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Investigation of red blood cell fractionation by gravitational field-flow fractionation

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ABSTRACT

Gravitational field-flow fractionation is used for the separation of particles according to their sizes in the range 1–100 μm: larger particles elute before smaller ones. This phenomenon can be explained as a result of the steric exclusion of the particles from the vicinity of the channel walls, and/or hydrodynamic effects supposedly associated with the inertia of the liquid. The method was used for the investigation of red blood cells. The dependence of the retention ratio on the flow-rate, sample volume, concentration of blood and relaxation time was studied. Analysis of fifteen individual fractions by Coulter counter and reinjection of three other fractions were studied in order to verify fractionation of red blood cells.

INTRODUCTION

Field-flow fractionation (FFF), an analytical method suitable for the separation of macromolecules and particles, is based on the simultaneous action of effective field forces and a liquid flow through a fractionation channel on macromolecules and particles [1]. Vectors of field forces and the liquid flow are mutually perpendicular, the forces act across the channel and the liquid flows along the longitudinal axis of the channel. A concentration gradient is formed across the channel due to the field forces. Simultaneously, a flow velocity profile is established due to the viscosity of the flowing liquid. Different elution velocities arise for various types of particle or macromolecule, and a separation results from these two gradients [2,3].

Gravitational field-flow fractionation (GFFF) is a relatively simple experimental technique. Retention of particles is observed under the Earth's gravitational field. Separation of micrometre-size particles is affected by the interplay of inertial and gravitational forces. The steric mode is effective if the mean Brownian displacement from the wall is less than the particle radius. In this region, particles extend to the flow stream primarily because of their own finite size. In this case the dif-

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ferences in radii of particles rather than in Brownian displacement are responsible for differential migration [4,5]. The deviations from the steric mode observed in GFFF are caused by hydrodynamic lift forces [6].

GFFF has been used for the separation of glass beads [4], chromatographic silica gel supports [7] and blood cells [8–11]. The steric mode of particle separation has been observed in various FFF techniques, *e.g.* centrifugation [12,13] and flow FFF [14]. Caldwell *et al.* [12] separated human and animal blood cells by using low centrifugal forces. They investigated the influence of the osmolality, flow-rate and sample size on retention. They succeeded in characterizing different sizes of red blood cells from various animal species. They obtained a "fingerprint" characteristic of the particular animal species. Nováková *et al.* [10] studied human blood cells by GFFF. They observed differences between "buffy coat" (corresponding to all species of white blood cells) and red blood cells, and studied the dependence of the retention time on the flow-rate and the ionic strength of the carrier liquid. Cardot *et al.* [11] employed GFFF to study red blood cells of healthy donors and of transfused patients. They found differences in the retention of fresh and older red blood cells of a human blood sample from a healthy donor whose diagnosis of a double red blood cell population had been demonstrated by the Beth–Vincent immunological technique.

This paper describes a study of the behaviour of red blood cells by GFFF under various experimental conditions (flow-rate, sample volume, concentration of blood, relaxation time). Characterization of fifteen individual fractions by particle size analysis (Coulter counter) and reinjection of three other fractions into the channel were used to verify the fractionation by GFFF.

EXPERIMENTAL

The separation channel was cut in 0.23-mm thick PTFE FEP foil (Bytax, Chemoplast, Wayne, NJ, USA) inserted between two glass plates. The dimensions of the channel were: width 12 mm, length 560 mm, volume 1.42 ml. The entire system was clamped between two plexiglass plates. The carrier liquid and the sample were introduced into the channel by an inlet capillary situated at the channel head, and taken out by an outlet capillary located at the end of the channel. The carrier liquid was introduced into the channel through an LD2 linear injector (Development Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia). The sample was injected into the separation channel using a microsyringe by an injection system with a septum placed directly in front of the inlet capillary. The flow-rate was maintained at 200 μl/min during the injection for 6 s. Then the flow was stopped for a period of time necessary for relaxation (1 min in most experiments).

