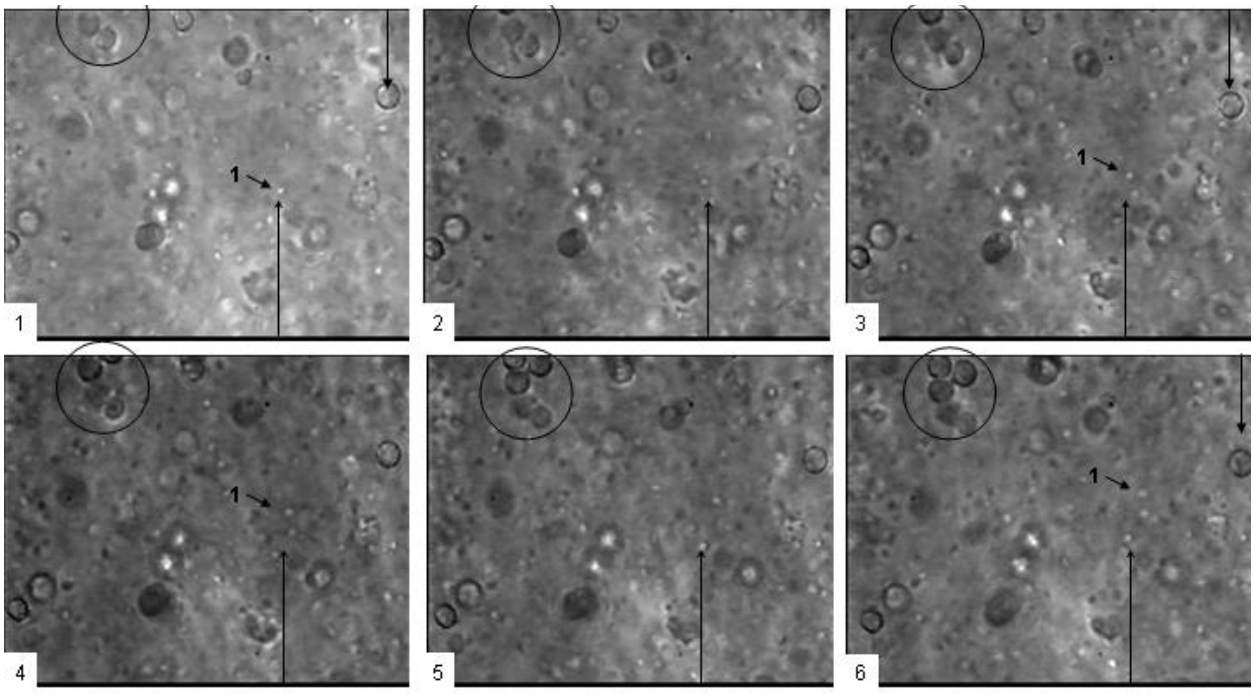

Figure 3: Movement of particles in plasma several millimeters above the plasma/cellular mass boundary 20 min after the start of sedimentation. Each image was collected for 40 msec, interval between frames 2 sec, field 167x125 mkm. Encircled is a group of falling down erythrocytes; downward directed arrow (left upper corner) points to a falling down erythrocyte. A small arrow No 1 points to one of bright particles floating upwards. Its movement may be tracked by the increase of distance between the particle and tip of the upwards directed arrow.

