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THE INTERACTIONS OF FIBRINOGEN AND DEXTRANS WITH ERYTHROCYTES

BY M. RAMPLING AND JOHN A. SIRS

From the Department of Biophysics, St Mary's Hospital Medical School, Paddington, London, W. 2

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SUMMARY

1. The rate of packing of erythrocytes in whole blood, under a centrifugal field of 200 *g*, has been studied using an automatic recording centrifuge.

2. Reduction of the supernatant fibrinogen concentration, by repeatedly washing the cells, lowers the rate of packing and reduces the cell flexibility.

3. Resuspending the cells in their own plasma or in isotonic solutions containing fibrinogen restores their flexibility.

4. Rouleaux formation has been shown to have no effect on the rate of packing by comparison of blood diluted with plasma, isotonic NaCl or Ringer–Locke solutions. While the degree of rouleaux formation varied with the diluent used, the rate of packing and packed cell haematocrit were the same, for the same dilution.

5. Both formalin and dextran altered the degree of rouleaux formation and reduced erythrocyte flexibility. Dextran was found to act indirectly on the erythrocyte flexibility by reducing the plasma fibrinogen concentration.

INTRODUCTION

When red blood cells are repeatedly washed, the rate of packing in a centrifuge of the final suspension is significantly slower than that of the initial blood (Sirs, 1971). This has been interpreted as a change of flexibility of the erythrocytes. The rate of packing returns to normal if the cells are resuspended in the original plasma. A similar loss of flexibility was observed by Rampling & Sirs (1970) after defibrinating blood with glass beads. These and other observations suggested that one of the factors involved in erythrocyte flexibility might be fibrinogen.

It has been accepted for some time that erythrocyte sedimentation rate (ESR) under gravity is influenced by the fibrinogen concentration. This in turn is considered to be related to the degree of rouleaux and aggregate formation in the blood (Dawson, 1960; Cutler, Park & Herr, 1938). Ponder

(1947) has argued, using a modified form of Stokes's Law, that with rouleaux formation the ESR rises due to the effective increase in the volume to surface area ratio of the cells. If this were the case it would apply to the early stage of packing in a centrifuge. Sirs (1968) and Rampling & Sirs (1970), however, have shown that the process of packing is not one of simple sedimentation and Stokes' Law is not applicable with blood at normal haematocrits.

This study was undertaken to investigate further the effect of fibrinogen on the rate of packing and whether rouleaux formation is directly related to the ESR. In pursuing these studies the effect of low molecular weight dextran has also been investigated, since its clinical use is mainly based on the supposition that by disrupting rouleaux and increasing the plasma volume it improves blood flow *in vivo*. Recent evidence (Eisenberg, 1969), however, suggests that dextran has little or no effect on blood viscosity *in vitro* and that the disruption of rouleaux is of questionable benefit in clinical use (Collins & Ludbrook, 1966).

MATERIALS AND METHODS

Centrifuge technique

The rates of packing of the erythrocytes were determined by means of the Automatic Recording Centrifuge. This device and its use has been described by Sirs (1970). The curves observed represent the rate of fall of the upper surface of the red cell column under a centrifuge field of about 200 *g*. The initial slope of these curves in terms of the percentage change of the length of the cell column per minute has been utilized as a quantitative index of the packing rate. To ensure that the differences were not due to variations of the haematocrit, the packed cell haematocrit, obtained at 12,000 *g* for 4 min, was measured with each sample.

The blood used in these experiments was taken I.V. and heparinized with 6 i.u./ml. It was stored at 4° C until required for the experiments, all of which were done at room temperature. In all cases the blood had normal haematocrits.

Fibrinogen experiments

The purified preparation of human fibrinogen used was supplied by the Lister Institute of Preventive Medicine (University of London). Of the total protein in the sample, 78% was clottable. This was diluted in the Ringer–Locke solution, as specified below, and heparin 6 i.u./ml. added to ensure no gel formation occurred on adding these solutions to red cell suspensions. It was shown, by varying the heparin concentration, that the heparin had no effect on the rate of packing in these circumstances. Measurements of the densities of the fibrinogen solutions similarly disclosed no differences large enough to affect the rate of packing. Solutions of fibrinogen were reconstituted from the dried powder just before the experiments. The total protein in the solutions was estimated and checked with an U.V. spectrophotometer.