The blood samples were obtained from patients under hospital control. The EDTA solution (30 mg of dipotassium EDTA per ml) was used as an *in vitro* anti-coagulation and calcium-complexing substrate. The concentration of blood in this solution was 90.9%. The carrier liquid was of the following composition: 8.5 g of NaCl, 1.1375 g of $Na_2HPO_4 \cdot 12H_2O$ and 0.135 g of KH_2PO_4 in 1000 ml of redistilled water. For the study of the dependence of the retention ratio on the blood concentration, 3 μl of differently diluted blood samples were injected into the channel at a flow-rate of 300 μl/min. The experiments were carried out at the following concentrations of blood in the sample: 7.5, 11.4, 15.0, 22.7, 30.0 and 45.5% (v/v). For the measurement of the dependence on the volume of the injected sample, a 15% (v/v) concentration of blood in the sample solution was chosen. Samples of different volumes (2, 4, 6, 8 and 10 μl) were fractionated at a flow-rate of 300 μl/min.

For the study of relaxation processes, 3 μl of the 15% (v/v) solution of blood were injected at a flow-rate of 300 μl/min. After injection the flow was stopped, and the sample was exposed only to the influence of the gravitational field for 0.5, 1, 2, 3, 5 and 10 min, respectively. After the relaxation time the linear flow was reapplied and fractionation of the sample was accomplished.

The influence of the flow-rate on the retention

ratio was studied at flow-rates of 200–2000 μl/min. Injected samples of blood solutions (3 μl; 15%, v/v) were allowed to relax for 1 min prior to fractionation.

Fractionation of red blood cells by GFFF was verified as follows. Samples of diluted blood (5 μl; 15%, v/v) were injected into the channel, allowed to relax for 1 min and fractionated at a flow-rate of 200 μl/min. After passing through the separation channel fifteen fractions of 1 ml each were collected. Individual fractions were characterized by the number, average size and distribution of particles (*i.e.* red blood cells) using a Coulter counter Model 2M with Coulter channelyzer 256 (Coulter Electronic Limited, Luton, UK).

In another experiment, 5-μl samples of 90.9% blood in EDTA solution were injected into the channel, allowed to relax for 1 min and fractionated at a flow-rate of 300 μl/min. After passing through the separation channel three fractions were collected, the first fraction (1.5 ml) corresponding to the beginning of the peak (retention time 10–15 min), the second fraction (0.6 ml) at the peak maximum (15–17 min) and the third fraction (1.5 ml) at the end of the peak (17–22 min). Non-equal volumes of fractions were selected because the fraction at the peak maximum contained more red blood cells than the fractions collected at the beginning and at the end of the peak. Red blood cells were allowed to settle, and 5-μl samples of the bottom layer were reinjected into the separation channel and fractionated.

In all experiments the UVM 4 detector (Development Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) equipped with an 8-μl flow-cell was used. The detection wavelength was 254 nm.

RESULTS

It was found that the time necessary for complete relaxation of samples was 30 s: longer relaxation times did not show any influence on the fractionation. We routinely used a relaxation time of 1 min.

FFF gives good results for the separation of small amounts of sample. Overloading effects were observed in thermal, flow and sedimentation FFF [15,16]. To determine a proper dilution of blood samples, we studied the dependence of the retention ratio on sample concentration and volume. Fig. 1 shows the decrease in the retention ratio with decreasing concentration of red blood cells in the sample. It is evident from Fig. 2 that the retention ratio decreases with decreasing volume of the injected sample.

The dependence of retention ratio of red blood cells on the flow-rate is presented in Fig. 3. The retention ratio increases with higher flow-rates.

As shown in Fig. 4a, the eluted sample was separated into fifteen fractions and these were characterized by Coulter counter analysis. Table I lists the numbers of particles in the individual fractions and their average diameter, and the standard deviations of number and size of particles taken from eight measurements. The results show that the fractions collected at the peak maximum exhibit an almost regular change of the particle size from the biggest to the smallest. The graph of the particle size distribution of these fractions (Fig. 4b) shows that it is possible to take

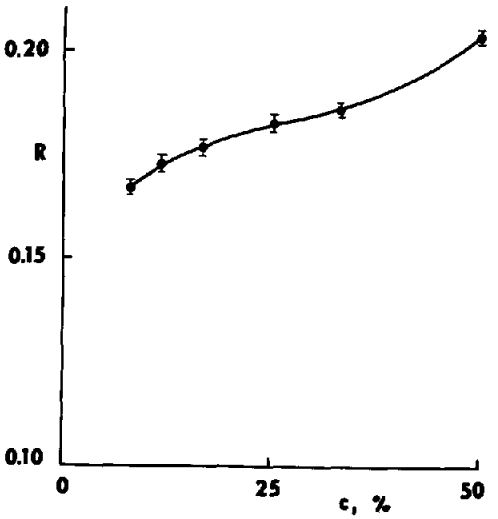

Fig. 1. Dependence of the retention ratio (R) on the concentration (c) of blood samples at a flow-rate of 300 μl/min; the S.D. was taken from five measurements. Injection volume, 3 μl; concentration, 7.5, 11.4, 15.0, 22.7, 30.0 or 45.5% (v/v); relaxation time, 1 min.