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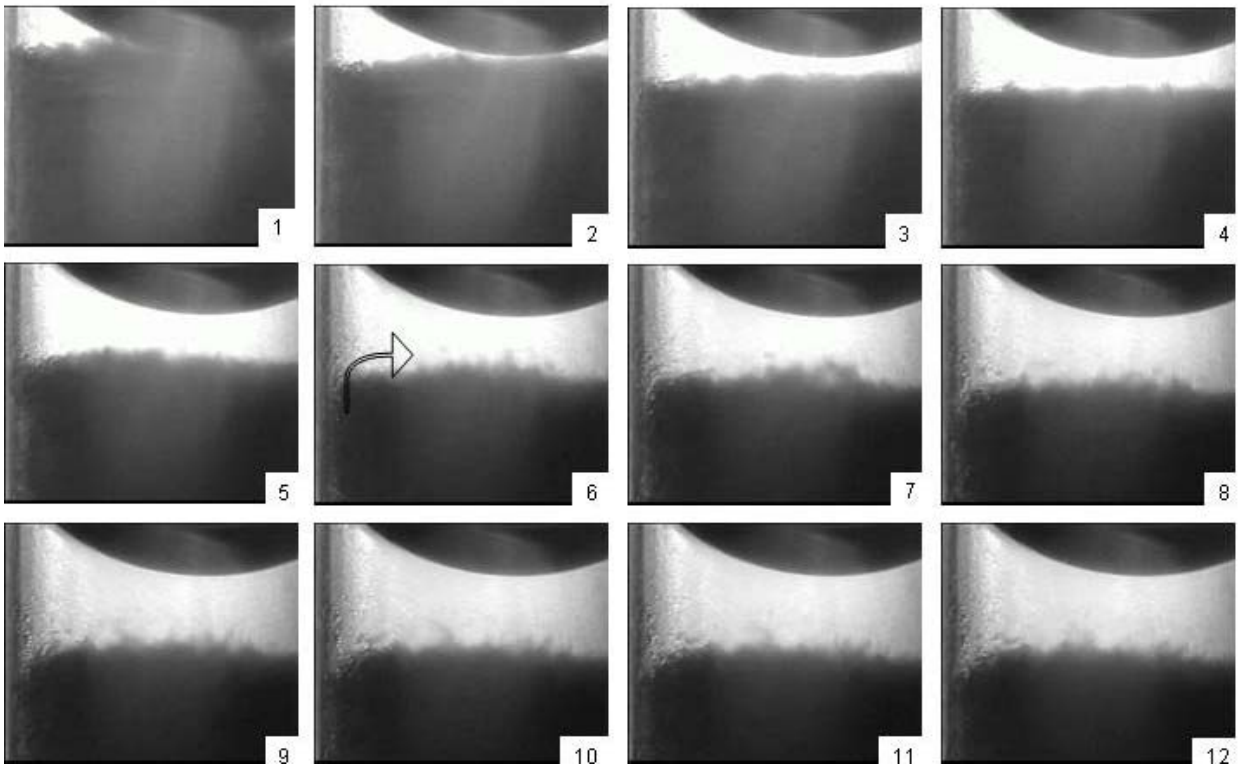

Figure 4: Sedimentation in a capillary (i.d. 0,6 mm). Frame No 1 – 1 min after the capillary filling with blood. Instants to which consecutive frames belong: 1) 0 sec (the start of the recording), 2) 60 sec, 3) 120 sec, 4) 160 sec, 5) 200 sec, 6) 220 sec, 7) 230 sec, 8) 240 sec, 9) 244 sec, 10) 248 sec, 11) 250 sec, 12) 252. An arrow at the frame No 6 indicates the emergence of an upward flow of substance along the capillary wall and rejection of cells and their associates upon the plasma/cellular mass boundary. Field – 800x600 mkm.

Patterns of sedimentation of blood in narrow capillaries differ from those in a rectangular cuvette. Separation of the plasma/cellular mass boundary from the meniscus (the blood/air interface) that can be seen in the upper part of images as a black hemisphere occurs much more slowly in a capillary than in a rectangular cuvette. Plasma/cellular mass boundary remains at the beginning rather clear-cut. At some moment an upwards flow appears near the wall of a capillary. It throws away even very big associates of erythrocytes. In a particular experiment illustrated in Figure 4 this flow becomes evident 4 min after filling the capillary with blood and its installation in a vertical position (frame No 6). At the same time small bright particles begin to release from the settling cellular mass.

Massive particles are thrown away from the cellular mass upon the surface of the boundary with plasma. Bright particles may be seen in the upstream. As the intensity of the upstream increases number and dimensions of these particles increase as well as the rate of the boundary sedimentation. Note that time intervals between successive frames in Figure 4 become shorter with time.

The rate of the boundary sedimentation is more than doubled with the appearance of the upstream – from 18 to 40 mkm/min with the tendency to further acceleration. It was noted also that that during the first 4 min of observation the meniscus also descended by 6,5 mkm. During the same period plasma/cellular mass boundary descended by 100 mkm.

If a fully filled capillary with blood was isolated from the air by plugging the upper orifice of a capillary the movement of the plasma/cellular mass boundary was much slower than in the same blood settling in an open capillary. During the first 20 min the rate of the plasma/cellular mass boundary settling does not exceed 20 mkm/min. This value is close to the one characteristic for the rate of the boundary sedimentation in an open capillary before the upstream movement of particles emerges in it. Besides, the boundary in the plugged capillary was much fuzzier than in the open one.