Formalin experiments

The test solutions consisted of various concentrations of formalin in 0·85% saline. The main control solution was plasma removed from blood which had been allowed to settle overnight. In some cases other control solutions were used; these were

0·85 % saline or Ringer–Locke (7·6 g NaCl, 0·24 g $CaCl_2$, 0·42 g KCl, 2·0 g $NaHCO_3$/l. distilled water equilibrated with 30 mm Hg P_{CO_2} for pH control).

The experiments consisted of adding known quantities of the formalin solutions to the whole blood and spinning on the automatic recording centrifuge to obtain the packing rate. Similar control checks were made by substituting plasma, saline or Ringer–Locke for the formalin-saline. After each addition a determination of the 'packed-cell' haematocrit was made using a Hawksely microhaematocrit centrifuge.

Dextran experiments

The dextran solution used was Rheomacrodex manufactured by Pharmacia. This is a clinically used saline solution containing 10 % dextran (w/v) with an average molecular weight of 40,000. Various concentrations of this solution in saline were compared with controls in the same way as described above for formalin.

Viscosity measurements

Samples of the dextran solutions were allowed to flow down wide vertical columns under their own hydrostatic pressure and to issue from a narrow orifice. By comparing these flow rates with those for water the relative viscosities of the dextran solutions were determined.

Rouleaux determination

Each sample of blood that was spun on the centrifuge was investigated under a microscope to ensure that there were no gross changes to the cells such as crenation. Estimates were also made of the degree of rouleaux formation. These estimates were based on a grading structure similar to that of Engeset, Stalker & Matheson (1966) but with their three and four gradings combined. The gradings were:

0, no rouleaux;

1, short loose rouleaux;

2, larger more closely packed rouleaux with occasional branching;

3, very large rouleaux which adhere to form clumps.

Aggregation, corresponding to large groups of cells adhering together with random orientation and no obvious rouleaux within these groups, was added as a separate category. Finally, it was possible to observe gross cellular inflexibility, if present, by watching the deformability during slow movement of the cells before rouleaux or aggregates had formed.

RESULTS

Experiments with fibrinogen

A 10 ml. sample of blood, collected by venepuncture using heparin to prevent clotting, was divided into five 2 ml. samples. The first of these provided a normal whole blood control. The remaining four samples were then centrifuged at 1000 *g* for 10 min and the clear plasma above the cells was pipetted off and replaced by an equal volume of Ringer–Locke solution. After mixing the cells with the Ringer–Locke solution by shaking, the suspensions were again centrifuged and the supernatant removed and replaced with fresh Ringer–Locke solution. This was repeated three times. After the final removal of the supernatant from the four packed-cell samples, the suspensions were separately reconstituted by adding to the

first Ringer–Locke solution, to the second a solution of 0·2 g of total fibrinogen protein in 100 ml. Ringer–Locke, to the third a solution of fibrinogen in Ringer–Locke 0·6 g/100 ml., and to the fourth a solution of fibrinogen 1 g/100 ml. The volume of solution added to the cells was adjusted until the packed cell haematocrit of the new suspension was comparable to that of the initial whole blood. The rate of packing of each sample was then determined using the automatic recording centrifuge and