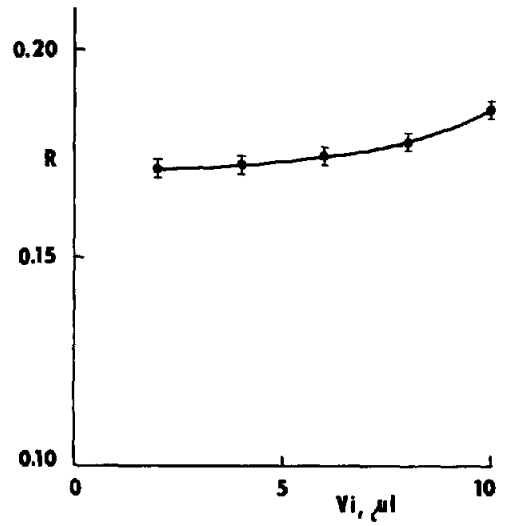

Fig. 2. Dependence of the retention ratio (R) on the volume injected (V_i) at the same flow-rate as in Fig. 1. Concentration, 15% (v/v); injection volume, 2, 4, 6, 8 or 10 µl; the S.D. was taken from five measurements; relaxation time, 1 min.

into account records only from fractions of the peak maximum, *i.e.* fractions 7–12, with a number of particles higher than 3000, because fractions obtained at the beginning and at the end of the peak, *i.e.* fractions 1–6 and 13–15, did not show a clear detector response.

After a whole blood sample had been passed through the separation channel, three fractions were collected as indicated in the fractogram in Fig. 5a. At lower blood concentrations the numbers of red blood cells were too low to be detected after reinjection. These fractions were reinjected individually into the channel. The fractograms of individual fractions are presented in Fig. 5b: they do not show a clear shift of the retention times of individual fractions. These three fractions were also characterized by Coulter counter analysis. The results indicated very small changes in the volume of particles, *i.e.* almost no fractionation

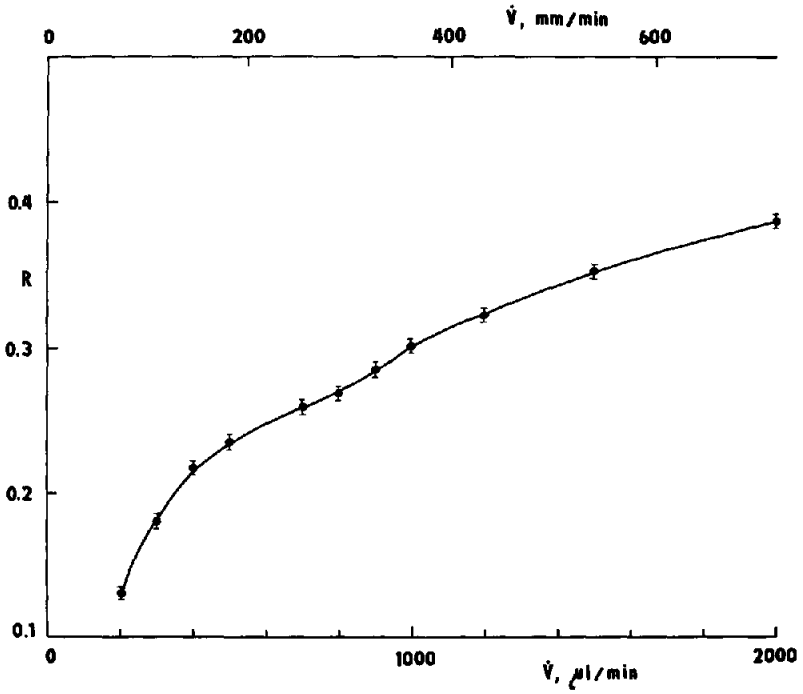

Fig. 3. Dependence of the retention ratio (R) of red blood cells on the flow-rate ($\dot{V}$); the S.D. was taken from four measurements. Injected volume, 3 µl; concentration, 15% (v/v); flow-rate, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500 or 2000 µl/min; relaxation time, 1 min.

