

STABILITY RATIO OF RED BLOOD CELLS: ITS DEPENDENCE ON TEMPERATURE AND ITS RELATIONSHIP TO THE ERYTHROCYTE SEDIMENTATION RATE

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SUMMARY

The theory of the coagulation of particles in laminar shear flow was applied in a study of the aggregation of red blood cells. The theory predicts that in a system of initially dispersed particles the total particle concentration would decay (because of aggregation) logarithmically with time with the decay rate, depending on the adhesiveness of the particles. The applicability of this theory was tested at 4 different shear rates ($G = 5.0 \text{ s}^{-1}$, 12.6 s^{-1} , 15.7 s^{-1} and 23.3 s^{-1}) for normal red blood cells in plasma. At every shear rate it was found that an equilibrium condition was approached after 10-20 min, suggesting that the equation is valid only during the first 10-20 min of the aggregation process. Having established the time period over which the theory is valid, values for the stability ratio of red blood cells were computed. The stability ratio, which is essentially a measure of the adhesiveness of the cells, is defined as the ratio of the number of effective collisions (collisions resulting in adhesion) to the total number of collisions.

A very simple shearing technique was used. The suspension of red blood cells was placed in a small weighing bottle and the shearing force was provided by a small magnetic stirring rod, whose rotational frequency could be controlled. The mixing chamber was clamped in a small water bath, which in turn rested on a heated magnetic stirrer. Samples for microscopic viewing and counting were prepared by transferring a very small volume of the suspension onto a microscope slide and preserving it by placing an identical slide on top.

The stability ratio (α) was found to have a negative linear relationship with shear rate (regression line $\alpha = 0.02640 - 0.00084 G$, $r = 0.94$) and extrapolation to $\alpha = 0$ suggests that a minimum shear rate of $\sim 32 \text{ s}^{-1}$ is required to prevent re-aggregation of the red blood cells. For 10 healthy men and women the stability ratio, calculated at a shear rate of 12.6 s^{-1} , was found to be 0.0129 ± 0.0009 (S.E.M.).

The stability ratio was also found to be temperature-dependent. It increased linearly with temperature from 4°C to 41°C and then decreased at higher temperatures, becoming zero at a temperature of 52°C .

Results obtained from a group of patients with high erythrocyte sedimentation rate (e.s.r.) were combined with those from normal, healthy subjects and a positive linear relationship between α and e.s.r. was obtained. This finding suggests that the stability ratio might be a valuable measurement in comparing red blood cells in disease with normal red blood cells.

INTRODUCTION

When red blood cells are suspended in plasma they form aggregates with their broad sides in apposition, a pattern commonly referred to as rouleaux formation (Fahraeus, 1929). The degree of rouleaux formation, as a function of time, depends

on the adhesiveness of the cells, which is related to the concentration of the fibrinogen and globulin fractions in the plasma (Fåhræus, 1921, 1929; Ponder, 1927). In laminar flow the degree of rouleaux formation depends also on the frequency of collisions between the cells and on their stability ratio (collision efficiency); i.e. the ratio of the number of effective collisions to the total number of collisions.

An expression for the collision frequency among particles in a laminar shear field has been derived by Smoluchowski (1917). When the particles are not in Brownian motion, he showed that

$$b = \frac{4}{3}Gn^2(2r)^3, \quad (1)$$

where b is the collision frequency, G the shear rate, and n the concentration of cells of radius r per unit volume. From equation (1), Swift & Friedlander (1964) obtained the following relationship:

$$\ln \frac{N_t}{N_0} = \frac{-4G\phi t}{\pi}, \quad (2)$$

where N_0 is the total concentration of single cells at time $t = 0$; N_t the total concentration of single cells and aggregates at time t ; and ϕ the volume fraction of the cells. Equation (2) is correct only if every collision results in adhesion; so to allow for situations where not every collision results in adhesion Swift & Friedlander (1964) introduced a factor, α , into the equation. Equation (2) then becomes

$$\ln \frac{N_t}{N_0} = \frac{-4G\phi\alpha t}{\pi}, \quad (3)$$

where α is the stability ratio of the cells. The values of α range from 0 ($N_t = N_0$ for all t values) to 1 (every collision is effective) and it gives the probability that 2 cells, or a cell and an aggregate, or 2 aggregates, adhere on collision.