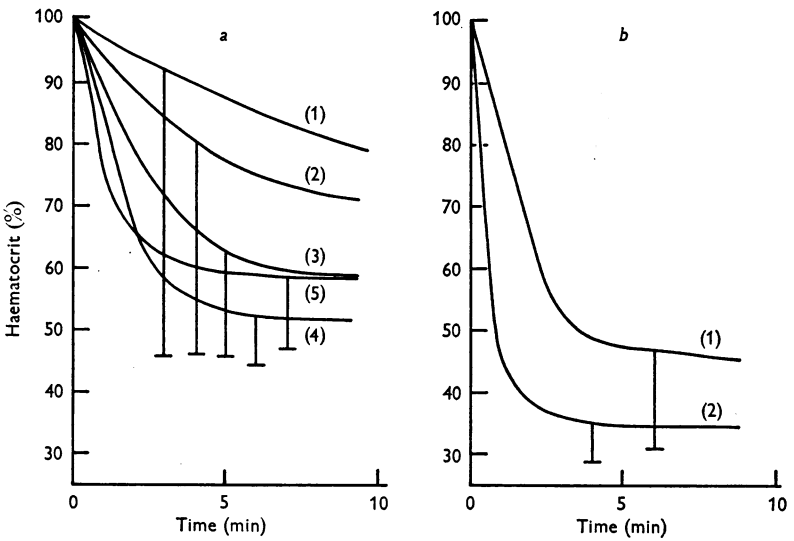

Fig. 1. The variation of haematocrit (percentage length of cell column to total length) with time at different fibrinogen concentrations in the suspending media. The base lines indicate the corresponding packed-cell haematocrit at 12,000 *g*.

a. The effect of adding fibrinogen solutions to washed cells relative to curve (5) for whole blood; (1) washed cells resuspended in Ringer–Locke solution (2) washed cells resuspended in Ringer–Locke containing 0·2 g fibrinogen/100 ml. solution; (3) after resuspension in 0·6 g fibrinogen/100 ml. solution (4) 1·0 g fibrinogen/100 ml. solution.

b. The effect of adding to 1 ml. whole blood, (1) ½ ml. Ringer–Locke solution, (2) ½ ml. Ringer–Locke solution containing fibrinogen at a concentration of 1·0 g/100 ml.

the results are shown in Fig. 1*a*. They demonstrate quite clearly the effect on the rate of packing of fibrinogen. The difference between whole blood and washed-cells suspended in Ringer–Locke solution cannot simply be explained by the fibrinogen effect. First, the shape of the whole blood curve is not obtained at any fibrinogen concentration; secondly, the concentration of fibrinogen necessary to obtain an equivalent initial packing rate is some three times greater than that in normal plasma. The effect

can, however, be almost completely reversed by resuspending the cells in their original plasma. This implies that a subsidiary effect must also be present in normal whole blood due to the presence of other plasma proteins. This has been confirmed by adding 0·5 ml. of fibrinogen solution 1 g/100 ml. to 1 ml. whole blood and comparing the rate of packing of this suspension with a similar dilution of whole blood with Ringer–Locke not containing any fibrinogen. The curves obtained are shown in Fig. 1*b*. The suspension simply diluted with Ringer–Locke solution is no different from that obtained by a similar dilution of the cells with their own plasma. Thus the addition of a small amount of fibrinogen in the presence of plasma even further increases the rate of packing and alters the observed haematocrit at all points. At the concentration of fibrinogen used the osmotic effects are negligible and there is no evidence to indicate the volume of the cells has changed. The curves demonstrate the error that can arise in estimation of the absolute haematocrit using the centrifuge technique.

Other tests have been made with the fibrinogen solutions to ascertain that these effects are directly attributed to the added fibrinogen and not some minor impurity present in this preparation. In the first experiment a solution containing 1 g fibrinogen/100 ml. Ringer–Locke was incubated at 40·5° C for 1 hr, a procedure which is known to inactivate the fibrin stabilizing factor. A comparison of the rate of packing, after adding this solution to washed packed cells, with that obtained after adding untreated fibrinogen solution disclosed no significant difference. After the fibrinogen solution was incubated at 52° C for 1 hr, which denatures fibrinogen, no significant increase in the rate of packing was observed relative to a similar control experiment using cells mixed with Ringer–Locke solution without fibrinogen. Similarly, adding thrombin to the fibrinogen solution (1 g/100 ml.) and removing the clot, before mixing with the packed red cells, completely inhibits the effect. A significant reduction of the effectiveness of the fibrinogen to increase the rate of packing is observed after the fibrinogen solution has been left standing at room temperature for 1 week. This is consistent with the report of Seegers, Nieft & Vandenbelt (1945) that decomposition of fibrinogen into α- and β-fractions due to a fibrinolytic enzyme occurs in these circumstances. The effect of an excess (250 i.u./ml. blood) of heparin was also studied. Whereas a significant increase in the rate of packing is obtained when an excess of heparin is added to whole blood, a similar addition has no effect on washed cells resuspended in either Ringer–Locke or fibrinogen solutions.

