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An In Vitro Study Of Rouleaux Formation In A Non-flowing Environment

Lionel Satyanand Sewchand

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AN IN VITRO STUDY OF ROULEAUX FORMATION IN A NON-FLOWING ENVIRONMENT

by

Lionel Satyanand Sewchand, B.Sc. (Hons.)

Department of Biophysics

Submitted in partial fulfillment

of the requirements for the degree of

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To my wife, Madalena Maria,

and son, John Carlos.

ABSTRACT

This research was carried out to examine the effects of polyvinylpyrrolidone and dextran, two neutral polymers, as rouleaux-inducing agents, to investigate the possible species-specificity in rouleaux formation, to study the modes of formation of rouleaux doublets and longer rouleaux, and finally to examine the effects of cellular surface charge, increased macromolecular concentrations, and other changes in the medium surrounding the cells on red-cell adhesion. The method of study involved observations of the red cells, in dilute suspensions, forming rouleaux on a plastic coverslip and recording these observations using photomicrography and cinemicrography for subsequent analysis.

The dextran fractions used in this study (Dx-70, Dx-110 and Dx-500) were found unable to induce aggregation of human cells at concentrations beyond 70g/l. The Dx fractions however induced aggregation of red cells of other species at all concentrations above a critical concentration level. Polyvinylpyrrolidone (PVP-40, PVP-360), on the other hand, was found to induce aggregation of all species cells, including human, at all concentrations above its critical rouleaux-inducing concentration level. These results suggest that rouleaux formation depends not only on the physicochemical properties of the rouleaux-inducing molecules but also on the nature of the adsorb-

bing surfaces.

In the PVP-induced aggregation of interspecies populations of red cells it was found that red cells of any one species adhered in rouleaux formation with red cells of any other species. However, a statistical analysis of the cells in mixed rouleaux showed that red cells preferred to adhere to cells of the same kind (species) rather than to red cells of another species. The preference could not be explained by differences in the cell sizes, but the apparent differences in the surface structure of the cells might play a responsible role.

Red cells lying on a coverslip in a solution of rouleaux-inducing agent, were observed to form doublets by either the sliding, cresting or flipping mode, as previously reported. The sliding mode was predominant in PVP solutions up to 5g/l concentration, while the other modes prevailed at higher concentrations. In Dx-70 and Dx-110 solutions, the non-sliding modes were predominant at a macromolecular concentration of approximately 40g/l while sliding was the principal mode at higher and lower concentrations. A model was proposed which attributed the net adhesive force between the cells as the main determinant in the transition from sliding to non-sliding. The tank-tread model of other investigators for explaining the process of alignment of the cells in rouleaux formation was discounted in this study. The mode of formation of long rouleaux (four or more cells) was

independent of the macromolecular concentration, but dependent on the orientation of the cell or rouleau when contact was first made. The formation of long rouleaux clearly illustrated that the process involves a surface energy with the general tendency to reduce this surface energy to a minimum.

The adhesiveness of the cells was found to increase linearly with the PVP concentration over the concentration range studied. The increased interaction of the cells with increased PVP concentration was also illustrated. A reduction in the surface charge of the cells was found to increase their adhesiveness. The study of red cells suspended in a no-Ca^{++} medium was not conclusive but suggestive that a reduction in the divalent ion concentration results in a decrease in the adhesiveness of the cells. Changes in the pH of the medium had no apparent effect on the adhesiveness of the cells, but the cells were found to be compressed, prior to sliding, for a relatively long duration (~75 seconds) at high pH's. Young cells were found to be more aggregable than old cells and this result was interpreted to be partly due to the greater deformability of the young cells.

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CHAPTER 1

INTRODUCTION

1.1. The Red Blood Cell

The red blood cell was first described in 1673 by the Dutch microscopist, Leeuwenhoek, who reported them as "small round globules" but the "ruddy globules" were probably first observed by Swammerdam 15 years earlier. (see Wintrobe, 1967, p. 63). The fact that red cells are really flat discs rather than globules was however recognised one hundred years later by William Hewson who suggested that they "must be of great use" in the body economy.

The discovery of these "great uses" came in the nineteenth century. Most notable were the contributions of vonLeibig (1852) who discovered that the red cells contain a compound of iron which can combine with O_2 and CO_2 in a reversible reaction, Hoppe-Seyler (1867) who demonstrated the oxidation-reduction potential of haemoglobin, and Neumann (1868) who discovered the hemopoietic function of the bone marrow. There were other contributions from Vierordt (1851), Welcher (1863), Malassez

(1874) and Hayem (1877) (see Bishop and Surgenor, 1964, p. 3). By the turn of the twentieth century, it was already common knowledge among researchers in blood that the red cells make up 45 to 50% of the total blood volume, and their main function is the transport of O_2 and CO_2.

Red cells are produced by the erythropoietic tissues in the body, mainly located in the marrow of long bones. Adult red cells, after having lost their nuclei and subnuclear particles (mitochondria, RNA granules, etc.) retain many enzymes, proteins, carbohydrates, lipids, anions and cations, and finally become simple cells structurally. The red cell contains haemoglobin in high concentration (Ponder and Barreto, 1955) and it is the haemoglobin which has a strong affinity for the O_2 it carries from the lungs to other cells. On the way back to the lungs it carries CO_2, a waste product of the body cells. The loading and unloading of the gases is a passive response of the haemoglobin to the gas concentrations in the environment of the red cells. This function is carried out by the red cell for a period of about 120 days, at which time it is removed from the circulation by the reticuloendothelial system of the body.

The normal human red cell has the shape of a biconcave disc whose diameter is 8.1μm±0.5 (S.D.), rim thickness 2.4μm±0.1 (S.D.), and "dimple" thickness 1.0μm±0.1 (S.D.) (Ponder, 1948, p. 14; Canham and Burton, 1968; Evans and Fung, 1972; Jay, 1975). What role the biconcave

shape of the cell plays in the diffusion of gases is unclear. What is clear though is that the "nonsphericity" (Canham and Burton, 1968) is essential to the tolerance of the erythrocytes to deformation in the circulation. The membrane of the red cell resists stretching and any small increase in area results in hemolysis (Canham and Parkinson, 1970; Evans and LaCelle, 1975). The biconcave discoidal shape is one which would permit shape changes without any stretching of the cell membrane, a feature which would be of obvious advantage to a cell which is constantly squeezing through passages of diameters less than its own in the microcirculation. The membrane of the red cell is very flexible (Brånemark and Lindstrom, 1963) and it is this additional property that enables it to rapidly change its shape and endure the tortures of the microcirculation.

Electrophoretic measurements of intact red cells indicate that they are negatively charged at physiological pH and ionic strength. The negative surface charge is primarily due to the carboxyl group of sialic acid in the cell membrane (Cook, Heard and Seaman, 1961; Eylar, Madoff, Brody and Oncley, 1962).

Of particular interest in this research is the reported adhesiveness of the red cells (Fåhraeus, 1921; George, Weed and Reed, 1971). They adhere to each other reversibly in plasma to form groups of cells stacked side to side. Even in the microcirculation the formation and

breaking up of these groups of cells is occurring continually as demonstrated by the motion studies of Bråne-mark (1971). A white cell (which is larger than the red cell) entering a small channel momentarily blocks the flow and creates an environment of very low shear rate for a few seconds. The stacking of the red cells, side-by-side, will take place under these conditions.

1.2. Rouleaux Formation

A. Definition and Meaning

A rouleau may be defined as a column-like structure of cells formed when red cells reversibly adhere to each other broadside against broadside (fig. 1). Recent electronmicrographic studies of Rowlands and Skibo (1972) have shown that the cells in rouleau are parallel-sided and the concavity seen in the single, separate cells is no longer evident when the cells are in rouleaux formation. Also, the cells in rouleaux are thinner but have a greater diameter than the single, separate cells (Thorsén and Hint, 1950).

The definition of rouleaux may be extended to include not only the coin-like arrangement, but also irregular aggregates which are formed by identical factors as those operating in the formation of simple rouleaux (Fåhraeus, 1921, 1929 and 1958). Knisely, Block, Eliot and Warner (1947, 1950) understood rouleaux to mean only the simple rouleaux, in which the red cells are stacked as coins on

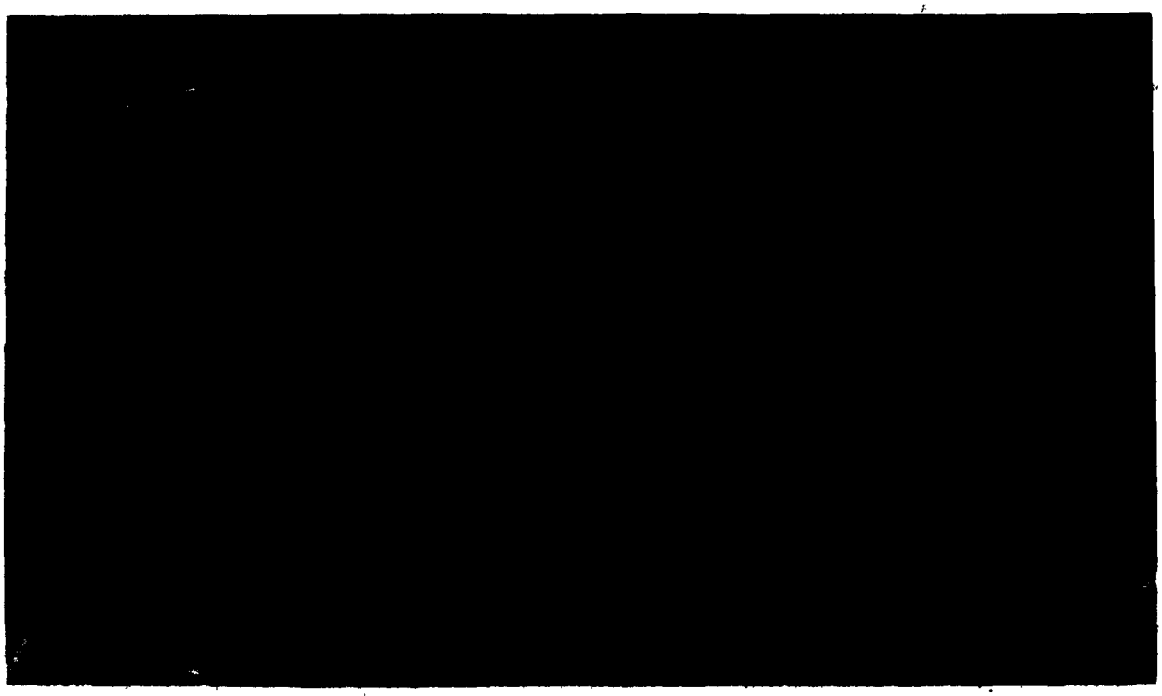
FIGURE 1

Human red cells in rouleaux.

a. Linear rouleaux (the scale represents 10μm)

b. A branched rouleau (the scale represents 10μm)

c. A rouleau clump (scale same as in b.)



one another. This interpretation led them to report their observations of aggregation in patients, having a variety of pathologic conditions, as red cells *in vivo* "agglutinated into masses (not rouleaux), changing the blood from its normal relatively fluid state to a circulating sludge" (Knisely *et al.*, 1947). The red cells in these "sludges" were reported to be arranged at all angles to each other within the masses, and the masses were apparently more rigid and tougher than the ordinary, flexible serpentine rouleaux. However, the *in vitro* study of Thorsén and Hint (1950) and the *in vivo* work of Odell, Aragon and Pottinger (1947), Hirschboeck and Woo (1950) and Ditzel (1959) showed that there is only a difference of degree, not of kind, between the simple rouleaux formation and the "sludge" or irregular aggregation. The degree of aggregation *in vivo* was found to be directly related to the abnormality of the concentration of the plasma proteins, while the *in vitro* study of Thorsén and Hint showed that at high colloid concentrations (rouleaux-inducing colloids), red cells which came into contact with one another no longer shifted about so that their flat surfaces came face-to-face but remained stuck to each other at whatever place they touched. So the *in vivo* sludge is rouleaux formation according to the more inclusive definition of Fahraeus. In this thesis, the terms "rouleaux formation" and "red cell aggregation" are interpreted to be equivalent.

Rouleaux formation can be easily studied *in vitro* because it occurs among red cells suspended in plasma and also among red cells suspended in solutions of various long chain asymmetric molecules like high molecular weight Dextran and polyvinylpyrrolidone.

Rouleaux formation should be distinguished from agglutination. It is a reversible phenomenon, i.e. mechanical agitation would reduce the rouleaux to single cells and as soon as it is removed the cells would get back into rouleaux formation. Agglutination, on the other hand, is an antigen-antibody reaction and it is irreversible.

B. Factors Affecting Rouleaux Formation

Rouleaux do not form in Ringer solution alone. Their formation necessitates the presence of long chain, asymmetric molecules for bridging the cells together. In addition, there are two other important considerations, namely, the surface charge of the cells and the deformability of the cells. The importance of these parameters will be discussed later.

Rouleaux formation occurs when the adjacent cells are brought into close range by gravitational sedimentation, thermal motion, Brownian motion, or mechanical agitation. The mechanics of the encounter of two spheres in a shear flow has been treated by Goldsmith and Mason (1967) and worked out in detail by Batchelor and Green (1972). The

spheres on streamlines less than one diameter apart will pass within a fraction of 1% of the diameter of each other. Application of such information to human red cells suggests that the adjacent cells may approach each other with a separation of the order of 10nm. Therefore, a slight degree of shearing, by increasing the probability of cell encounter, may favour red-cell aggregation. A larger increase in shear rate, however, causes cell disaggregation by increasing the deforming stress acting on the cells. In the normal blood, red-cell disaggregation is essentially complete when the apparent shear rate is increased above 50 sec^{-1} (Schmid-Schönbein, Gaehtgens and Hirsch, 1968; Brooks, Goodwin and Seaman, 1970).

i. Influence of Macromolecules on Rouleaux Formation

Red cells suspended in plasma form rouleaux in zero flow or low shear conditions (Fåhraeus, 1929). Such aggregation depends on the presence of fibrinogen and some of the serum globulin fractions. A comparison of the relative effectiveness of the various plasma proteins in inducing rouleaux formation shows that the effectivness is strongly dependent on the molecular size (Chien, Usami, Dellenback and Gregersen, 1970). Similar results have been obtained with neutral polymers such as dextran (Dx) and polyvinylpyrrolidone (PVP) (Thorsén and Hint, 1950).

The adsorption of plasma proteins (negatively charged

at physiological pH) and neutral polymers to the red cell surface is probably by van der Waals force or hydrogen bonding. The adsorption force due to these bonding mechanisms is much weaker than those due to electrostatic attraction or covalent bonding. Since the red cell surface is negatively charged, positively charged macromolecules can bridge red cells by electrostatic attractive force. Examples of such positively charged macromolecules are polylysine (Nevo, deVries and Katchalsky, 1955; Katchalsky, Danon, Nevo and deVries, 1959), polyglutamic acid (Kulkarni and Blout, 1962) and polybrene (Lalezari, 1968).

ii. Influence of Surface Charge of the Cells

The red cell surface is negatively charged, mainly as a result of the presence of N-acetylneuraminic acid (sialic acid) (Seaman and Uhlenbruck, 1963). The negative surface charge provides an electrostatic repulsive force which tends to cause disaggregation. This disaggregating influence is most significant when the bridging macromolecule is neutral or negative in charge and short in molecular length, and when the cationic strength in the suspending medium is reduced (Jan and Chien, 1973a, b). A reduction of surface charge, for example by Neuramindase treatment, increases red-cell aggregation by neutral polymers. A reduction of the surface charge, however, causes a decrease in the aggregation induced by positively

charged macromolecules (Jan and Chien, 1973a).

iii. Influence of Red Cell Deformability

Ultrastructural studies on rouleaux have shown a deformation of adjacent cells to form parallel surfaces which make possible the existence of multiple macromolecular bridges. Therefore, red cell deformability plays an important role in rouleaux formation (Chien and Jan, 1973). A reduction in deformability is associated with a decreased tendency of red-cell aggregation in several physiological and pathological conditions. Examples are found in the normal red cells of goat (Chien, Usami, Dellenback and Bryant, 1971), human red cells hardened in aldehydes (Chien, 1973) or treated with surface-active agents (Ehrly, 1968), and the irreversibly deformed cells from patients with sickle cell anemia.

C. In Vivo Observations

There is ample evidence that rouleaux formation is not a phenomenon occurring exclusively in artificial circumstances, but that it occurs readily in the circulation in man and in animals, particularly when there is stagnation of blood flow.

According to Ditzel (1959), Coccius in 1852 was the first to observe that under certain conditions the circulating red cells in man will form clumps (irregular aggregates) in the smaller vessels. This intravascular

clumping has since been observed repeatedly at the microscopic level in different tissues of man and animals. The methods used for in vivo studies include direct visualization with the microscope, television microscopy, adsorption microspectroscopy, dynamic pressuregrams and high speed cinemicrography (Bloch, 1962). Observations are usually made of the bulbar conjunctival vessels which are superficial and parallel to the surface, thereby making possible observation of the flow of blood from the arterioles through the capillaries and into the venous channels without interruption or loss of detail (Knisely et al., 1947). The architectural composition of the aggregates differs depending upon their location in the microcirculation. In the terminal arterioles where the shearing forces are great and the calibre small, the cells either circulate individually or in simple rouleaux. In contrast, in the venules where the shearing forces are minimal and the calibre greater the red cells would stick together, not only as rouleaux, but also as clusters of rouleaux or irregular masses (Ditzel, 1959).

Erythrocyte aggregation (or rouleaux formation) is observed in practically all diseased conditions (Knisely et al., 1947; Weis-Fogh, 1957) but the degree of aggregation varies considerably from one disease to another and from individual to individual, and is rather closely correlated with the general condition of the disease process (Odell et al., 1947; Ditzel and Moinat, 1957). Using

paper electrophoresis Ditzel (1959) showed that the degree of aggregation is related directly to an increase in the high molecular weight serum proteins, the globulin fractions, and a decrease in the smaller molecular weight proteins, the albumin. Changes in the amount of lipoproteins appear to have little influence on the formation of aggregates.

Intravascular aggregation is not restricted to disease conditions; it also occurs in normal health. Ditzel (1956) observed rouleaux formation in the venules of supposedly healthy people, with the incidence increasing with age. Weis-Fogh (1957) reported that among sixty persons who had normal erythrocyte sedimentation rates (ESR) and haemoglobin values, 20% showed intravascular erythrocyte aggregation, but only 3% to a pronounced degree. These observations suggest that even the normal concentration levels of plasma proteins are capable of inducing rouleaux formation.

Observations have also been made in animals where rouleaux formation is induced artificially. Intravascular aggregation has been observed after intravenous injection of type III pneumococcus polysaccharide into albino mice (Younger and Nungester, 1944), after intravenous injections of high molecular weight dextran and human fibrinogen into rats, hamsters and rabbits (Thorsén and Hint, 1950; Cullen and Swank, 1954; Gelin, 1956), and after digestion of a highly fatty meal, leading to lipemia, in

hamsters (Swank and Cullen, 1953; Cullen and Swank, 1954). These observations all re-enforce the fact that intravascular erythrocyte aggregation is caused by an increase of highly asymmetric molecules of large sizes in the circulating blood.

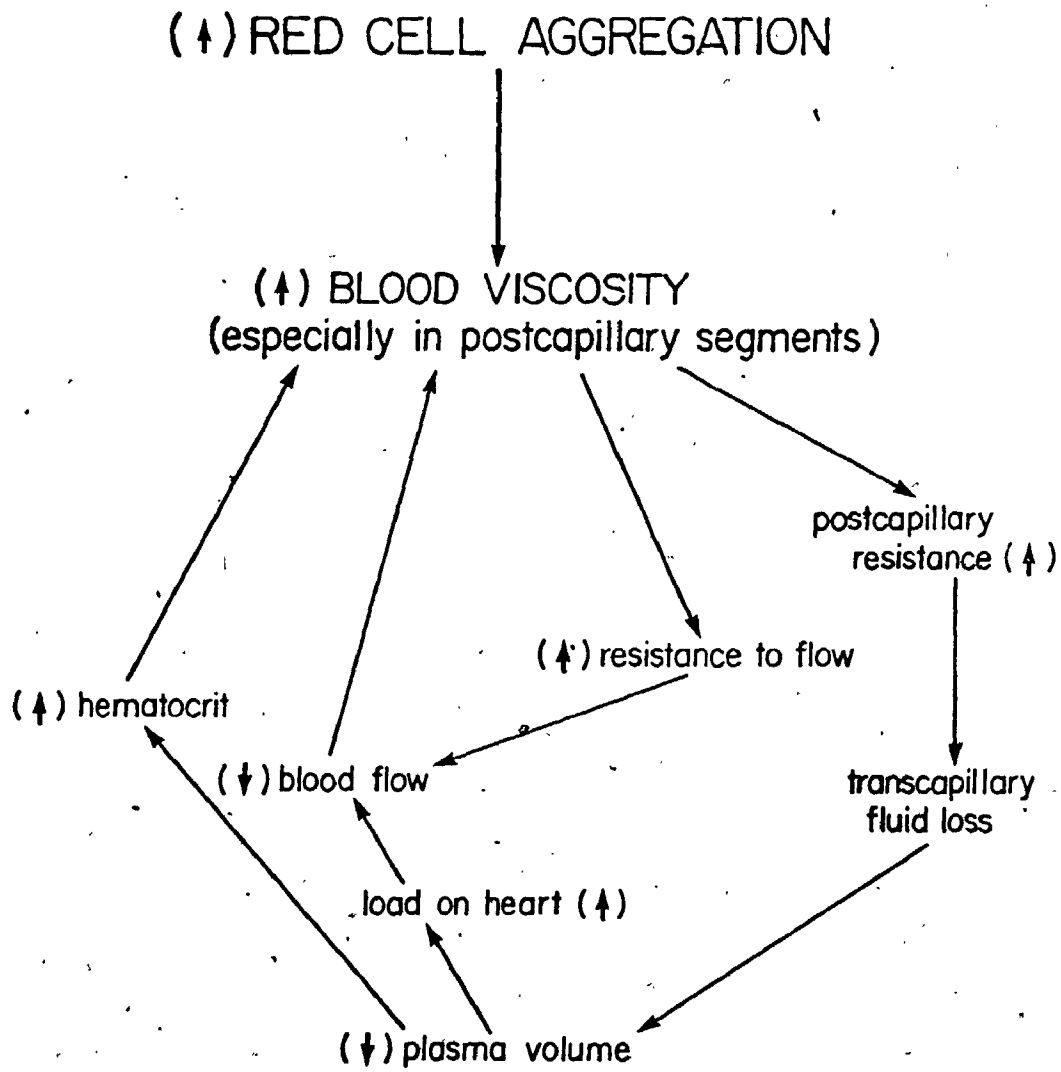
D. Pathological Significance

Abnormal changes in the circulation as a result of rouleaux formation or red cell aggregation are indicated by a decrease in the flow rate of the red cells, especially in the postcapillary venules and sinusoids (Knisely et al., 1947). This is attributed to an increase in the viscosity of blood. The aggregates may also obstruct or occlude the capillaries, sinusoids and venules as reported by Matsumoto, Hardaway, McClain and Margetis (1968), and Vanecko, Szanto, and Shoemaker (1969) who observed microscopically the plugging of the microcirculation. Stasis of cells relative to plasma occurs, with consequent decreased venous return of cells (Gelin, 1956). This is known as plasma skimming where the true capillaries may contain a practically red cell-free plasma (Gelin, 1956, 1959). The significance of rouleaux formation or red-cell aggregation with respect to the circulation is perhaps best explained in figure 2.

The following pathophysiological conditions, resulting from elevated red-cell aggregation (rouleaux formation) have been enumerated: increased load on the heart and in-

FIGURE 2

Flow sheet showing the significance of rouleaux formation in the circulation. (This figure is modified from Chien, 1974). Symbols: (↑) means increase; (↓) means decrease.


(↑) RED CELL AGGREGATION
(↑) BLOOD VISCOSITY
(especially in postcapillary segments)
postcapillary
resistance (↑)
(↑) resistance to flow
(↑) hematocrit
(↓) blood flow
transcapillary
fluid loss
load on heart (↑)
(↓) plasma volume

creased peripheral resistance (Bergentz, 1961; Swank and Escobar, 1957); decreased tissue oxygenation (Lofstrom and Zederfeldt, 1957) and decreased CO_2 elimination with acidosis (Bergentz, 1961); retarded wound healing (Zederfeldt, 1957); tendency to develop venous thrombi (Bergentz, 1961; Borgstrom, Gelin and Zederfeldt, 1959); and finally, anoxic damage in parenchymal tissues (Fajers and Gelin, 1959).

In the postcapillary venules and in the venous limbs of the capillary, hypoxic damage to vessel walls is more easily evidenced. This hypoxic damage is due to restricted blood flow after vasoconstriction or intravascular aggregation, or by the combined effects of both. The walls of postcapillary venules are very thin, in some tissues consisting merely of endothelium with but very few surrounding supportive cells. Anatomically, these vessels have the least ability of any to retain blood plasma proteins. It has been described that the decreased rates of supply of O_2 to endothelium for any great period of time result in an alteration of the endothelium such that it is no longer able to retain all of the blood plasma proteins (Starr, 1926; Bor, Stewart, Cho, Kirkardesler and Eren, 1962). Protein-containing fluids pass out through the vessel walls thus initiating edema. So, the impaired O_2 supply caused by red cell aggregation leads to the production and maintenance of interstitial edema.

The influence of intravascular red cell aggregation

on thrombus formation was experimentally demonstrated by Borgstrom et al. (1959) in a lengthy series of studies involving hundreds of rabbits; thrombus was induced by the combination of venous stasis (induced by ligation of femoral veins) and red cell aggregation (induced by high molecular weight colloids). The conclusions from these studies clearly demonstrated that intravascular aggregation induces thrombosis, and is not a consequence of it.

Rouleaux formation also initiates damage to the heart. This is known from the work on dogs (Swank and Escobar, 1957) and rabbits (Stalker, 1967) where aggregation was induced by high molecular weight dextrans. In hearts affected by coronary atherosclerosis (induced by a daily diet of cholesterol) strong aggregation produces lasting ischemic myocardial damage (Bicher and Beemer, 1967a, b; Bicher, 1968).

Bergentz and Danon (1965) and Stalker (1967) described the histological changes induced by intravascular aggregation of long duration and reported microscopic damages in all parenchymal tissues. Hypoxic tissue destruction, especially in liver and heart, is the most prominent lesion. Animals in a state of shock are more susceptible, but the damage occurs even in normal animals. Lesions in the kidney which are clearly recognizable in histologic sections are also initiated by intravascular aggregation (Fajers and Gelin, 1959).

E. In Vitro Observations

i. Historical Review

William Hewson (1773) was credited with the first observation of rouleaux formation. Using a primitive microscope he observed rouleaux formation in fresh blood smears: "I have seen them (red blood cells) with their sides parallel, like a number of coins, laid one against another."

In the nineteenth century a considerable amount of work was reportedly done to understand the phenomenon. From the work of Lister (1857) it was learned that rouleaux formation is enhanced in diseased blood. Norris (1869), in an effort to explain the mechanism of rouleaux formation, stated that the phenomenon was caused by surface tension forces. He reasoned that the interfacial tension between the red cells and plasma tends to force the cells together in such a way that the amount of cell surface exposed to plasma is minimized. This is interesting in that there is not a great deal more known about this possibility even now.

By 1912 when Wiltshire published his studies on rouleaux formation it was already known that rouleaux formation could be induced in isotonic solutions of gum acacia, gelatin and other macromolecules in place of plasma. Wiltshire isolated two factors or forces influencing rouleaux formation. One was the attractive force between the cells, due to surface tension; the second was a frictional

force tending to prevent rouleaux formation. Wiltshire described the frictional force as arising from a "viscous or sticky envelope surrounding the corpuscle" and suggested that the relative magnitudes of the two forces would determine whether rouleaux would form. Wiltshire (1912) also studied the effect of pH and temperature on rouleaux formation. He reported that pH had no effect on the degree of rouleaux formation but increasing temperatures increased the rouleaux-inducing capacity of serum.

Following the classic paper on the suspension stability of blood by Fåhraeus (1921) studies on the phenomenon of rouleaux formation intensified. Fåhraeus showed that the red cells in the blood of many patients sedimented more rapidly than those in normal blood. This test has since become established as a valuable clinical aid, especially in identifying patients with inflammatory disease and assessing improvement or deterioration. An increased sedimentation rate (ESR) is associated with the aggregation of red cells into abnormally large rouleaux (Fåhraeus, 1921, 1929). While variations in size and shape of the cells might influence their capacity to form rouleaux, the essential cause of the excessive aggregation in disease lies in the plasma, since the red cells of a normal person form larger aggregates, and sediment more rapidly, when transferred into the plasma of a diseased person (Fåhraeus, 1921, 1929; Hardwicke and Squire, 1952). It has been established that the responsible factors in

the plasma are fibrinogen and the globulins (Fåhraeus, 1929; Hardwicke and Squire, 1952).

The ESR method has been used extensively for determining quantitatively the aggregation tendency of human erythrocytes in various clinical conditions (Frimberger, 1939; Wunderly and Wuhrmann, 1944; Hirschboeck, 1947; Mlczoch, Wunderly and Wuhrmann, 1949). Thorsén and Hint (1950) and Richter (1965) also used this method for determining the aggregation tendency of normal human erythrocytes suspended in solutions of dextran fractions with varying molecular weight, and for quantitatively measuring the relative aggregation tendency of erythrocytes from man and from a number of laboratory and domestic animals (Richter, 1966).

The results of the work of Thorsén and Hint (1950) showed the importance of the colloid constituents of the plasma in determining the stability of the red cells in suspension. Their findings led to the development and introduction of dextran 40 (molecular weight 40,000; Rheomacrodex) a dextran fraction of low molecular weight which is claimed to have an important effect in improving blood flow in the microcirculation (Gelin, 1956; Gelin and Ingelman, 1961). The specific property attributed to dextran 40 is the prevention and counteraction of red cell aggregation (Engeset, Stalker and Matheson, 1966). The work of Thorsén and Hint (1950) was also the first to show that for any rouleaux-inducing polymer a certain concen-

tration of the polymer is necessary for inducing the formation of rouleaux. Above this critical concentration, the extent of rouleaux formation increases with increasing concentration of the polymer.

Since the introduction of the ESR method (Fåhraeus, 1921) for quantitatively measuring aggregation other methods have also been developed. These will be discussed in the next section.

Investigators in the last 15 years or so have been very interested in understanding and explaining the mechanism of rouleaux formation. One hypothesis proposed for explaining the mechanism is based on the colloid stability theory of Derjaguin, Landau, Verwey and Overbeek (Weiss, 1967). On the basis of this theory the cells adhere to one another at the specific intercellular distance at which the attractive van der Waals force is balanced by the electrostatic repulsive force between the cells. This theory accounts for the relatively large intercellular spacing between the cells in rouleaux formation of approximately 200 Å (Rowlands and Skibo, 1972). However, Chien and Jan (1973) studied by electron microscopy the intercellular relationship in rouleaux formed by dextrans with different molecular sizes and found that the distance between the cells in rouleaux increased with the size of the dextran molecule. Their results led them to postulate a model of red cell aggregation by monolayer bridging of the macromolecules. Contrary to the colloid stability theory

in which the force of aggregation is proposed to result from attraction between the cell surfaces, this new model attributes the aggregation force to an attraction between the macromolecules and the cell surface.

The effects of various factors on rouleaux formation have been studied in the past few years. These include the red cell shape and deformability (Goldstone, Schmid-Schonbein and Wells, 1970), surface charge (Jan and Chien, 1973a), shearing forces of the medium (Goldsmith, 1967; Goldstone et al., 1970) and the ionic strength of the medium (Jan and Chien, 1973b). The results of all these studies undoubtedly contribute to the understanding of rouleaux formation. However the true nature of the mechanism of the phenomenon still remains unclear, and its study is a challenging area of research.

ii. Approaches of Study

The various techniques employed for studying red-cell interactions are summarized in figure 3.

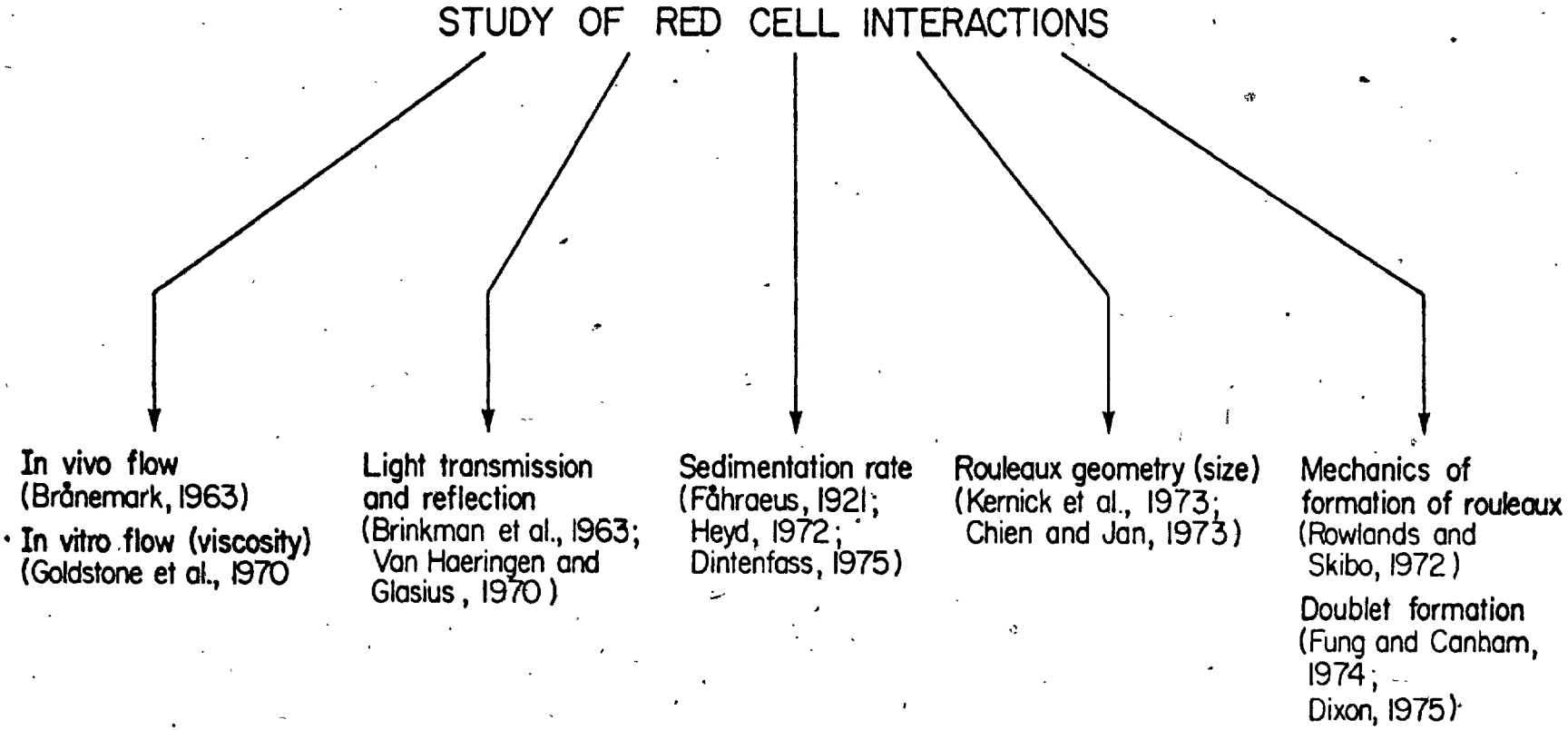
The ESR (Fåhraeus, 1921) became the first real method for quantitatively measuring the aggregation of red cells. The physical basis of this method lies in the fact that the terminal velocity of a spherical particle falling through a continuous medium under the effect of gravity is governed by Stoke's law

$$V = \frac{2}{9} g \frac{\rho 1 - \rho 2}{\eta} r^2$$

FIGURE 3

The various methodsused in the study of red-cell interactions.