Swift & Friedlander (1964) used equation (3) in their study of the coagulation of hydrosols. Curtis (1969) used the same equation to find values for α for cells obtained from the organs of embryonic chickens and from tissue cultures. From these values of α he was able to calculate corresponding values for the London-Hamaker force constant and for the adhesive energy between the cells. He later used equation (3) to investigate tissue specificity in the adhesion of chick embryo cells, species specificity among sponges and strain specificity in the fresh water sponge, *Ephydatia fluviatilis* (Curtis, 1970*a, b*; Curtis & van de Vyver, 1971).

Equation (3) is obviously not valid for large values of t . It suggests that if $\alpha \neq 0$ aggregates will build up as collisions take place; and eventually aggregation might be expected to lead to the accumulation of all the cells into a single aggregate. This never occurs in practice because of the dispersing effect of the shearing forces. Albers & Overbeek (1960) showed that the shearing forces acting on an aggregate will increase as the square of its radius; consequently, for a given α value and for a given shear rate, G , there will be an equilibrium stage where the rate of aggregation equals the rate of break-up. As the equilibrium conditions are approached the aggregation kinetics will diverge from that indicated by equation (3).

In the present study values of α for red blood cells are computed over a time

period for which equation (3) is valid. The dependence of α on temperature and a possible relationship between α and the erythrocyte sedimentation rate (e.s.r.) are also investigated.

MATERIALS AND METHODS

Blood was taken from each donor by venipuncture into heparinized vacutainers. Aliquots were taken and used to measure e.s.r. (Westergren) and haematocrit (microhaematocrit method). (The e.s.r. is the distance in mm sedimented by the blood-cell column in 1 h when the blood is put into a long, vertical, narrow-bore tube. The haematocrit is the volume per cent of blood cells.) A few drops of the remainder were retained for subsequent dilution. The rest was centrifuged and the plasma fraction removed. Red blood cells were added to the plasma fraction to give a haematocrit of 0.5%. The cell/plasma suspension (4 ml) was placed in a small weighing bottle containing a magnetic stirring rod 8 mm \times 1.5 mm. The mixing chamber was clamped in a small water bath, which in turn rested on a heated magnetic stirrer. The position of the chamber relative to the stirrer was adjusted so that the stirrer rotated about its centre, ensuring complete mixing. The rotation frequency was initially set at maximum (30 rev./s) for \sim 30 s and a sample observed microscopically to ensure that all rouleaux had been broken up. The frequency was then adjusted to the desired rate (shear rate, $G = 2\pi \times \text{rev./s}$) and a stopwatch was started. The frequencies used were low enough to ensure laminar flow. Samples were taken by surface tension, a short column being drawn into a pipette of diameter 1 mm. A very small volume of suspension was quickly transferred to a clean microscope slide by holding the pipette perpendicular to the slide, making contact, and then tapping the other end of the pipette with a finger. The resulting sample was 1 mm in diameter and a few tens of microns in thickness. An identical slide was placed on top of the first to preserve the specimen. No grease or other seal was used. When this last manoeuvre is watched on an inverted microscope it is seen that the spreading is small and slow and produces no break-up of rouleaux or collisions among cells and rouleaux. Observations were made with a Nikon (model M) inverted microscope at \times 400 magnification and the number of cells (single and multiple) were counted in several fields, taken at random, to a total count of \sim 100 cells, i.e. $N_0 \approx 1000$. Plots were then made of $\ln N_0/N_t$ versus t from which values of α were calculated (eqn (3)). All experiments were performed at room temperature (22 °C) unless otherwise specified.