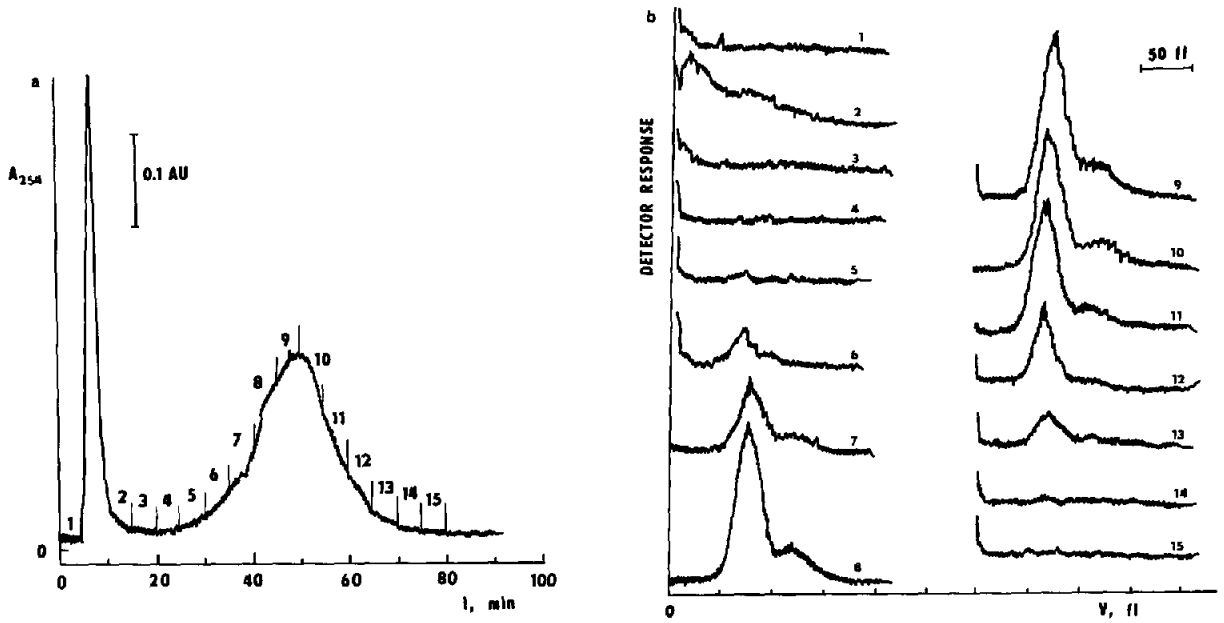

Fig. 4. (a) Fractogram of red blood cells at a flow-rate of 200 μl/min. Concentration, 15% (v/v); injection volume, 4 μl; relaxation time, 1 min. Numbers indicate individual fractions. (b) Characterization of individual fractions obtained by Coulter counter analysis.

TABLE I

CHARACTERIZATION OF INDIVIDUAL FRACTIONS

No.	Number of particles	σ_N[a] (%)	Mean volume (fl)	σ_V[b] (%)
1	2268	1.6	104	3.0
2	11 536	0.6	65	1.2
3	3040	1.0	72	1.6
4	826	1.2	83	2.5
5	883	2.1	87	2.0
6	3036	1.2	86	0.4
7	6455	2.1	92	0.7
8	14 294	2.0	88	0.5
9	15 593	3.4	88	0.6
10	11 809	1.3	84	0.4
11	10 732	1.4	82	1.3
12	5661	1.2	82	1.0
13	3230	1.8	87	0.6
14	1184	2.5	89	1.6
15	1363	1.4	86	0.6

[a] σ_N is the S.D. of the number of particles taken from eight measurements.

[b] σ_V is the S.D. of the size of particles taken from eight measurements.

(see Table II). The fraction taken at the peak maximum (fraction 2) contained a much greater number of particles than the fractions at the beginning (fraction 1) and the end (fraction 3) of the peak. It is clear that it is possible to obtain reasonable fractionation of red blood cells only with diluted blood samples.