In this study, the methods involving rouleaux size (Chien and Jan, 1973) and mechanics of rouleaux formation (Rowlands and Skibo, 1972; Fung and Canham, 1974; Dixon, 1975) were used.


STUDY OF RED CELL INTERACTIONS
In vivo flow (Brånemark, 1963)
In vitro flow (viscosity) (Goldstone et al., 1970
Light transmission and reflection (Brinkman et al., 1963; Van Haeringen and Glasius, 1970)
Sedimentation rate (Fåhraeus, 1921; Heyd, 1972; Dintenfass, 1975)
Rouleaux geometry (size) (Kernick et al., 1973; Chien and Jan, 1973)
Mechanics of formation of rouleaux (Rowlands and Skibo, 1972)
Doublet formation (Fung and Canham, 1974; Dixon, 1975)

where V denotes the terminal velocity of the falling particle, g the acceleration due to gravity, ρ_1 and ρ_2 the density of the particle and the medium respectively, η the coefficient of viscosity of the medium, and r the radius of the particle.

This equation strictly applies only to spherical particles, and to particles falling without edge effects or the effects of other particles causing upward movement of the suspending medium, but it also gives us an idea of how the size of other roughly globular particles will affect the velocity of their fall through a continuous medium. In blood the sedimentation rate is governed by the same factors as in the above expression but in a slightly modified way because red cells are not spherical. Fåhraeus (1929) found that in human blood variations in the density of the red cells and the plasma, as well as the viscosity of the plasma, do not influence the ESR significantly. The important factor is the radius (r) of the falling particles. The radius in itself does not vary greatly among red cells of a particular blood, but if the red cells are aggregated into rouleaux, then the resultant r of an aggregate will be increased many times and this aggregate will settle much more quickly in the plasma than single cells or smaller rouleaux. The ESR therefore reflects the extent of rouleaux formation.

That an increase in ESR is related to an increase in the size of the red cell aggregates has been widely

accepted (Ponder, 1947; Thorsén and Hint, 1950) but this tenet has recently been questioned by Rampling and Sirs (1972) who suggested that cell flexibility is the key factor in the packing speed of the cells, with increased plasma protein concentration increasing the flexibility of the membrane. The ESR is still however used today as a measure of the degree of red cell aggregation (Dintenfass, 1975). ESR is measured in mm/hr and for normal subjects this value ranges from 1 to 9 mm/hr for men and 1 to 15 mm/hr for women, according to Wintrobe (Wintrobe, 1967). In many disease states the ESR is greatly increased and can rise above 100 mm/hr (Wintrobe, 1967, pp. 360-362).

Another method used for measuring rouleaux formation is one called "syllectometry". This method was first introduced by Brinkman, Zijlstra and Jansonius (1963) who made use of the fact that rouleaux formation diminishes the reflection of light by erythrocytes because the convex rim of an erythrocyte reflects much less light per unit of surface area than the concave surfaces, which become unavailable for reflection in building up rouleaux. The decrease in light reflection thus depends on the degree of rouleaux formation. In this method the light reflection of blood was recorded during rapid stirring and also during 60 seconds immediately after stirring is stopped. The curve (syllectogram) obtained, which resembles an exponential decay, is a quantitative expression of the process of rouleaux formation. The half-time, which is the time

in which half of the reflection decrease is reached, is considered to be a measure of the rate of rouleaux formation. The percentage of reflection decrease is a measure of the extent of rouleaux formation (VanHaeringen and Glasius, 1970).

In the 1960's, viscometry at very low rates of shear was widely used for quantitatively measuring aggregation (Wells, Gawronski, Cox and Perera, 1964; Chien, Usami, Taylor, Lundberg and Gregersen, 1966; Merrill, 1969). The relative viscosity of the red cell suspensions at very low rates of shear ($\sim 0.1\ sec^{-1}$) is used as an index of the degree of aggregation (Chien, Usami, Dellenback, Gregersen, Manninga and Guest, 1967).

In 1973, Chien and Jan introduced another method for measuring the degree of red cell aggregation. They described it as MAI - microscopic aggregation index. It involves taking photomicrographs of the cells in rouleaux after a certain length of time, and counting the number of cells and units (each unit is either a single isolated cell or rouleau) in each photomicrograph. This method will be discussed in more detail in chapter 4. The MAI is a measure of the average length of each rouleau after a certain length of time.

The methods described above may be referred to as statistical approaches to the study of rouleaux formation. While they may accurately evaluate the degree of rouleaux formation, they are unable to reveal precise information

about the process occurring at the cellular level. That is, they (except perhaps the MAI method) do not permit direct observation of the cells as they are participating in the adhesion process. Realising this fact, recent investigators (Rowlands and Skibo, 1972; Fung and Canham, 1974; Dixon, 1975) studied the interactions of the cells in the process of rouleaux formation. Rowlands and Skibo (1972), using a micropipette technique, studied the manner in which the cells, suspended in native plasma, join together in rouleaux formation. They reported the process to be "sliding* or tilting of one cell with respect to another". Fung and Canham (1974) came to the same conclusion in their study of the modes and kinetics of doublet formation for cells suspended in solutions of varying concentrations of polyvinylpyrrolidone (PVP-360), a rouleaux-inducing agent, and also for cells suspended in native plasma. Fung and Canham also reported that the incidence of tilting or "cresting" (as they described it) increased for cells suspended in plasma from subjects with elevated ESR, and also in PVP solutions of concentrations between 4 and 7g/l. This was explained by a possible increase in the friction between the cells, arising from the increased macromolecular adsorption on the cell surface. This hypothesis was supported by the work of Dixon (1975, Chap.3) who found that increasing the relative viscosity of the rouleaux-inducing medium increased the incidence of cresting.

*"sliding" and "cresting" are explained in Section 6.3A.

F. Current Knowledge (Summary)

A review of the literature tells us of a number of conclusions drawn from the studies of rouleaux formation. Some of the conclusions made from the in vitro studies are summarized as follows:

1. The plasma proteins, fibrinogen and globulins, are responsible for the formation of rouleaux (Fåhraeus, 1921, 1929; Hardwicke and Squire, 1952). The low molecular weight albumin and lecithin (Fåhraeus, 1929; Stats and Wasserman, 1943) are inhibitory to the process.

2. Rouleaux formation can be induced artificially by various long chain asymmetric molecules, e.g. gum acacia, gelatin, dextran, and polyvinylpyrrolidone (Wiltshire, 1921; Thorsén and Hint, 1950). Rouleaux do not form in Ringer alone.

3. For each colloid, a critical mol. wt. and a critical concentration are necessary for inducing rouleaux formation (Thorsén and Hint, 1950).

4. In dextran solutions at high concentrations, human red cells do not aggregate (Chien and Jan, 1973).

5. Electron micrographs have shown the cells to be parallel-sided in rouleaux (Rowlands and Skibo, 1972; Chien and Jan, 1973); the separation between the cells increases with the molecular weight of the dextran fraction (Chien and Jan, 1973).

6. The red cells of different species aggregate to different degrees under similar conditions (Richter, 1966;

Berman and Fuhro, 1973).

7. Factors like cell shape, cell deformability (Goldstone et al., 1970), surface charge (Jan and Chien, 1973a), temperature, hematocrit (Kernick, Jay, Rowlands and Skibo, 1973) and viscosity of the medium (Dixon, 1975, Chap. 3) affect the mechanics of formation of rouleaux.

1.3. Aims of the Research

The study of rouleaux formation may serve to provide basic information for many facets of the microcirculation and help to elucidate the fundamental physico-chemical basis of cellular adhesion. This fact is known by every investigator involved in red-cell aggregation research. Still, there are many aspects of the phenomenon that remain unresolved.

Different species' red cells have been known to have different aggregating tendencies under similar conditions (Richter, 1966; Berman and Fuhro, 1973). Differences in the aggregating tendencies cannot be explained by differences in the surface charge density because cat cells and mouse cells have almost identical surface charge density but cat cells are more aggregable. There is some unrecognized property of the cell surface that is responsible for the difference in aggregability (Berman and Fuhro, 1973). No one has been able to point out what this surface property is, but the general evidence suggests that there is a difference in molecular adsorption. The

inevitable question therefore arises as to the specificity of rouleaux formation. Is it a species specific type of phenomenon? In other words, would human cells adhere only to human cells or would they also adhere to cells of other species?

Of interest also is the fact that human cells do not aggregate in dextran solutions of high concentrations (Chien and Jan, 1973). Is this fact attributable to some property of the human red cell itself or is it the rouleaux-inducing molecules, dextran, or is it both? This question is answered by studying the aggregation of human and other species' cells in various concentrations of dextran and polyvinylpyrrolidone solutions. This study also answers the question of the existence or non-existence of a single mechanism of rouleaux formation in terms of macromolecular adsorption.

As mentioned previously, not much has been done to explain the behaviour of the membranes of the adhering cells at the microscopic level. Fung and Canham (1974) have illustrated microscopically the sliding and non-sliding mechanisms of cells forming doublets in normal plasma and plasma from subjects with high ESR, and also in solutions of polyvinylpyrrolidone (PVP-360). The non-sliding mechanism of doublet formation, reportedly, dominates in high ESR plasma and also in higher concentrations of PVP-360. It is of interest therefore to study the formation of longer rouleaux and see whether the mechanism

is dependent on the macromolecular concentration. The rolling of one cell on another in a "tank-tread" motion involving no slip between the membranes has also been proposed as a possible process for cells aligning in rouleaux (Hummel, 1969; Chien and Jan, 1973). These studies by Hummel (1969) and Chien and Jan (1973) however did not involve actual observation of the cells participating in the adhesion process. The possibility of the existence of "tank-tread" could therefore be investigated in our study. Also, the question of whether the mode of rouleaux formation differs with the nature of the inducing agent is considered.

Finally, a model of forces is considered at the microscopic level to show the effect of surface charge and membrane flexibility on doublet formation. The forces affecting cell adhesion in our non-flowing system are restricted to the macromolecular bridging force, the electrostatic repulsive force between the cells, and the resisting forces of membrane deformation. Under conditions of a flowing medium, one has to consider the additional factors of the mechanical shearing force and the increased probability of cell contact. The biophysical factors affecting the force balance described here may also be applicable to other biological systems, e.g., the interaction of actin and myosin filaments in the muscle sacromere, the fusion and release of vesicles in secretory cells and nerve endings, or even drug-receptor interactions

(Chien, 1973).

The aims of this research could therefore be summarized as follows:

1. Induced rouleaux formation in interspecies populations of red cells.

2. The contrasting of polyvinylpyrrolidone (PVP-360) and various dextran fractions as rouleaux-inducing agents.

3. The mechanics or modes of human red cell rouleaux formation induced by PVP-360 and various dextran fractions.

4. A consideration of the forces at the cellular level in the sliding mode of doublet formation.

CHAPTER 2

GENERAL METHODS AND MATERIALS

2.1. Blood Samples and Suspending Medium

Fresh samples of blood were obtained from healthy humans, cats, rats, mice, dogs, rabbits and guinea pigs and immediately introduced into heparinized glass vacuum containers. Some of the rats and mice were anaesthetized with Nembutal. All blood was refrigerated and used within two hours. In some cases where just a drop of human blood was needed, blood was obtained by finger prick and used immediately.

For experimental observation normal red cells or Neuraminidase-treated red cells were suspended in an environment similar to that of plasma. Such an environment was provided in the form of isotonic Ringer solution buffered by tris hydroxy-aminomethane and hydrochloric acid.

The buffered isotonic Ringer solution was prepared as follows. First, three stock solutions were made: A - 8.32 g/l NaCl, 0.42 g/l KCl, 0.24 g/l $CaCl_2$, 0.20 g/l NaHCO, and 1.0 g/l dextrose; B - 5.66 g/l HCl;

C - 37.6 g/l tris hydroxymethyl aminomethane (THAM). Solutions A, B, and C were made up with twice-distilled water and had a calculated tonicity of 310±5 mOsm. A Fiske osmometer was used to make minor adjustments in the concentrations of the solutions to ensure isotonicity. The buffer was prepared by mixing 120 ml of solution B with 80 ml of solution C. The mixture was adjusted to pH 7.40±0.02 by small additional amounts of solution B or C, using a Beckman pH meter. The 200 ml buffer was then added to 800 ml of solution A. The resulting solution was one litre of isotonic (310±5 mOsm), buffered (pH 7.40±0.02) glucose-Ringer solution. This solution was filtered through a 0.22 um millipore filter (Millipore Filter Corp., Bedford, Mass., U.S.A.) before use. When not in use it was refrigerated at 4°C for use later.

For inducing rouleaux formation either of two agents was used. These two agents are polyvinylpyrrolidone (PVP) (Sigma Chemical Co., St. Louis, Missouri, U.S.A) and dextran (Pharmacia Fine Chemicals, Uppsala, Sweden). These rouleaux-inducing agents, obtained in powder form, were dissolved in isotonic Ringer solution to any desired concentration. These rouleaux-inducing solutions would later be referred to as either PVP-Ringer or Dx-Ringer. The various PVP and Dx fractions together with their corresponding molecular weights used in this study are summarized in Table 1.

Table 1. Types of Rouleaux-Inducing Agents Used and their Average Molecular Weight.

Polymer Type	Average Molecular Weight
PVP-360	360,000+
PVP-40	40,000+
DX-500	500,000*
DX-110	110,000*
DX-70	70,000*

+Data supplied by Sigma Chemical Co., Missouri, U.S.A.

*Data supplied by Pharmacia Labs., Uppsala, Sweden.

2.2. Preparation of Neuraminidase-Treated Cells

Only human cells were treated with Neuraminidase in this study. Vibrio cholerae Neuraminidase (500U/ml) was obtained from BDH Chemicals Ltd., Orangeburg, New York, U.S.A. The cells were treated with Neuraminidase according to the method of Eylar et al. (1962). About 5 ml of blood was obtained by venipuncture and immediately introduced into a heparinized glass vacuum container. The blood was centrifuged and the red cells were washed three times with isotonic buffered Ringer solution. The washed cells were then incubated at 37°C for one hour with Neuraminidase at a concentration of 30U/ml of packed cells. After incubation the cells were again washed twice before they were ready for use as N-treated cells. A Neuraminidase concentration of 30U/ml of packed red cells ensured that most of the sialic acid content was removed from the cells (Eylar et al., 1962; Gattegno, Bladier and Cornillot, 1975).

2.3. Properties of Polyvinylpyrrolidone (PVP) and Dextran (DX)

A. Polyvinylpyrrolidone

PVP is non-toxic, relatively inert and water soluble. It can be made with sufficiently high molecular weight so that it will be retained in the circulation after transfusion. These properties led to the development of PVP as a plasma expander during the Second World

War when there was a need for blood substitutes. Several investigators however, using PVP as a plasma expander, noticed that it accelerated the erythrocyte sedimentation rate. This observation led to the discovery that PVP induced rouleaux formation (Guillot and Fiehrer, 1948), and investigators since then began using PVP to induce rouleaux for in vitro study.

PVP is a long chain polymer. Although its structure indicates that it should be neutral in aqueous solution, its solutions have been found to be slightly acidic (pH4-5). This acidity has been attributed to a dialysable impurity in commercially available PVP (Mancewicz, Hoerman and Forziati, 1962). PVP is able to bind with a large variety of other molecules. For unknown reasons, it binds preferentially to neutral or anionic molecules (Morawetz, 1965).

According to Hengsfenberg and Schuch (1951) PVP exists in a random coil conformation in aqueous solutions. Ferguson and Nuki (1971) pointed out, however, that the coil is not completely random. Steric hindrance between the pyrrolidone rings tends to give the molecule some linear rigidity. The molecular shape is therefore asymmetric. Miller and Hamm (1953) have estimated that a molecule of PVP with a molecular weight of 40,000 has an axial ratio of approximately 22 (i.e. the average molecular shape is ellipsoidal with the ratio of the major axis to the minor axis equal to 22). Solutions of

molecules in such conformation behave generally as Newtonian fluids. Ferguson and Nuki (1971) have shown that up to a 10% solution of PVP with molecular weight 360,000 has Newtonian and non-elastic flow properties.

B. Dextran

From the work of Gronwall and Ingelman (1945), and Thorsén and Hint (1950) it is known that Dx, like other macromolecules, can change the suspension stability of blood. Thorsén and Hint (1950) showed that the increase in the ESR of human red cells was proportional to the concentration and to the molecular weight of the colloid. Below a certain molecular weight, there was no increase in the ESR even at very high concentrations. This "critical" molecular weight was found to be about 60,000 for Dx. The work of Thorsén and Hint also led to the development and introduction of Dx-40 (mean molecular weight 40,000; Rheomacrodex) a dextran fraction of low molecular weight, claimed to have an important effect in improving blood flow in the microcirculation (Gelin and Ingelman, 1961).

Low molecular weight Dx is still used today as a plasma expander. Dx is a chemically indifferent, electrically uncharged polysaccharide composed of glucose molecules mainly linked by 1.6-D-glucosidic bonds. Native Dx is derived from the fermentation of sucrose by Leuconostoc Mesenteroides NRRL B-512. Parial hydrolysis

and fractionation of the high mol. wt. Dx yields products having mol. wts. in the range suitable for clinical use.

Information on the true structure of Dx is lacking. Wales, Marshall and Weissberg (1953) considered structures consisting of a linear chain with branches of uniform length, uniformly distributed along the chain. According to Senti et al. (1955) however, some branches might be longer than others; some branches might even be longer than the interval separating them along the main chain, and these long branches might also be branched. The structure of the molecule or the degree of branching, nevertheless, is of no importance to its aggregating ability (Thorsén and Richter, 1959).

2.4. Microscopy and Photography

The general procedure was to suspend a small sample of blood in a solution of PVP or Dx in Ringer solution. The cell suspension was prepared by introducing a small drop of blood into a 1 ml plastic syringe. The PVP-Ringer or Dx-Ringer solution was then drawn into the syringe and the suspension was mixed by introducing a small bubble into the syringe and slowly inverting the syringe several times. The volume concentration of the cells was adjusted to approximately 0.2%.

Special well slides were made for holding a drop of red-cell suspension. The slides were made by attaching small plastic rings onto glass microscope slides with

epoxy cement. To prepare a slide for viewing, a drop of red cell suspension was placed on a plastic coverslip. This was then inverted onto a well slide filled with paraffin oil. The whole preparation was then re-inverted for viewing as shown in figure 4.

Observation was made with a Nikon model M inverted microscope, using a 100X bright field oil immersion objective or a 40X objective with either 10 or 15X eye-piece.

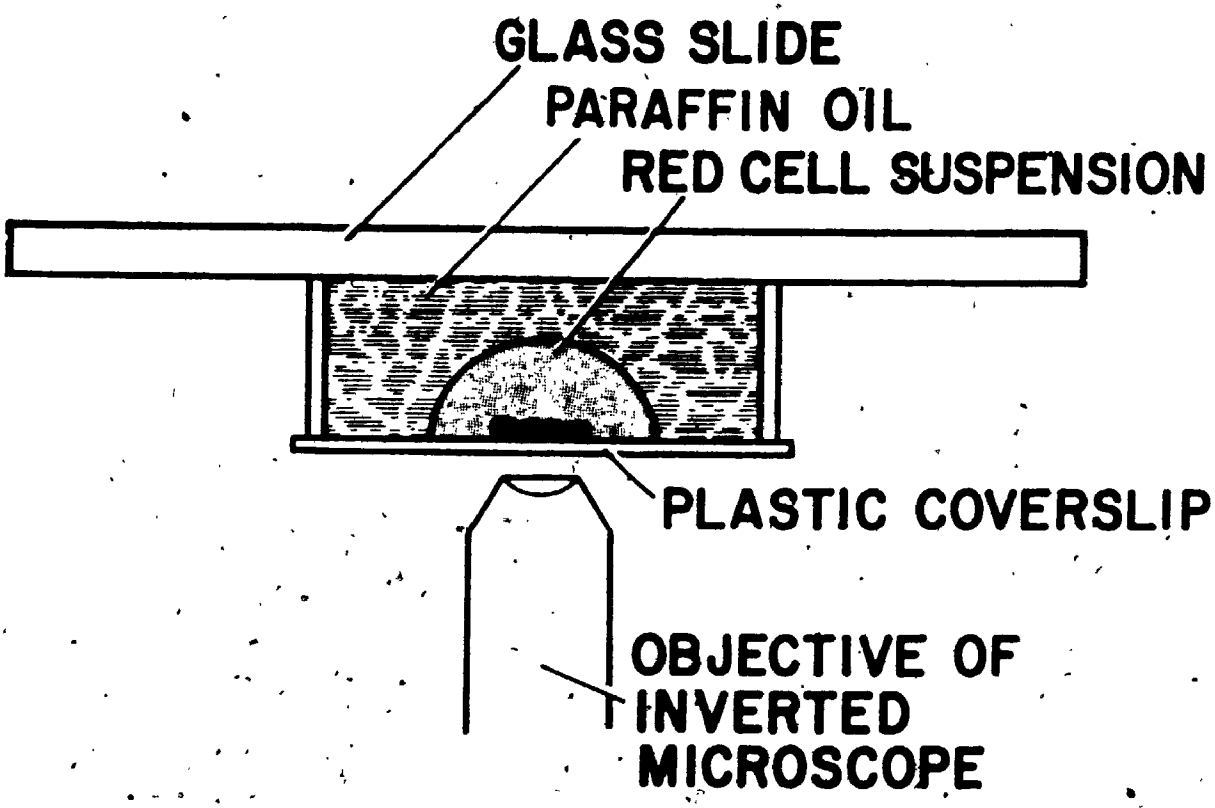
In part of the photomicrography a 35mm camera was used. High magnification prints were made from the 35mm film and the magnification of the image was determined by a calibrated stage micrometer (Leitz Wetzler, 1.00mm divided into 100 parts).

For cinemicrography, 16mm movie film was used with a Bolex camera (model H16M). The Bolex camera was coupled to the microscope with a Nikon EFM photomicrographic unit and was driven by an R.J. Matthias time lapse camera drive and control unit at 2.33±0.01 frames/second. Measurements were made directly from the film projections on a ground glass screen using a motion analysing projector (L-W Photo Inc.). The image size was approximately 15cm by 20cm. The final magnification was 7,500X. The cine-film was also used for making high magnification prints.

FIGURE 4

Schematic of preparation used to observe the cells.

The cells are observed from below through the inverted microscope. They are seen lying broadside down on the coverslip. Two cells are shown here greatly exaggerated in size.


GLASS SLIDE
PARAFFIN OIL
RED CELL SUSPENSION
PLASTIC COVERSLIP
OBJECTIVE OF
INVERTED
MICROSCOPE

CHAPTER 3

GEOMETRY OF THE CELLS IN ROULEAUX

3.1. Measurement and Calculation

Red cells of all the species mentioned in chapter 2 were photographed lying on the plastic coverslip of the sample chamber. Photomicrographs of the cells in Ringer and in PVP-Ringer (where the cells are aggregated into rouleaux) were prepared, from which measurements of the dimensions of the individual cells were made using a rule with 1/60 inch graduations. These measurements were converted to the proper units (um) by using the calibrated stage micrometer which was photographed and printed at the same magnification.

For the monodispersed cells (cells in Ringer) measurements were made of the diameter only. The monodispersed cells have maximum thickness at the rim and minimum thickness at the centre but, because they no longer retain their biconcave shape in rouleaux, measurements of their thicknesses were thought unnecessary. The cells lying flat on the coverslip did not all appear circular, so the diameter of each cell was taken as the

average of the maximum and minimum diametral measurements. In rouleaux the cells appear to have lost their biconcavity as evident from the electron micrographs of Rowlands and Skibo (1972) and Chien and Jan (1973). In this work, the cells in rouleaux are assumed to be flat-sided surfaces of revolution with parabolic rims (figure 5b). Canham and Burton (1968) assumed a circular rim, while Houchin, Munn and Parnell (1958) assumed that the cells are elliptical in cross-section. The assumption of a parabolic rim rather than a circular rim is justified, because an examination of the cells in rouleaux on electron micrographs shows the rim to be more parabolic rather than representative of an arc of a circle.

The assumption that the cells in rouleaux are flat-sided made the mathematics for the calculations of cellular volume and surface area relatively simple. The corresponding equations for cellular volume and surface area were derived to be:

Cellular Volume,

$$V = \pi h[0.25d^2 - 0.17hd + 0.05h^2] \quad (1)$$

Surface Area,

$$A = 0.5\pi[d^2 + 0.96hd - 0.22h^2] \quad (2)$$

where h (μm) is the thickness and d (μm) is the diameter of each cell in rouleaux. For the derivation of the above equations see appendicies A and B.

FIGURE 5

Cells in rouleau.

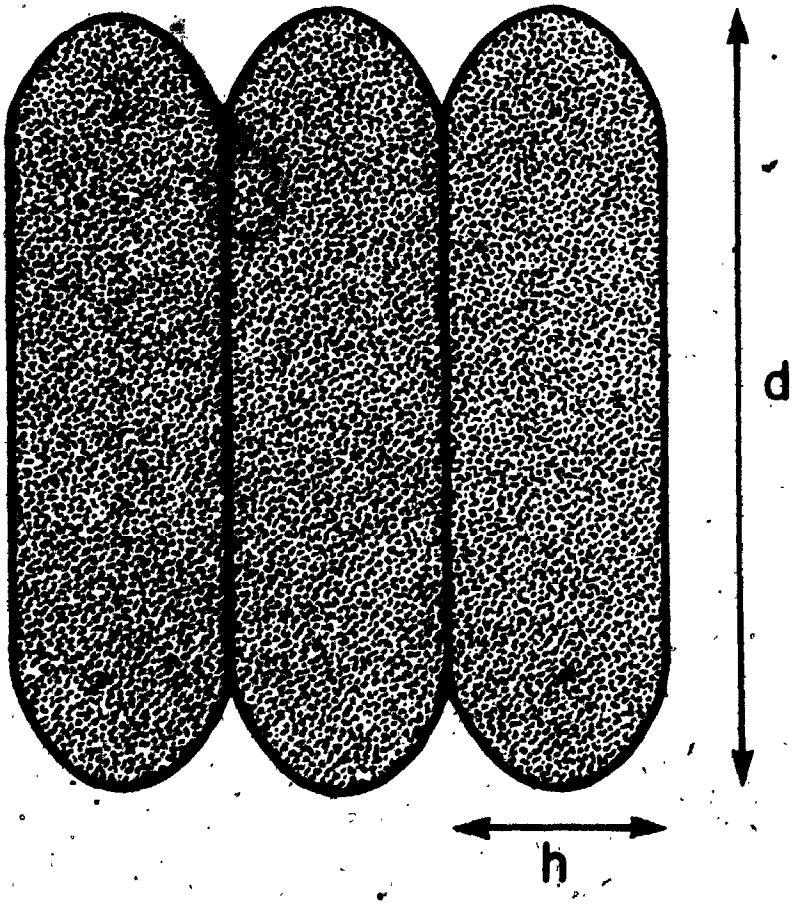
a. Photomicrograph of a linear rouleau. It is difficult to decide from a photomicrograph what shape or shapes the rims of the cells in rouleau assume. However, electronmicrographs of Rowlands and Skibo (1972) and Chien and Jan (1973) have shown them to be more like a parabola than an arc of a circle.

b. A schematic presentation of the cells as flat-sided structures with parabolic rims. This is the configuration assumed in this study when calculating cellular volume and surface area.

(The scale in fig. 5a represents 10μm).

a)

b)



The cell dimensions (h and d) were measured one-third the way in from the outside of the image of the cell membrane. To make the calculations less tedious, the above equations were programmed into a PDP-10 time-sharing computer which was then fed with the measured values of h and d.

3.2. Results and Discussion

The equilibrium shape of a normal red cell in isotonic Ringer solution is consistently a biconcave disc. The formation of rouleaux results in some distortion of the red cell's shape with probably a complete removal of its biconcavity. Due to compression the cells are flattened and their diameter is slightly increased. Figure 6 shows histograms illustrating the increase in the diameter measured in this study for human red cells in rouleaux. The diameter of the cells increases by approximately 5%. For the cells treated with Neuraminidase (these cells will be referred to as N-treated cells) the diameter has the same value as that of the untreated cells (figures 6b and 6c). This finding suggests that an increase in the net attractive force between the cells, resulting from a decrease in the surface charge density after N-treatment (Cook et al., 1961; Eylar et al., 1962), has no effect on the diameter of the cells in rouleaux. In fact, the diametral dimension of the cells in rouleaux has been found in this study to be unaffected by the type and concentration of the

FIGURE 6

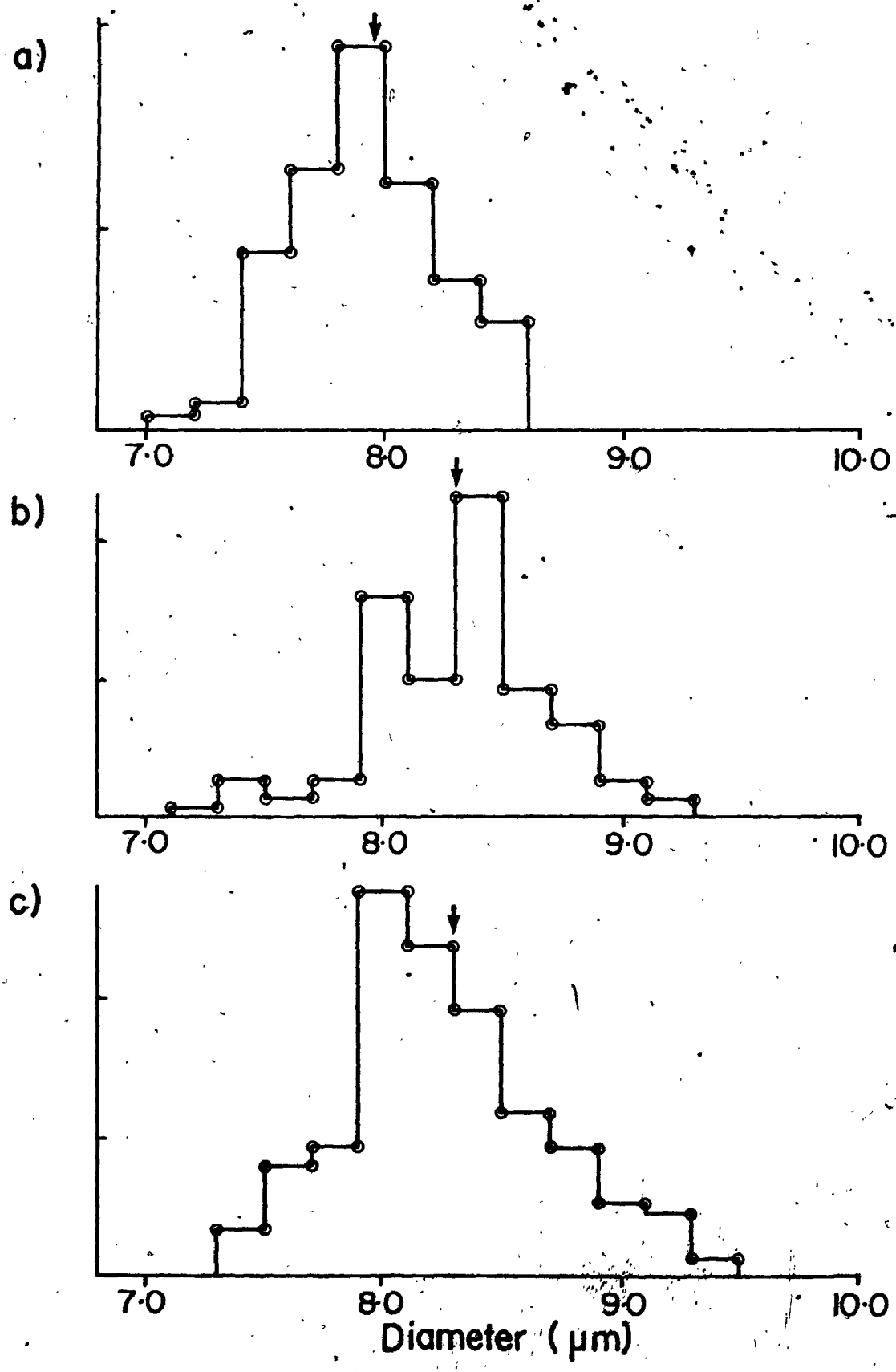
Histograms of the diameter of the cells. The interval on the ordinate represents 15 cells.

a. Human cells in Ringer solution. The mean diameter = 7.96μm±0.32 (S.D.), n = 100

b. Normal human cells in rouleaux. The mean diameter = 8.32μm±0.42 (S.D.), n = 115

c. N-treated human cells in rouleaux. The mean diameter = 8.30μm±0.46 (S.D.), n = 187

These histograms show the diameter of the cells increases in rouleaux formation. Also, neuraminidase-treatment has no effect on the diameter of the cells in rouleaux.


a)
b)
c)
7·0
8·0
9·0
10·0
Diameter (μm)

macromolecules used.

The value obtained here for the mean diameter of the cells in rouleaux (8.32μm±0.42) agrees extremely well with the widely accepted value of 8.28μm±0.10 obtained by Houchin et al. in 1958. The average thickness (see Table II) of the human cell in rouleaux is however about 14% larger than the value given by Houchin and associates. The complete results of Houchin et al. are:

Diameter = 8.28μm±0.01

Thickness = 1.71μm±0.06

Surface Area = 134μm^2±2.8

Cellular Volume = 82μm^3±2.7

In their study, Houchin et al. made measurements on cells "in straight rouleaux of 7 or more cells not touched by other rouleaux". For mean cellular thickness the average length of each rouleau was divided by the number of cells contained in it. The obvious flaw in their method is that the separation between the cells is also included. If they had allowed for the separation between the cells their value for the mean thickness would have been less than 1.71μm±0.06, which is even smaller than the value obtained in this study. One possible explanation for the larger value obtained in this study is that the unnatural cellular environment might have caused some swelling to occur; Houchin et al. made their measurements on cells suspended in native plasma.

Table II lists the mean values of the geometric

Table II. Geometric Parameters of Red Cells in Rouleaux.

Species	Diameter, μm (mean±SD)	Thickness, μm (mean±SD)	Cellular Volume, μm^3 (mean±SD)	Surface Area, μm^2 (mean±SD)
Human	8.32±0.42	1.96±0.22	91.4±12.4	131.5±12.0
Rabbit	6.73±0.43	2.00±0.23	58.7±8.90	90.5±10.2
Dog	7.38±0.38	1.77±0.18	66.7±9.51	104.4±10.2
Cat	5.64±0.40	2.04±0.31	40.2±8.20	66.1±8.90
Mouse	6.01±0.30	1.90±0.18	43.5±4.40	72.8±5.90
Rat	6.87±0.29	1.82±0.16	58.3±7.57	91.5±7.51
Guinea pig	7.92±0.36	1.86±0.19	81.2±6.00	124.4±7.30
Mouse*	6.38±0.28	1.90±0.17	49.9±6.30	81.2±6.80
Rat*	7.40±0.36	1.83±0.15	67.2±8.50	106.0±9.50

*Anaesthetized with Nembutal.

parameters measured and calculated for the various species in this study. With the exception for human cells, published information on the dimensions of red cells in rouleaux formation was not found. Besides, the published geometric values of single, biconcave cells of various species vary considerably (Schalm, 1965; Altman and Dittmer, 1972). It will therefore not be possible to discuss the values in Table II comparatively. The values given in the Table for the diameter of the various species' cells in rouleaux represent an increase between 5 and 10% of the diameter of the undeformed cells measured in this study. Note the increase in the dimensions of the cells of the anaesthetized animals (mouse and rat). The increase is consistent with the finding of Seeman, Kwant, Sauks, and Argent (1969) in which anaesthetic and anaesthetic-like agents were found to expand the membrane area by a few per cent.