Most of the experimental technique above has been published (Kernick, Jay, Rowlands & Skibo, 1973). As indicated by these authors the method using 2 microscope slides is much better than the coverslip-slide method (Ponder, 1927), which causes considerable spreading and further mixing. A haemocytometer chamber, perhaps, would be better still (Chien *et al.* 1967), but it has the disadvantage in that it is \sim 100 μm deep and additional rouleaux can form as sedimentation occurs. Nonetheless, the double-slide method is cheaper and it proved reproducible in the present study.

RESULTS

Fig. 1 shows typical plots of $\ln N_0/N_t$ versus t at 4 different shear rates for red blood cells suspended at a concentration of 0.5%. The volume fraction of the cells was obtained directly from the haematocrit of the stirred sample. At $t = 0$ there were no aggregates present, i.e. $N_0 = N_t$. In every case the equilibrium conditions are approached after 10–20 min. Although not entirely obvious from the graphs, it is believed the equilibrium is reached sooner at the higher shear rates. Also, the equilibrium size of the aggregates decreases as the shear rate is increased. Using a time-period of 10 min, over which equation (3) is applicable, values of α were calculated and plotted as a function of G (Fig. 2). A linear relationship was obtained (regression line: $\alpha = 0.02640 - 0.00084 G$, correlation coefficient, $r = 0.94$) and extra-

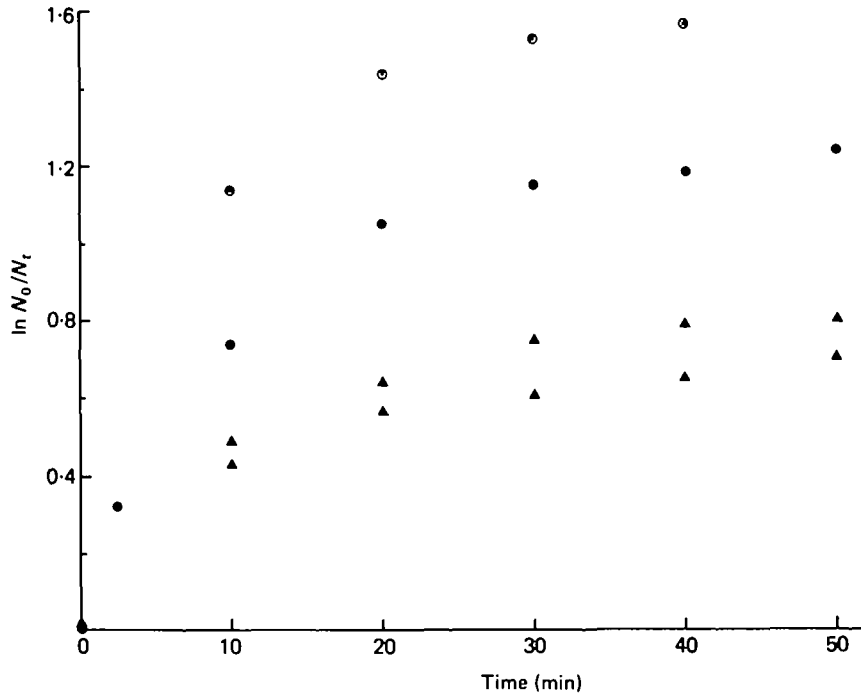


Fig. 1. Typical plots of $\ln N_0/N_t$ versus time for the aggregation of initially dispersed red blood cells at 4 different shear rates: (\odot) 5.0 s^{-1} ; (\bullet) 12.6 s^{-1} ; (\triangle) 15.7 s^{-1} ; (\blacktriangle) 23.3 s^{-1} .