DISCUSSION

The separation of biological particles is a specific problem in the analytical application of separation methods. This type of analyte is characterized by its biological activity in the native state. The native state can be changed and the biological activity lost by relatively small changes of physical and chemical conditions. These changes may be caused by the separation process itself, owing perhaps to adsorption or a change of temperature, pressure or solvent composition. Thus, the analysis of biological particles differs from the analysis of inorganic or synthetic particles and, therefore, it demands a special ap-

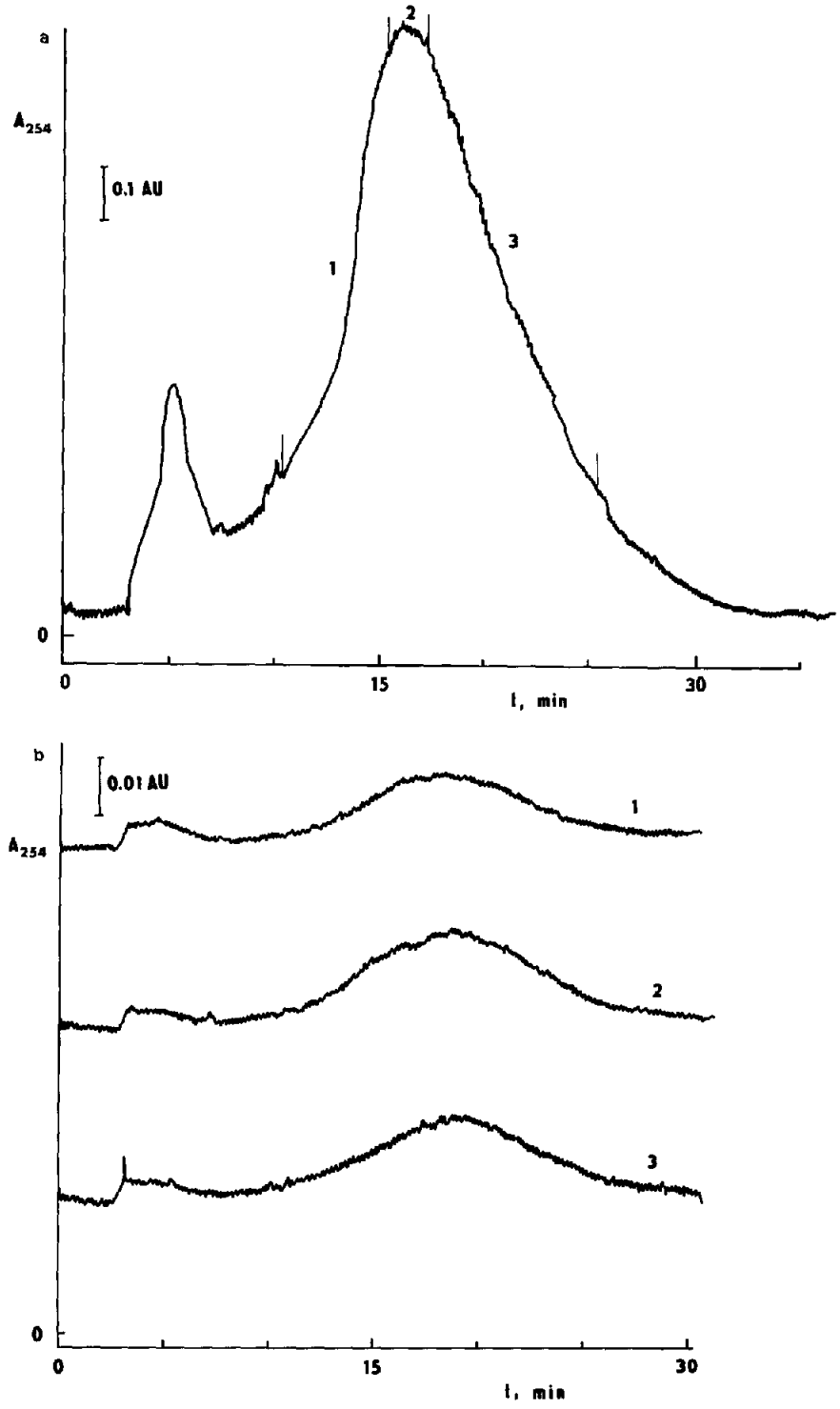

Fig. 5 (a) Fractogram of red blood cells at a flow-rate of 300 μl/min. Concentration, 90.9% (v/v); injection volume, 5 μl; relaxation time, 1 min. Numbers indicate individual fractions. (b) Fractograms of three reinjected individual fractions measured under the same conditions as in (a).