Previous studies of changes of the rate of packing using the automatic recording centrifuge are consistent with a change of flexibility of the red blood cells. In ESR studies at different fibrinogen concentrations the effects have been interpreted as due to rouleaux formation. At least over

the major portion of the packing curves obtained with the recording centrifuge any effect of rouleaux appears to be unlikely as the cells are so closely packed together. The following experiments were therefore undertaken to more clearly establish if there is any correlation between the rate of packing and rouleaux formation.

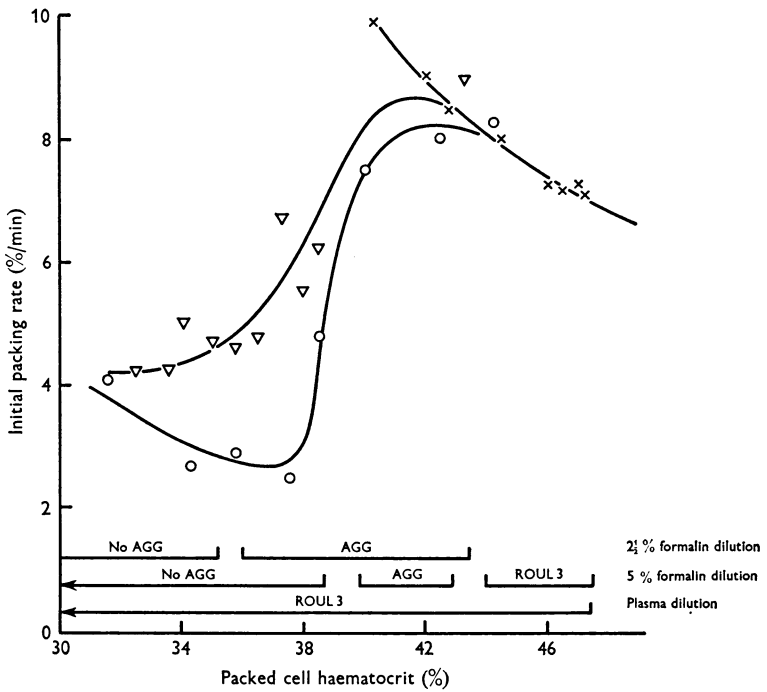

Fig. 2. The effect on the packing rate of diluting blood with formalin solutions compared with the same dilution with plasma. ×—× plasma, ▽—▽ 2½ % formalin, ○—○ 5 % formalin. The brackets below the graphs indicate the degree of aggregation and rouleaux formation for each sample. ROUL 3 indicates the presence of grade 3 rouleaux, AGG the presence of aggregates and No AGG the total disappearance of any cellular adhesion.

Formaldehyde and rouleaux formation

The addition of formaldehyde to blood is known to affect both the degree of rouleaux formation and rate of clotting. It can also be shown directly, such as by fixing sickle-cells, to make erythrocytes inflexible. Experiments were therefore undertaken to compare the effect of formaldehyde on the rate of packing and rouleaux formation relative to a control sample to which only plasma or Ringer–Locke was added. Typical results of these tests are shown in Fig. 2. The 'packed cell' haematocrits that were measured, even after the maximum addition of formalin, were not signi-

ficantly different from those calculated from the known dilution of the original blood suspension, in spite of the appreciable inflexibility induced by the formalin.

There were three distinct stages in the appearance and behaviour of the blood to which formalin was added which were common to all the subjects investigated. As the formalin concentration was increased there followed

(1) an initial phase during which the samples showed no differences in packing rates or degree of rouleaux formation from the control samples;

(2) this was followed by a total loss of any organized rouleaux type of interaction, instead the cells adhered in disorganized aggregates with very indistinct cell boundaries. The rouleaux formation of the control sample at a similar dilution was unaffected. It was during the beginning of this stage that differences in packing rate between the test and control solutions were first noted;

(3) sudden and complete loss of the aggregates in the formalin samples, but no change in the control samples.