Even though red cells change their shape when they go into rouleaux formation, there is no reason for them to increase in volume or surface area. The surface area and volume of the cells might then be regarded as constant. In fact, the values given in Table II for the surface area and volume of human cells in rouleaux agree very well with the published data of Evans and Fung (1972):

Surface Area = $135\mu m^2 \pm 16$ (SD)

Cellular Volume = $94\mu m^3 \pm 14$ (SD)

Evans and Fung used improved resolution microscopy for

determining the dimensions of single, isolated red cells.

The value of 82μm^3 obtained by Houchin et al. (1958) for the cellular volume is about 12% less than the mean value obtained in this study. Their value of the surface area however agrees with our value of 132μm^2. The cellular volume, rather than surface area, is more sensitive to changes in the thickness of the cells (see eqn. 1) and this helps to explain the disparity. Of added interest is the fact that Houchin et al. assumed the cross-section of the cells in rouleaux to be elliptical, but, from their published pictures and the pictures obtained in this study, stacked pancake-shaped discs rather than spheroids seem to make a more reasonable model.

In summary, the geometric parameters of various species' red cells in rouleaux were measured. From these measurements, the surface area and volume of the cells were calculated on the assumption that the cells in rouleaux were parallel-sided structures with parabolic rims. For each species, the mean diameter of the cells in rouleaux was found to be significantly larger than that of the undeformed, biconcave cells. The surface area and cellular volume, however, remained constant.

These results were used in the study of mixed-species rouleaux formation (see chapter 5) for identifying the cells in mixed rouleaux.

CHAPTER 4

THE CONTRASTING OF PVP AND Dx AS ROULEAUX-INDUCING AGENTS

4.1. Introduction

If red cells are suspended in their own plasma they will form rouleaux. The plasma proteins, fibrinogen and the various globulin fractions, are responsible for the formation of these column-like structures (Fahraeus, 1921, 1929; Hardwicke and Squire, 1952; Dintenfass, 1974). Rouleaux formation is also induced by synthetic polymers (Guillot and Fiehrer, 1948; Thorsén and Hint, 1950; Hummel, 1963; Chien and Jan, 1973; Brooks and Seaman, 1973). The formation is greatly dependent on the degree of asymmetry of the colloid molecules and likewise on their average molecular weight and molecular weight distribution. The clinical implication of this fact is that the sedimentation rate is not only determined by the concentration of the fibrinogen and globulin fractions but also by the molecular properties and distribution of the fractions in question.

Among the non-physiological macromolecules used for

promoting reversible red cell adhesion are the high molecular weight Dx's and PVP. The behaviour of these two types of polymer, both electrically neutral at physiological pH, was investigated and is reported in this chapter. The mechanism by which proteins and other macromolecules induce rouleaux formation has not been clearly understood. While this fact is acknowledged, this study does not attempt to propose a mechanism of the phenomenon but merely relates the observed differences in the rouleaux-inducing properties of Dx and PVP. The results of this study hopefully will add some light on the existing, proposed models of the phenomenon.

4.2. Method

Red cells from normal, healthy blood and red cells that have been treated with Neuraminidase were used in these experiments. They were suspended in isotonic Ringer solution at pH 7.4. The Ringer solutions contained various concentrations of Dx fractions or PVP fractions. The volume concentration of the cells in the suspensions was adjusted by colour to approximately 0.2%. One drop of the suspension was placed in a non-flowing well-chamber and observed as explained in section 2.4.

The aggregation of the cells was measured quantitatively by direct microscopic observation according to the method of Chien and Jan (1973). After the cells had settled onto the coverslip, about ten minutes were

allowed to elapse before photomicrography, at 400X magnification, was taken from at least seven different fields. In some cases, for example at high Dx concentrations, a time of approximately thirty minutes was allowed to elapse. The number of cell units in each field was counted according to the definition that one "unit" was either a single, separate cell or one aggregate. The average number of cells in each unit was calculated as the ratio

$$MAI = \frac{\text{No. of cells in Ringer solution}}{\text{No. of units in Dx or PVP solution}}$$

where MAI is the microscopic aggregation index. An MAI value equal to unity implies the absence of aggregation. The concept of microscopic aggregation index is better explained in figure 7.

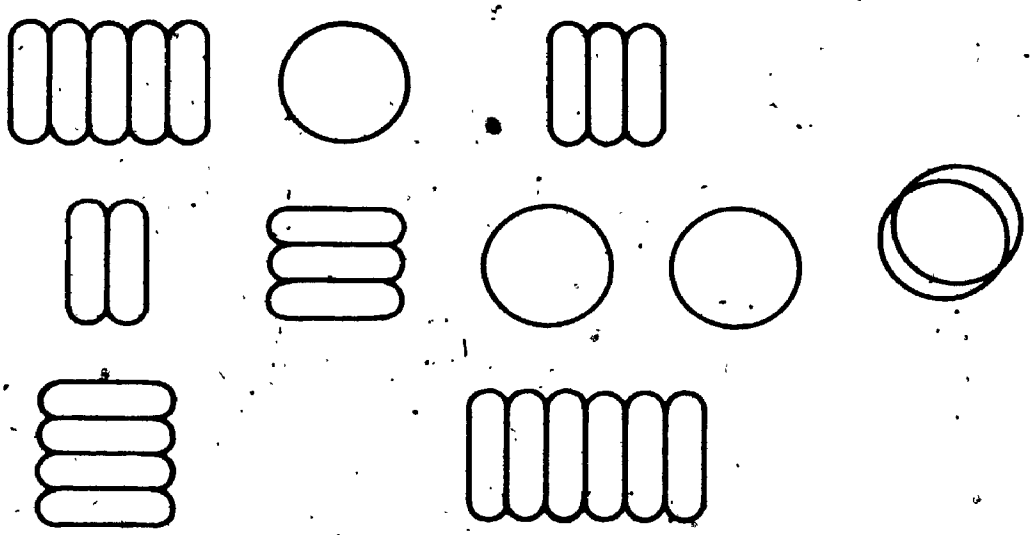
The use of MAI for quantitatively measuring the aggregation of the cells is a good one because the pattern of the "MAI vs. macromolecular concentration" graphs agrees extremely well with the patterns obtained when using sedimentation rate or low-shear viscosity for quantitatively measuring aggregation (Chien and Jan, 1973).

4.3. Results and Discussion

All the graphs in this study represent the smoothest curves drawn by "eye". The points plotted represent the mean and S.E.M. for measurements between eight and twelve

FIGURE 7

Explanation of the "microscopic aggregation index" method (Chien and Jan, 1973) for measuring quantitatively the degree of rouleaux formation. The MAI is defined as: $MAI = \frac{\text{No. of cells in Ringer solution}}{\text{No. of units in the rouleaux-inducing medium}}$ where each unit is either a single, separate cell or one aggregate. An MAI value of unity means that no aggregates are present. This method is sensitive to the concentration of the red cells and can only be used for dilute suspensions (hematocrit < 1%).



$$MAI = \frac{\text{Total Number of Cells}}{\text{Total Number of Units}}$$

$$= \frac{28}{10}$$

$$= 2.8$$

values, i.e. n ranges between 8 and 12.

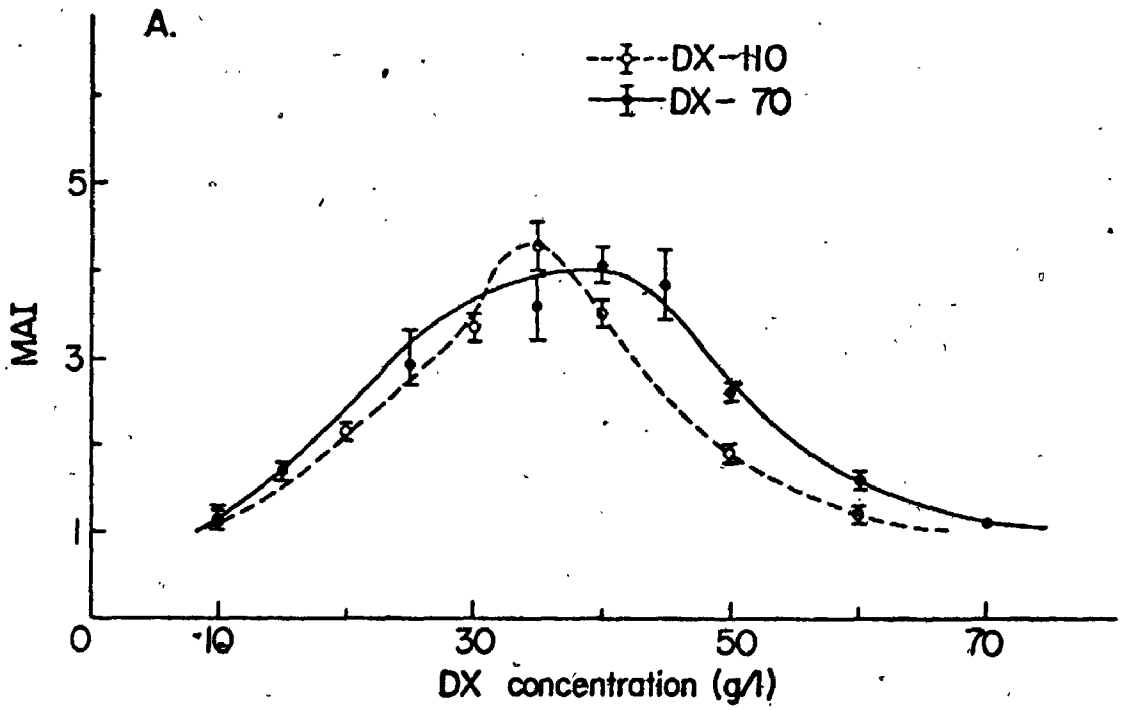
Figures 8A and 8B show the results of this study for Dx fractions inducing aggregation of human red blood cells. In the case of Dx-70 and Dx-110, aggregation "peaked" between concentrations of 30 g/l and 45 g/l. For Dx-500, aggregation "peaked" between concentrations of 8 g/l and 15 g/l. Dx concentrations greater than 70 g/l (not shown in diagrams) were also used in this study. For all three Dx fractions, aggregation of human cells was not observed at concentrations greater than 70 g/l. These results are consistent with those obtained by Chien, Luse, Jan, Usami, Miller, and Fremount (1970) and Chien and Jan (1973).

Of interest to this study is the fact that Dx's are unable to induce aggregation at concentrations greater than 70 g/l. Since the results were comparable with various Dx's, the next question was whether other neutral polymers behaved in the same way. PVP, another neutral polymer, was used in this investigation. Figures 9A and 9B illustrate the results obtained for the PVP study on normal human red blood cells. The MAI increased to a certain value, decreased slightly, remained constant and then began to rise. In contrast to the dextran studies, aggregation was always observed in these experiments above the critical PVP concentrations (~1.0 g/l for PVP-360 and ~5 g/l for PVP-40) at which aggregation first occurred. PVP concentrations as high as 150 g/l (not shown in

FIGURE 8

A. Effect of increased concentrations of Dx-70 and Dx-110 on the aggregation of human red cells. Aggregation "peaked" at a concentration of ~35g/l for Dx-110, and at a concentration of ~40g/l for Dx-70. Both fractions failed to induce aggregation at concentrations beyond 70g/l.

B. Effect of increased concentrations of Dx-500 on the aggregation of human cells. Aggregation "peaked" at a concentration of approximately 10g/l. Dx-500 failed to induce aggregation of human cells at concentrations beyond 70g/l.


A.
DX-110
DX-70
MAI
5
3
1
0
10
30
50
70
DX concentration (g/l)

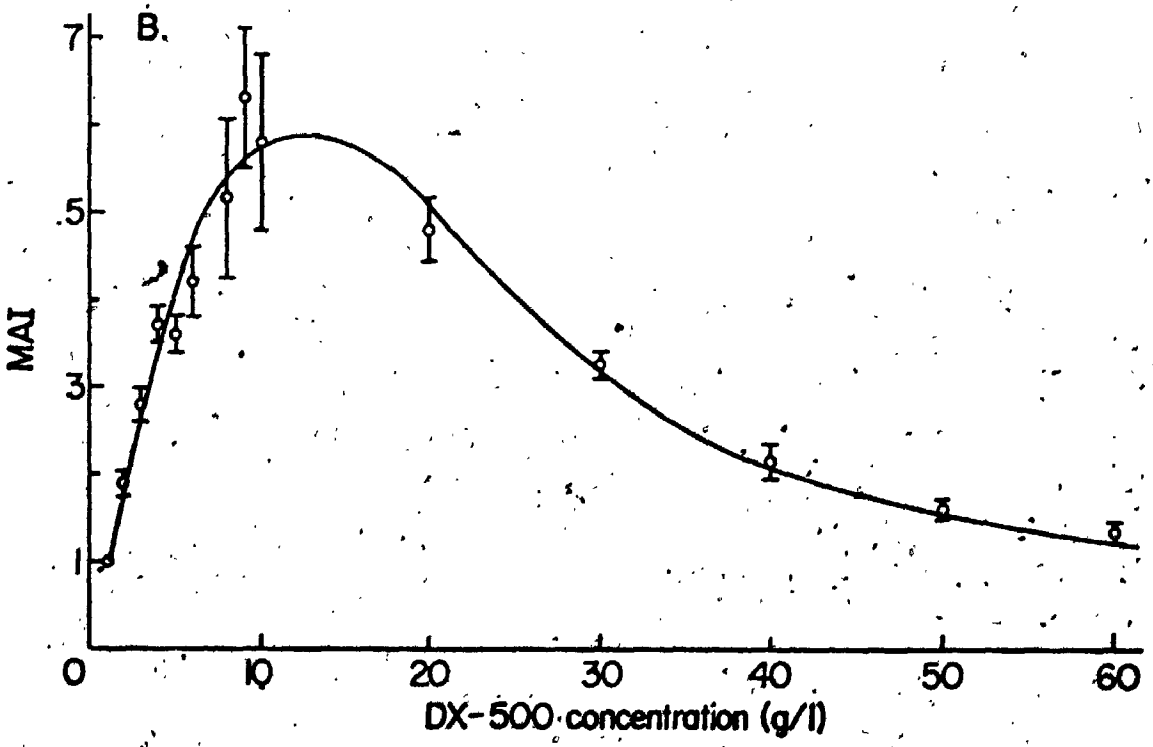
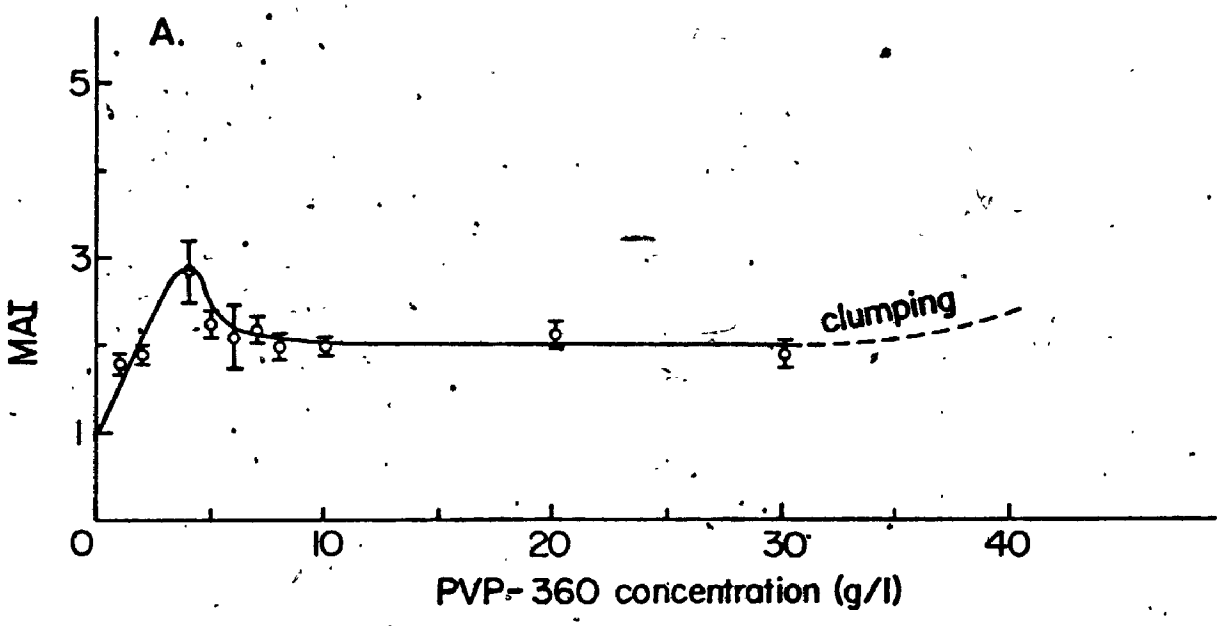
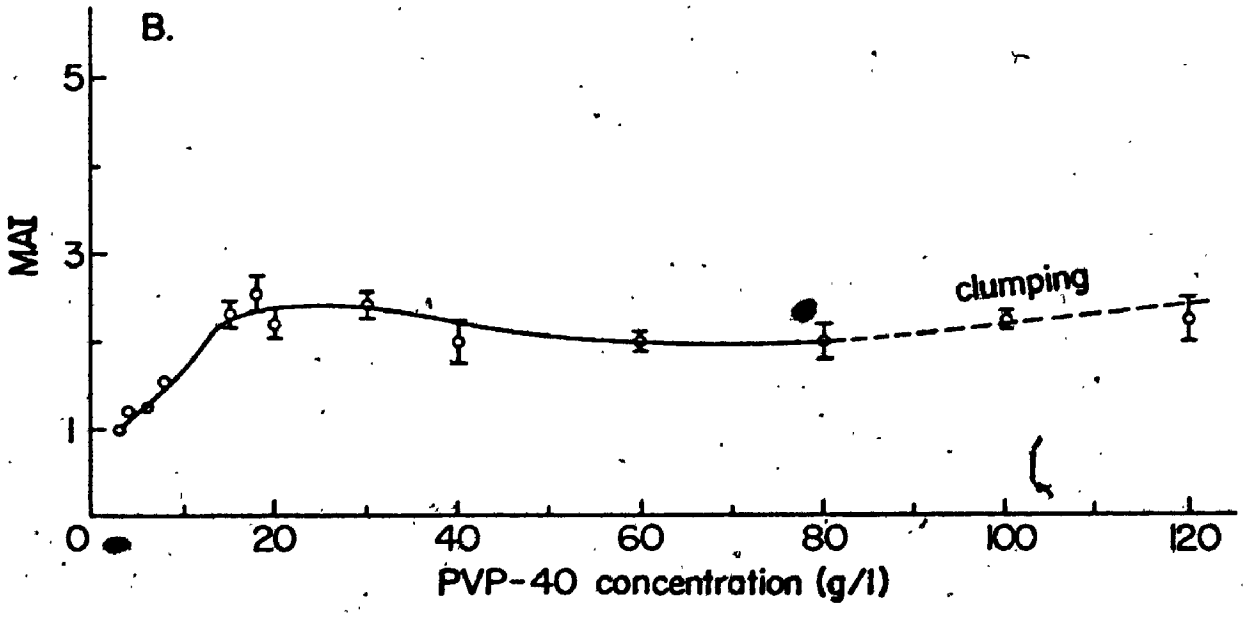

B.
MAI
7
5
3
1
0
10
20
30
40
50
60
DX-500 concentration (g/l)

FIGURE 9

A. Effect of increased concentrations of PVP-360 on the aggregation of human red cells. Aggregation "peaked" at a PVP concentration of 4g/l, dropped significantly at a concentration of 5g/l and then levelled off until at a concentration of approximately 30g/l clumping began.

B. Effect of increased concentrations of PVP-40 on the aggregation of human red cells. Aggregation reached a maximum at a concentration of approximately 20g/l and levelled off until clumping began at a concentration of approximately 80g/l. Clumping is denoted by the dotted line.


A.
MAI
5
3
1
0
10
20
30
40
clumping
PVP-360 concentration (g/l)


B.
MAI
5
3
1
0
20
40
60
80
100
120
clumping
PVP-40 concentration (g/l)

diagrams) were used in this study and there were no signs of the absence of aggregation. Instead, clumps (irregular structures of cells as compared with the normal column-like rouleaux formation) were observed. For PVP-360, severe clumping began to occur at a concentration as low as 30 g/l. Clumping also occurred in the low molecular weight PVP-40 solutions, but at a much higher concentration (~80 g/l).

The MAI values for the clumps or irregular aggregates were difficult to obtain because of the difficulty in counting the number of cells in each clump. The dotted lines in figures 9A and 9B represent severe clumping where the true values of MAI were difficult to compute. The two points plotted in the "clumping" area of figure 9B represent the MAI values computed for the regular rouleaux (not clumps) observed at the respective concentrations.

Photomicrographs of human red cells in various solutions of Dx-Ringer and PVP-Ringer are shown in figure 10. The absence of aggregation of human cells can be seen in Dx solutions at concentrations of 80 g/l and 120 g/l. Although a time of approximately 30 minutes was allowed for the cells to form rouleaux at these high concentrations aggregation never occurred. This was in contrast to the results obtained for the lower Dx concentrations where the usual time of only 10 minutes was allowed. In the case of PVP-360, aggregation always occurred if the concentration was above 1.0 g/l. In fact, some mild

FIGURE 10.

Photomicrographs of human cells in various solutions of PVP-Ringer and Dx-Ringer.

Figures 10A - 10E: Human cells in 10g/l, 30g/l, 60g/l, 80g/l and 120g/l respectively of Dx-110.

Figures 10a - 10e: Human cells in 1g/l, 3g/l, 6g/l, 10g/l and 50g/l respectively of PVP-360.

(The scale represents 10μm).


A
B
C
D
E

clumping was observed at a concentration as low as 6 g/l (figure 10C).

The fact that PVP at high concentrations was able to induce aggregation of human red cells while Dx could not, suggests some difference in the nature of adsorption of the two types of macromolecules. PVP and Dx have different structures in that PVP is a linear polymer while Dx is branched (Wales et al., 1953; Senti et al., 1955). To determine if Dx and PVP would interact in the same way with red cells of other species, these experiments were then repeated for red cells other than human.

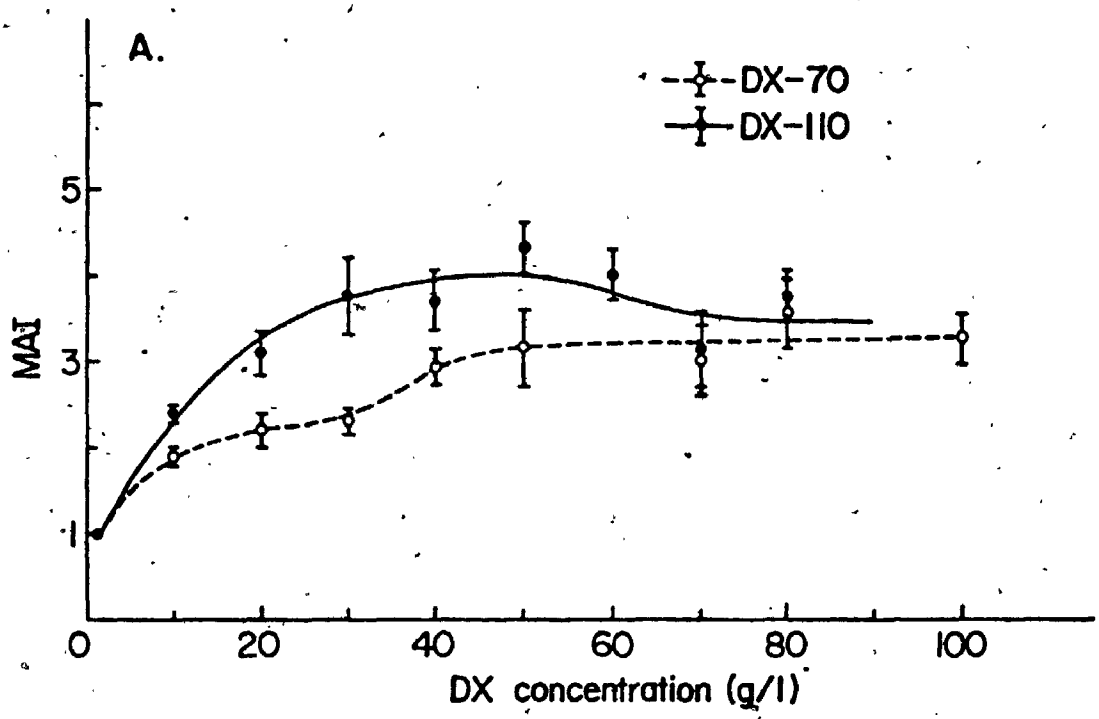
Figures 11A and 11B show the results obtained for the effects of Dx-70 and Dx-110 on the aggregation of cat cells and rabbit cells respectively. The MAI rose in each case with increased Dx concentration and then levelled off. Dx concentrations greater than 100 g/l were used and aggregation always occurred with both cat cells and rabbit cells. Severe clumping was observed at high polymer concentrations. Figure 12 shows the effect of increased PVP-360 concentration on the aggregation of cat cells. As in the case of human cells, aggregation reached a peak, decreased slightly and then levelled off before clumping began. It seems that the interaction of the PVP molecules with cat cells is quite similar to that with humans.

Photomicrographs of human cells and cat cells in various concentrations of Dx-Ringer are shown in figure 13.

FIGURE 11

A: Effect of increased concentrations of Dx-70 and Dx-110 on the aggregation of cat cells. Aggregation increased to a maximum and then levelled off. Clumping began at a concentration of approximately 100g/l.

B: Effect of increased concentrations of Dx-70 and Dx-110 on the aggregation of rabbit cells. As for cat cells, aggregation increased to a maximum and then levelled off with clumping occurring at high concentrations.


A.
DX-70
DX-110
MAI
5
3
1
0
20
40
60
80
100
DX concentration (g/l)

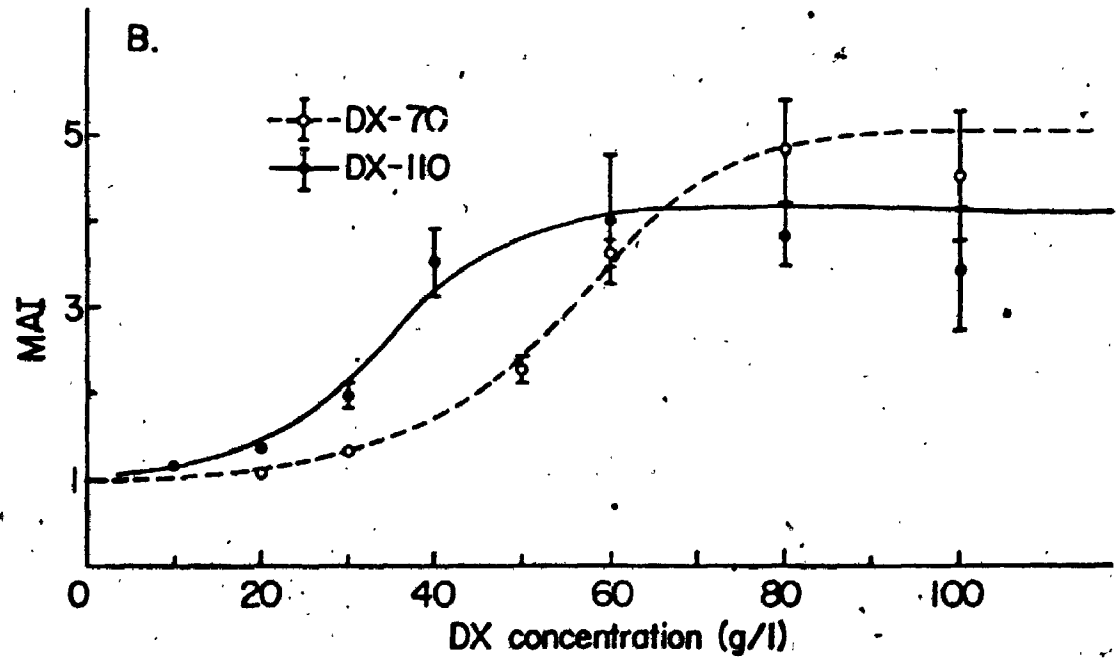

B.
DX-70
DX-110
MAI
5
3
1
0
20
40
60
80
100
DX concentration (g/l)

FIGURE 12

Effect of increased concentrations of PVP-360 on the aggregation of cat cells. Similar to the effect of PVP-360 on the aggregation of human cells, aggregation increased to a maximum at a concentration of 4g/l and then dropped significantly at a concentration of 5g/l before levelling off. Clumping began at a concentration of approximately 20g/l. Clumping is denoted by the dotted line.

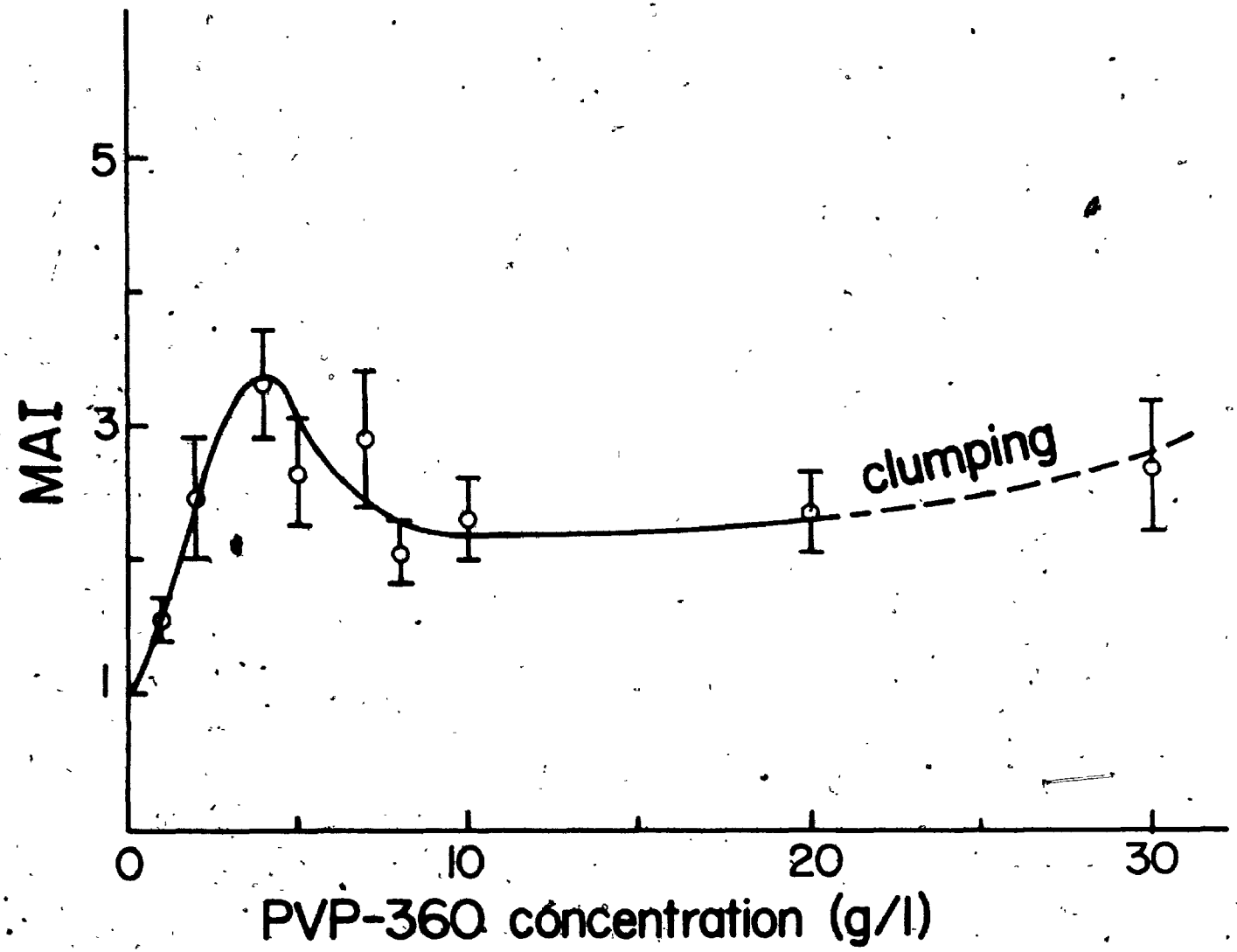

MAI
5
3
1
0
10
20
30
clumping
PVP-360 concentration (g/l)

FIGURE 13

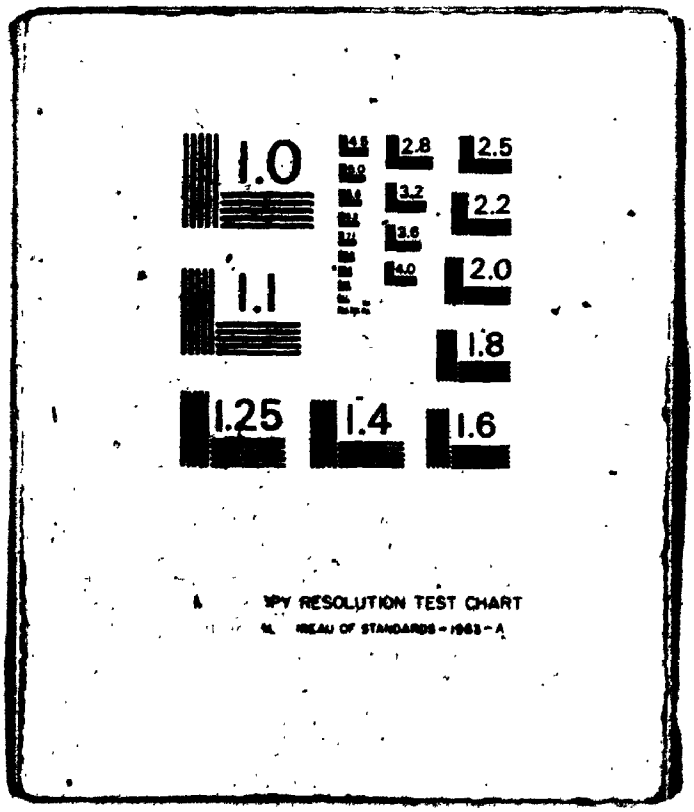
Photomicrographs of human cells and cat cells in various concentrations of Dx-Ringer.