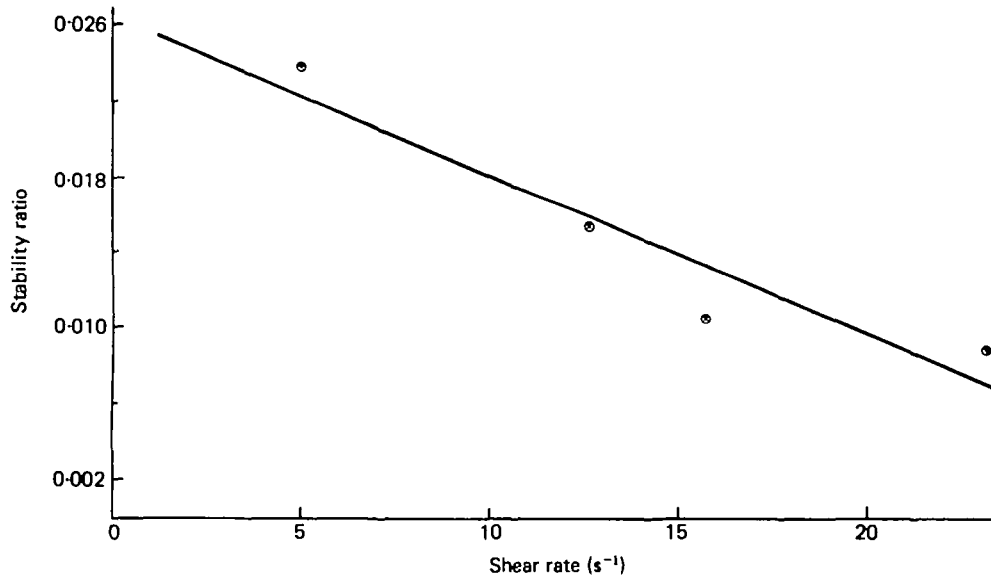


Fig. 2. The relationship between the stability ratio of the red blood cells and the shear rate. The regression line is: $\alpha = 0.02640 - 0.00084 G$, correlation coefficient, $r = 0.94$. Extrapolation to $\alpha = 0$ gives a G value of 32 s^{-1} .

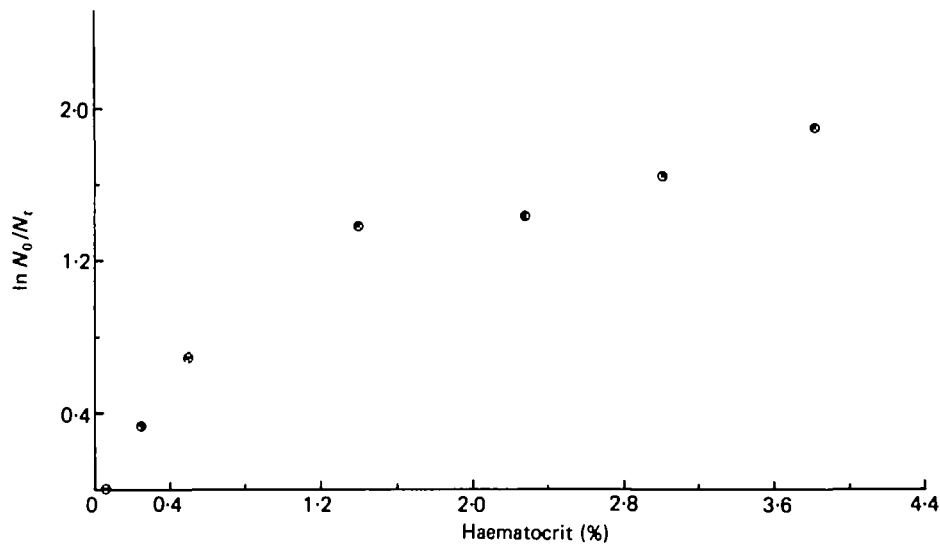


Fig. 3. The relationship between $\ln N_0/N_t$ and the volume concentration of the red blood cells in plasma at a shear rate of 12.6 s^{-1} .