The determination of the point at which the cells could be seen to be inflexible by microscopic observation was not very precise. However, in all cases this occurred in a region close to the end of stage 2 and the beginning of stage 3. This coincided with that region at which the most marked changes in packing rate occurred.

The formation of rouleaux and aggregates after the addition of the small quantities of formalin required to produce these effects, fall within sharp divisions; a range of 0–0·4 % of formalin in whole blood was compatible with stage 1, 0·4–0·8 % with stage 2 and > 0·8 % with stage 3. The effects of formaldehyde and the quantities required were not significantly different for the three subjects investigated, and for the same subject on different occasions. Furthermore, the action of formaldehyde was independent of its original concentration and depended only on the total quantity added to the blood.

It is quite clear from these results that a change from rouleaux to aggregates has no effect on packing rates. This was so in spite of the greater volume-to-surface ratio of the aggregates. Also throughout the stage at which aggregates appeared, the degree of aggregation was unchanged, but the packing rates still fell progressively. This is quite contrary to what Stokes' Law would suggest. Furthermore, on their disappearance there was no break in the curves. Only continuously changing flexibility can explain these results.

Low molecular weight dextran and rouleaux formation

Another agent known to alter rouleaux formation and aggregation is dextran. The first experiments consisted of a comparison of the packing

rates of blood diluted by adding Rheomacrodex with the rates of controls. A typical set of results shown in Fig. 3.

In control experiments whole blood was similarly diluted with plasma, Ringer–Locke and isotonic saline. For the same dilution, there was no difference in the packing rates of these samples though significant differences in rouleaux gradings between plasma and the other diluents were present. The results for the Rheomacrodex, however, were very different even for the smallest dilution where there was no obvious change in rouleaux formation.

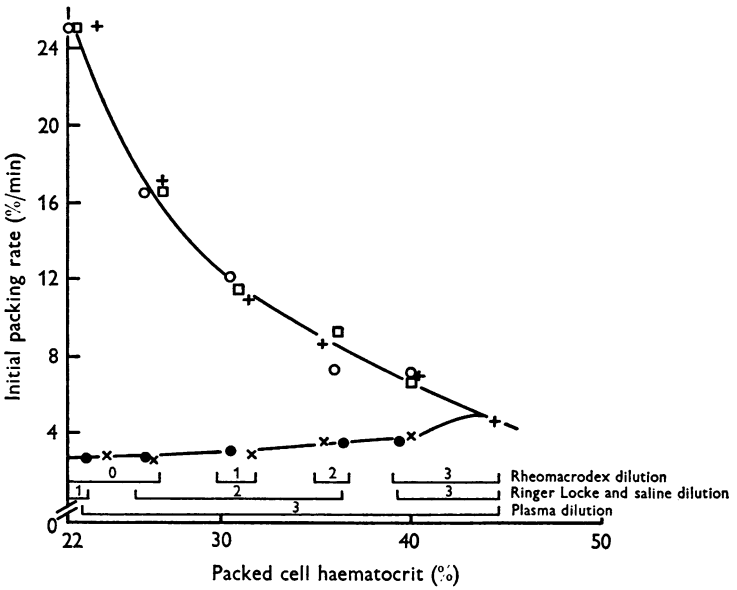

Fig. 3. The effect of Rheomacrodex on the packing rate compared with plasma, saline and Ringer–Locke controls. +—+ plasma, ○—○ Ringer–Locke, □—□ saline, ×—× Rheomacrodex pH 4·5, ●—● Rheomacrodex pH 7·4. The brackets below the graphs indicate the rouleaux gradings for each sample.

The pure dextran solution was found to have a pH of about 4·5, so experiments were also done with the same solution to which had been added a small quantity of 0·15 N-NaOH to bring the pH to 7·4. There was no difference between these packing rates and those of the original Rheomacrodex solution.

Again the results indicate that the degree of rouleaux formation and rate of packing are not directly related. They also, surprisingly, suggested that the addition of dextran made the erythrocytes inflexible.