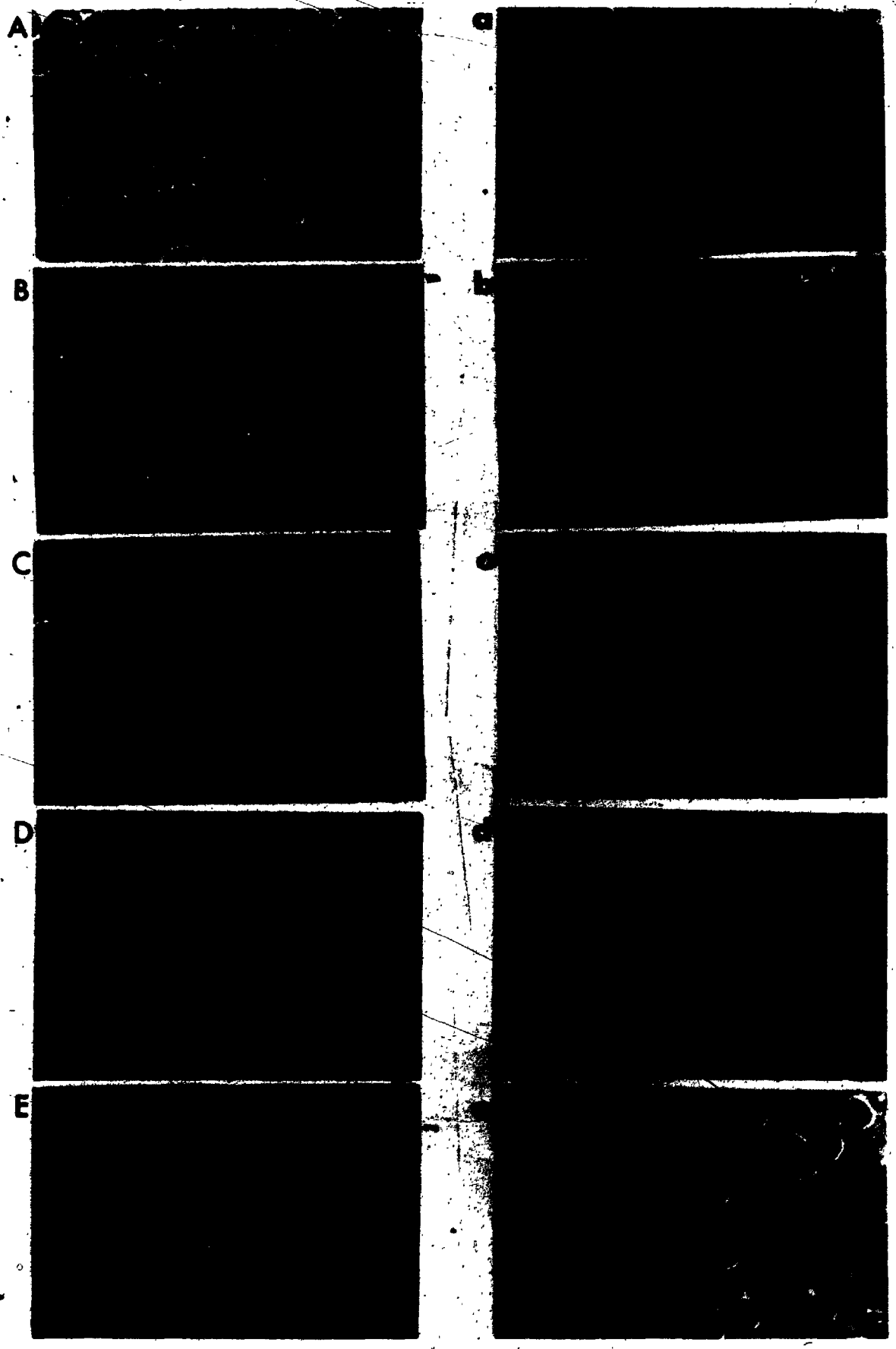
Figures 13A - 13E: Human cells in 10g/l, 30g/l, 50g/l, 70g/l and 80g/l respectively of Dx-70.
(The scale represents 10μm).

Figures 13a - 13e: Cat cells in 20g/l, 50g/l, 70g/l, 100g/l and 120g/l respectively in Dx-70.
(The scale represents 10μm).

2 3

OF /DE


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
IPV RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS - 1963 - A


A
B
C
D
E

Note the severe clumping of cat cells (figure 13e) at high Dx concentrations.

The aggregation "peaks" in figures 9A and 12 were consistently obtained at a PVP-360 concentration of approximately 4 g/l. To determine if this was due to the effect of the PVP molecules on the electrostatic potential of the adhering cells (Castaneda, Bernstein and Varco, 1965), the PVP-360 experiments were repeated on N-treated human cells. After N-treatment most of the surface negative charge on the cells is lost (Eylar et al., 1962; Durocher, Payne and Conrad, 1975), and hence the effect of the electrostatic potential on aggregation would be less pronounced. Figure 14 shows the result of the effect of PVP-360 on the aggregation of N-treated human cells. The aggregation "peak" is no longer evident. In addition, the N-treated cells began clumping at a lower concentration (~16 g/l) as compared with normal cells (~30 g/l). These findings emphasize the role of the surface electric charges on the aggregation of red cells.

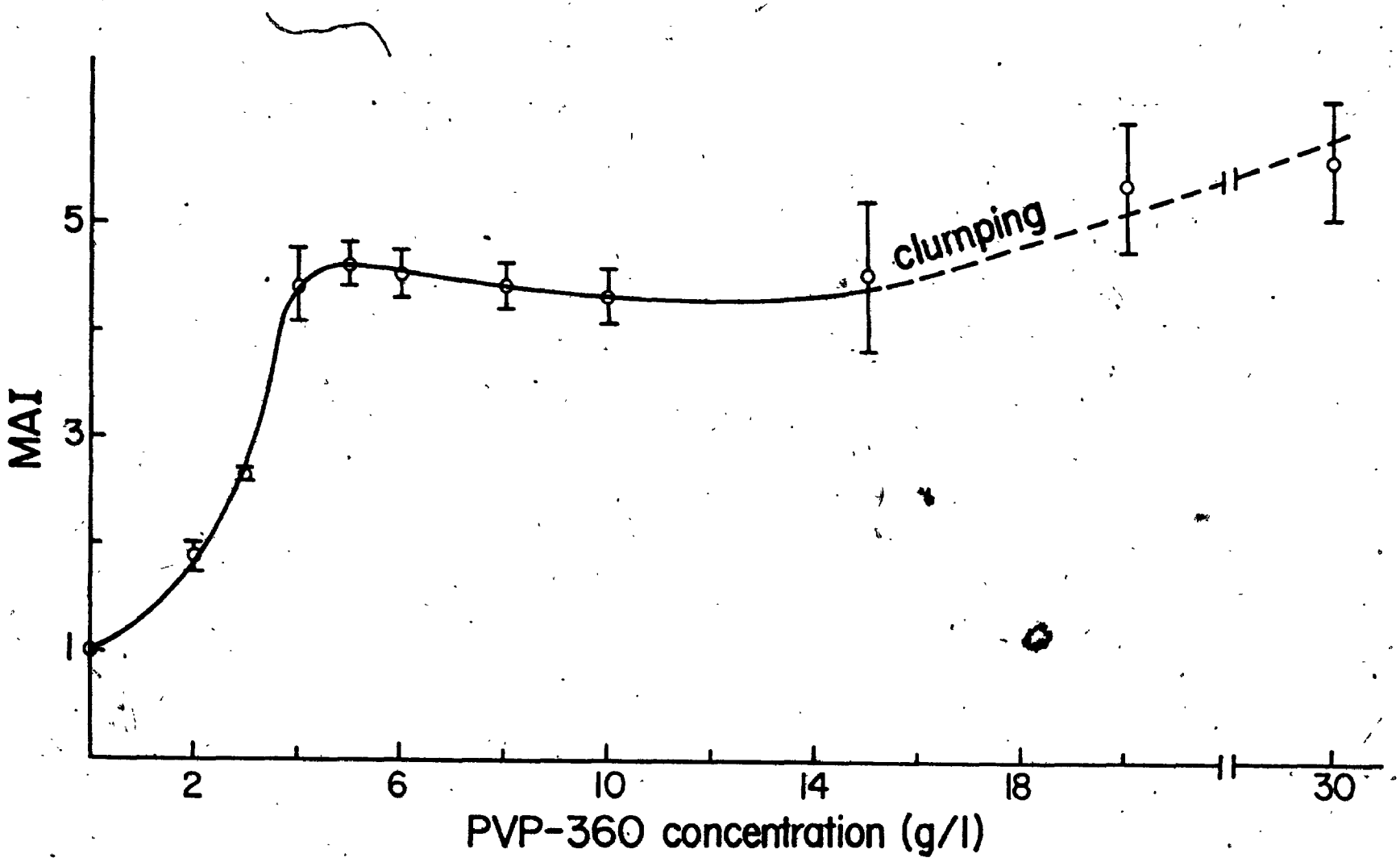
4.4. Discussion

It must be made clear that in this study interest is not focussed on a comparison of the degrees of aggregation induced by the various types of macromolecules. The main interest in this study was to investigate whether or not aggregation would be induced at high polymer concentrations. It is for this reason that not too much importance

FIGURE 14

Effect of increased concentrations of PVP-360 on the aggregation of N-treated human cells. The "peak" for the normal human cells (see figure 9A) is no longer evident for the N-treated cells.

Note that clumping began at a concentration of about 15g/l as opposed to 30g/l for normal human cells.


MAI
5
3
1
clumping
2
6
10
14
18
30
PVP-360 concentration (g/l)

was placed on the concentration of the red cells in the suspending media. In every case, the cell concentration was judged by the colour of the suspending medium which, from experience, denoted a cell concentration of approximately 0.2%. The scatter in the MAI values shown in most of the figures might then be due to the inconsistency in the red cell concentration.

No attempt will be made here to explain the contrasting properties of Dx and PVP in inducing rouleaux formation. The particular structural organization of macromolecular chains is responsible for important physical and biological properties. As pointed out by Bruck (1974, p. 34), however, the conformations of adsorbed molecules remain essentially unknown. In view of these difficulties, it is perhaps more advisable to examine the theories proposed for explaining the absence of aggregation of human cells at high Dx concentrations.

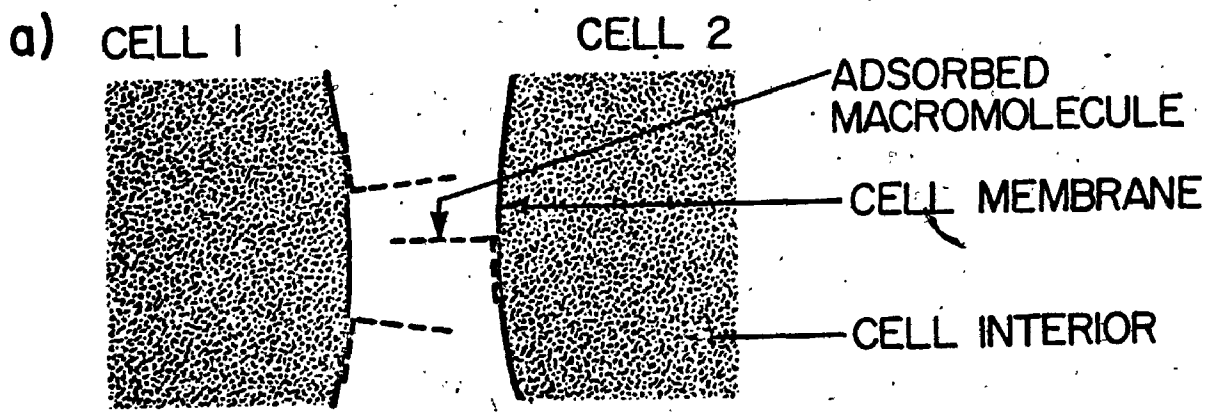
Chien et al. (1970) used the bridging model of LaMer and Healy (1963) to explain the absence of aggregation of human cells at high Dx concentrations. In the bridging model, the terminal segments of the bridging macromolecule adsorb onto the surfaces of two adjacent cells and the central segment occupies the intercellular space (see figure 15). The existence of intercellular spaces with relatively uniform distance led Chien et al. (1970) to conclude that the monomolecular layer of macromolecules formed parallel bridges between the cells. Chien and

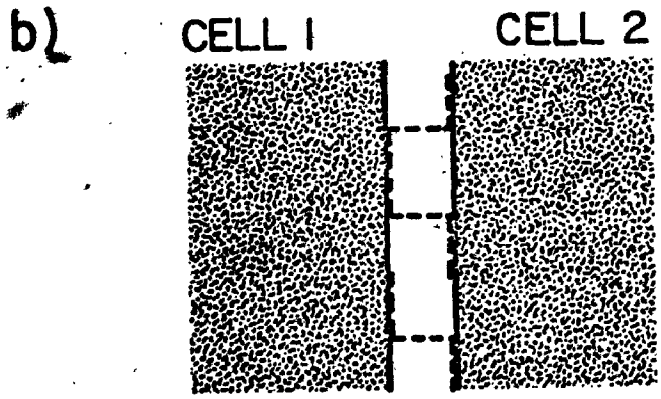
FIGURE 15

Cells in rouleau: explanation of the bridging model (adapted from Chien *et al.*, 1970).

a. Adsorption of macromolecules onto red-cell surface.

b. Rouleau formation by macromolecular bridging.


a)
CELL 1
CELL 2
ADSORBED MACROMOLECULE
CELL MEMBRANE
CELL INTERIOR


b)
CELL 1
CELL 2

associates (1970) also used this model to explain why Dx-20 (average molecular length of 25 nm) and serum albumin (average molecular length of 15 nm) do not induce aggregation. The average molecular lengths of these molecules are relatively short (Ingelman and Halling, 1949), and the segment available for bridging after subtraction of the adsorbed segments from the total molecular length is probably too short to overcome the force of electrostatic repulsion. The monolayer bridging model also suggests that at high macromolecular concentrations, aggregation should not occur because the sides on the red cell surface area which are available for bridging of the adsorbed macromolecules become saturated.

In 1973 Jan and Chien (1973a), studying the effects of various Dx fractions on the aggregation of N-treated human cells, found that aggregation did occur at high polymer concentrations. No matter how high the polymer concentrations were, the Dx fractions were found to be very effective in inducing aggregation of human cells. This finding made the saturation model (LaMer and Healy, 1963) questionable for explaining the lack of aggregation. Thus the electrostatic repulsive force between the cells appears a more reasonable model.

Brooks and Seaman (1973) and Brooks (1973b), studying the effects of Dx on red cell interactions, found

that the relative zeta potential* of the cells increased with rising Dx concentrations and also with increasing Dx molecular size. Brooks (1973b) hypothesized that the adsorption of neutral polymers caused a decrease in the effective counterion concentration near the red cell surface, and that this led to an increase in the surface potential, that is, an increase in the electrostatic repulsive force between the cells (Verwey and Overbeek, 1948). The lack of aggregation of human cells at high Dx concentrations was therefore explained by Brooks (1973c) as being due to the significantly increased force of electrostatic repulsion, resulting from a reduction in the effective ionic strength near the cell surface.

While the model of Brooks (1973b) seems plausible, it is difficult to imagine why the cells of other species still aggregate at high Dx concentrations. In other words, it is difficult to understand why the effective ionic strength near the human red cell surface is significantly reduced, after polymer adsorption, while it is not in the case of the red cells of other species. Brooks (1973b) referred to the reduction in the effective ionic strength near the cell surface as the "volume exclusion effect". He attributed the increase in the surface

---

*The relative zeta potential has been defined (Brooks, 1973b) as the ratio $Z = \frac{\eta_\beta U_\beta}{\eta_o U_o}$, where η is viscosity (cP), U is electrophoretic mobility ($\mu m.s^{-1}/Vcm^{-1}$), and the subscripts β and o represent respectively the presence and absence of the macromolecules.

potential (zeta potential*) of the cells entirely to this "volume exclusion effect" and did not consider in his model the possibility that the interaction of the macromolecules with the cell surface was specific.

Brooks (1973b, 1973c), on the basis of his model, postulated that the relative zeta potential of the cells should always increase in the presence of any neutral polymer. In other words, his model implies that the lack of aggregation of red cells should be observed at high concentrations of any neutral polymer. However, it has been shown in this study that PVP, a neutral polymer, does induce aggregation of human and other species' red cells very effectively at high concentrations. The results of this study suggest that the interaction of the macromolecules with the cell surface, and whatever effect it has on the surface charge density, is a more important consideration than the "volume exclusion effect". The fact that Dx, at high concentrations, does not induce aggregation of human red cells while it does for cat cells and rabbit cells suggests that the interaction of Dx with the human cell surface is different from that with red cells of other species.

---

*Zeta potential, ζ, is defined (Brooks, 1973b) as $\zeta = \frac{360\pi\,\eta\,\mu}{D}$, where ζ is in mV, η is the viscosity (cP) of the medium, μ the electrophoretic mobility ($\mu m\ s^{-1}/V\ cm^{-1}$), and D the dielectric constant of the medium.

It is possible that the interaction of the cell surface proteins with the polymer produces a re-arrangement of the interfacial region as proposed by Ross and Ebert (1959). Such a re-arrangement might expose previously undetected charged groups, in the case of the interaction of human cells with Dx, thus increasing the surface charge density and consequently the zeta potential. The interaction of the Dx molecules with red cells of other species possibly results in a partial screening of the charged groups, with a decreased effect on the zeta potential. This hypothesis however needs further investigation. As far as the author is aware, there is no way, at the present time, in which the interaction of the macromolecules with the cell surface can be fully understood. The reason for this is that the surface structure of red cells still remains unknown.

In conclusion, the results of this study suggest that the process of rouleaux formation involves a specific interaction between the macromolecules and red cell surface. The results suggest that the interaction of Dx with the human red cell surface is different from that of PVP. The results also suggest that the interaction of Dx with the human red cell surface is different from that of Dx with red cells of other species. Thus the saturation model of LaMer and Healy (1963) and the "volume exclusion" model of Brooks (1973b) seem unsatisfactory in explaining why Dx's, at high concentrations, fail to induce aggrega-

tion of human red cells. A model incorporating the specific interaction of the macromolecules with the cell surface seems more reasonable.

CHAPTER 5

PVP-INDUCED ROULEAUX FORMATION IN INTERSPECIES POPULATIONS OF RED CELLS*

5.1. Introduction

A big difference in the degree of aggregation between various species was first observed by Hirschfeld (1907) who, from experimental evidence, concluded that the degree of aggregation was a function both of the aggregability of the red cells and of the aggregating potency of the plasma. Eliasson and Samelius-Broberg (1965) and Richter (1966) investigated the species difference in a little more detail, using various Dx fractions, and came to the same conclusion. Berman and Fuhro (1973) concluded from their study that interspecies differences in red cell aggregation are the result of some property of the red cell itself rather than the plasmatic environment. Berman and Fuhro did their study on human cells and hamster cells, which were suspended either in their own plasma or in plasma from the other

*The principal part of this chapter has been published in Can. J. Physiol. Pharmacol. 54: 437-442, 1976.

species; in each case, human cells aggregated at the same rate and to the same extent but significantly more than the hamster cells.

In view of the above findings and of the apparent differences in the surface structures of the various species' red cells, regarding the arrangement of the surface components (Seaman and Uhlenbruch, 1963), it was thought of interest in this study to investigate whether rouleaux formation was a species-specific type of phenomenon. In other words, would human cells form rouleaux among themselves only or would they also adhere to red cells of other species? If mixed rouleaux were formed, then a statistical analysis would be done to see whether a preference was demonstrated for like cells to adhere to each other.

5.2. Method

Fresh samples of blood were obtained from humans, cats, rats, mice, dogs, rabbits and guinea pigs and immediately introduced into heparinized glass vacuum containers. The red cells of all species were washed twice with isotonic Tris-buffered Ringer solution (pH 7.4, 310 mOsm).

Samples of blood were obtained from two species for each experiment. The number of cells per millilitre for each species was calculated using measured values of hematocrit and the previously calculated cell volumes

(see Table II). The two samples were then mixed in such a proportion that there were approximately equal numbers of cells of each type in the mixture. Blood from this mixture was introduced into PVP-Ringer solution (PVP-360, 4g/l) to prepare suspensions of red cells of a volume concentration of approximately 0.2%.

Observation was made as explained in section 2.4. A time of approximately 30 minutes was allowed for the cells to form rouleaux. High magnification prints were made from the 35-mm film and the magnification of the image was determined by the calibrated stage micrometer. From these prints of the cells in rouleaux, each cell in a rouleau was labelled for species type on the basis of its geometric parameters (Table II).

5.3. Results and Discussion

In this study a PVP-360 concentration of 4g/l was used to induce rouleaux formation. Preliminary studies showed that the cells of each species formed neat, long and regular rouleaux at this concentration, and indicated no apparent differences in the aggregating tendencies of the different kinds (species) of red cells. Sliding was observed to be the principal mode of doublet formation for all the different types of cells.

Table II gives the mean and standard deviation of the red cell geometric parameters measured and calculated for the various species studied. The cells of the

anaesthetized animals did not appear to have different properties from the normal cells in the process of rouleaux formation.

The procedures of the single-species experiments were repeated for the mixed-species experiments, and each cell in a mixed rouleau was identified for species type on the basis of its geometric parameters. Figure 16 shows photomicrographs of examples of mixed rouleaux. Cellular volume was the key parameter used for distinguishing a species type from the other (Figure 17c). The few cells of the mixed aggregates that fell in the volume overlap region were identified for species type by the additional geometric data on surface area and diameter (Figures 17d and 17a). As seen in Figure 17b, thickness is an unsuitable parameter for species type identification.

Figure 18 shows the method used for presenting the preference of cells to form rouleaux with cells of the same species. The X-axis denotes the number of adjacent pairs of cells in each rouleau of mixed cells, that is, a rouleau of 10 cells has 9 adjacent pairs (X=9). The Y-axis denotes the ratio of the number of like adjacent pairs to the number of adjacent pairs. The Y-axis is the measure of preference: a value of unity indicates the highest preference with cells touching only cells of their own species; a value of zero indicates that a rouleau was made up of mixed cells with no cell touching a cell from

FIGURE 16

Photomicrographs of mixed cells in rouleaux.

a. Dog and cat cells in mixed rouleaux.
(The scale represents 5μm).

b. Human and mouse cells in mixed rouleaux.
(The scale represents 5μm).

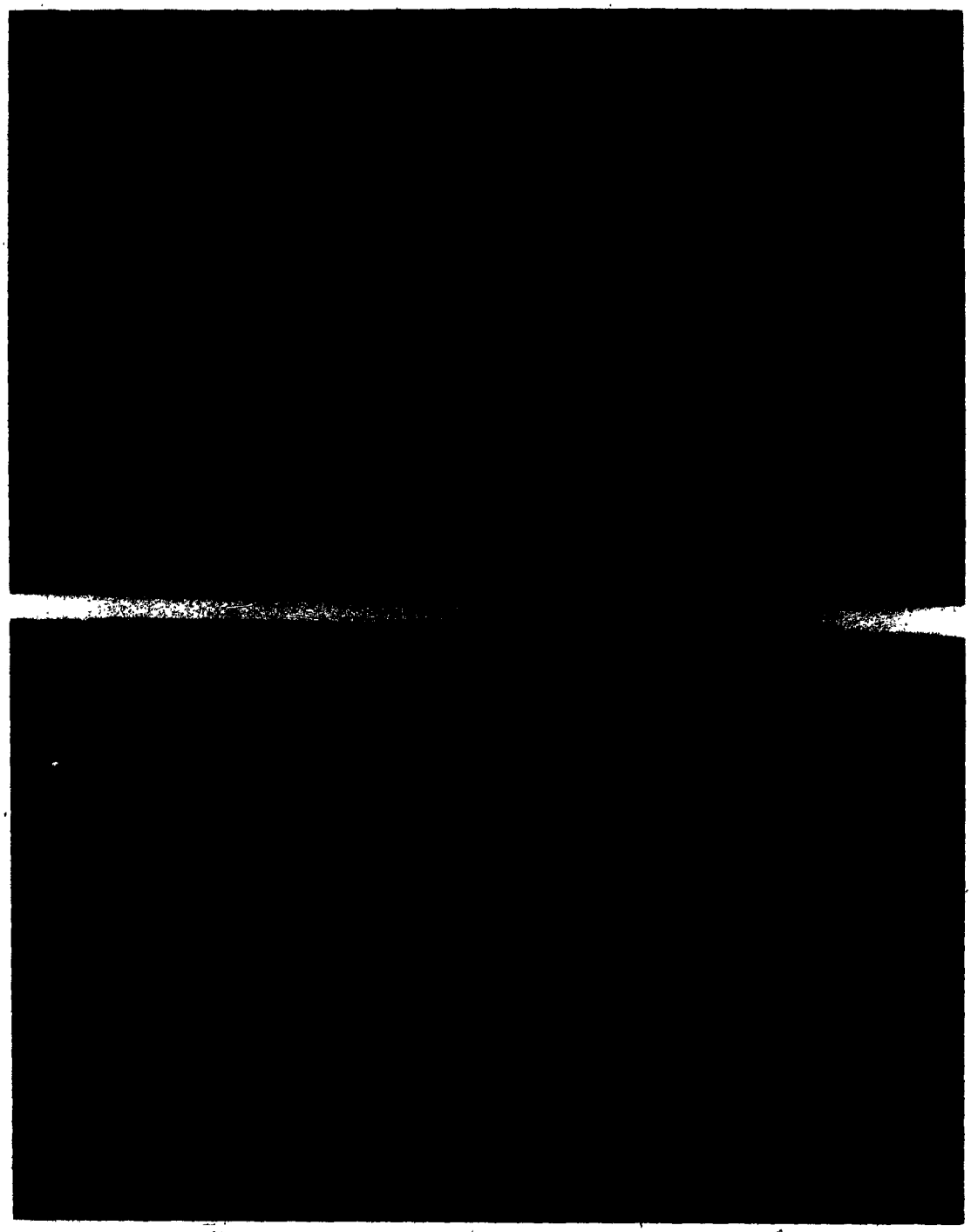


FIGURE 17

Histograms of the ranges of values of diameter, thickness, volume and surface area for mouse and human red cells in rouleaux.

Volume was used as the key parameter for identifying the cells in mixed rouleaux.

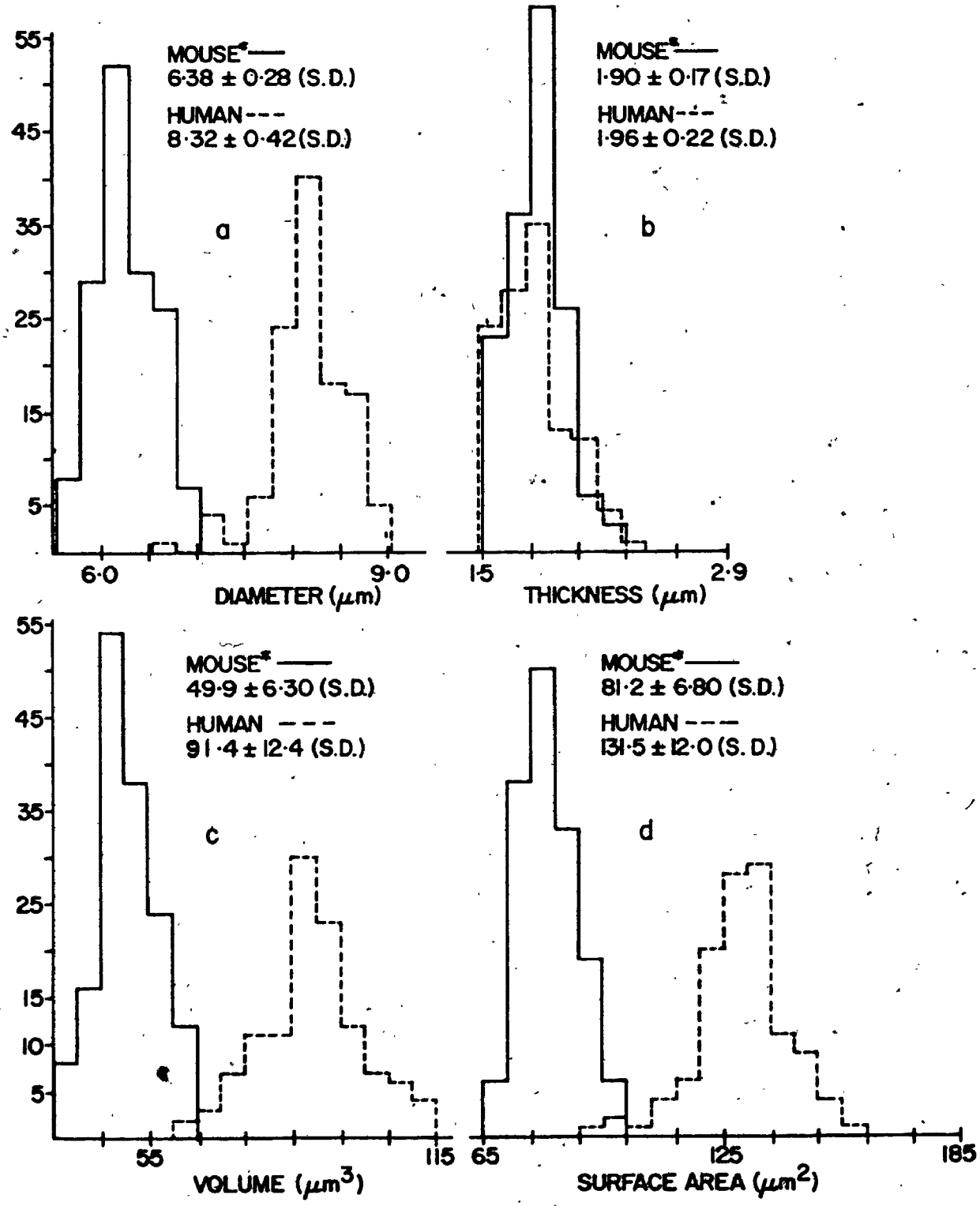
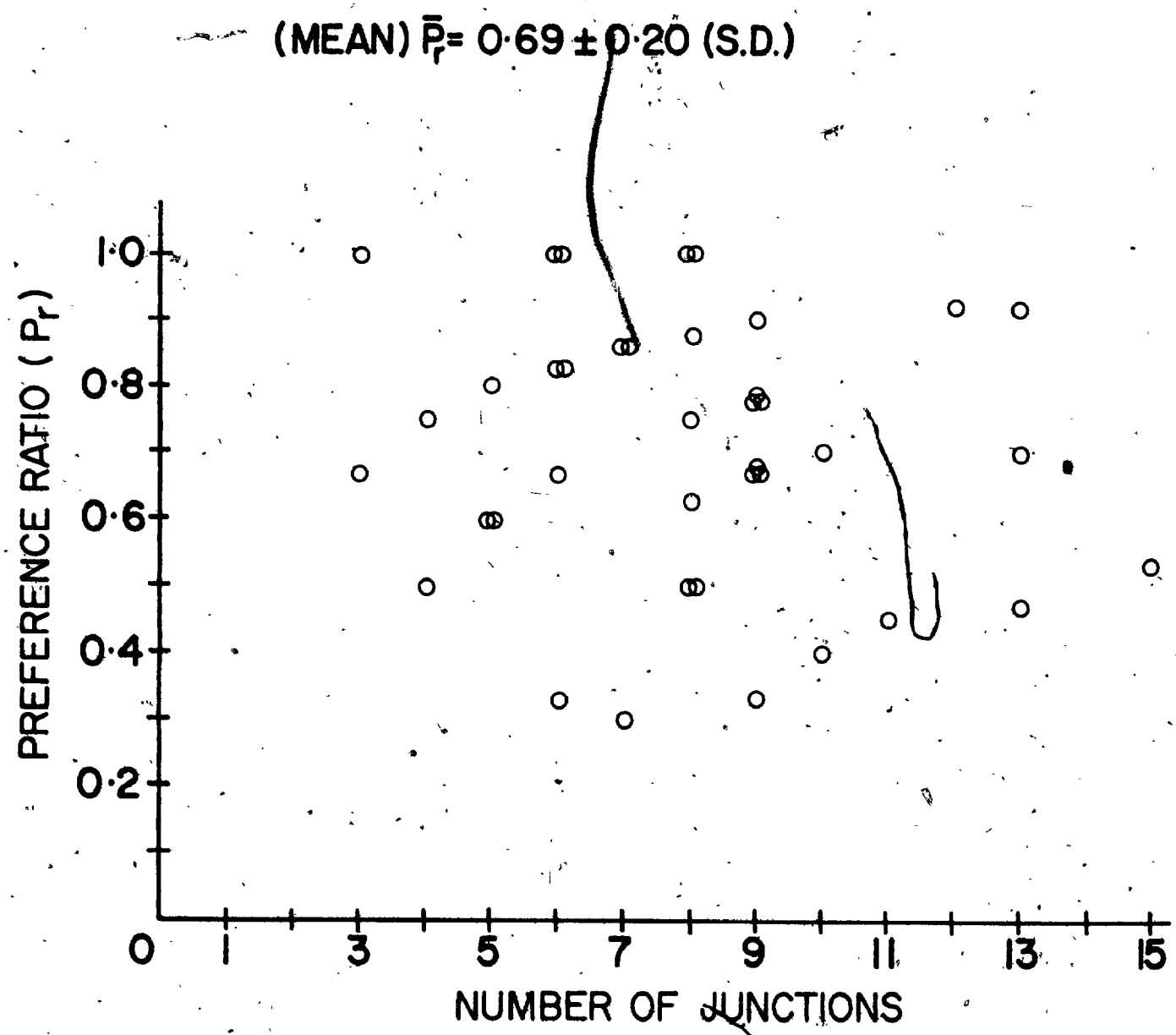

MOUSE
6·38 ± 0·28 (S.D.)
HUMAN
8·32 ± 0·42 (S.D.)
a
55
45
35
25
15
5
6·0
9·0
DIAMETER (μm)
MOUSE
1·90 ± 0·17 (S.D.)
HUMAN
1·96 ± 0·22 (S.D.)
b
1·5
2·9
THICKNESS (μm)
MOUSE
49·9 ± 6·30 (S.D.)
HUMAN
91·4 ± 12·4 (S.D.)
c
55
45
35
25
15
10
5
55
115
VOLUME (μm³)
MOUSE
81·2 ± 6·80 (S.D.)
HUMAN
131·5 ± 12·0 (S.D.)
d
65
125
185
SURFACE AREA (μm²)

FIGURE 18

A measure of preference for red cells of one species to form rouleaux with red cells of the same species rather than with red cells of another species.

The abscissa represents the number of adjacent pairs of cells in rouleaux. The ordinate represents the ratio of the number of like adjacent pairs to the total number of adjacent pairs of cells in rouleaux.


(MEAN) $\bar{P}_r$= 0·69 ± 0·20 (S.D.)
PREFERENCE RATIO (P_r)
1·0
0·8
0·6
0·4
0·2
0
1
3
5
7
9
11
13
15
NUMBER OF JUNCTIONS

the same species.

It was evident from these experiments that cells of one species did form rouleaux with cells of another species. It was therefore necessary to compare the sequencing of cells in the rouleau studied with a purely random sequence, in order to determine if preference was demonstrated for cells to adhere to their own kind. The random number tables were used with even numerals representing one species and the odd numerals another. The model rouleaux lengths were set to match the actual number of cells in the experimentally observed rouleaux. Figure 19 shows the result for a simulated random placing of two species of cells in model rouleaux formation.

The mean preference value obtained for the case of human cells mixed with mouse cells (figure 18) is 0.69. For the corresponding model rouleaux (random numbers) the mean preference value is 0.37 (figure 19). Using the Student's t-test, these two values were statistically compared and were found to be significantly different ($p < 0.01$); that is, human cells demonstrated a preference to form rouleaux with human cells, and similarly for mouse cells. Table III gives the results of the ten combinations studied. Nine of the ten combinations demonstrated a preference, different from the preference calculated for the randomly occurring sequence ($p < 0.01$). The rat-mouse combination was the only one that showed no preference ($p>0.01$), possibly because both animals are

FIGURE 19

Measure of preference for a simulated random placing of two species of cells in model rouleaux formation.

This figure represents a plot similar to that of figure 18 but using random numbers, where even numbers represent one species and odd numbers another.

(MEAN) $\bar{P}_r = 0{\cdot}37 \pm 0{\cdot}16$ (S.D.)