Discussion

Direct visualization of blood settling shows that processes taking place in it have little in common with usual sedimentation of particles in a medium the density of which is lower than their buoyant density. Previously we came to the same conclusion basing on the analysis of time-resolved patterns of plasma/cellular mass boundary sedimentation ("ESR-grams") obtained under the standard conditions of ESR evaluation but with the help of the automatic device for high-temporal resolution of the process of red blood sedimentation [1-3]. Using ESR-graphy several distinct stages may be

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resolved in the process of the boundary sedimentation in healthy donors' blood: a lag period during which the boundary just forms and practically does not move (τ 1 at Figure 5), period τ 2 – start of sedimentation at low rate, period τ 3 – acceleration of sedimentation rate and period τ 4 – deceleration of the rate of a boundary movement.

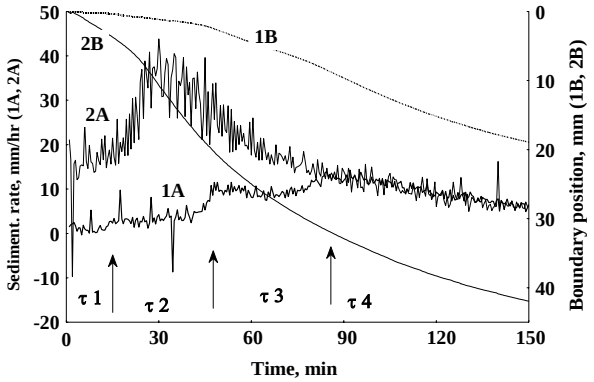

Figure 5: Velocity-time curves – «ESR-grams» (1A and 2A, left ordinate) and change in plasma/cellular mass boundary position (1B and 2B, right ordinate) in blood of a healthy donor (curves 1A and 1B) and of a diabetes patient (curves 2A and 2B). Arrows separate different stages in the process of the boundary sedimentation in blood of a healthy donor.

One can see from Figure 5 that patient's blood is characterized by a higher average rate of sedimentation and much earlier shift from the stage τ 3 to the stage τ 4. The stage τ 1 is lacking in it and the stage τ 2 is very short. At the early stages of sedimentation apparent reverse movement of the boundary is often observed. In particular experiments illustrated in Figure 5 the "negative" velocity event in healthy donor's blood is observed some time before the shift from the stage τ 2 to the stage τ 3 (ESR-gram 1A) and shortly after the start of sedimentation of diabetic patient's blood (ESR-gram 2A).

One may also observe oscillations of sedimentation rate at ESR-grams; their amplitudes depend upon the stage of the process and properties of blood. It is much higher at ESR-grams for patients' blood in comparison to those for healthy donors' blood. Our studies have shown that the intensity of such oscillations in fact reflects the blurring of the plasma/cellular mass boundary: the fuzzier is the boundary, the higher is the amplitude of oscillations. It turned out that the level of blurring correlated with the state of health of a donor and could be influenced by different treatments of blood. In particular a systematic study of blood of ischemic patients has shown that blurriness of the boundary significantly increased on the days with geomagnetic storms coinciding with deterioration of their state of health; on geomagnetically calm days boundary blurriness was less [5].

All these and other phenomena revealing themselves in ESR-graphy studies are difficult to explain in frame of a simple hydrodynamic model according to which blood is just a colloidal solution in which particles sediment due to gravity in a liquid at a rate dependent on their size and difference of their intrinsic buoyant density and density of a medium.

However if to consider that blood contains also a third phase – a gaseous phase represented with nano- and micro-bubbles of gas all these phenomena find logical explanation.

Direct visualization of processes taking place in freshly obtained blood reveals phenomena indicating of gas evacuation from blood. The presence of gas bubbles is especially evident when sedimentation takes place in a rectangular cuvette where gas evacuation is expressed as a stormy movement of particles during the formation of a plasma/cellular mass boundary and boiling-like phenomena after the boundary has been formed. The ratio of surface area of blood/air interface to the height of blood pillar is larger in a rectangle cuvette than in a narrow capillary. Thus in the first case a big surface area allows for faster evacuation of gas bubbles from blood. Besides as the height of blood layer in a rectangular cuvette is smaller than the height of blood pillars in capillaries the hydrostatic pressure of the liquid in cuvettes is lower than in capillaries providing for easier formation and for growth of bubbles. In fact we found in ESR-graphy experiments that initial rates of the boundary sedimentation in blood taken to pipettes with equal diameter was increasing with the decreasing of the height of a blood pillar in full agreement with the above reasoning.