Dextran and erythrocyte flexibility

More detailed investigations of the effects of dextran were carried out in an attempt to clarify the results reported above and, in particular, to determine whether the cells suffered reduced flexibility and the effects of the change of plasma viscosity.

Typical results for various concentrations of Rheomacrodex in saline are shown in Figs. 4 and 5. Fig. 5*B* shows the relative viscosity of the solutions used.

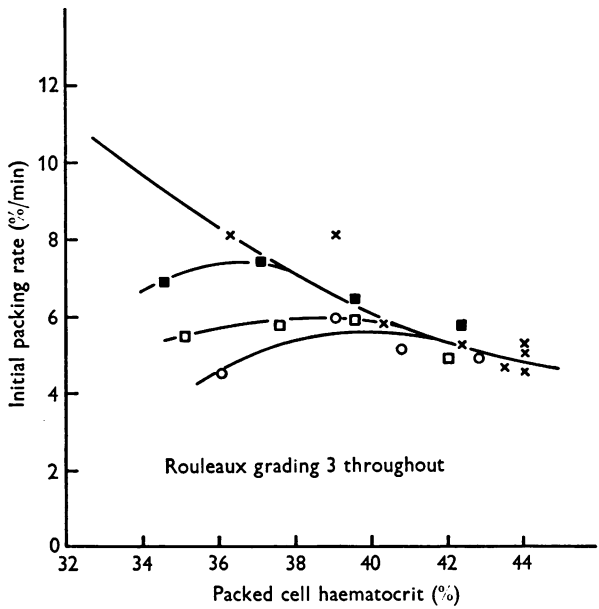

Fig. 4. The effect of dilution with dextran solution up to 25 ml./100 ml. of whole blood on the packing rate compared with plasma controls. ×—× plasma, ■—■ 5% dextran solution, □—□ 6⅔% dextran and ○—○ 10% dextran.

The results for small dextran additions are shown in Fig. 4. As expected the variation from the plasma controls becomes more pronounced as the concentration of Rheomacrodex in the test solution increased. Even the smallest concentration deviates from the controls, though its relative viscosity is about 2 which is very close to that of plasma (McDonald, 1960; Usami, Chien & Gregersen, 1969) and the density too is not significantly different from that of plasma. Furthermore, throughout this test no observable change in rouleaux formation occurred. This is indicative of inflexibility having been caused by the dextran and only requires the dextran in 8% (with respect to the whole blood) of Rheomacrodex to begin it.

Fig. 5 shows the effect on the packing rate of much larger additions, in particular in the region where dextran-induced rouleaux disruption occurred. The results for the 5 % solution are particularly significant since this is very similar to plasma as far as rheological properties are concerned. The results are consistent with a progressive decrease of flexibility, though it could not be seen by microscopic investigation. Even the lowest concentration used, i.e. $1\frac{2}{3}$ %, shows significant slowing of packing rate though there was no rouleaux change and the relative viscosity was only 1·2. The curve for the raw Rheomacrodex, i.e. the 10 % solution, shows the