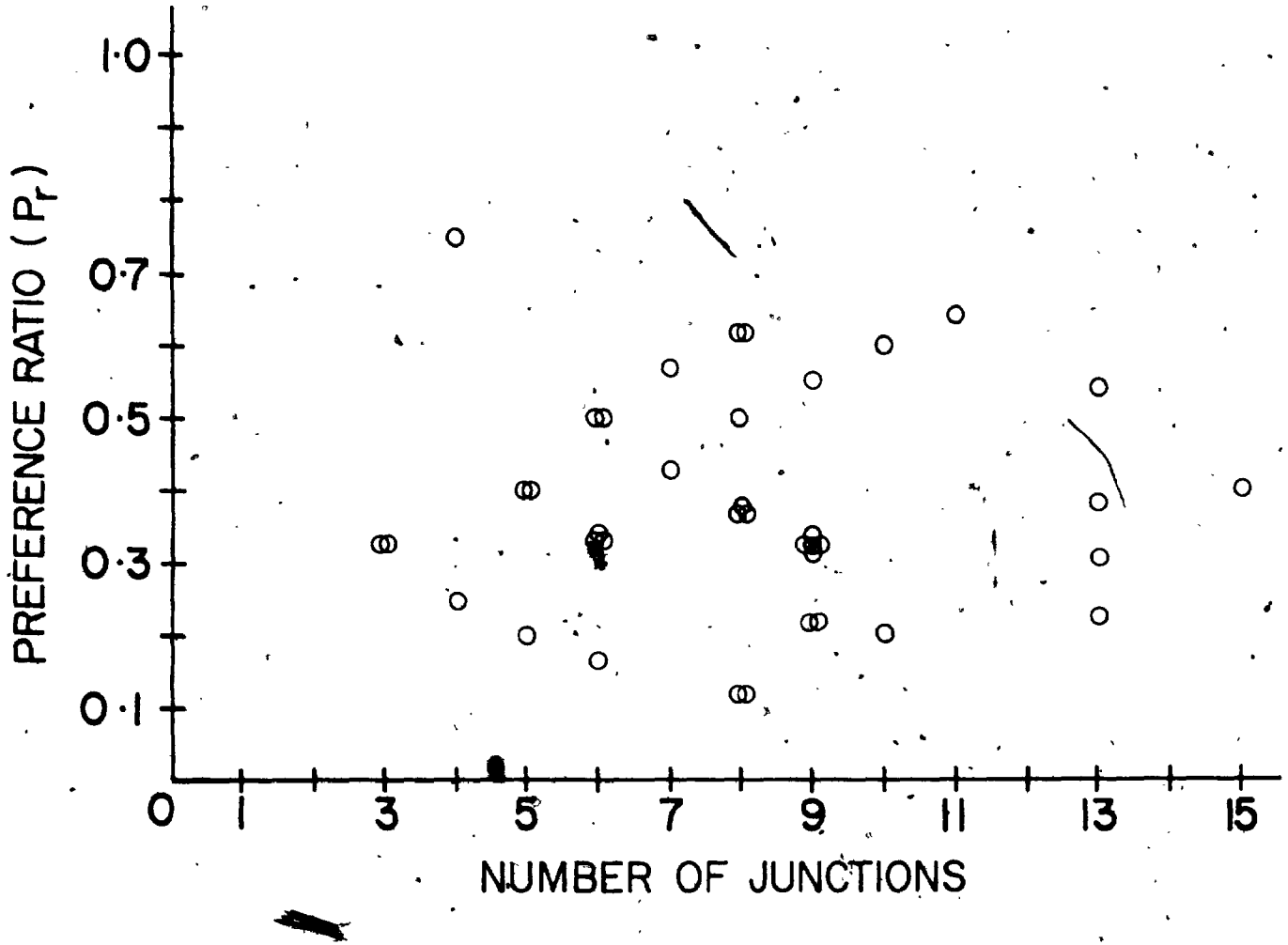


Table III. Preference parameters, $\bar{P}_r$, for various species combinations. Each of the preference values is significantly higher ($p < 0.01$) than for the equivalent random sequence, except the rat*-mouse* combination.

Combination	No. of rouleaux	$\bar{P}_r$ value (red cells) ± SEM	$\bar{P}_r$ value (random numbers) ± SEM
Dog-Cat	27	0.82±0.03	0.46±0.03
Guinea pig-Dog	32	0.64±0.04	0.41±0.03
Dog-Rat	20	0.58±0.04	0.43±0.03
Human-Rat	13	0.85±0.06	0.39±0.03
Human-Cat	13	0.75±0.03	0.39±0.03
Human-Dog	11	0.72±0.07	0.41±0.04
Rabbit-Cat	15	0.73±0.05	0.38±0.04
Human-Mouse*	40	0.69±0.03	0.37±0.03
Rabbit-Mouse*	23	0.73±0.03	0.39±0.03
Rat*-Mouse*	28	0.52±0.04	0.43±0.03

*Anaesthetized with Nembutal.

of the same family.

5.4. Discussion

The results of these experiments have three-fold significance. First, there is a statistical preference for the cells of one species to form rouleaux with cells of the same kind (species), although cells of two species do mix in rouleaux formation. Secondly, the preference shown is not simply a reflection of like sizes being more inclined to adhere to one another, since the rat-mouse mixed cell population was the only one which demonstrated no preference although there is an obvious difference in size between these two types of cells (Table II). Some of the other combinations tested were more similar dimensionally than this mixture. Thirdly, the results indicate that rouleaux formation may not completely be the result of intercellular attraction of specific membrane sites which would probably be species-specific in location and pattern.

CHAPTER 6

THE MECHANICS OR MODES OF ROULEAUX FORMATION OF HUMAN RED CELLS

6.1. Introduction

The techniques generally employed for studying red cell aggregation involve light transmission and reflection (Brinkman et al., 1963; VanHaeringen and Glasius, 1970), rate of erythrocyte sedimentation (Fåhraeus, 1921) and measurement of relative, apparent viscosity (Chien et al., 1967). These methods are statistical approaches to the problem of understanding the phenomenon of rouleaux formation. While they may accurately evaluate the degree of rouleaux formation, they cannot reveal direct information about the process or processes occurring at the cellular level. That is, they do not permit direct observation of the cells as they are participating in the adhesion process. Consequently, any model of the modes or mechanics of rouleaux formation, emerging from studies involving the above methods, will be hypothetical.

The first report on a direct study of the interactions of the red cells in the process of rouleaux forma-

tion was made by Rowlands and Skibo (1972) who studied the modes of rouleaux formation for red cells suspended in their native plasma. Fung and Canham (1974) also made an independent study of the modes and kinetics of doublet formation for red cells suspended in their own plasma and also in various solutions of PVP-360. Fung and Canham (1974), in addition to confirming the findings of Rowlands and Skibo (1972), found a good correlation between the results obtained for red cells suspended in plasma (plasma obtained from blood with differing ESR) and the results obtained for red cells suspended in solutions of increasing concentrations of PVP-360.

The study reported in this chapter is an extension of the work commenced by Fung (Fung, 1971; Fung and Canham, 1974). The motives for carrying out this study are as follows:

1) Fung and Canham found that the mode of doublet formation was dependent on the macromolecular concentration; at PVP-360 concentrations greater than 6g/l, doublets were formed only by the non-sliding modes. This was explained as being due to the increased friction between the cells resulting from the increase in the viscosity of the medium. This explanation received support from the work of Dixon (1975, Chap. 3). If this explanation were correct, does it follow that the formation of longer rouleaux in high macromolecular concentrations is confined to the non-sliding modes?

2) Should the net attractive force or adhesive force between the cells be considered as an important parameter in determining the transition from sliding to cresting in doublet formation?

3) Complete overlap of the cells on completion of the sliding mode of doublet formation is seldom observed. Can this be the result of an optical problem?

4) "Tank-treading", where the cell membrane rotates around the cell contents, has been proposed by some investigators (Chien and Jan, 1973) as the mechanism by which the cells align themselves in rouleaux formation. This study will provide the opportunity for examining the possibilities of the "tank-tread" model.

5) It is known that there are apparent differences in the physicochemical properties of PVP and Dx in inducing rouleaux formation of human cells (see chapter 4). Can we observe anything noteworthy in examining the interactions between the cells when suspended in solutions of these rouleaux-inducing agents? Besides, a comparison of the results of this study with those obtained by Rowlands and Skibo (1972) will help establish the credibility of using these substances (PVP and Dx) as suitable substitutes of the plasma proteins in model studies of rouleaux formation.

6) Why do the cells align themselves as a roll of coins? Can we observe this phenomenon as a general tendency among the cells?

6.2. Method

The modes of rouleaux formation were observed for red cells suspended in two different media namely, Ringer solution containing PVP-360 and Ringer solution containing dextran (Dx-70, Dx-110). The concentration of the PVP-Ringer solution ranged from 1.0g/l to 10g/l while the range for the Dx-Ringer solution was 1.0g/l to 100g/l.

Sample preparations and observations were made as explained in section 2.4. The observations were recorded on cine-film (Kodak TRI-X, 16mm) run at 2.33±0.01 frames per second. The cinefilm was also used as negatives from which high magnification glossy prints were made.

6.3. Results and Discussion

Red cells, suspended in any of the solutions, would settle to the bottom of the sample chamber onto the coverslip and then exhibit a random motion (believed to be due to Brownian motion). This "jiggling" motion brought about the chance contact between the cells which was the first step leading to doublet formation. Longer rouleaux were then formed in similar fashion. All the various processes observed in doublet formation and formation of longer rouleaux were filmed and analysed.

A. Doublet Formation

Doublets are formed by any of three modes - sliding, cresting and flipping. These three modes were discussed

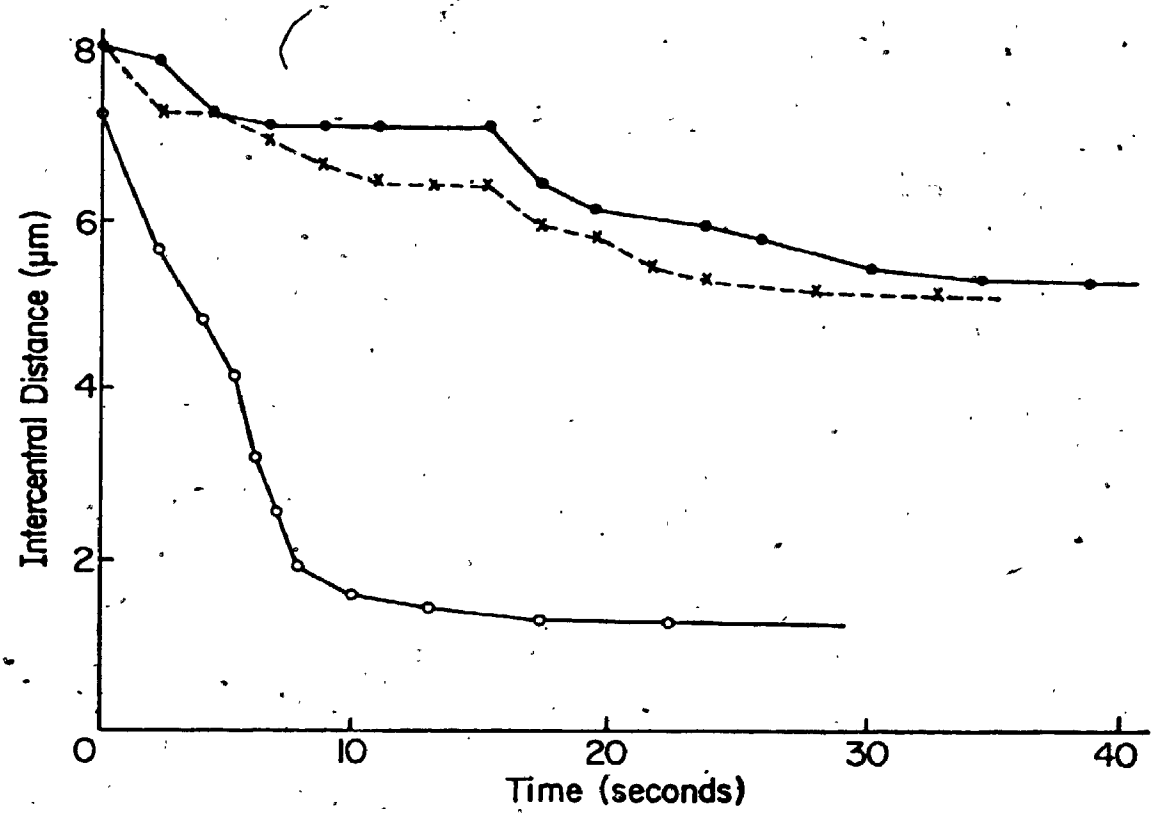
in detail by Fung (1971) and Dixon (1975, Chap. 1). In sliding, two cells make contact at their rims which become flattened in the region of contact and then one cell slides over the other. In the cresting mode, the cells pivot at the area of contact which rises above the coverslip as the cells go into broadside against broadside apposition. Flipping occurs when one cell pivots like a hinged lid about the area of contact between the cells, rises through the vertical position and finishes broadside down on the stationary cell.

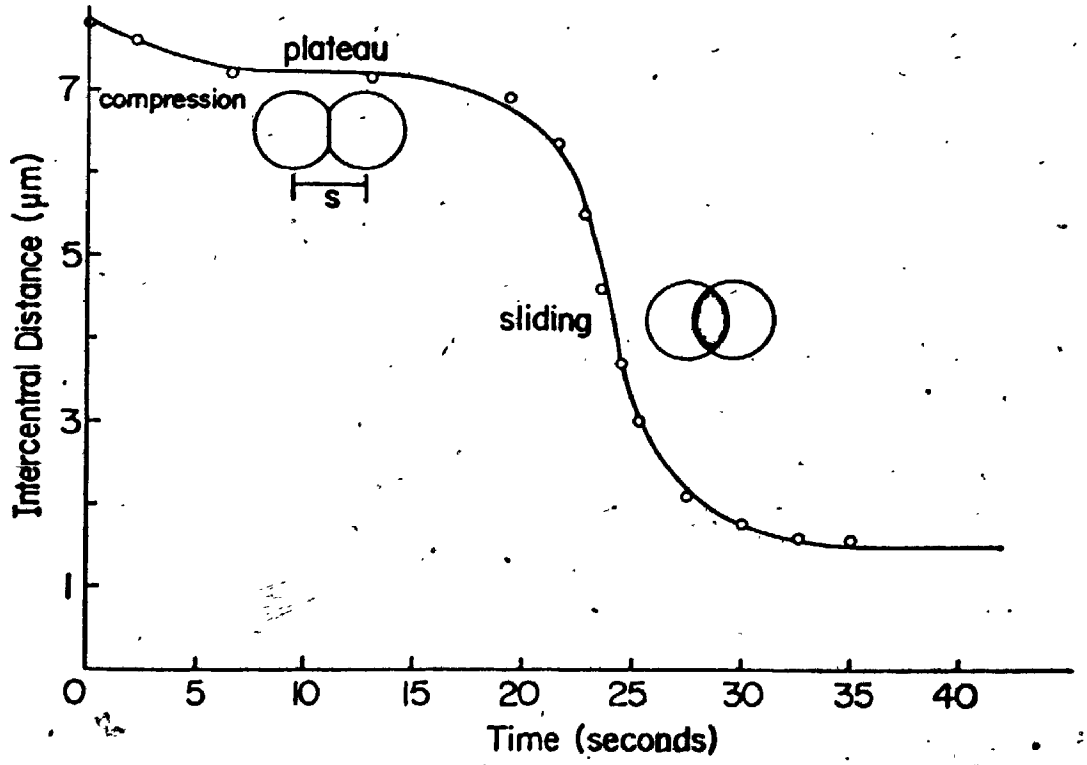
The sliding mode of doublet formation is predominant in PVP concentrations ranging from 1.0g/l to 5g/l while the other modes prevail at higher concentrations. In the Dx solutions, the three modes of doublet formation were again observed but the sliding mode started differently. There was very little compression or flattening of the membranes before sliding began; sliding was slower than in PVP solutions and was often observed to stop when the cells were half way to complete overlap. In addition, two adjacent cells, seemingly touching each other, would jiggle around for a relatively long time before they would make adhesive contact. Figure 20a shows the kinetics of three sliding events in Dx-induced doublet formation. Compare this figure with figure 20b which shows the kinetics of sliding for PVP-induced doublet formation. Sliding is much more uniform in PVP-induced doublet formation. Another interesting finding is that while

FIGURE 20

A. Kinetics of three sliding events in Dx-induced doublet formation. The full lines represent two sliding events in a Dx-70 concentration of 50g/l. The dotted line represents a sliding event in a Dx-70 concentration of .40g/l. As seen in these figures, the kinetics of sliding are irregular for Dx-induced doublet formation.

B. Kinetics of a typical sliding event in PVP-induced doublet formation. Three distinct phases exist: compression, plateau and finally the sliding phase.


Intercentral Distance (μm)
8
6
4
2
0
10
20
30
40
Time (seconds)


plateau
compression
s
sliding
Intercentral Distance (μm)
7
5
3
1
0
5
10
15
20
25
30
35
40
Time (seconds)

cresting and flipping were predominant (92%, n=25) in Dx-Ringer at a Dx concentration of approximately 40g/l, sliding prevailed at the other Dx concentrations.

The transition from sliding to non-sliding was explained by Fung (1971) and Fung and Canham (1974) as being due to the increased frictional force arising in the area of contact between the cells. The increased frictional force was attributed to rising concentrations of PVP-360 solutions which increased the viscosity of the medium between the cells. The model of Fung and Canham (1974) was supported by evidence presented by Dixon (1975). Dixon, using glycerol to increase the relative viscosity of the suspending medium, studied the effect of viscosity of the medium on the modes of doublet formation. He found that the percentage of non-sliding events increased from 12% to 34% when the relative viscosity of a PVP-Ringer solution (4g/l) was increased by approximately 90%. The results of Dixon (1975), however, are difficult to draw conclusions from, because very little is known about the effect of glycerol on the red cell geometry. Glycerol molecules are able to penetrate the cell membrane (Jacobs, Parpart and Corson, 1937) and this might affect the deformability of the cell. Besides, there is the possibility of the binding of glycerol to the PVP molecules (Kassem and Mattha, 1970), a result which might affect the rouleaux-inducing properties of the PVP molecules.

While the effects of viscosity on the modes of

doublet formation are acknowledged, it is felt in this study that the net attractive force between the cells is a more influential determinant. The reason for suggesting this is that in part of this study PVP-360 solutions, devoid of calcium ions, were used and doublet formation by the sliding mode was predominant even at a PVP concentration as high as 7g/l (see chapter 8, Table IV). In a normal solution (i.e. with Ca^{++} ions) sliding does not occur at this PVP concentration. In fact, sliding made up approximately 60% of the modes of doublet formation in a PVP-Ringer solution (no Ca^{++} ions) of concentration 10g/l. There is no documented evidence that the lack of calcium ions reduces the viscosity of the medium. From the work of Brooks (1973c) and Jan and Chien (1973b), it is inferred that the divalent cations; for example calcium, in the medium act to screen the negative charges on the cell membranes, thus reducing the electrostatic repulsive force between the cells. The lack of calcium ions, therefore, causes an increase in the electrostatic repulsive force between the cells (reducing the net adhesive force), and it is believed for this reason that the sliding mode of doublet formation still persists at high PVP concentrations.

Further evidence in support of the contention here that the net attractive force is a more important factor in determining the transition from sliding to non-sliding, comes from the results of the Dx experiments. As seen in

chapter 4, red cell aggregation peaked at a Dx concentration of approximately 40g/l (Dx-70, Dx-110). And, in this study, the percentage of the non-sliding events peaked at the same Dx concentration. This finding suggests a correlation between the net attractive force and the mode of doublet formation. This concept will be further discussed in the next sub-section.

A popular consideration among investigators interested in the rheology of the red cell is the concept of "tank-treading". Tank-treading is a term introduced for the hypothetical motion of the moving cell on the stationary cell when the membrane of moving cell rolls rather than slides on the non-moving membrane. Such a motion would involve no sliding or slippage between the points of contact on the two membranes. This mechanism is however not supported by us. As Fung and Canham (1974) pointed out, it is not possible for two reasons. Firstly, the "tank-tread" model makes it very difficult to explain the transition to cresting as observed in doublet formation induced by increased PVP concentrations. Secondly, the membrane of the moving cell would undergo considerable shear caused by the continual rearrangements of the elements of the viscoelastic membrane, in that the central part of the tank-tread would roll with a lower angular velocity than the two sides of the tank-tread. The different parts of the tank-tread, moving at different rotating speeds, would be sheared relative to each other.

This is explained in figure 21A. There is a third reason why tank-treading is not possible. It is common observation that two multicellular rouleaux join together with the end cell of one rouleau sliding over the end cell of the second rouleau. Tank-treading therefore would not be possible since the cell, next to the end cell of the moving rouleau, would prevent the end cell from tank-treading. This is illustrated in figure 21B.

B. Models of the Side-Views of Doublet Formation

For cells forming rouleaux on a coverslip complete overlap in the sliding mode of doublet formation is seldom observed (figure 25d, long arrow). This observation was first made by Fung (1971) who used a similar technique to mine for studying the modes and kinetics of doublet formation. With this technique, the red cells were always seen to lie in such a manner on the coverslip that few were seen edge-on but most flat. In doublet formation, the cells orient themselves flat with respect to the coverslip before they make adhesive contact and slide or crest; edge-on cells forming doublets or attempting to form doublets were observed only once in this study. In this one instance, the cells were flattened in the region where the rims were in contact, but remained stuck with neither sliding nor cresting taking place.

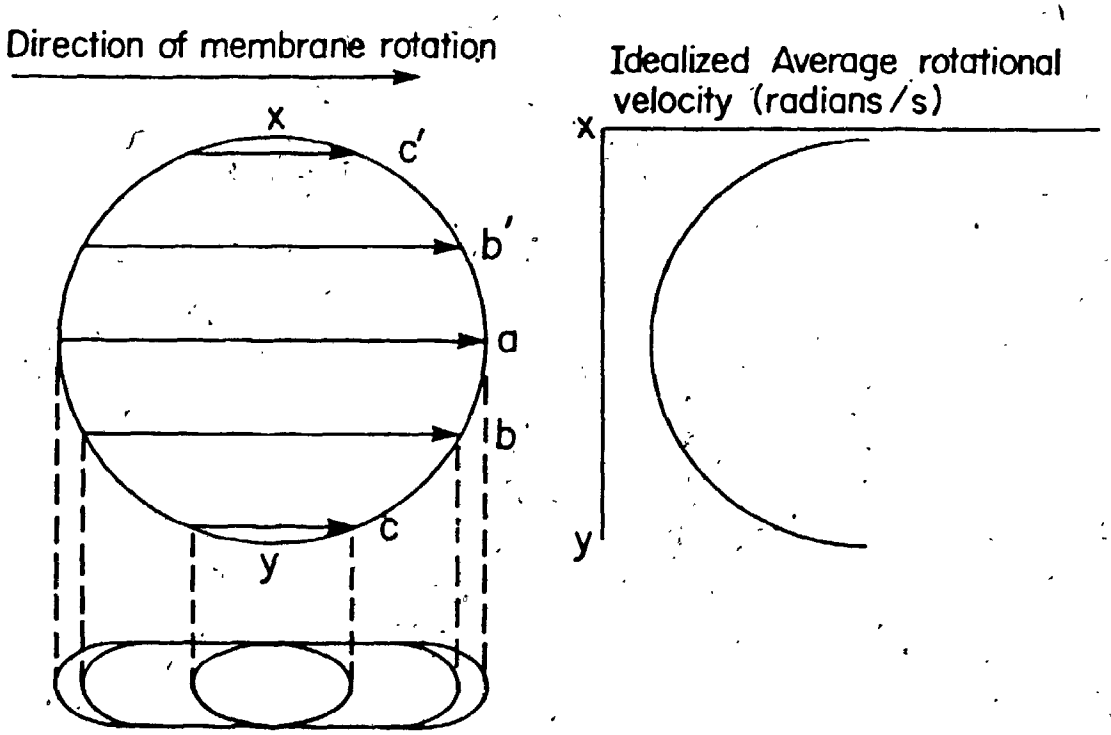
In view of the observed fact that cells forming doublets could not be seen from the side, models of the

FIGURE 21

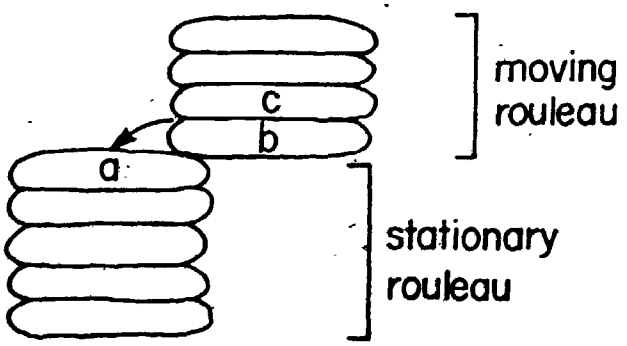
A. Shearing of the red cell membrane in tank-tread motion. As seen in this figure (cell shown flat and also from the side, schematically) the point c will rotate with a greater angular velocity than point b which in turn will rotate with a greater angular velocity than the central point a. Tank-treading therefore means that the membrane of the moving cell would have to undergo considerable shear caused by the continual rearrangement of the elements of the viscoelastic membrane.

B. Movement of the end cell of one rouleau over the end cell of a stationary rouleau. Cell c of the moving rouleau would prevent cell b from tank-treading over cell a of the stationary rouleau.

A.


Direction of membrane rotation
x
c'
b'
a
b
c
y
Idealized Average rotational velocity (radians/s)
x
y

B.


c
b
a
moving rouleau
stationary rouleau

side-views have been proposed (Fung, 1971; Fung and Canham, 1974; Dixon, 1975). These models however did not explain why incomplete overlap of the cells is frequently observed. Figures 22A, 22B and 22C respectively represent the side-views of sliding, cresting and flipping as proposed by Fung and Canham (1974) and Dixon (1975). In the sliding mode the broadside plane of the cells remains parallel to the coverslip, while in the cresting mode the cells buckle at the point of contact and rise off the coverslip until they are erect, broadside against broadside. Flipping which makes up less than 10% of the non-sliding modes occurs when one of the cells is apparently stuck to the substrate (coverslip). The transition from sliding to cresting was explained as being due to the increased viscosity of the medium resulting from the increased macromolecular concentration (Fung, 1971; Dixon, 1975). The increased viscosity reportedly increases the friction in the area of contact between the cells thus preventing slippage between them.

My model of the side-views of the principal modes of doublet formation (figures 23A and 23B) is slightly modified from that of Fung and Canham (1974) and Dixon (1975). The red cells after making contact will buckle during compression, but the extent to which they buckle will depend on the net attractive force between the cells. In sliding the cells buckle during compression and rise to a certain point (figure 23A, t=5 sec.), remain stationary

FIGURE 22

Models of the side-views in doublet formation. The models of sliding and cresting were proposed by Fung (1971) and supported by Dixon (1975). The model of flipping was proposed by Dixon (1975) and is supported by the author.

(Modified from Dixon, 1975, Chaps. 1 and 2)

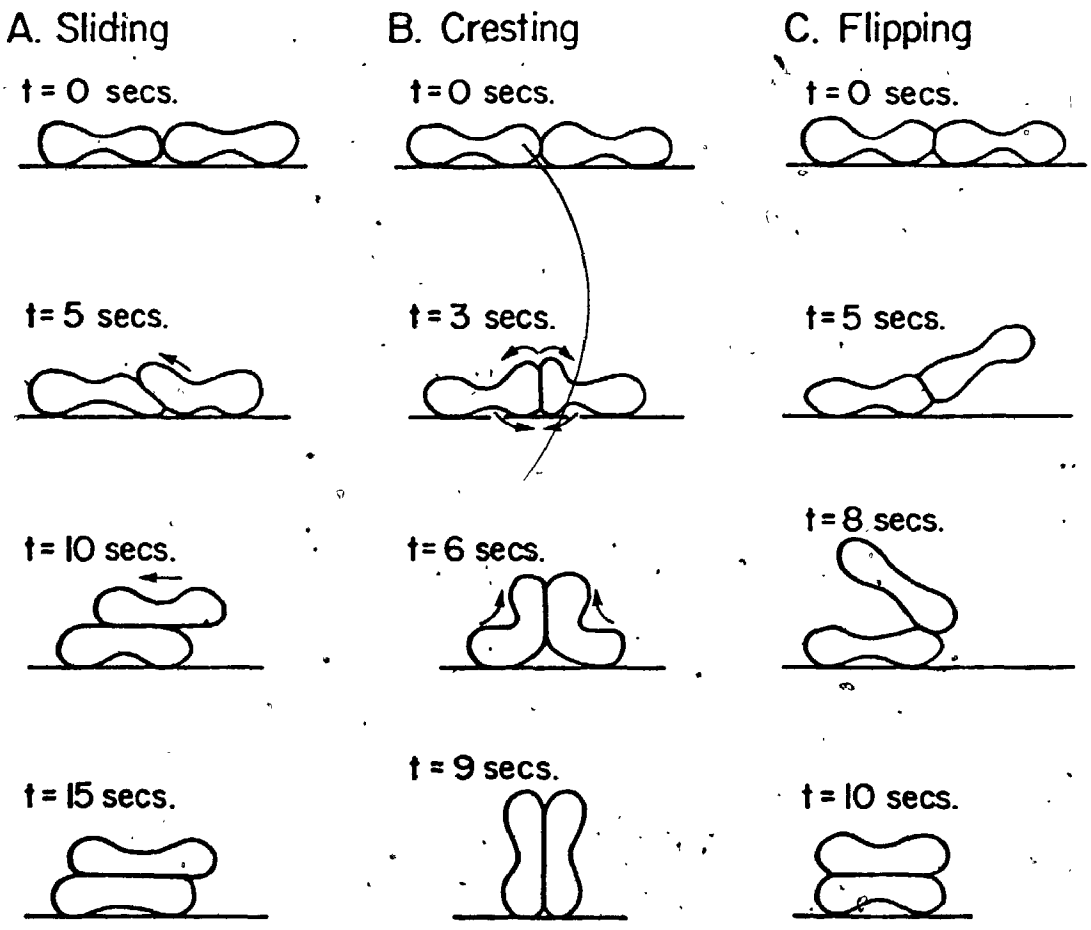

A. Sliding
t = 0 secs.
t = 5 secs.
t = 10 secs.
t = 15 secs.
B. Cresting
t = 0 secs.
t = 3 secs.
t = 6 secs.
t = 9 secs.
C. Flipping
t = 0 secs.
t = 5 secs.
t = 8 secs.
t = 10 secs.

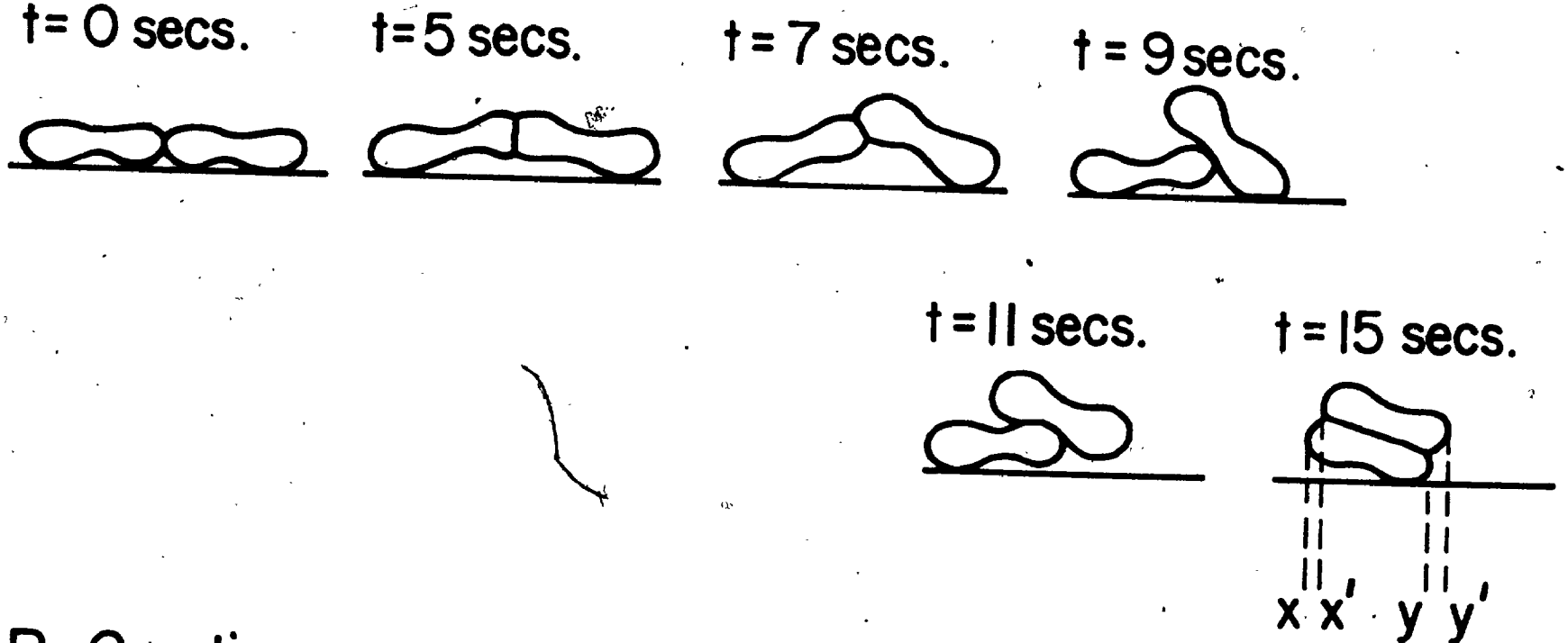
FIGURE 23

The author's model of the side-views of the principal modes of doublet formation.

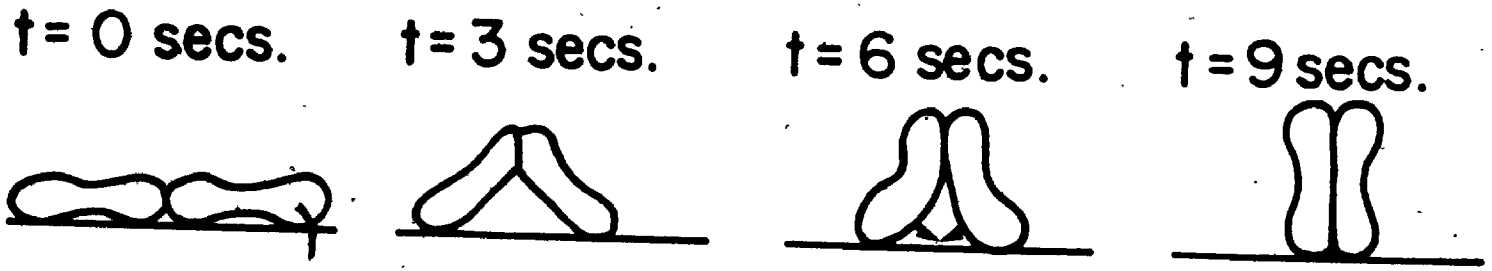
In the sliding mode, the cells after making contact buckle to a certain point during compression and remain like that for a while. When the metastable equilibrium is disturbed, the cells "pop" out of the compression and then one cell slides to complete overlap over the other. However, as shown, complete overlap could not be seen from the bottom because of the doublet's angle of inclination to the flat, horizontal coverslip.

In cresting, the cells buckle beyond a critical point and then the bottom ends "close" in as shown.