TABLE II

CHARACTERIZATION OF FRACTIONS

No.	Number of particles	Mean volume (fl)
1	8382	89
2	57 710	86
3	37 622	86

proach. Unfavourable influences must be minimized, and the method selected must be gentle enough for the particular biological analyte. As the Earth's gravitational field appears to be sufficiently intense to allow the separation of blood cells [8–11] by GFFF, this technique seems to be a promising tool in cell biology.

FFF is an analytical separation method that is best adapted for separating small samples. With large samples, the particles are likely to interfere with one another's movement, thus distorting the migration process. For even larger samples, not all particles will find a place at the crowded wall, and they will migrate ahead until they find an unoccupied region. This overlying migration process is expected both to broaden the zone and increase the average migration rate. The latter prediction is verified by dependences (see Figs. 1 and 2) of the retention ratio on both the injected sample volume and the dilution of the blood sample. These figures show that the retention ratio decreases with decreasing amount of blood cells injected into the channel. Therefore, it is evident that, for the study of blood cells, it is convenient to work with sufficiently diluted solutions. Cardot *et al.* [11] also studied blood cells in these concentration ranges; they diluted blood 100–1000 times and injected 100 μl of the sample into the channel.

The dependence of the retention ratio of red blood cells on the flow-rate was studied in order to examine the possible influence of the lift-forces [6,17] on the sample at higher flow-rates. The results showed that, at higher flow-rates, lift-forces seem to be activated (see Fig. 3).

Polydisperse samples can be considered as mixtures of many disperse fractions. Therefore, the fractogram of a polydisperse sample can be considered as the sum of monodisperse fractograms. When the sample passes through the separation channel, the particles are fractionated, *i.e.* separated according to certain properties. In GFFF the sample is separated according to the particle size. Particle size (Coulter counter) analysis of fifteen fractions collected from the sample of the red blood cells after passing through the separation channel (Table I, Fig. 4b) shows that the fractions collected at the peak maximum (fractions 7–12) exhibit an almost regular change of the particle size from the biggest to smallest ones. The fractions obtained at the beginning (fractions 1–6) and at the end (fractions 13–15) of the peak exhibit certain irregularities, which can be explained by very low numbers of particles in these regions. In this case, small amounts of other cells in these fractions have an effect, and the average volume of the particles does not correspond to the theoretically assumed value. Differences in the shape, density and rigidity of individual subpopulations of red blood cells may also be important [8,9].

Direct confirmation of the fractionation of polydisperse materials can be based on the collection of fractions from the elution stream and the reinjection of these fractions into an FFF system for a second run. Reinjection of three fractions (Fig. 5a and b) did not clearly prove fractionation, but it must be remembered that the blood sample has a relatively low distribution of cell sizes, especially if the sizes are expressed in radii and not in volumes, as in Tables I and II. In the case of diluted blood samples used for the first fractionation, the reinjected samples were too dilute to obtain a good detector response. For this reason we performed this experiment with undiluted blood samples. It is clear from a comparison of results obtained for concentrated and diluted samples (see Tables I and II) that, in the case of the diluted (15%) sample, fractionation was clearly shown by Coulter counter results, whereas almost no fractionation was found by Coulter counter in the case of the concentrated (90.9%) sample. The different shapes of the frac-

tograms of concentrated blood samples were observed previously [10].

Although the experimental arrangement for GFFF is very simple, interpretation of the results obtained is difficult. It has been found [4] that the larger particles elute before the smaller ones. This phenomenon can be explained either by the steric exclusion of the particles from the vicinity of the channel walls [18] or by hydrodynamic effects associated with particle–liquid flow interactions [19]. However, one must keep in mind that the particles in GFFF are separated according to a discriminating parameter that combines the size, shape and density of particles [14,20,21], and that the difference between rigid spherical latex particles and soft discoidal red blood cells is certainly significant.

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