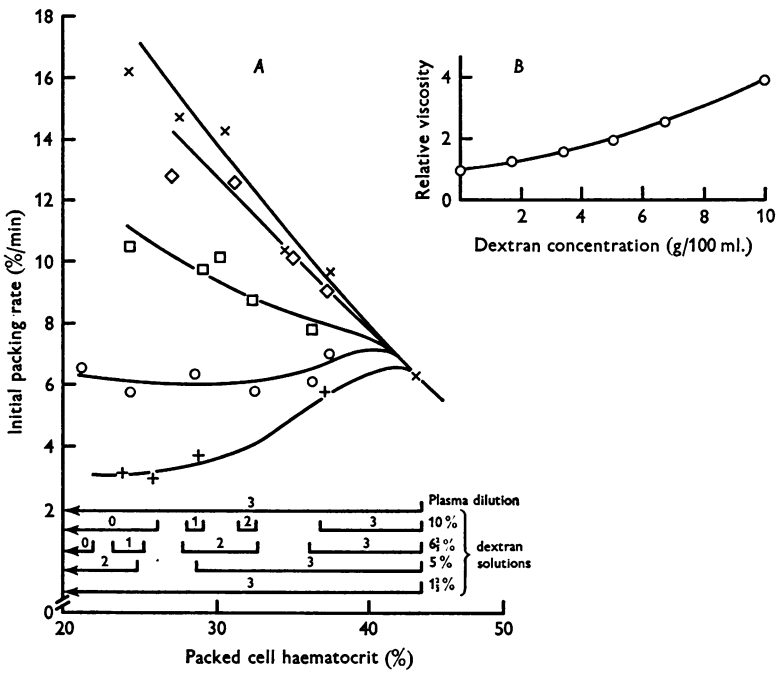

Fig. 5. *A*, the effect of dilution with dextran solution up to 100 ml./100 ml. of whole blood on the packing rate compared with plasma controls. × — × plasma, ◇—◇ $1\frac{2}{3}$ % dextran solution, □—□ 5 % dextran, ○—○ $6\frac{2}{3}$ % dextran, and + — + 10 % dextran. The brackets below the graphs indicate the rouleaux gradings for each sample.

B, relative viscosity of solutions containing various concentrations of dextran.

gross effect due to the combination of viscosity and inflexibility. It was found that additions of dextran solution equivalent to 1·6 g dextran/ 100 ml. whole blood were compatible with rouleaux grading 3. Additions of 2·3–3·8 g/100 ml. were compatible with grading 2, and up to 5·2 g/

100 ml. were compatible with grading 1. All rouleaux were disrupted by the addition of 7·0 g/100 ml. These results are comparable with those reported by Engeset *et al.* (1966).

DISCUSSION

These results show that rouleaux have no effect on the rate of packing produced by a centrifugal field of about 200 *g*. Similarly, aggregate formation has been shown to be of no influence in this context. Hence the use of Stokes' Law to explain this form of packing is untenable. In view of the constancy of all other factors which could affect packing rates, only increasing stiffness can explain the results reported here. Thus the recording centrifuge offers a technique which gives an index of flexibility.

The effect of decreasing or increasing the fibrinogen concentration is to correspondingly lower or increase the cell flexibility. While this also alters the degree of rouleaux formation the rates of packing at 200 *g* do not quantitatively correlate with this factor. The only anomaly occurs at the highest fibrinogen concentration, 1 g/100 ml., used, where the initial slope of the curve is slower and the final haematocrit is lower than that for whole blood. This cross-over effect may in part be due to the higher visocity of the fibrinogen solution but it is more likely to be caused by the fibrinogen making a greater number of cross-linkages between the cells which have to be initially broken before efficient packing can occur. Though fibrinogen is probably the main factor in plasma responsible for this effect, it is also evident that other proteins in plasma must also contribute to stabilizing and aiding its action. The ease by which it can be removed, by the shear of centrifugation, suggests it is located in the 'buffy coat' surrounding the cell in such a way to utilize its elastic and tensile properties, which are similar to myosin and collagen. Merrill, Gillilard, Lee & Salzman (1966) have shown that a relationship exists between the yield stress of blood and the fibrinogen content of the plasma which they ascribe to rouleaux formation. Chien, Usami, Dellenback & Gregersen (1970) have similarly investigated the shear dependence of blood viscosity due to the interaction of plasma proteins with erythrocytes. Their results also indicated a secondary effect of other plasma proteins on the change of viscosity with fibrinogen concentration. The present results suggest that the effect of erythrocyte flexibility, rather than rouleaux formation, is the more important factor in accounting for these viscosity changes and why whole blood exhibits such a relatively low viscosity relative to the individual viscosities of its component parts.

The force acting during the ESR test is some 200 times smaller than that used with the recording centrifuge in the above experiments. None-theless, it is felt that the same general conclusions must apply to this mode of

packing and that Stokes' Law is not applicable. The flexibility of the red blood cell must also be a major factor contributing to the rate of fall. It has always been difficult to understand with the previous interpretation, that changes of this rate were due to rouleaux formation, why the rate of fall of the cell boundary, which corresponds to the slowest falling fraction (i.e. individual cells), should have altered. The present interpretation is consistent with the erythrocytes being in contact with each other, even over the initial packing phase. The effect of rouleaux formation could, however, influence the rate of fall when only the weak force of gravity is used, due to the bonding between the cells preventing packing. The mechanism of the ESR test is thus likely to be more complicated than the situation with the recording centrifuge and further investigation is necessary to elucidate this point.