A. Sliding



B. Cresting

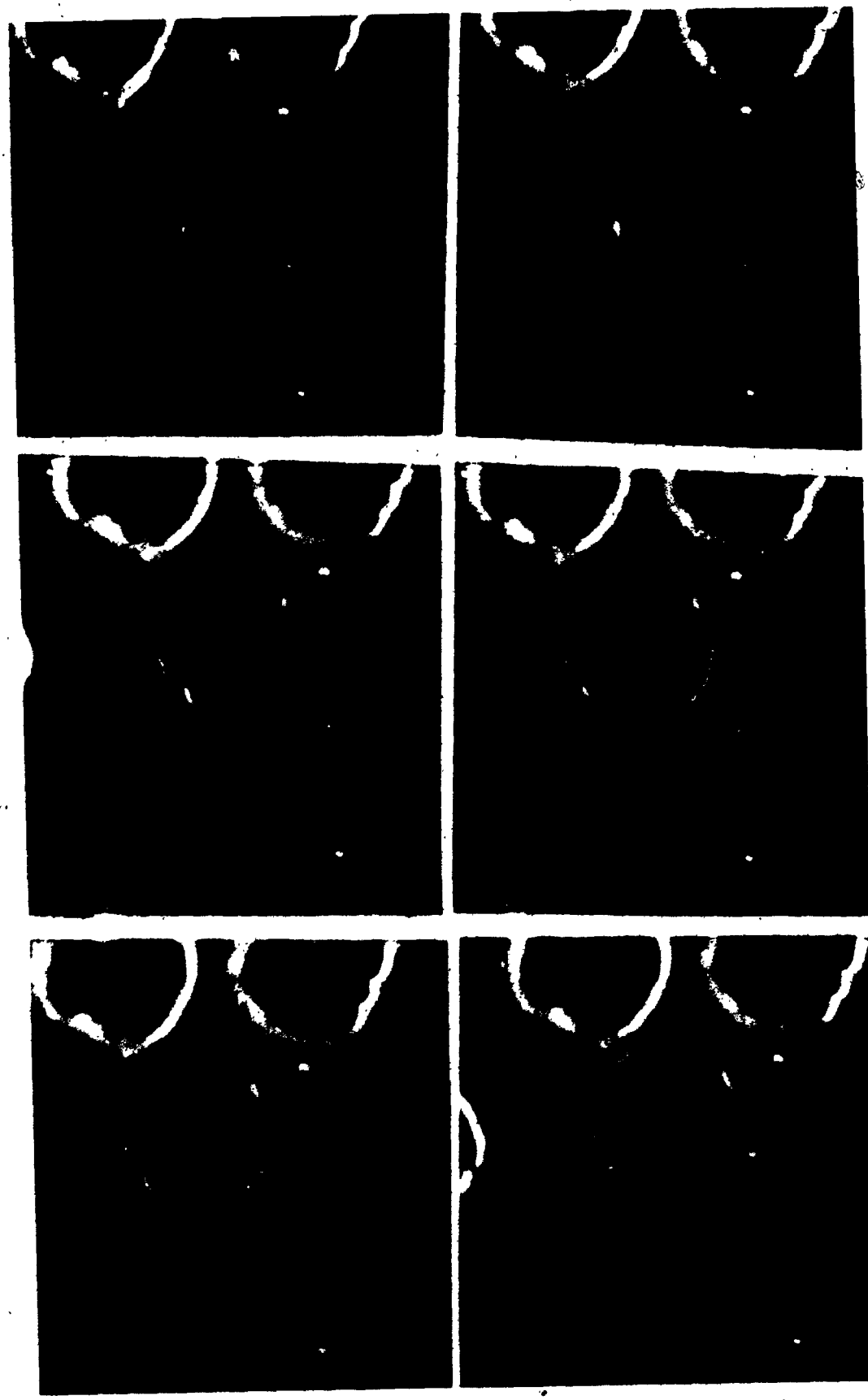


for a while (plateau phase lasting a variable period of time), after which one cell slides over the other until complete overlap is reached. As shown in figure 23A (t=15 secs.) complete overlap would not be observed when viewing from above or below. The rotational motion of the cells during sliding differs from doublet to doublet. In a few cases, just after sliding has stopped, the doublet's angle of inclination (angle of the broadside plane of the doublet) to the flat, horizontal coverslip is so small that almost complete overlap can be observed from either above or below. Evidence to show that the cells do not remain with their broadside plane parallel to the coverslip during sliding is given in figure 24. The visual decrease in the diameter of the cells in the direction of sliding, with no apparent change in the diameter perpendicular to the direction of sliding, implies that the cells do not remain flat during sliding.

In the cresting mode of doublet formation the implication of our model (figure 23B) is that the net attractive force between the cells is large enough for the cells to buckle beyond a certain critical point. After the cells have risen above the critical point, the bottom ends of the cells "close" in as shown in figure 23B (t=6 secs.). It is believed that the net attractive force between the cells increases as the PVP adsorption increases (Hummel, 1963, 1969; Hummel and Szczepanski, 1963) and it is for this reason that the cresting mode of doublet

FIGURE 24

The sliding mode of doublet formation. This is an example for cells suspended in a PVP-Ringer solution of concentration 4g/l.
(The scale represents 10μm).



formation prevails beyond a PVP concentration of 5g/l.

C. Formation of Longer Rouleaux

The modes of triplet formation both in PVP-Ringer solution and in Dx-Ringer solution were observed to be the same as for doublet formation in the corresponding solution. As in doublet formation, the modes are dependent on macromolecular concentration. Figure 25 (central cells, short arrow) is an example of the sequence of the cresting mode of triplet formation - the whole process was completed in approximately 15 secs.

The modes of formation of longer rouleaux (4 or more cells) were observed to be the same for both types of rouleaux-inducing polymers. The modes were independent of the macromolecular concentration, but were found to be dependent on the orientation of the cell or rouleau when contact was first made. The modes of formation of long rouleaux are sliding and clapping or "penknife-closure" as Rowlands and Skibo (1972) described it. The fact that sliding was observed to occur even at high macromolecular concentrations is evidence against the explanation of Fung and Canham (1974) and Dixon (1975) to account for the transition from sliding to cresting in doublet formation.

Figure 26 is an example of a single cell attaching itself to the end cell of a branched rouleau in a PVP-Ringer solution. The single edge-on cell is deformed

FIGURE 25

The cresting mode in triplet formation (central cells, short arrow).

The long arrow shows an example of incomplete overlap in doublet formation.

This is an example for cells in a 40g/l Dx-110 solution.

(The scale represents 10μm).

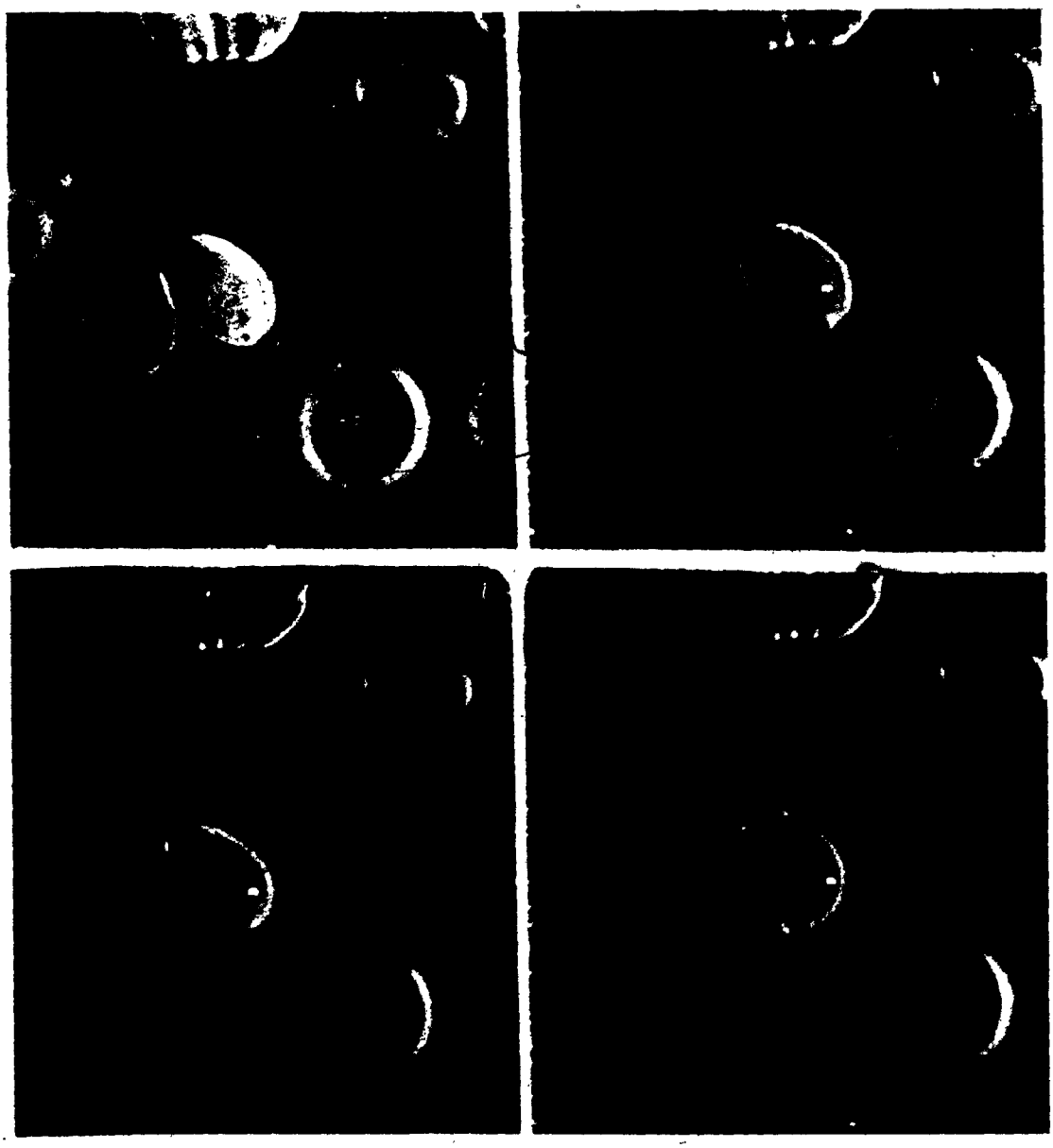
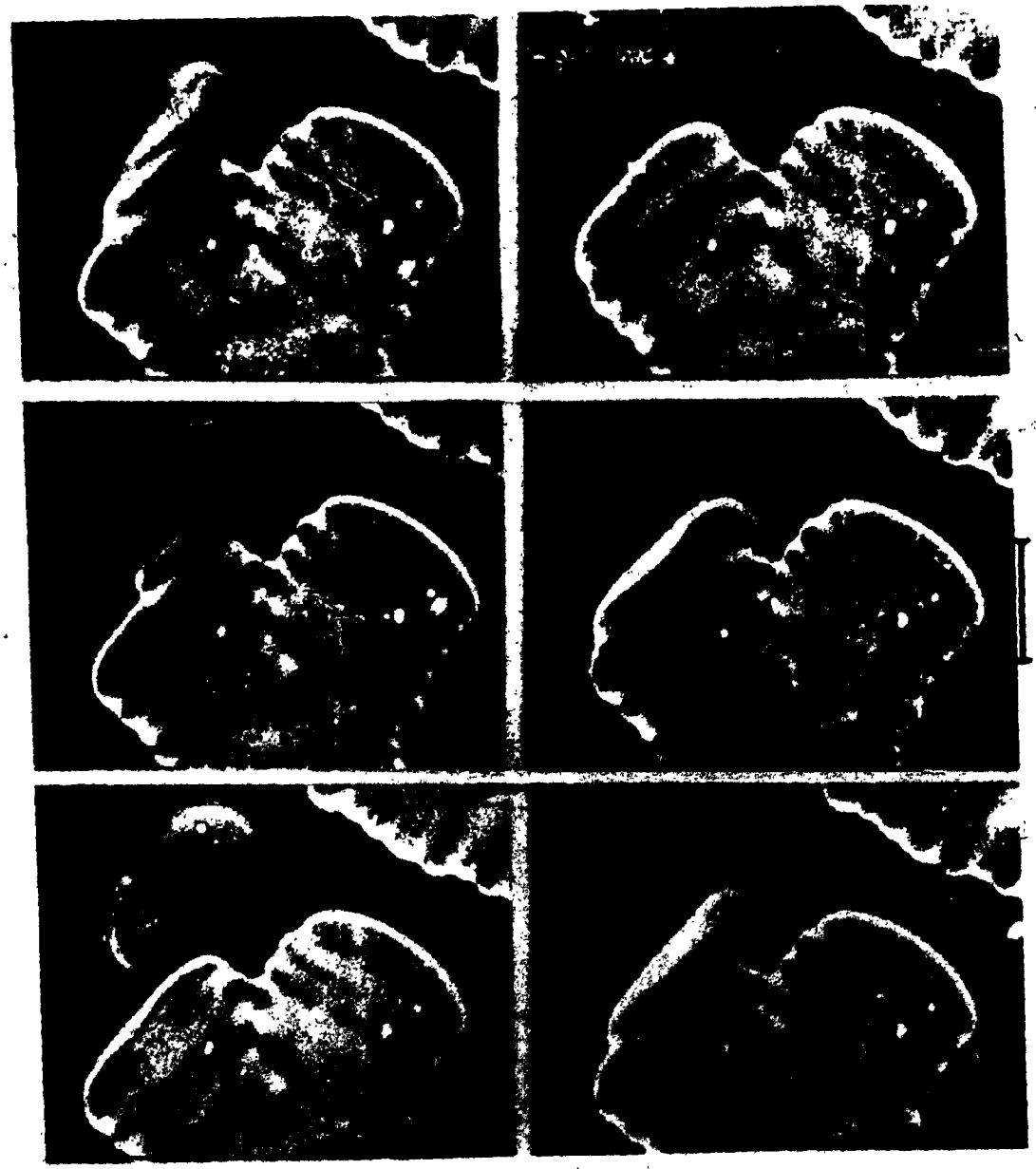


FIGURE 26

Photomicrographs showing a single, edge-on cell sliding over an end cell of a branched rouleau. Note the deformation of the single cell as it made contact (figure 26b) with the end cell.

This is an example for cells suspended in PVP-Ringer solution of concentration 6g/l.

(The scale represents 5μm).



(figure 26b) after making contact and then slides over the end cell of the stationary rouleau. The whole process was completed in about 10 seconds after the initial contact. The average sliding velocity was computed to be 0.76 μm/s which is comparable with that obtained by Fung (1971) who measured the average velocities in doublet formation. Figure 27 shows a single cell flat on the coverslip appearing to make contact with the end cell and penultimate cell of a 7-cell rouleau (figure 27a), the rouleau rotating counterclockwise through an angle of nearly 30° (figure 27e) before the single cell exhibits the clapping or "penknife-closure" action (f → i). The flat cell did not orient itself edge-on as if destined to slide over the end cell of the rouleau. The mode depended both on the related orientation of the cell and rouleau when contact was made, and on the position of the initial contact.

Figure 28 shows a doublet sliding over the end cell of a rouleau. The process is the same as that shown in figure 26 for a single cell where contact was first made with the rim of the end cell before sliding occurred. The average sliding velocity is about 1.0 μm/sec which is comparable with the average velocity in doublet formation. The clapping or flipping of doublets onto the end cells of rouleaux was also frequently observed in this study. Sliding of triplets with the resulting formation of a single rouleau is shown in figure 29. This is an

FIGURE 27

The attachment of a single, flat cell to an end cell of a linear rouleau. The rouleau rotates counter-clockwise through an angle of about 30° before the single cell flips over the end cell.

An example for cells in 4g/l PVP solution.

(The scale represents 10μm).

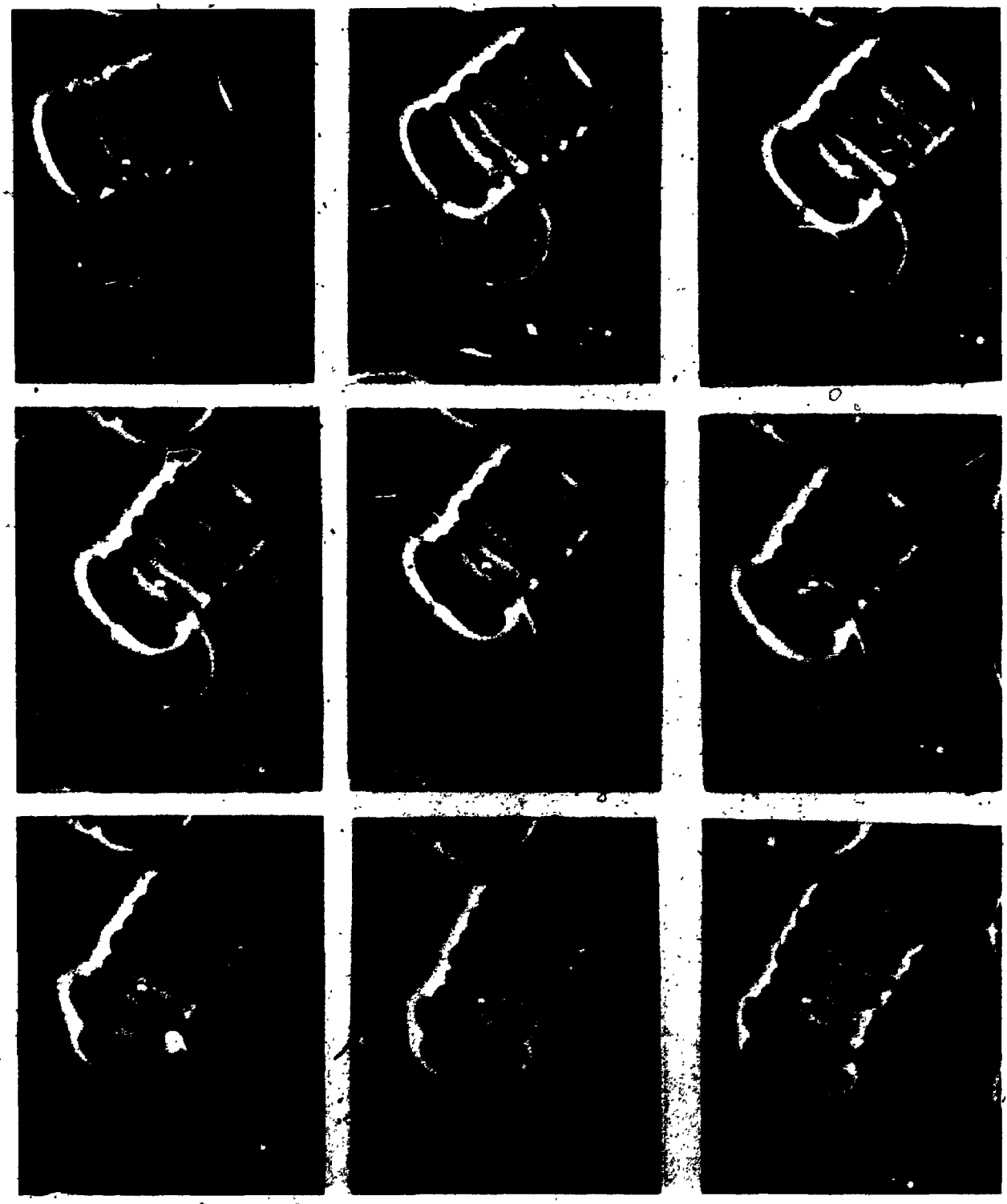


FIGURE 28

Photomicrographs showing a doublet sliding over the end cell of a stationary rouleau in a PVP solution of concentration 6g/l.

(The scale represents 10μm).

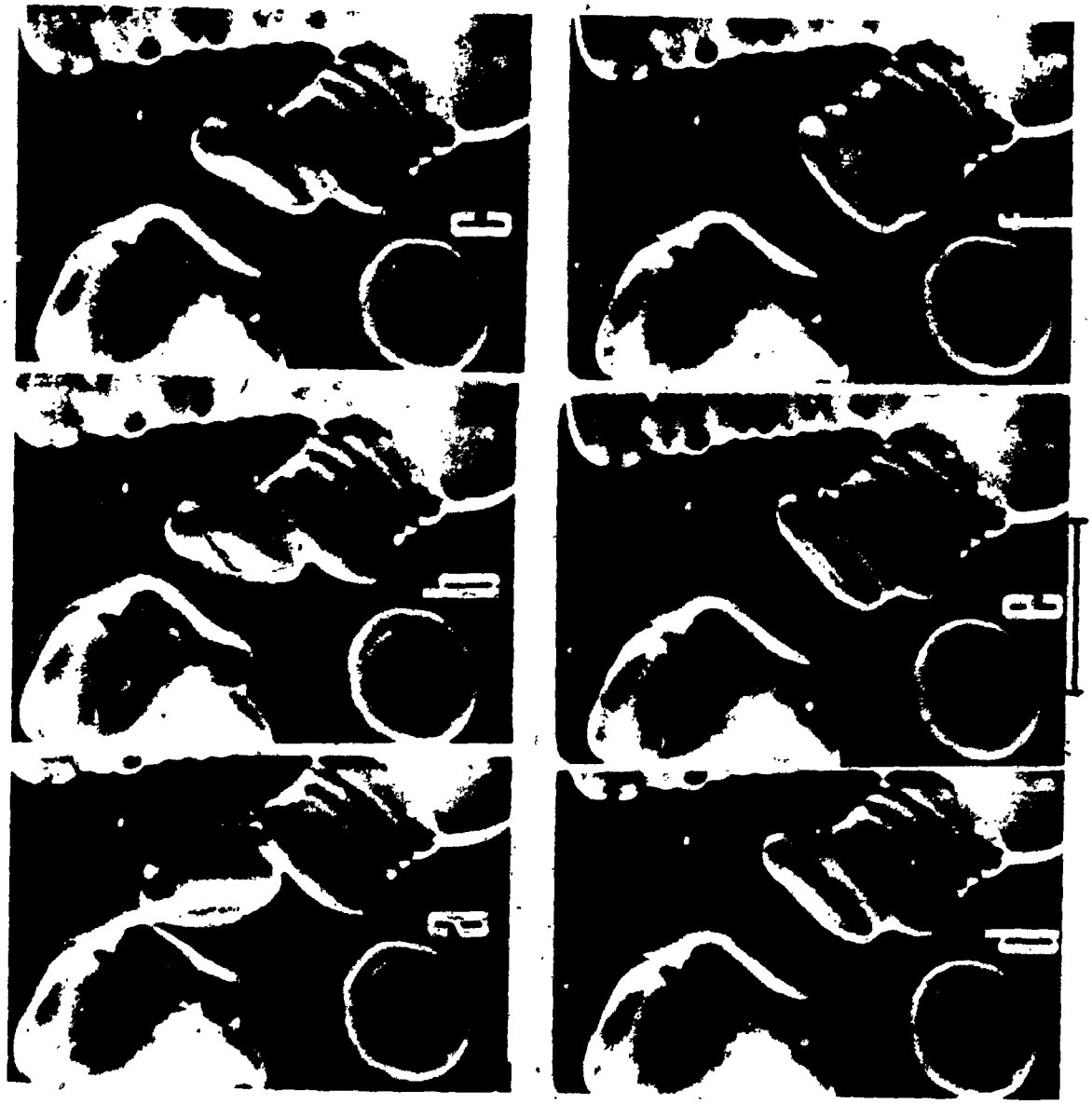
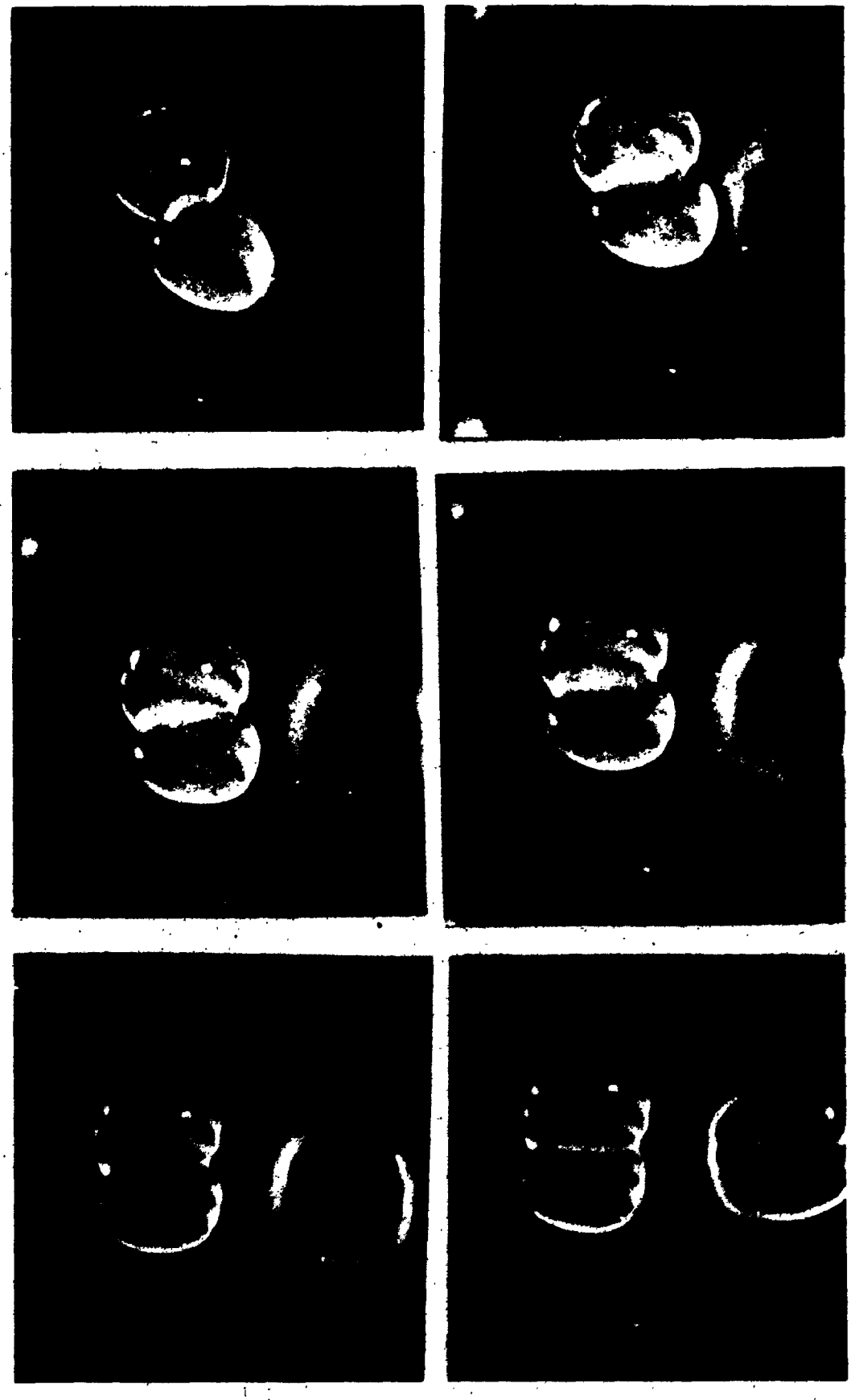


FIGURE 29

The sliding of triplets with the resulting formation of a single rouleau, in a Dx-110 solution of concentration 40g/l.

(The scale represents 10μm).



example of the formation of longer rouleaux in Dx-Ringer solution; the time sequence is comparable with that in PVP-Ringer solutions. The "penknife-closure" action of rouleaux formation is illustrated in figure 30. The two rouleaux first make contact as shown in figure 30d and then close in to form a single rouleau (figure 30f). Note how the adhering surfaces align themselves parallel to each other (figure 30c), a configuration which would facilitate bridging of the adsorbed macromolecules.

Figure 31 shows a 4-cell rouleau appearing to make contact with the side of a longer rouleau before moving over to the rim of the end cell (figure 31c) and sliding into a perfect single rouleau alignment (figure 31f). Stable attachment of a single cell (shown in this figure) or rouleau to the side of another rouleau is a rare occurrence at low macromolecular concentrations; it occurs frequently at PVP concentrations above 10g/l. The process of rouleaux formation seems to involve a surface energy with the general tendency to minimize this energy by reducing the amount of red cell membrane in contact with the colloid-Ringer solution. In this study, some of the observed instances of the formation of branched rouleaux were also filmed. Figure 32 represents such an example, where each frame is separated from the preceding frame by approximately 4 seconds. Here, a single rouleau sediments onto three separate rouleaux in close proximity and joins them into a branched rouleau. Note how the

FIGURE 30

Illustration of the clapping or "penknife-closure" action of rouleaux formation, in a PVP solution of concentration 6g/l.

Note how the adhering surfaces align themselves parallel to each other (figure 30c), a configuration which would facilitate bridging of the adsorbed macromolecules.

(The scale represents 5μm).

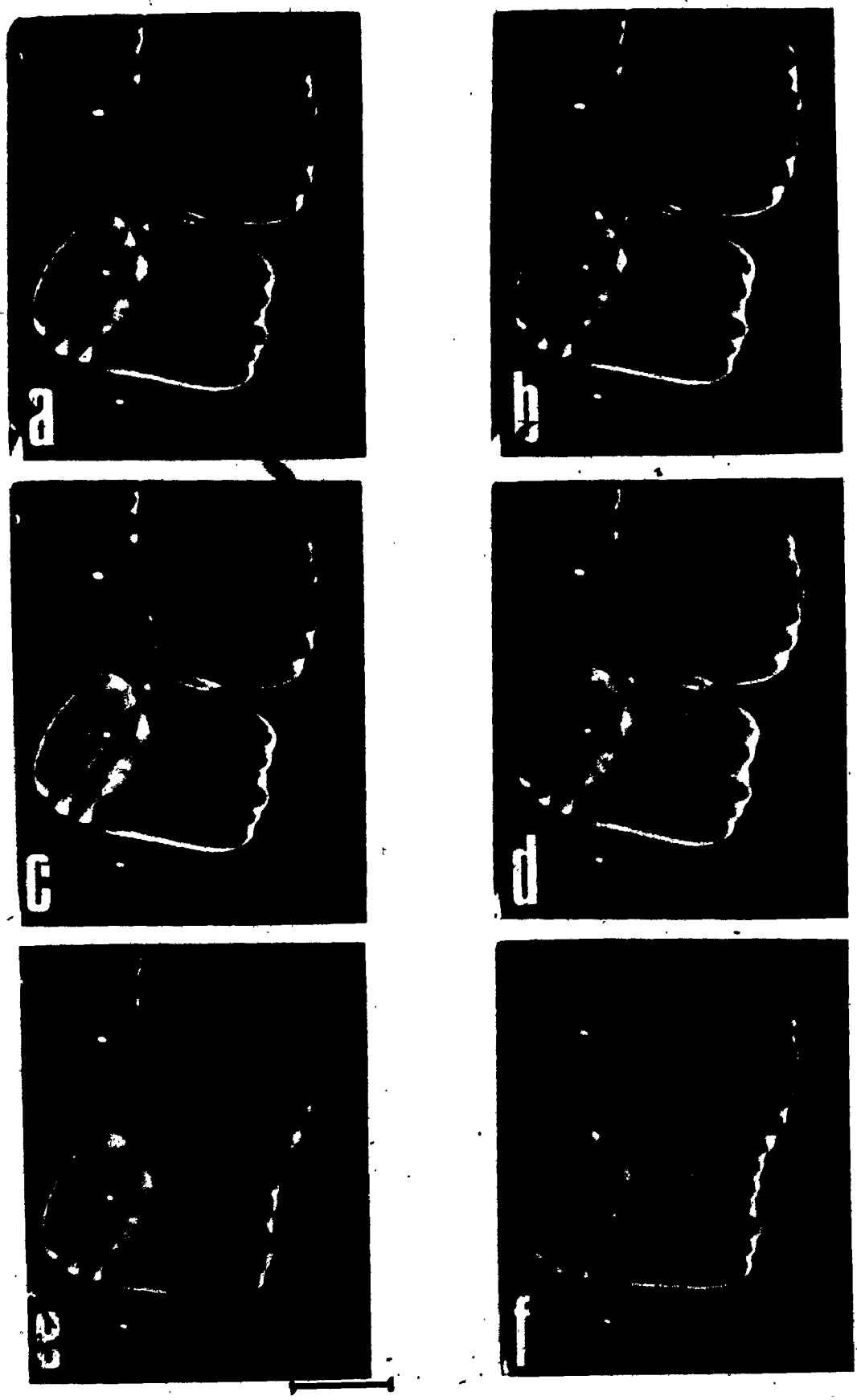

a
b
c
d
e
f

FIGURE 31

Photomicrographs showing a four-cell rouleau appearing to make contact with the side of a longer rouleau before moving over to the rim of the end cell and sliding into a perfect single rouleau alignment (6g/l PVP-Ringer).

Stable attachment of a single cell (shown in this figure) to the side of a rouleau is a rare occurrence at low macromolecular concentrations.

(The scale represents 5μm).

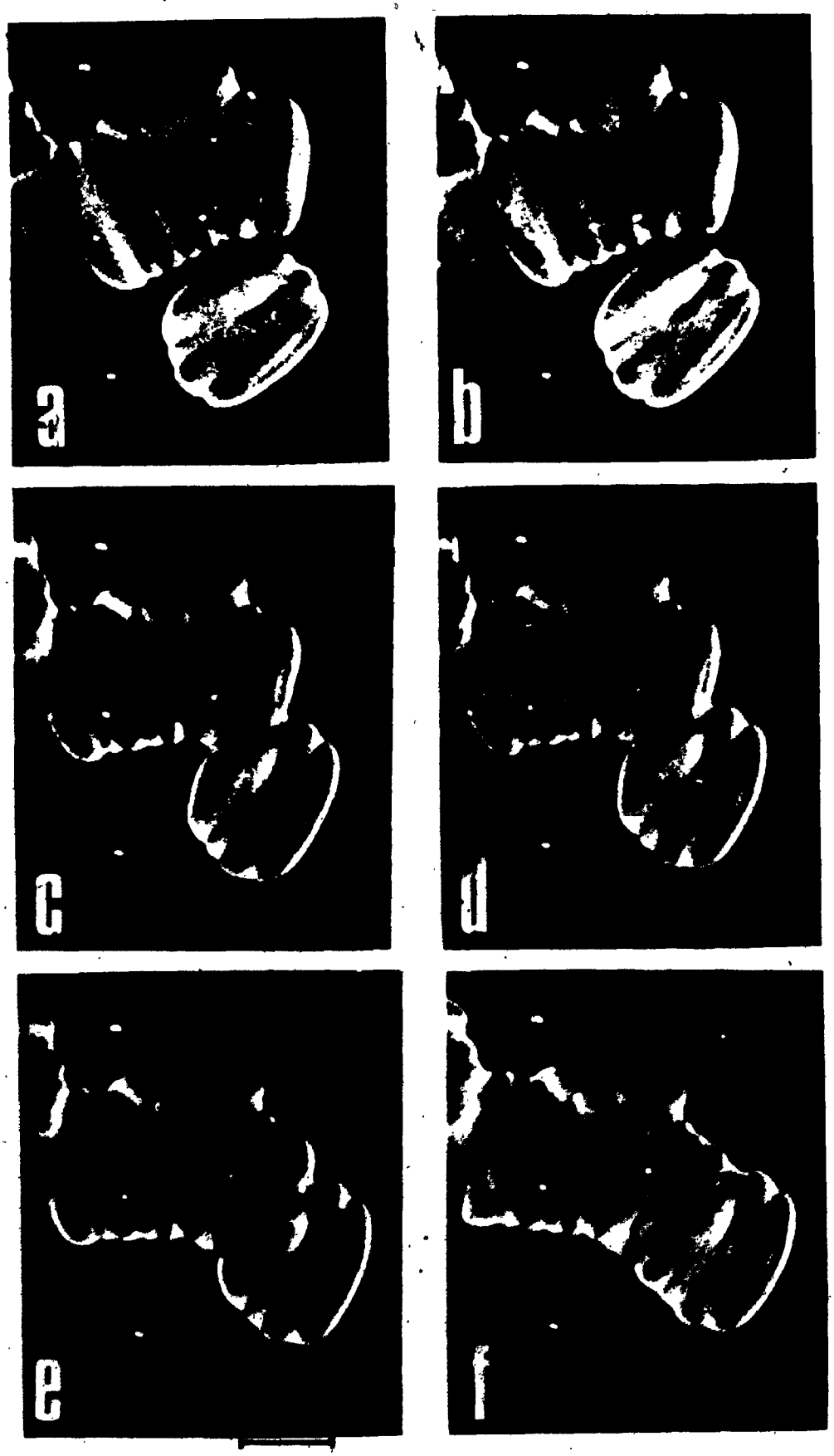
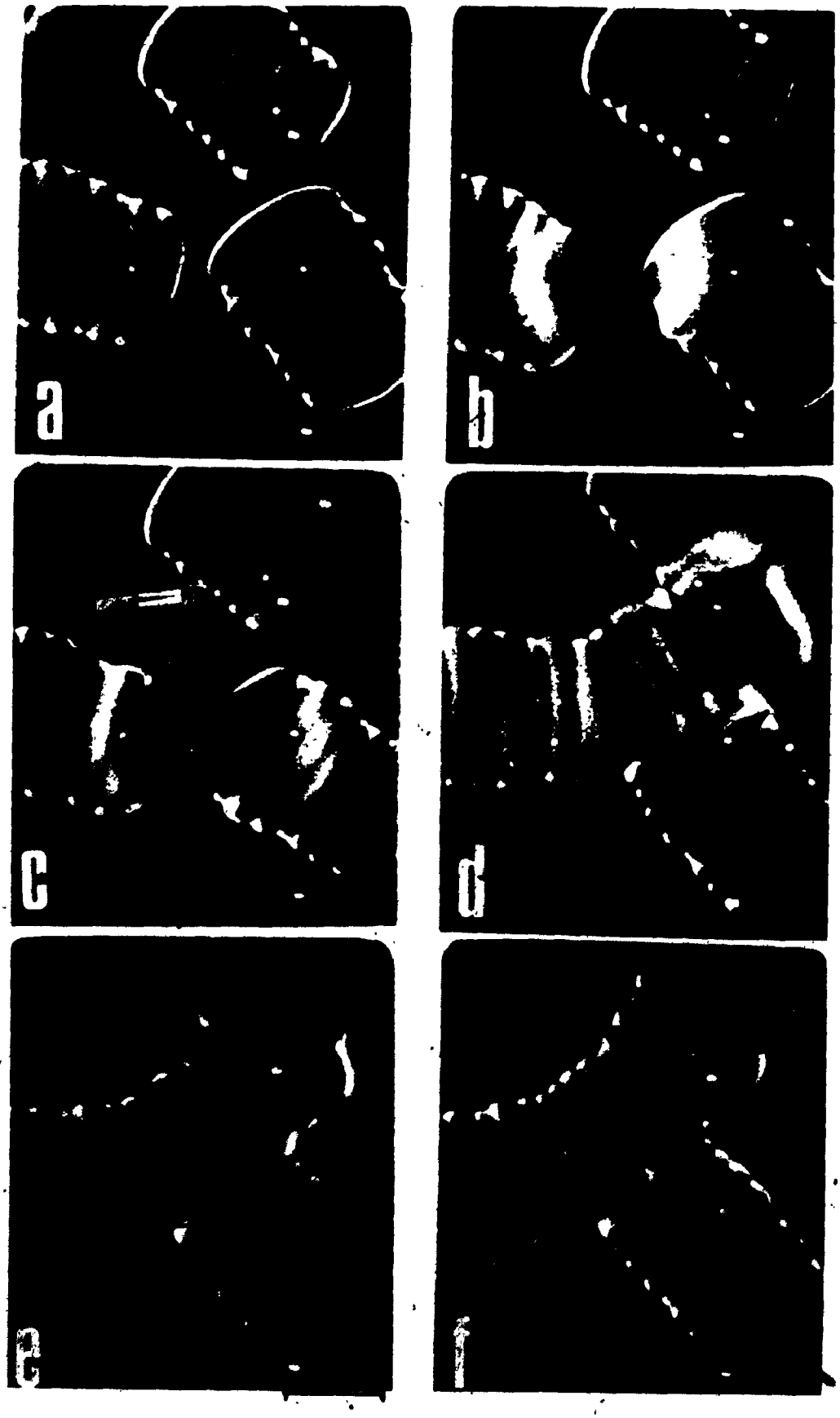

a
b
c
d
e
f

FIGURE 32

Formation of a branched rouleau, where a single sedimenting rouleau joins three separate rouleaux resting on the coverslip.