Table 1. Values for the stability ratio (α) of red blood cells from healthy men (*e.s.r.* < 7 mm/h) and women (*e.s.r.* < 20 mm/h) calculated at a shear rate, $G = 12.6 \text{ s}^{-1}$ and over a time period of 10 min (cell concentration = 0.05%)

| Subjects | No. of measurements | Stability ratio, α |
|---------------|---------------------|---------------------------|
| 4 Men | 6 | $0.0114 \pm 0.0011^*$ |
| 6 Women | 16 | 0.0134 ± 0.0011 |
| Men and women | 22 | 0.0129 ± 0.0009 |

* \pm S.E.M.

polation to $\alpha = 0$ shows that a minimum shear rate of $G \simeq 32 \text{ s}^{-1}$ is required to prevent re-aggregation of the red blood cells.

All subsequent experiments were done at a shear rate of $G = 12.6 \text{ s}^{-1}$. The relationship between $\ln N_0/N_t$ ($t = 10 \text{ min}$) and the haematocrit is shown in Fig. 3. Again, as expected, an equilibrium is approached indicating that there is an equilibrium aggregate size associated with the shear rate. For a shear rate of 12.6 s^{-1} the average (equilibrium) aggregate size is 4–6 cells.

Table 1 gives values for the stability ratio of red blood cells from healthy men and women calculated at a shear rate, $G = 12.6 \text{ s}^{-1}$, and over a time period of 10 min. The volume fraction of cells was 0.005 (unless otherwise stated; all suspensions had a cell concentration of 0.5%). The women gave a slightly higher ($\sim 18\%$) value than men but the difference was not significant ($P > 0.15$, Student's *t*-test). The combined value for all 10 subjects was 0.0129 ± 0.0009 (S.E.M.).

The stability ratio was found to be temperature-dependent. Experiments were

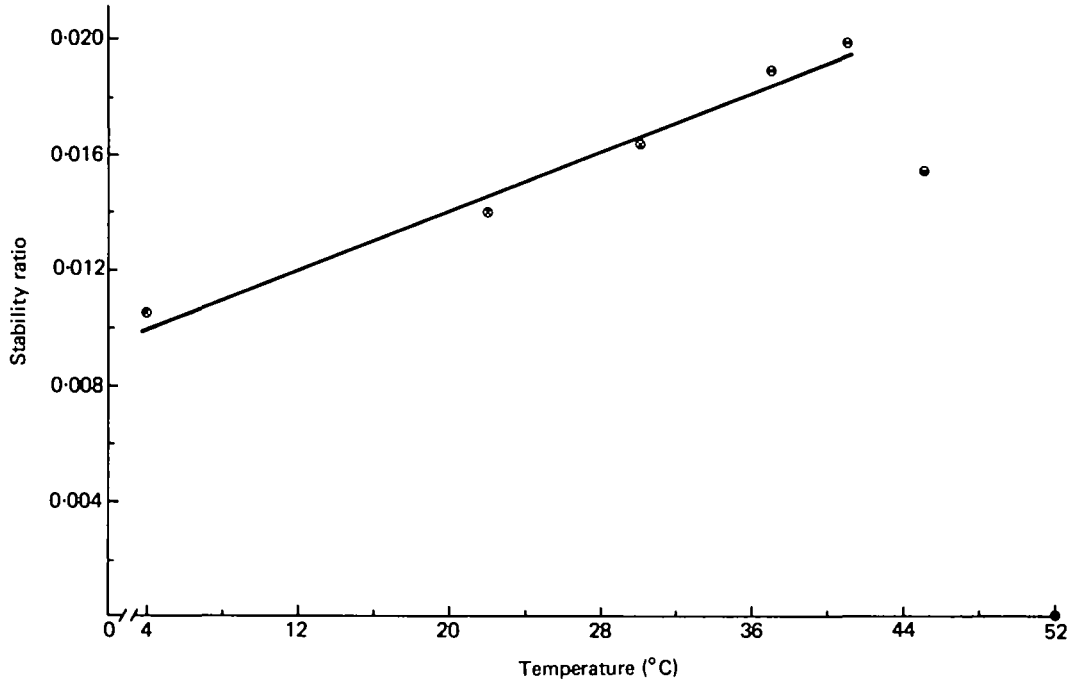


Fig. 4. Effect of temperature on the stability ratio of red blood cells in plasma ($G = 12.6 \text{ s}^{-1}$). The stability ratio reached a maximum at $\sim 40^\circ \text{C}$.