Any factor which affects the plasma fibrinogen concentration in blood will in turn alter the erythrocyte flexibility. The action of dextran in altering the rate of packing can mainly be interpreted in this way. There are many reports which show (Swank, 1958; Semple, 1955) that dextran may complex with or even precipitate fibrinogen from solution. In particular, Kroll & Dybkaer (1964) have investigated quantitatively the efficiency of dextran in Rheomacrodex to do this. Previously dextran has been used because of its action in disrupting rouleaux and aggregates and its ability to lower blood viscosity by plasma volume expansion. Thus in the experiments reported above and those of Engeset *et al.* (1966), the addition of 9–13 g dextran/100 ml. plasma totally disrupts all rouleaux formation. On the basis of Kroll & Dybkaer's results this corresponds to the inactivation of 0·12–0·16 g fibrinogen/100 ml. plasma. The blood used in the centrifuge experiments had normal clotting times, so the plasma fibrinogen level was about 0·24 g/100 ml. (Litwin, Chapman & Stoliar, 1970). After the addition of dextran 9–13 g/100 ml. plasma only 0·08–0·12 g fibrinogen/100 ml. plasma remains. The viscosity measurements of Merrill *et al.* indicate that the yield stress falls effectively to zero at a concentration of 0·08–0·12 g fibrinogen/100 ml. plasma in agreement with the results quoted here. At these concentrations of fibrinogen, as indicated by the fall in the rate of packing, erythrocytes are relatively inflexible. A fall in the rate of packing was observed after adding 0·8 g dextran/100 ml. whole blood, which by calculation corresponds to a reduction of only 10% in the plasma fibrinogen content. This would indicate that even with these small differences of fibrinogen concentration membrane stiffening is present. This is not due to simple dilution, as is indicated by adding Ringer–Locke and saline, shown in Fig. 3.

It has been proposed in the past that fibrinogen causes rouleaux by bridging between sites on the membranes of adjacent cells (Merrill *et al.*

1966). The electrical repulsive force due to the charge on the membranes is considered to be insufficient over the length of the molecule to overcome the binding forces of the fibrinogen. It has further been argued that dextran acts by competition for these binding sites. The results reported above show that the complexing ability of dextran for fibrinogen can explain rouleaux disruption simply by lowering the concentration of the free fibrinogen and that attached to the membranes.

The manufacturers of Rheomacrodex suggest that the maximum infusion given in one dose should be 1000 ml. in 30–60 min. This would result in a normal adult having a dextran concentration in the plasma of about 3 g/100 ml. This is not enough to have a very significant effect on rouleaux formation, however it will cause fibrinogen inactivation especially at the site of infusion. Furthermore, it will cause erythrocyte inflexibility. Though the effect of this on the bulk viscosity is probably overcome by the volume expansion induced in the blood stream, an effect of erythrocyte inflexibility on blood flow will be present in the microcirculation. At no time should the infusion be greater than that required to produce the first signs of inflexibility, i.e. the Rheomacrodex concentration should be kept below 16 ml./100 ml. of plasma. This is because the volume expanding ability of the dextran falls in a few hours due to renal excretion while the inflexibility induced by fibrinogen loss continues as long as the fibrinogen is lacking. Continued infusion of dextran on subsequent days will continue to deplete the fibrinogen level and cause further inflexibility. Thus dextran should be used sparingly and only to act as a volume expander.

The mode of action of the formalin is much less clear. Certainly inflexibility and aggregate formation begin at the same time. But ultimately the aggregates disappear though the inflexibility continues to increase until at a concentration of about 3% (v:v) of formalin in the plasma total fixing occurs. The stiffening action is possibly a direct one on the membrane, probably as a result of protein cross-linkage as occurs in the tanning process (Bjorksten, 1951). Perhaps small amounts of formaldehyde induces stronger bonding of the fibrinogen to the membrane, but larger amounts denature the fibrinogen.

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