Note how the third rouleau (shown by the arrow) rotates through an angle of nearly 90° to align itself neatly with the rest of the branched structure.

(The scale represents 5μm).


a
b
c
d
e
f

rouleau, denoted by the arrow, rotates through an angle of nearly 90° to align itself neatly with the rest of the branched structure. This is further evidence to show that the whole process of rouleaux formation involves a minimization of the red cell surface area exposed to the plasma-like environment.

6.4. Discussion

The modes of rouleaux formation in Dx-70, Dx-110 and PVP-360 solutions have been filmed and analysed. The striking difference between Dx and PVP as rouleaux-inducing polymers is that rouleaux formation of human red cells is not induced in Dx solutions at concentrations beyond 70g/l while it occurs in any PVP-360 solution above 1.0g/l concentration (chapter 4). Cresting doublet formation is predominant in the Dx solutions at a concentration of approximately 40g/l while sliding is the main mode at lower or higher Dx concentrations. In the PVP-360 solution, the transition from sliding to cresting occurs at a concentration of about 5g/l, but the cresting mode persists at higher concentrations. The difference encountered in the sliding modes of doublet formation for red cells suspended in Dx and PVP solutions is perhaps due to some difference in the structural property of the two types of polymer; a relatively rigid Dx molecule might account for the slow rate of sliding. Another reason might be due to possible differences in the adsorption of

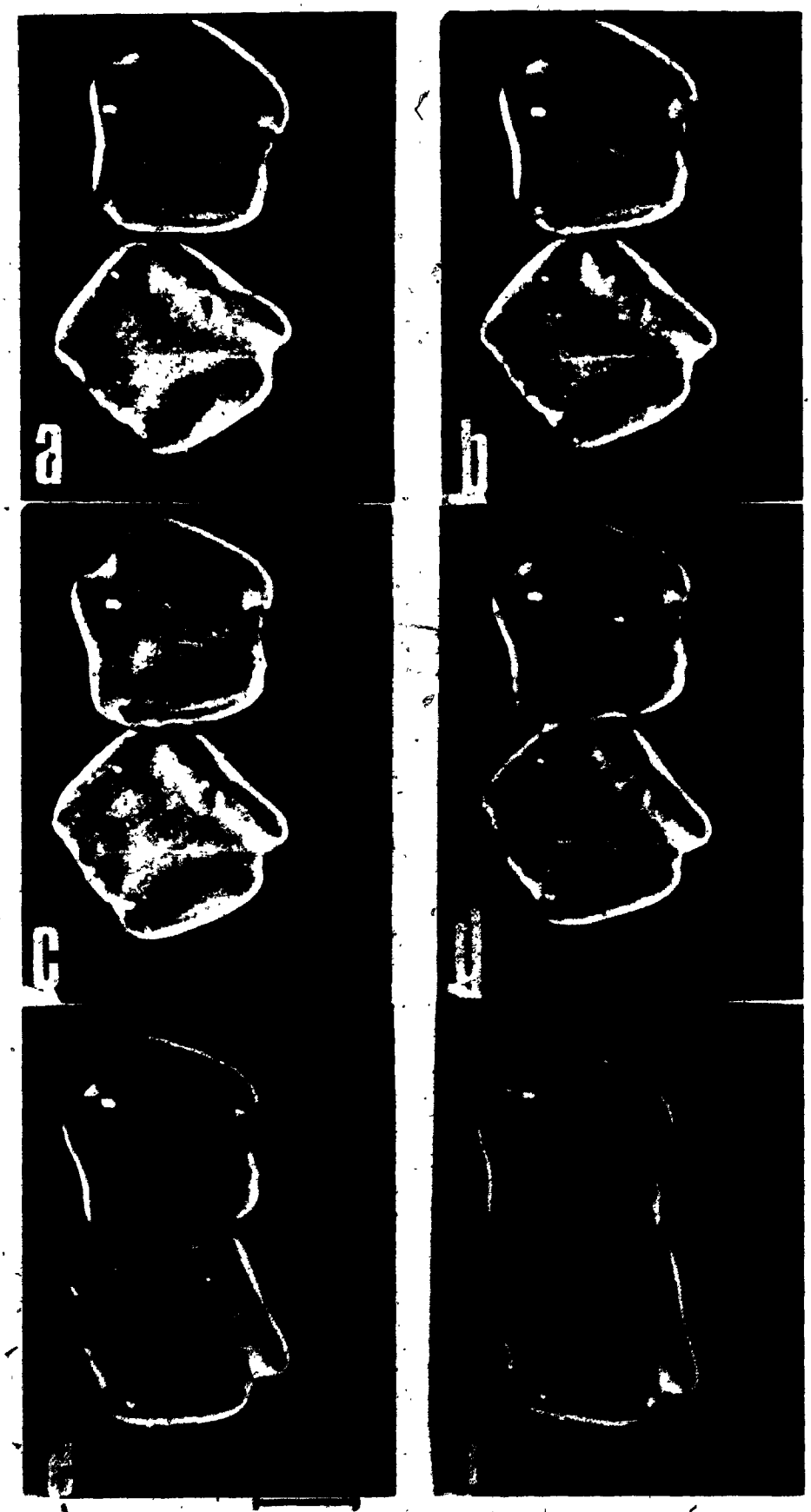
the two types of polymer to the cell surface. So far, the nature of the adsorption of polymers still eludes investigation (Bruck, 1974, p. 34).

Consistent with the findings of Thorsén and Hint (1950) it was found that aggregation or rouleaux formation only occurred above critical concentrations of the colloid solutions. Concentrations of 5.0g/l and 1.0g/l were critical for inducing rouleaux formation for the Dx and PVP solutions respectively. At concentrations slightly above the critical levels, cells coming in contact with one another move into short, loosely constructed rouleaux. At higher colloid concentrations the cells seemed to be more firmly attached to each other in rouleaux with apparent increase in their diameter and decrease in their thickness. The formation of film in colloid solutions at phase boundaries between the red cell surface and colloid medium has been reported (Thorsén and Hint, 1950; Hummel, 1963; Brooks and Seaman, 1973; Scherer, Morarescu and Ruhenstroth-Bauer, 1976). This coating of the red cell surface was also evident in our observations of red cells suspended in high concentrations of PVP. Figure 33 shows two rouleaux sliding to form a single rouleau in a PVP solution of concentration 10g/l. As seen in this photomicrograph, the outlines of the cells in rouleaux are hardly visible; it is believed that this is due to the thickness of the surface film which overlaps in the peripheral region between the cells. This photomicrograph

FIGURE 33

The sliding of two rouleaux in a PVP-Ringer solution of relatively high concentration (10g/l). The outlines of the cells are hardly visible, possibly because of the coating of adsorbed molecules.

(The scale represents 5µm).


a
b
c
d

might also serve as good evidence for disproving the contention of Fung and Canham (1974) and Dixon (1975, Chap. 3) that friction, resulting from the increased viscosity of the medium, prevents the cells from sliding over each other.

The modes of rouleaux formation observed in this study are the same as those observed for red cells suspended in their own plasma (Rowlands and Skibo, 1972; Fung, 1971; Fung and Canham, 1974). Fung studied the modes of doublet formation in normal plasma and in plasma from patients with elevated ESR. Sliding was reported to be the principal mode of doublet formation in normal plasma, while cresting made up the main mode for the "high ESR" plasma. In the study of Rowlands and Skibo, single cells were ejected from micropipettes onto rouleaux and their modes of attachment to the rouleaux were observed. In addition, the attachment of multicellular rouleaux to other multicellular rouleaux was observed in their study where they reported sliding and "penknife-closure" (clapping) as being the modes of formation.

In summary, the modes of rouleaux formation were studied for human cells suspended in varying concentrations of PVP-360 and various Dx solutions. The modes of doublet formation and triplet formation were found to be dependent on the macromolecular concentration; the modes of formation of longer rouleaux were independent of the concentration but dependent on the orientation of the cell or rouleaux. In Dx solutions, sliding in doublet format-

tion or triplet formation was not as smooth or uniform as that for PVP solutions or plasma (Fung and Canham, 1974). This fact coupled with the finding that high Dx concentrations do not induce rouleaux formation of human red cells (chapter 4) perhaps suggests that PVP is a much better substitute of the plasma proteins in model studies of rouleaux formation. The "tank-tread" model of rouleaux formation has been discounted, and a new model has been proposed for explaining the transition from sliding to cresting in doublet formation. This new model also explains why complete overlap of the cells is seldom observed. Finally, the column-like arrangement of the cells in rouleaux formation suggests that the process of rouleaux formation is directed towards reduction to a minimum of the surface area exposed to the medium as if there is a surface energy associated with the cells in contact.

CHAPTER 7

THREE PARAMETERS IN THE STUDY OF RED-CELL ADHESION: AVERAGE RATE OF SLIDING, ADHESION LENGTH RATIO, AND COMPRESSION TIME

7.1. Introduction

The parameter, average rate of sliding, was introduced for the first time by Fung (1971) in his study of the modes and kinetics of red-cell doublet formation. Dixon (1975) also used this parameter in his study of the effects of pH and viscosity of the medium on red-cell adhesion. The other parameters are, however, introduced for the first time in this study.

The two new parameters can only be applied in doublet formation, and in some cases triplet formation. The reason why they are not applicable in the formation of longer rouleaux will be obvious after reading the discussions in sections 7.2 and 7.3.

The study of the formation of doublets, the simplest of rouleaux, perhaps provides the best possible method for understanding the mechanism of rouleaux formation. Its study affords the opportunity for one to examine closely

the actual cell-to-cell interactions and make measurements which relate the effects of changes in the surrounding medium and changes of the intrinsic cellular properties on the cell-to-cell interactions.

7.2. The Three Parameters

A. Average Rate of Sliding

The mechanics of sliding are briefly discussed in section 6.3 and more extensively discussed by Fung (1971) and Fung and Canham (1974). As seen in figure 34, a plot of the intercentral distance of the cells (in the sliding mode of doublet formation) as a function of time yields a sigmoid curve. Three distinct phases exist. First, there is a compression phase in which the membranes are flattened against each other in the area of contact; secondly, there is a plateau phase lasting a variable period of time in which no apparent movement between the cells occurs; and lastly, there is the sliding phase where one cell slides over the other.

Fung (1971) used the average rate of sliding as a measure of the attractive force between the adhering cells. The average rate of sliding was calculated by dividing the total distance travelled from the start of sliding to the finish, by the time taken during sliding, i.e. - $(S_f-S_i)/(t_f-t_i)$ in figure 35. The difficulty in determining t_f and t_i was accommodated by using the defined value of 0.05μm/sec (the approximate error in the determination

FIGURE 34

A plot of the intercentral distance of the cells as a function of time in the sliding mode of PVP-induced doublet formation. The sliding event is divided into three phases: compression, plateau, and sliding.

This figure is the same as figure 20B.

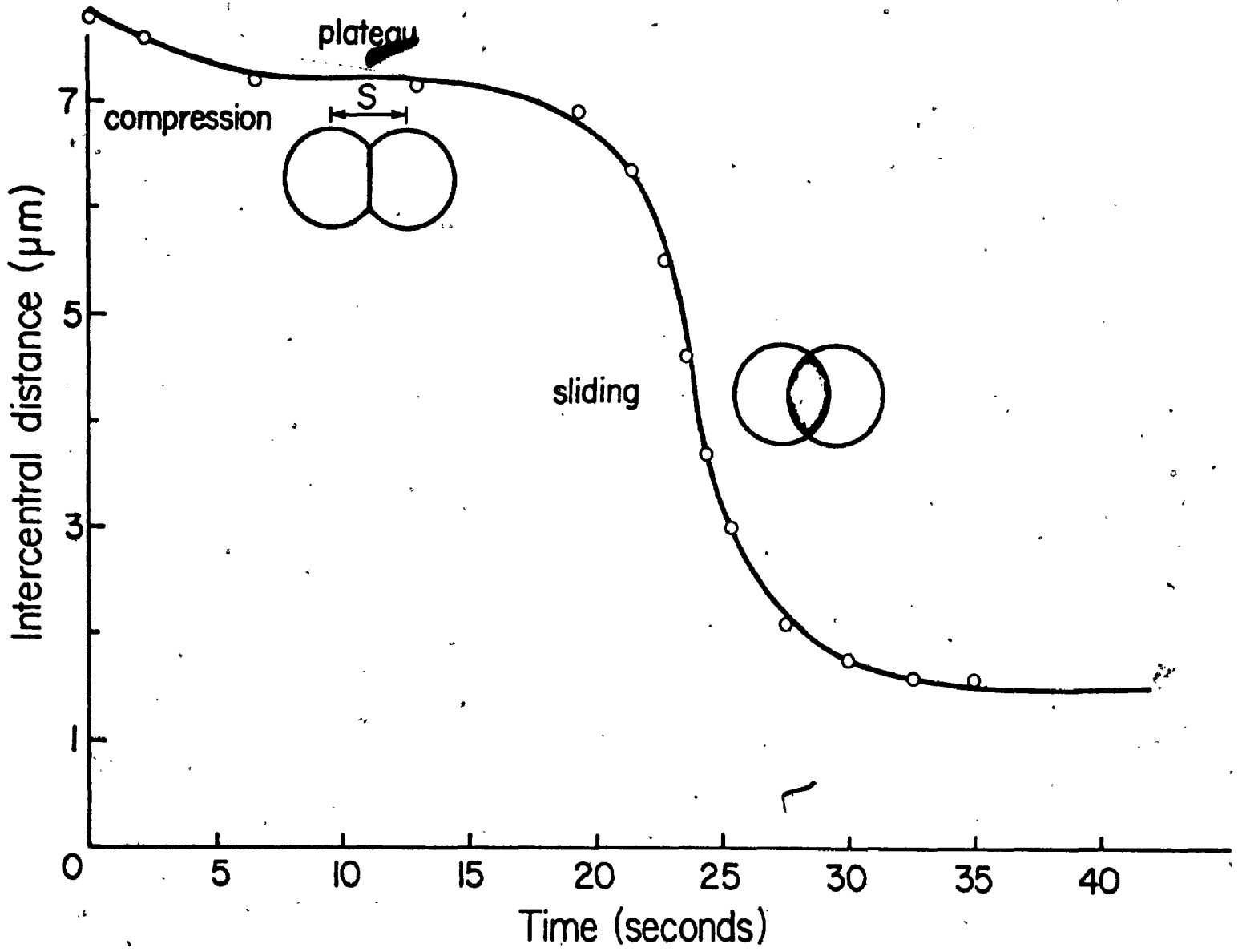
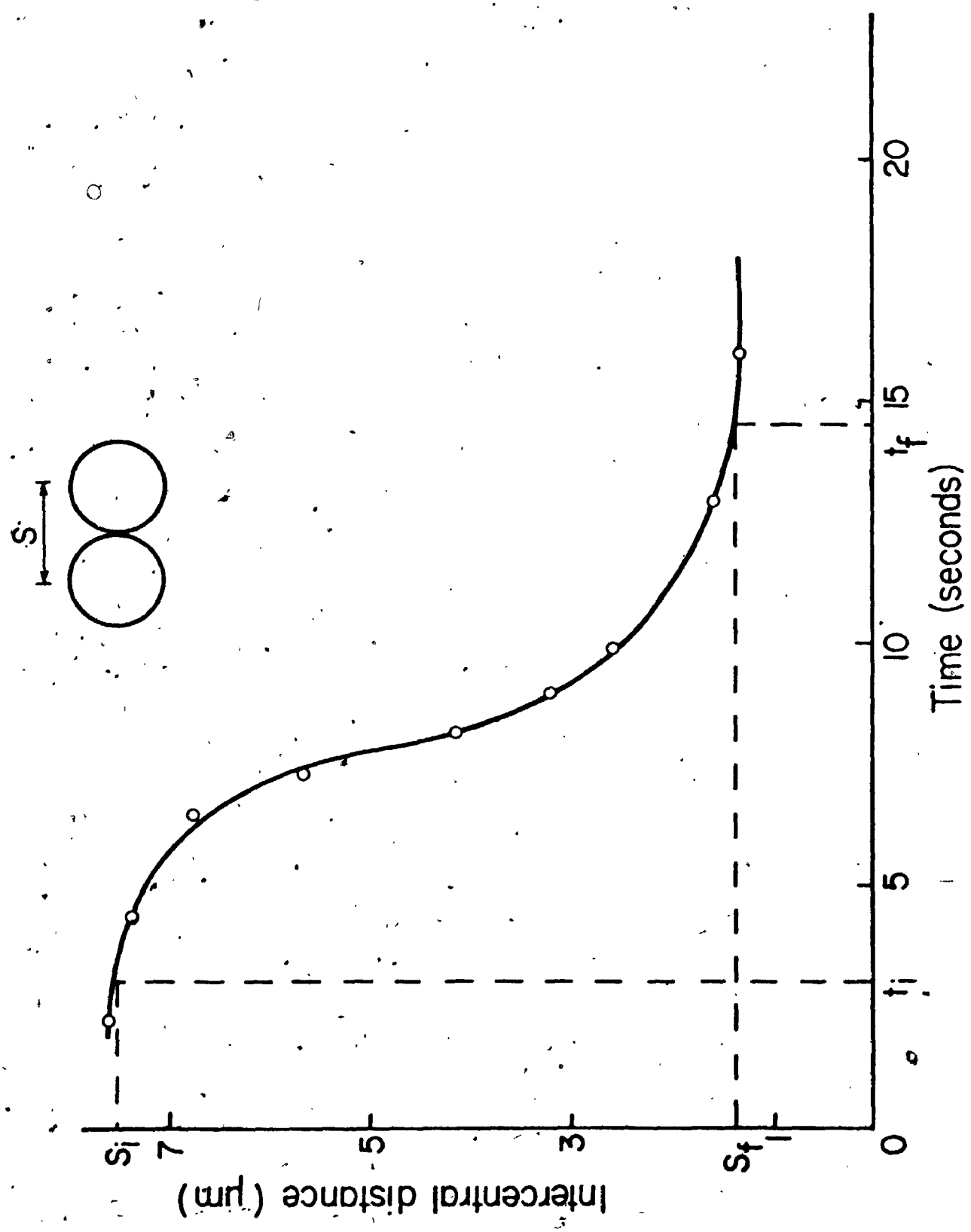

plateau
compression
S
sliding
Intercentral distance (μm)
7
5
3
1
0
5
10
15
20
25
30
35
40
Time (seconds)

FIGURE 35

Illustration of the determination of the average velocity of sliding.

(S_i, t_i) and (S_f, t_f) are points on the graph where the tangent of the slope has a value of 0.05μm/S. The average rate of sliding was defined as $-\left[\frac{S_f - S_i}{t_f - t_i}\right]$ by Fung (1971), and this definition is used in this study.


Intercentral distance (μm)
Time (seconds)
S
S_i
S_f
t_i
t_f
0
5
10
15
20
7
5
3
1

of the slope) in place of zero slope. Fung's definition of the average rate of sliding was also used in this study.

B. Adhesion Length Ratio

This measurement came about as a result of the presence of a plateau phase in the sliding mode of doublet formation. The plateau phase lasts a variable period of time and is believed to represent a metastable equilibrium between the adhesive force of the cells and the resisting forces due to membrane deformation. This equilibrium is perhaps disturbed by Brownian motion in the system before the cells go into their sliding motion; when the equilibrium is broken the cells "pop" out of the compression like the release of compressed springs.

As seen in figure 36, the contact area, A, in the plateau phase is proportional to LL_1 where L is the contact length for the cells seen flat, and L_1 is the contact length if the cells were viewed from the side. Assuming that the plateau phase represents a metastable equilibrium between the adhesive force (F_a) of the cells and the resisting membrane forces (F_d), the contact area, A, is then a measure or index of the adhesive force between the cells; it is also an index of the membrane deformability.

For equally deformable cells (F_d contant), changes in the contact area, measured in the plateau phase, indicate changes in the adhesive force between the cells, that is,

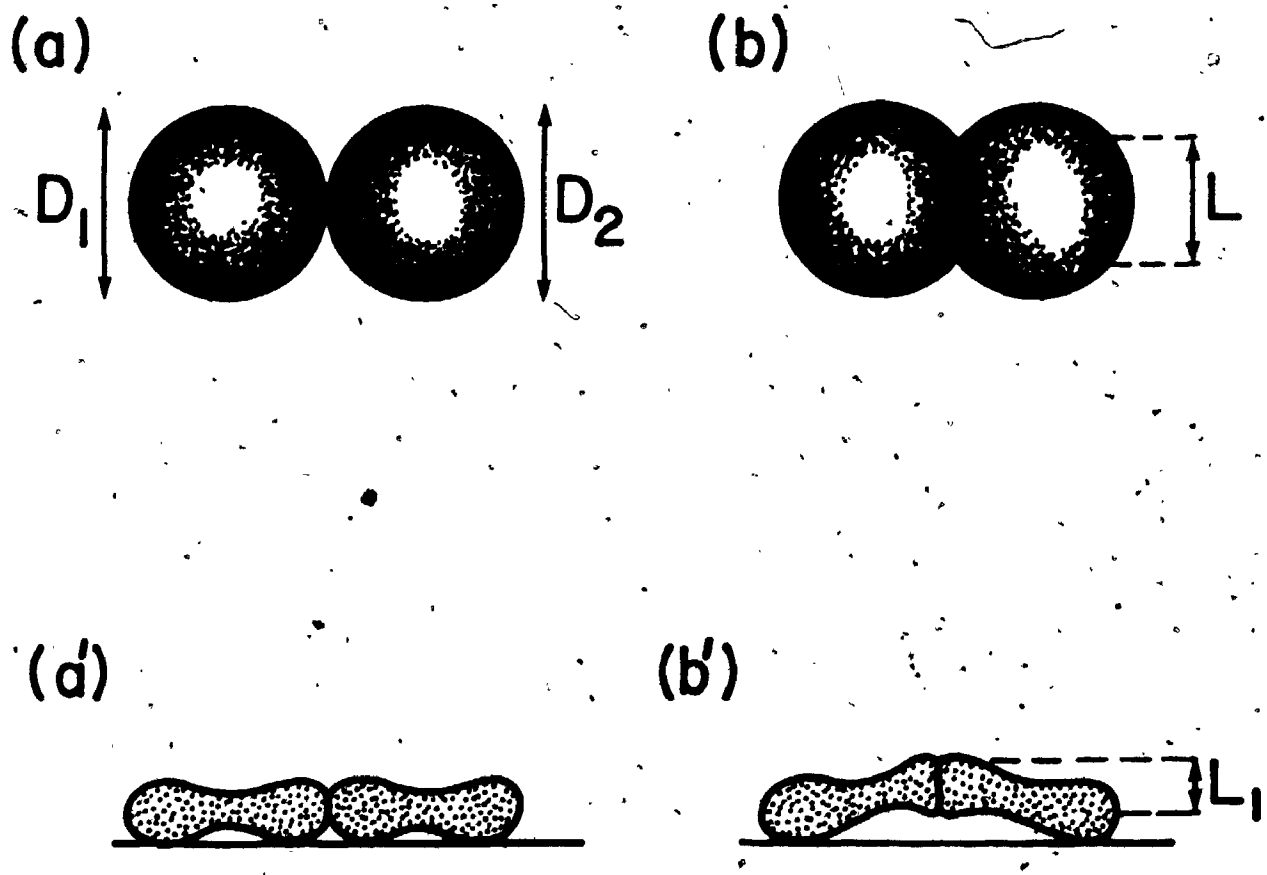
$$A\uparrow\downarrow \Rightarrow F_a\uparrow\downarrow, \quad (\uparrow \text{increase}; \downarrow \text{decrease}).$$

FIGURE 36

Determination of adhesion length ratio and compression time.

Adhesion length ratio is defined as $\frac{2L}{D_1+D_2}$.

Compression time is defined as the time between initial contact (a) and the beginning of sliding (b).


(a)
D1
D2
(b)
L
(d')
(b')
L1

Similarly, if the adhesive force (F_a) is kept constant, changes in the contact area will indicate changes in membrane deformability, that is,

$A \uparrow\downarrow \Rightarrow \uparrow\downarrow$ cell deformability.

In this study the cells could only be viewed flat in the process of doublet formation and this made the parameter, L_1, physically impossible to measure. It was not unreasonable, nonetheless, to assume L_1 constant because from the studies of Jay (1975) the thickness of individual cells in a population is one of the least varying measurements. The parameter, L, was therefore used to indicate changes in the contact area, A.

The diameter of the cells in a population varies between 7μm and 9μm and this meant that the parameter, L, would be a function of the size of the cells (figure 37). The dependence of L on the size of the cells therefore necessitated the use of the dimensionless ratio, $2L/(D_1+D_2)$, which is termed the adhesion length ratio. $(D_1+D_2)/2$ is the mean diameter of the adhering cells, measured before contact was made. The adhesion length ratio is independent of the size of the cells (figure 38). This new parameter was then used as an index of the adhesive force between the cells, and also an index of the cellular deformability.

C. Compression Time

This parameter is defined as the time between initial

FIGURE 37

The relation between maximum adhesion length (L) and the mean initial cell diameter $\left(\frac{D_1+D_2}{2}\right)$. (The regression line is L = 0.58D-0.16, r = 0.82).

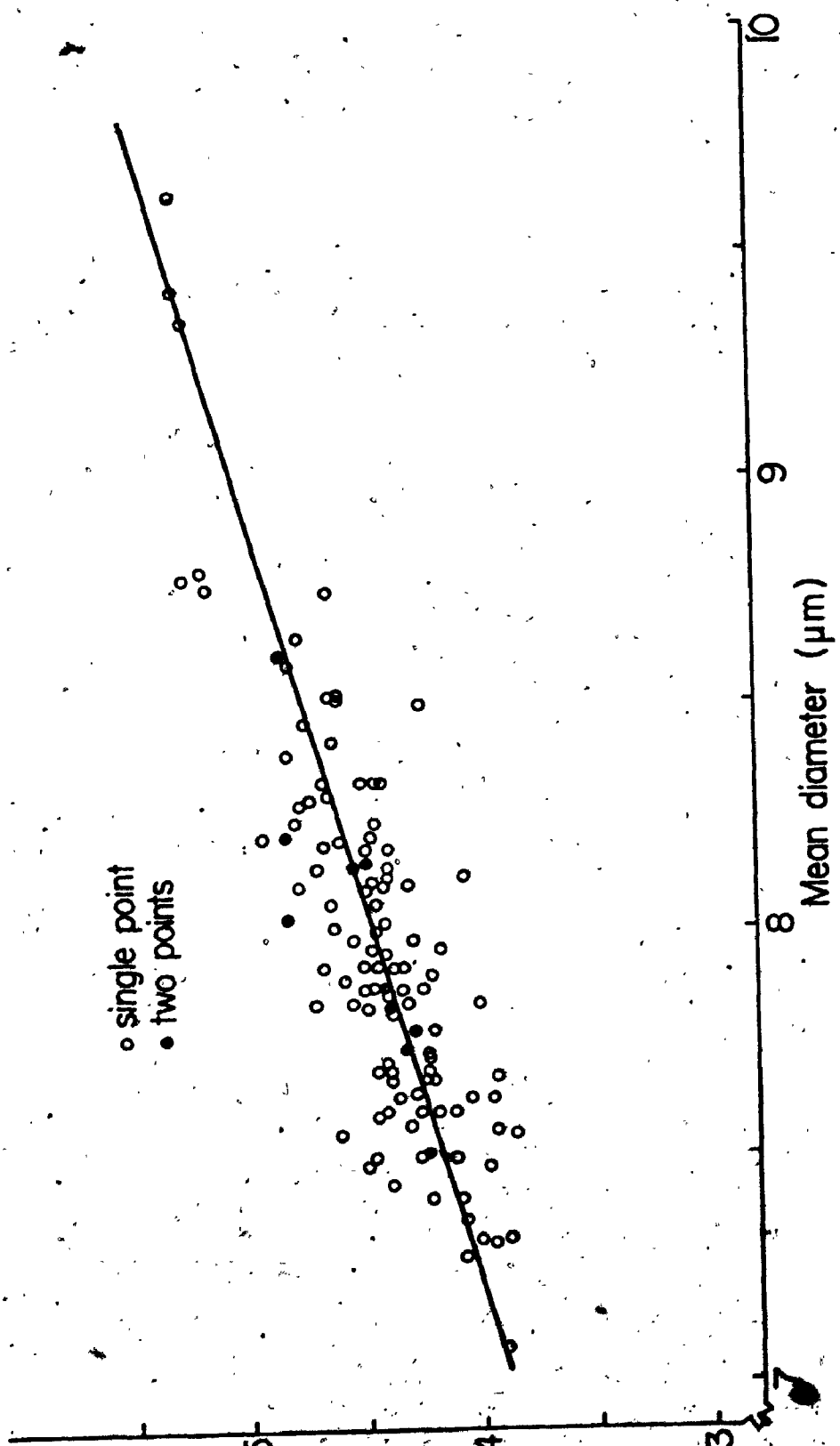
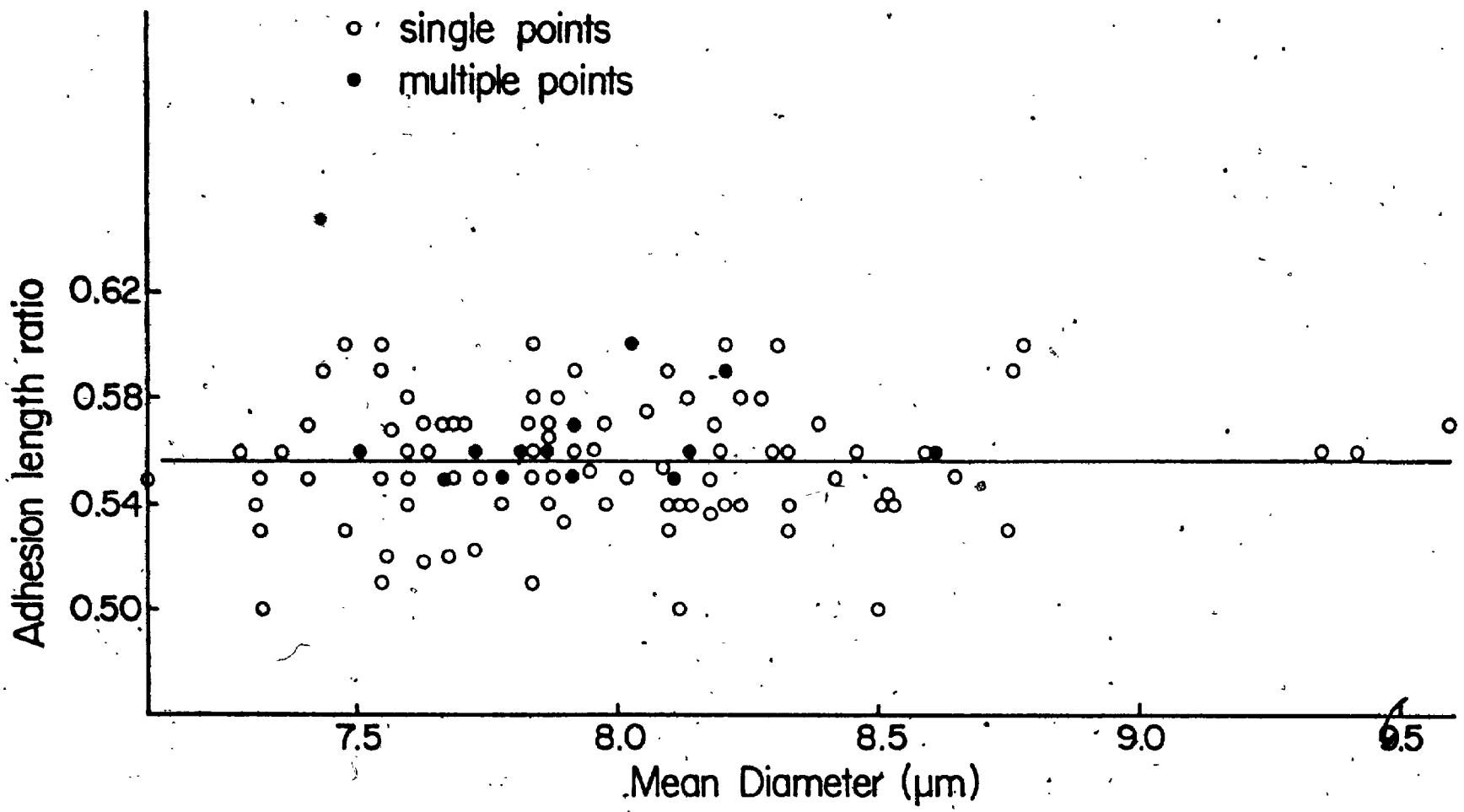

Adhesion length (μm)
5
4
3
Mean diameter (μm)
7
8
9
10
single point
two points

FIGURE 38

The relation between the adhesion length ratio and mean initial cell diameter. The adhesion length ratio is independent of the size of the cells.


single points
multiple points
Adhesion length ratio
0.62
0.58
0.54
0.50
7.5
8.0
8.5
9.0
9.5
Mean Diameter (μm)

contact of the adhering cells and the onset of sliding. (Note that it includes the time of the plateau phase, as well as the compression phase).

7.3 Discussion

The advantage of the non-flowing chamber used in this study is that the factors affecting red cell adhesion are restricted to the macromolecular bridging force, the electrostatic repulsive force between the cells, and the resisting force of membrane deformation. In a flowing medium one has to consider the additional factors of the mechanical shearing force and the increased probability of cell contact.