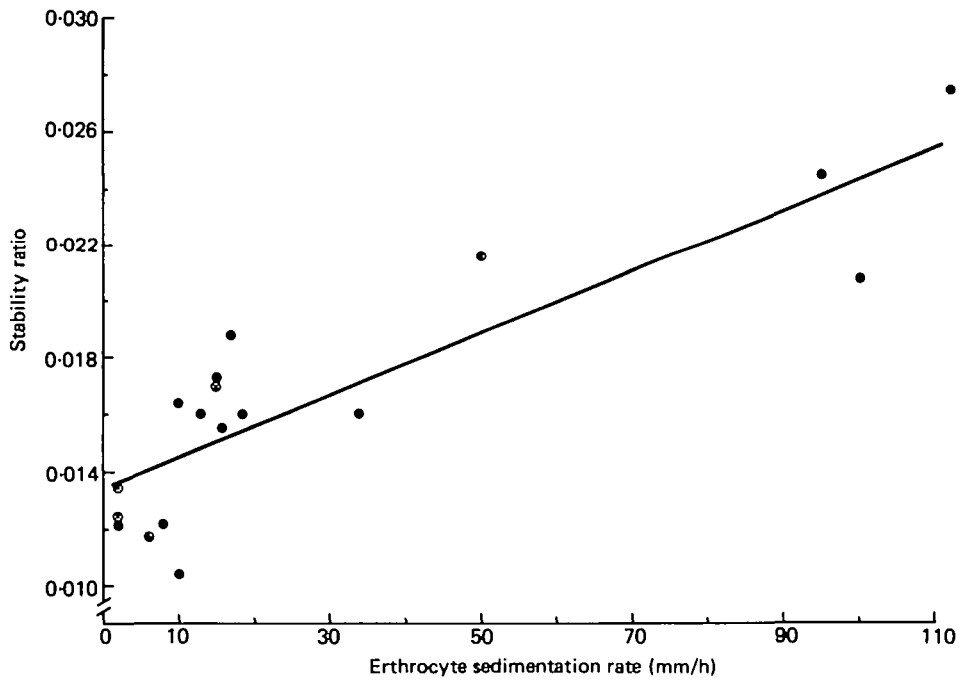


Fig. 5. Stability ratio *versus* erythrocyte sedimentation rate for a group of normal, healthy subjects (low e.s.r.) and a few patients (high e.s.r.). (⊙) Male; (●) female.

done with the water bath at 6 different temperatures and the stability ratio was found to increase linearly with temperature (linear regression: $\alpha = 0.00890 + 0.00026 t$, correlation coefficient = 0.99) up to $T = 41^\circ\text{C}$, beyond which it dropped drastically and had a value of zero at $T = 52^\circ\text{C}$ (Fig. 4). Red blood cells heated to 50°C sphere and do not form rouleaux (Ponder, 1949).

To investigate the relationship between α and e.s.r. the results obtained from healthy subjects were combined with those from a few patients (courtesy of Foothills Hospital, Calgary). Even though the number of subjects with high e.s.r. values was small, the results show that a trend exists and the stability ratio increases linearly with e.s.r. (Fig. 5). The regression line is $\alpha = 0.01333 + 0.00011$ (e.s.r.), correlation coefficient, $r = 0.87$.