Due to smaller surface area of blood contacting with air and higher hydrostatic pressure in the lower part of a capillary gas evacuation in capillaries starts after a lag-period during which buoyancy of bubbles or to be more precise of the particles to which they stick reaches the critical value allowing them to float. Bubbles may make their way in a viscous cellular mass predominantly in the vicinity of a capillary wall because of small cross-section of a capillary.

This mechanism allows suggesting an explanation to the existence of distinct stages of sedimentation process observed on ESR-grams – of a lag period (τ 1) and of a period of slow sedimentation (τ 2). The first one reflects the period of a boundary formation, the second – the situation before the up flow of particles emerges and the third one – settling of blood associated with gas evacuation. If for any reasons stability of gas vesicles in blood diminishes stages "τ 1 and τ 2" may be lacking because gas evacuation starts nearly immediately after filling of a pipette with blood. Such a situation is illustrated by the plot 2A at Figure 5, and it is characteristic for blood of patients with different pathologies.

The patterns of emergence of gas phase and evacuation of gas bubbles from blood allow to explain opposite effects of even slight dilution of blood with physiological solution or with own plasma that were observed using ESR-graphy [1]. In the first case the rate of the plasma/cellular mass boundary movement is usually slower that in the control, while addition of own plasma to blood results in a significant acceleration of

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blood settling. One may suggest that addition of physiological solution to blood retards the formation of bubbles because of gas dilution. Plasma added to blood increases gas concentration because it itself contins dissolved gas and the probability of gas bubbles formation is thus increased.

The level of fuzziness of the boundary between cellular mass and plasma may also very much depend on the pattern of gas evacuation: the more vigorous is it the blurrier is the boundary and the larger is the amplitude of apparent high frequency oscillations of sedimentation rate observed on ESR-grams.

Comparison of blood behavior in open and sealed capillaries also shows that the major factor determining the rate of erythrocytes sedimentation in whole blood is the rate of evacuation of gases from blood. Gas does not have an opportunity to evacuate from the sealed capillaries, thus the pressure in blood stays constant and bubble formation is retarded. If buoyant density of particulate matter in blood is not a constant parameter, dependent only upon the average weighted density of their molecular components, but it also depends upon the gas phase attached to them the rate of their sedimentation (or flotation) will be determined by this gas. The larger is the volume of gas layer absorbed by the particles, the lower is the density of the bubble, the lower is the buoyant density of a particle. Thus any particle present in plasma may sediment, be in a suspended state or float to the surface, that can be clearly seen as an oncoming traffic of different particles in one and the same field of vision.

Microscopic visualization of the process of sedimentation in capillaries allowed to support the concept of A.I. and S.A. Goncharenko [6] that blood volume in a body is provided not only by the volume of a liquid part of it containing solid inclusions but also by the gas phase of a variable volume. Slow subsiding of a meniscus separating blood and air indicates of a decrease of the volume of blood taken into the capillary in the process of sedimentation of solid particles in it. One should realize that some 4-5 liters of water in adult human blood hydrates surfaces of myriads of solid particles – cells, protein and lipid micelles the total area of which should exceed 1000 m^2. Hence water in plasma may be looked upon as a two-dimensional film with inclusions of small ions and gas molecules. Of all major gases present in blood the best candidate for a gas whose state may easily change from a dissolved state to a gas phase state is N_2 the presence of which in the inhaled air is about 79%. Its solubility in water is rather low and it may easily serve a seed for cavity pockets that will arise under changes of pressure.

Conclusions

Fresh native blood represents a complex aerated liquid due to natural presence of gas (presumably nitrogen) microbubbles in it. Gas phase may play significant role in determining the parameters of ESR. Since the volume of gas phase unlike liquid and solid phase may significantly change the volume of the same mass of blood in cardiovascular system may also change significantly dependent on the conditions determining the stability of micro- and nanobubbles and ambient pressure. This factor may play an important role in rheological behavior of blood in cardiovascular system.

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