DISCUSSION

It has been assumed all along that shear conditions are alone responsible for the production of collisions between the cells. However, in all suspensions Brownian motion tends to produce collisions between particles. The collision frequency, due to Brownian motion, of cells of equal size is given by the relationship (Smoluchowski, 1917):

$$B = \frac{8kT}{3\eta} n^2, \quad (4)$$

where B is the collision frequency, k the Boltzmann constant, T the absolute temperature, η the viscosity of the suspension and n the concentration of cells per unit volume. From equations (1) and (4) the ratio of the collision frequencies due to Brownian motion and shearing forces is

$$B/b = \frac{kT}{4G\eta r^3}.$$

Thus for a shear rate, $G = 1 \text{ s}^{-1}$, $T = 295 \text{ K}$ and for cells of radius $4 \mu\text{m}$ less than 1% of the collisions is due to Brownian motion. Brownian motion, therefore, did not play a significant role in the shear rates used in this study. It is also interesting to note that, contrary to Brownian motion, the collision frequency in shear flow is independent of the viscosity of the suspending medium (Smoluchowski, 1917).

The findings here provide a test for the applicability of equation (3) to the aggregation of red blood cells. It is shown that the aggregation kinetics diverge from that predicted by equation (3) when the constant shearing force is applied for a period greater than ~ 15 min. Calculations of the stability ratio α , therefore, could only be made, using equation (3), during the first 15 min of the aggregation process.

The effect of the applied shear on the initially dispersed cells is 2-fold: first, it brings the cells together, and secondly it tends to re-separate them, as the cells that travel more rapidly pass the others. At low shear rates the cells, if their adhesive force is sufficient, may reach a closeness of approach such that their adhesiveness can prevent the break-up of the newly formed aggregate. At higher shear rates few cells, if any, may get close enough (and for a long enough time) for stable

aggregates to form. One would therefore expect the stability ratio to decrease with increasing shear rate. Fig. 2 shows that this actually occurs. The regression line shows that a minimum shear rate of $\sim 32 \text{ s}^{-1}$ was required to prevent re-aggregation of the red blood cells. This value is less than the minimum shear rate of 46 s^{-1} (Goldstone, Schmid-Schönbein & Wells, 1970) normally required to break up aggregates into single cells, but this is not unexpected because the force required to break up an aggregate is generally larger than that necessary to prevent its formation (Weiss, 1961). Moreover, the minimum G value of 32 s^{-1} should not be taken as exact because of the uncertainties inherent in our simple shearing technique.

The stability ratio (α) is essentially a measure of the adhesiveness between cells as Curtis (1969) pointed out. It is useful in comparing the adhesiveness between different cell types and also for testing species specificity in adhesion (Curtis & van de Vyver, 1971; Curtis, 1970*a*, *b*). In blood it should be a useful tool in comparing red blood cells in disease with normal cells. Infectious diseases are usually associated with high e.s.r. values (Fåhræus, 1921, 1929) and the relationship between α and e.s.r. (Fig. 5) suggests that the stability ratio might be a valuable measurement.

In the present experiments the value of α varied very little from individual to individual in the population of normal subjects studied. Also, for any one individual the variability in α was even less, which attests to the reproducibility of our method. An increase in adhesiveness of red blood cells with temperature has been reported (Ponder, 1947; Dintenfass & Forbes, 1972) and while this is confirmed here it is also shown that the relationship is linear (up to $\sim 40^\circ \text{C}$). The decrease in α beyond $\sim 40^\circ \text{C}$ might be related to possible physical changes in the red blood cells and/or configurational changes in the aggregating proteins in the plasma.

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The quantitative relationship of the fibrillar centres and other nucleolar components to changes in growth conditions, serum deprivation and low doses of actinomycin D in cultured diploid human fibroblasts (strain MRC-5). EG Jordan, JH McGovern. Journal of Cell Science 1981 52: 373-389

The Erythrocyte Sedimentation Rate (ESR), also branded as sedimentation rate or Biernacki Reaction, is the rate at which red blood cells sediment in a time of 1 hour. It is an ordinary hematology test that is a distracted evaluate of inflammation. To execute the test, anti-coagulated blood is positioned in an erect tube, known as a Westergren tube, and the rate at which the red blood cells fall is calculated and accounted in millimeters per hour (mm/h). This test was originated in 1897 by the Polish doctor Edmund Biernacki.