Chien (1973), in considering the force balance at the surfaces of aggregating cells, postulated that the net force of aggregation, in the absence of bulk fluid motion, is given by the difference between the bridging force of the macromolecules (adsorption force) and the electrostatic repulsive force between the cells. This postulate is applicable in this study when considering the plateau phase and the measurement of the adhesion length ratio. The adhesive force, F_a, between the cells in this study is synonymous with the force of aggregation to which Chien (1973) had referred. For the adhering cells in doublet formation, the adhesive force (F_a) is given by

$$F_a = F_b - F_e \qquad (1)$$

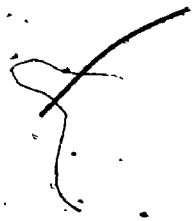
where F_b is the bridging force of the macromolecules and F_e is the electrostatic repulsive force between the cells. As the cells make contact and compress, the adhesive force would be counteracted by the resisting force (a combination of the elastic and viscous forces) of membrane deformation until the membranes are compressed or flattened enough (beginning of plateau phase) when the adhesive force is completely counterbalanced. F_a can be varied by varying F_b or F_e or both simultaneously. The results of changes in F_a, quantitatively measured by the adhesion length ratio, are reported in the next chapter.

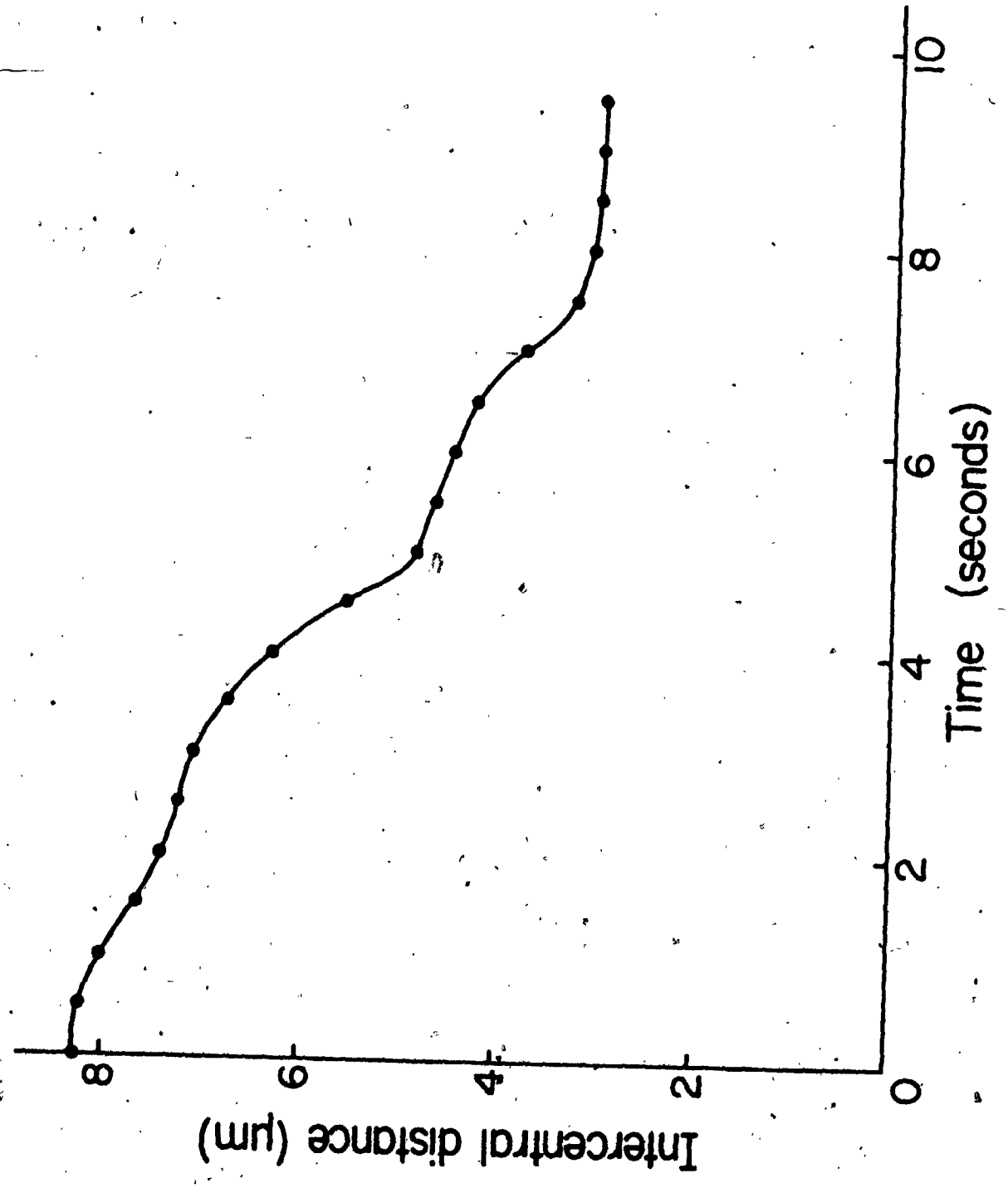
Measurements of the adhesion length ratio were not possible in this study for doublets formed by the non-sliding modes. The reason for this is that, in the non-sliding mode, the end of the plateau phase was not distinguishable from the onset of the final phase of the doublet formation. Figure 39 shows the kinetics of a typical cresting event. Recall from figure 34 that, in the sliding mode of doublet formation, the sliding phase is easily discernible from the plateau phase.

The adhesive force or the driving force responsible for the sliding of one cell over the other is different and more complicated to compute than the adhesive force considered in the compression and plateau phases of doublet formation. The difficulty arises from the existence of two additional forces which oppose motion. These two forces are a) the hydrodynamic drag force exerted by the

FIGURE 39

The kinetics of a typical cresting event (cells suspended in 5.0g/l PVP-Ringer).




Interkentral distance (μm)
8
6
4
2
0
2
4
6
8
10
Time (seconds)

fluid medium on the moving cell, and b) the frictional force arising in the area of contact between the cells. The force in a) can be treated approximately by assuming a thin disc for the moving cell (Lamb, 1945; p. 605). The force in b) cannot be treated directly because this requires a knowledge of the nature of adsorption, and the conformation of the adsorbed molecules which we do not possess. The thixotropic viscosity of the medium between the sliding cells is dependent on the specialized nature of the adsorbed molecules.

It is not the purpose of this work, however, to deal with these forces (during sliding) in any detail. The average rate of sliding is an index of the adhesive force of the cells in the sliding phase of doublet formation, and this parameter was used in this study for comparison purposes.

The parameter, compression time, seemed to be a variable with a considerable range. It was recorded in order to test the hypothesis that the onset of sliding was induced randomly, or alternatively, by changes in the cells, or medium between the cells.

The results of measurments of these three parameters are reported and discussed in the next chapter.

CHAPTER 8

EFFECTS OF SEVERAL FACTORS ON RED CELL ADHESION: PVP CONCENTRATION, REDUCED SURFACE CHARGE, NO-CALCIUM MEDIUM, pH OF THE MEDIUM AND CELL AGE

8.1. Method

The preparation of cell suspensions and observations in this study are the same as described in chapter 2. Only normal, healthy human cells were studied. Observations of cells forming doublets were recorded on 16-mm cinefilm which was analysed using an L-W Photo Analyser, and measurements of the three parameters, average rate of sliding, adhesion length ratio and compression time, were made as discussed in chapter 7.

In preparation of the no-calcium medium the osmolarity of the medium was maintained by adding an equivalent amount of choline chloride to compensate for the absence of calcium chloride. There was no scientific reason for using choline chloride; it was realized afterwards that increasing the amount of sodium chloride in the Ringer's solution would have done the same job. Nevertheless, there is no evidence that choline affects the membrane in any

way.

In the pH study, the pH of the Ringer solution was varied by altering the relative proportions of HCl solution and THAM solution in the buffer. The pH's of the resulting solutions were found to remain constant within 0.1 pH unit.

The effects of cell age on red cell adhesion were studied from 16-mm films of Dixon (1975). Dixon (1975, Chap. 5) had studied the effect of cell age on the average rate of sliding in, and the modes of, doublet formation. Young cells were separated from old cells according to density by centrifugation (Westermann, Pierce and Jensen, 1963). Red cell density increases linearly with cell age (Danon and Marikovsky, 1964)). The top 10% of the packed cell column was taken as the young cell sample; the bottom 10% was taken as the old cell sample. As a check on the separation procedure, Dixon (1975) did reticulocyte counts on both the young and old cell samples and found that satisfactory separation of the cells had taken place. Only 8% of the young cell sample were found to be reticulocytes and, as expected, no reticulocytes were observed in the old cell sample.

8.2. Results and Discussion

A. PVP Concentration

Fung (1971) found that there was a general increase in the rate of sliding in doublet formation as a function

of PVP concentration. The mean rate of sliding increased from 0.25μm/sec±0.03 (S.E.M.) for a PVP concentration of 2g/l to 0.48μm/sec±0.02 (S.E.M.) for a PVP concentration of 4g/l. The mean cresting velocity increased from 0.43μm/sec±0.01 (S.E.M.) to 1.30μm/sec±0.13 (S.E.M.) for a corresponding PVP concentration increase of 4g/l to 7g/l. The increase in the sliding velocity with PVP concentration was not linear but that of the cresting velocity was. This finding emphasized the importance of the "friction" between the membranes in the sliding phase of doublet formation.

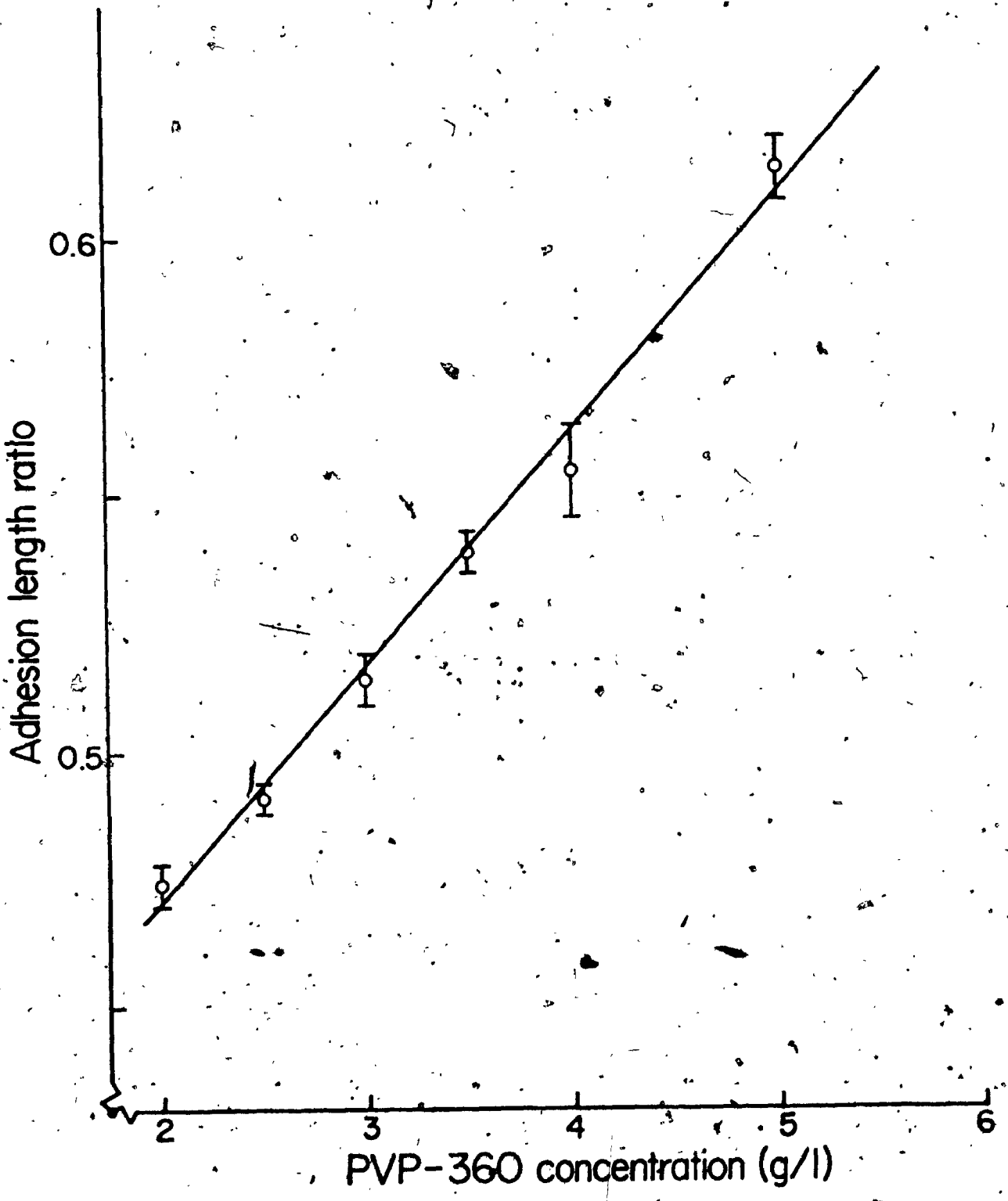
In this study the average rate of sliding was calculated for a PVP-360 concentration of 4g/l and found to be 0.41μm/sec±0.03 (S.E.M.). This value agrees well with the value obtained by Fung (1971) for the corresponding PVP concentration.

Measurements of adhesion length ratio and compression time of the cells suspended in the varying PVP-360 solutions were made in this study as discussed in chapter 7. Figure 40 shows the effect of increased PVP concentration on the adhesion length ratio. Beyond a PVP concentration of 5g/l measurements of adhesion length ratio were not done because more than 70% of the doublets were formed by the non-sliding modes (see Table IV). The adhesion length ratio was found to be proportional to the PVP concentration. Since there is no significant change in the surface potential of the cells over this PVP concentration range

FIGURE 40

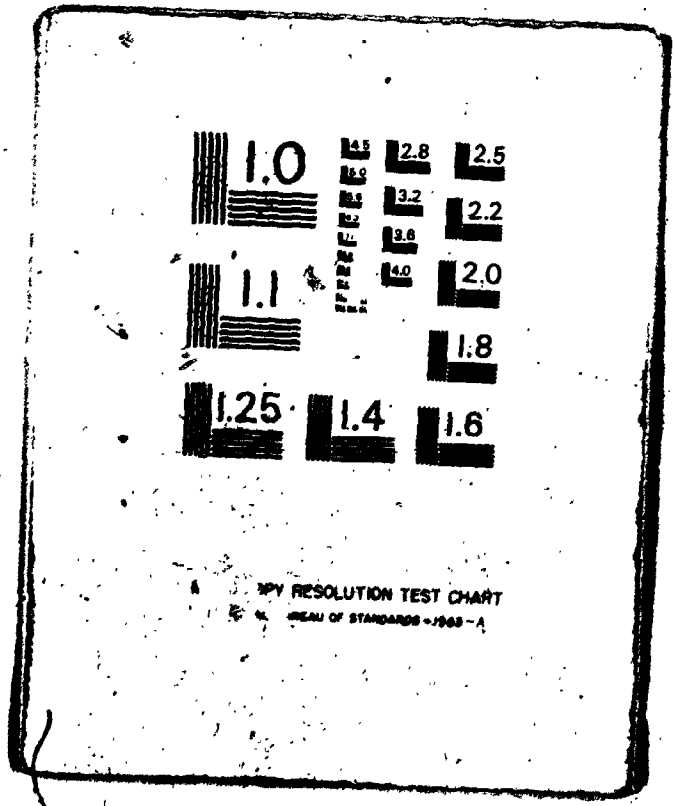
Effect of increased PVP-360 concentration on the adhesion length ratio of normal human cells. (The regression line is: adhesion length ratio = 0.05 x (PVP concentration, g/l) + 0.38, r = 0.99).

(The error bars are S.E.M.).


Adhesion length ratio
0.6
0.5
2
3
4
5
6
PVP-360 concentration (g/l)

3 3

OF/DE


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
MPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

(Castaneda et al., 1965) then, from equation 1 of chapter 7, the adhesive force (F_a) between the cells is proportional to the briding force of the PVP molecules. That is, F_a is a direct function of the number of adsorbed PVP molecules. Hummel and Szczepanski (1963), using radioactively labelled PVP, found that the amount of PVP molecules adsorbed to the red cell membrane is directly proportional to the PVP concentration, at least over the PVP concentration range used in this study. The linear relationship in figure 40 is therefore suggestive that the adhesion length ratio is a reliable measure of the adhesive force (F_a) or net attractive force between the cells.

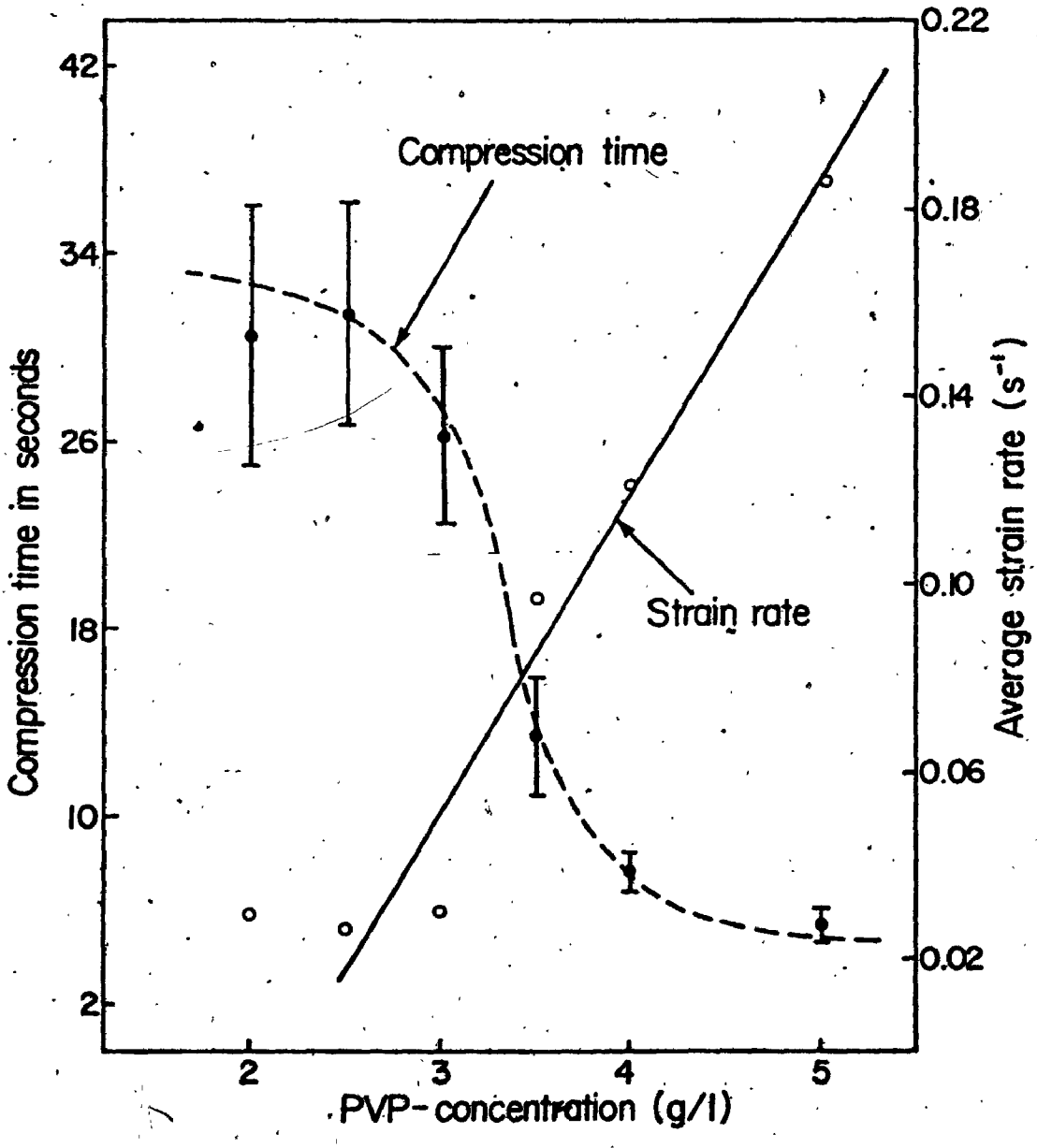
There is no direct evidence that the flexibility of the cells changes with the adsorption of the PVP molecules. Rampling and Sirs (1972) recently suggested that the flexibility of human red cells increases with increased adsorption of the plasma proteins. However, their method of measuring flexibility is indirect, and possibly not reliable; they measured flexibility as a function of the rate of packing of the red cells at low centrifugal forces (~200g). The rate of packing of the red cells is not only dependent on their flexibility but also depends on the interaction of the cells in the presence of rouleaux-inducing agents. As shown in figure 41 the strain rate of the cells (defined as the ratio: Adhesion length ratio/Compression time) increases linearly with the

FIGURE 41

Compression time and strain rate of the cells as a function of PVP concentration.

Note the wide scatter in the compression time at low PVP concentrations.

(The error bars are S.E.M.).


Compression time
Strain rate
Compression time in seconds
Average strain rate (s⁻¹)
PVP-concentration (g/l)
42
34
26
18
10
2
0.22
0.18
0.14
0.10
0.06
0.02
2
3
4
5

PVP concentration at relatively high concentrations. The fact that there is an increase in the strain rate does not necessarily suggest that the flexibility of the cells increases, but that there is an increase in the interaction between the cells resulting from the increased intercellular bridging of the adsorbed PVP molecules.

Figure 41 shows how the compression time varies with the PVP concentration. Note the wide scatter in the values of compression time at low PVP concentrations. This scatter is perhaps mainly contributory to the uncertainty in the strain rate at low PVP concentrations. As a possible explanation of the scatter in the compression time, one has to consider the potential energy stored in the compressed cells during the plateau phase. The membranes of the cells are elastic (Evans and LaCelle, 1975; Evans, Waugh and Melnik, 1976) and are therefore capable of storing energy. If the potential energy is high (as in the case where the adhesion length ratio is high), any slight disturbance in the system (for example, the random Brownian motion) could upset the equilibrium of forces in the plateau phase and cause sliding to occur. On the other hand, if the potential energy is low (low PVP concentration) the equilibrium in the plateau phase would be relatively more difficult to disturb, and this should account for the observed variability in the compression time.

B. Reduced Surface Charge

The surface charge of the cells was reduced by incubating them at 37°C with Neuraminidase as explained in section 2.3. These N-treated cells were studied within one hour after preparation.

Table IV shows how the percentage of sliding events in doublet formation decreases with increased PVP concentration over the concentration range studied. A comparison of this result with that obtained for normal cells shows that there are relatively fewer sliding events in the doublet formation of N-treated cells at high PVP concentrations (5.0 and 6.0g/l). Since the net attractive force between the cells is expected to be greater for the N-treated cells, then this result is in support of the hypothesis in chapter 6 (section 6.3A) that the net attractive force between the cells is a very important parameter in determining the transition from sliding to non-sliding in doublet formation.

The average sliding velocity for the N-treated cells is significantly greater ($p < 0.01$) than that for the normal cells at the same PVP concentration (see Table V). This is perhaps due to the greater attractive or adhesive force (F_a) between the cells which is reflected by the increase in the adhesion length ratio, also shown in Table V. For the same PVP concentration, the adhesion length ratio, obtained for the N-treated cells was found to be significantly greater ($p < 0.01$) than that for normal cells. This

increase in the adhesion length ratio is interpreted to be due to an increase in the adhesive force (F_a) between the cells and not to a possible increase in the deformability of the cells after Neuraminidase treatment; the micropipette work of LaCelle (1969) and the filterability studies of Durocher, Payne and Conrad (1975) have shown that Neuraminidase treatment has no significant effect on the deformability of the cells.

The results reported above for the N-treated cells are also not due to any changes in the dimensions of the cells. The diameter of N-treated cells in Ringer is 7.92μm±0.35 (S.D.) and the dimensions of the N-treated cells in rouleaux are

Thickness = 1.93μm±0.15 (S.D.)

Diameter = 8.30μm±0.46 (S.D.)

Cellular Volume = 89μm^3±9.4 (S.D.)

Surface Area = 131μm^2±10.5 (S.D.)

which agree very well with the values for normal cells given in Table II.

C. No-Calcium Medium

The reason for using a no-calcium medium in this study is because of the role the divalent ions in the solution play in the interaction of the red cells as reported by Brooks (1973c) and Jan and Chien (1973b). According to these investigators, a reduction in the concentration of the divalent ions in the medium reduces

the screening effect on the surface charge of the cells thus resulting in an increase in the electrophoretic mobility of the cells; that is, the electrostatic repulsion between the cells increases. Jan and Chien (1973b) reported that the rate of rouleaux formation decreases for red cells suspended in solutions of reduced concentrations of divalent ions (for example Ca^{++}, Ba^{++}, Mg^{++}).

Assuming that there is in fact an increase in the electrostatit repulsive force between the cells, that is, a decrease in the adhesive force, then this will serve as added evidence for the hypothesis in section 6.3A that the net attractive force between the cells is the main determinant in explaining the transition from sliding to non-sliding. In a no-calcium medium the percentage of sliding events in doublet formation is more than 60% at a PVP concentration as high as 10g/l (Table IV), while in a normal Ringer solution it is only 25% at a PVP concentration of 6g/l.

There is no significant difference between the average rates of sliding for red cells in no-calcium medium and red cells in normal PVP-Ringer (see Table V). Assuming that the adhesiveness of the cells is indeed reduced for the cells in the no-calcium medium, then one would expect the average velocity of sliding to be significantly reduced. It is not known, however, how the increased sphericity index of the cells in no-calcium Ringer affects the sliding velocity.

A decrease in the adhesion length ratio (also shown in Table V) does indicate a possible decrease in the adhesive force between the cells. However, it will be difficult to draw conclusions from these results of the adhesion length ratio because very little is known about the effect of calcium or lack of calcium on the deformability of the cells. From the micropipette studies of LaCelle and Weed (1971) it was learned that the Ca^{++}/Mg^{++} ratio in the red cell membrane has an important influence on its elastic behaviour. An increase in the Ca^{++}/Mg^{++} ratio causes an increase in membrane rigidity as indicated by an increased resistance for the passage of the red cell through micropipettes and an elevated viscosity of the cell suspension (Weed, LaCelle and Merrill, 1969). This effect was demonstrated for red cells from blood stored at 37°C for over six hours or at 4°C for over three weeks, during which there was a depletion of the red cell ATP (adenosine triphosphate) by more than 30%. Apparently, ATP acts as a chelating agent to reduce the calcium concentration in the membrane (LaCelle and Weed, 1971). The increase in membrane rigidity after ATP depletion was reversed following the restoration of intracellular ATP by incubation with adenosine or by the intracellular introduction of the chelator EDTA (ethylenediaminetetraacetic acid) or magnesium (Weed et al., 1969). These results, and the interpretation of Weed et al. (1969) and LaCelle and Weed (1971) could, however, be criticized in view of

the recent finding by Jay and Canham (1977) that the deformability of the cell or its resistance to passage through micropipettes is dependent on the size and geometry of the cell. A thorough investigation of the distribution in size and shape of the cells was not done by Weed and his co-workers.

Weed and Chailley (1972), in a study of the production of shape change in the red cells, found that pre-incubation of red cells in a no-calcium medium rendered them less susceptible to echino-cytogenic agents. They interpreted this result as being due to a loss of calcium from the membrane. A loss of intramembrane calcium might result in a decrease of the Ca^{++}/Mg^{++} ratio. It is conceivable, therefore, that there might be an increase in the flexibility of the cell membrane, assuming that the hypothesis of Lacelle and Weed (1971) is valid. For a fixed adhesive force (that is, F_a constant), the adhesion length ratio is then expected to be larger for the cells in the no-calcium medium. The fact that there is instead a decrease in the adhesion length ratio (see Table V) suggests that the adhesive force (F_a) between the cells is not constant for the same PVP concentration, but that the possible increase in the electrostatic repulsive force (Brooks, 1973c; Jan and Chien, 1973b) causes a decrease in F_a. This interpretation however might not be valid because the cells in the no-calcium Ringer were found to have a greater sphericity index, implying that

Table IV. Effects of PVP Concentration, Reduced Surface Charge, and No-Calcium Medium on the Percentage of Sliding Events in Doublet Formation.

PVP concentration (g/l)	Normal cells	N-treated cells	Normal cells in no-calcium medium
2.0	100% (n=13)	-	-
3.0	94% (n=17)	93% (n=30)	-
4.0	96% (n=21)	80% (n=49)	95% (n=20)
5.0	92% (n=24)	57% (n=14)	100% (n=13)
6.0	25% (n=12)	13% (n=15)	80% (n=9)
7.0	-	-	86% (n=7)
10.0	-	-	62% (n=24)
30.0	-	-	0% (n=6)

Table V. Measurements of Average Rate of Sliding, Adhesion Length Ratio and Compression Time.

Cells	PVP concn. (g/l)	No. of measure-ments	Avg. Rate of Sliding ±(S.E.M.)	Adhesion Length Ratio ±(S.E.M.)	Comp. Time (seconds) ±(S.E.M.)
Normal	3.0	23	-	0.51±0.01	18.0±4.0
	4.0	20	0.41±0.06	0.56±0.01	7.9±0.7
	5.0	24	-	0.61±0.01	5.5±0.7
N-treated	3.0	17	-	0.55±0.01	10.3±2.4
	4.0	22	0.63±0.04	0.60±0.01	5.5±1.6
In no-Ca^{++} medium	4.0	13	0.39±0.06	0.52±0.01	7.9±1.8
	5.0	11	-	0.55±0.01	4.5±1.3
	6.0	6	-	0.57±0.01	3.9±1.0

the cellular deformability is decreased. The cells in the no-calcium medium were found to have a mean diameter of 7.63μm±0.37 (S.D.) which is significantly less ($p < 0.05$) than 7.96μm±0.35 (S.D.), the mean diameter of the cells in normal Ringer. The dimensions of the cells in rouleaux (for no-calcium medium) are:

Thickness = 2.26μm±0.22 (S.D.)

Diameter = 7.80μm±0.50 (S.D.)

Cellular Volume = $88.0\mu m^3 \pm 16.0$ (S.D.)

Surface Area = $120\mu m^2 \pm 14.5$ (S.D.)

With the exception for cellular volume, all the other dimensions are significantly different from the corresponding values obtained for the cells in normal Ringer (see Table II). The decrease in surface area indicates that the sphericity index (defined as the ratio $4.84V^{2/3}/A$, Canham and Burton, 1968) of the cells in the no-calcium medium increases.

D. pH of the Medium

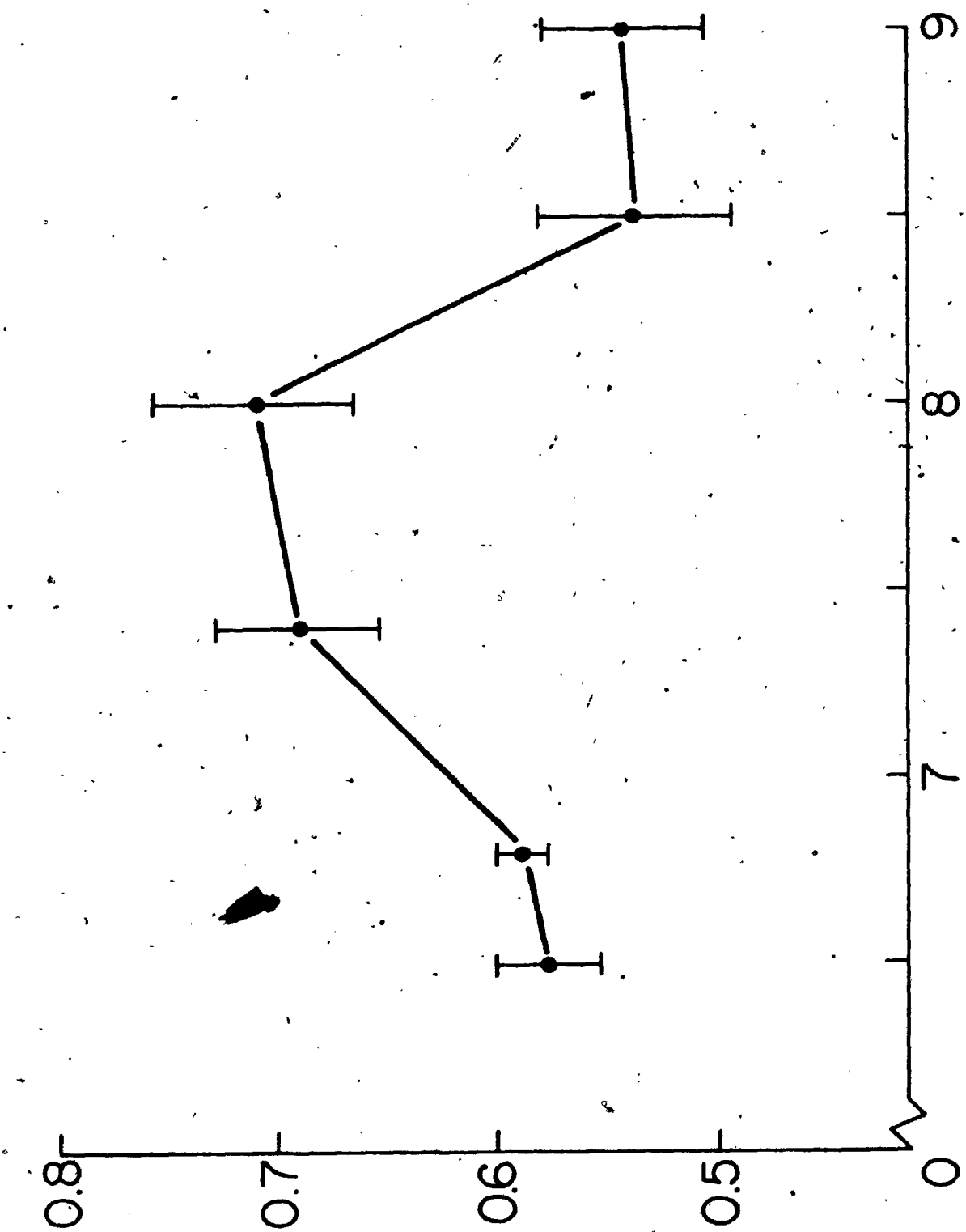
A pH range of 6.5 to 9.0 was chosen for study because the majority of red cells remain discocytes (biconcave) over this range. Below pH 6.5, over 50% of the red cells become stomatocytes (cup-shaped); above pH 9.0, over 50% of the red cells become echinocytes (crenated) (Weed and Chailley, 1972).

Figure 42 shows how the average intercellular velocity varies with the pH of the medium for red cells

FIGURE 42

Variation in the average intercellular sliding velocity with the pH of the medium (from Dixon, 1975, p. 73).

(The error bars are S.E.M.).


Average rate of sliding (μm s⁻¹)
0.8
0.7
0.6
0.5
0
6
7
8
pH of medium

suspended in PVP-Ringer of concentration 4g/l. This figure was obtained from Dixon (1975). The average sliding velocity showed a maximum between a pH of 7.4 and 8.0 which was about 25% higher than the average velocity at the extreme high and low pH's.

Table VI summarizes the results showing the effect of pH on the mode of doublet formation and the adhesion length ratio, while figure 43 represents a graphical presentation of the effect of pH on the compression time. It should be noted that the experiments carried out at the various pH's were all done for red cells suspended in PVP-Ringer at a PVP concentration of 4g/l.

The percentage of sliding events decreased slightly at high and low pH's. This indicates that the range of pH studied had little effect on the mode of doublet formation.

The adhesion length ratio was found to remain constant at 0.56 over the pH range studied. The compression time remained constant between pH 6.5 and pH 8.0, increased slightly at pH 8.2 and then markedly at pH's above 8.3.

The results of this pH study are difficult to interpret in view of the fact that the little documented evidence available on the effect of pH on cellular deformability is not conclusive, and the effect of pH on the conformation of the PVP molecules in the medium is still unknown. As a result, any interpretation and discussion

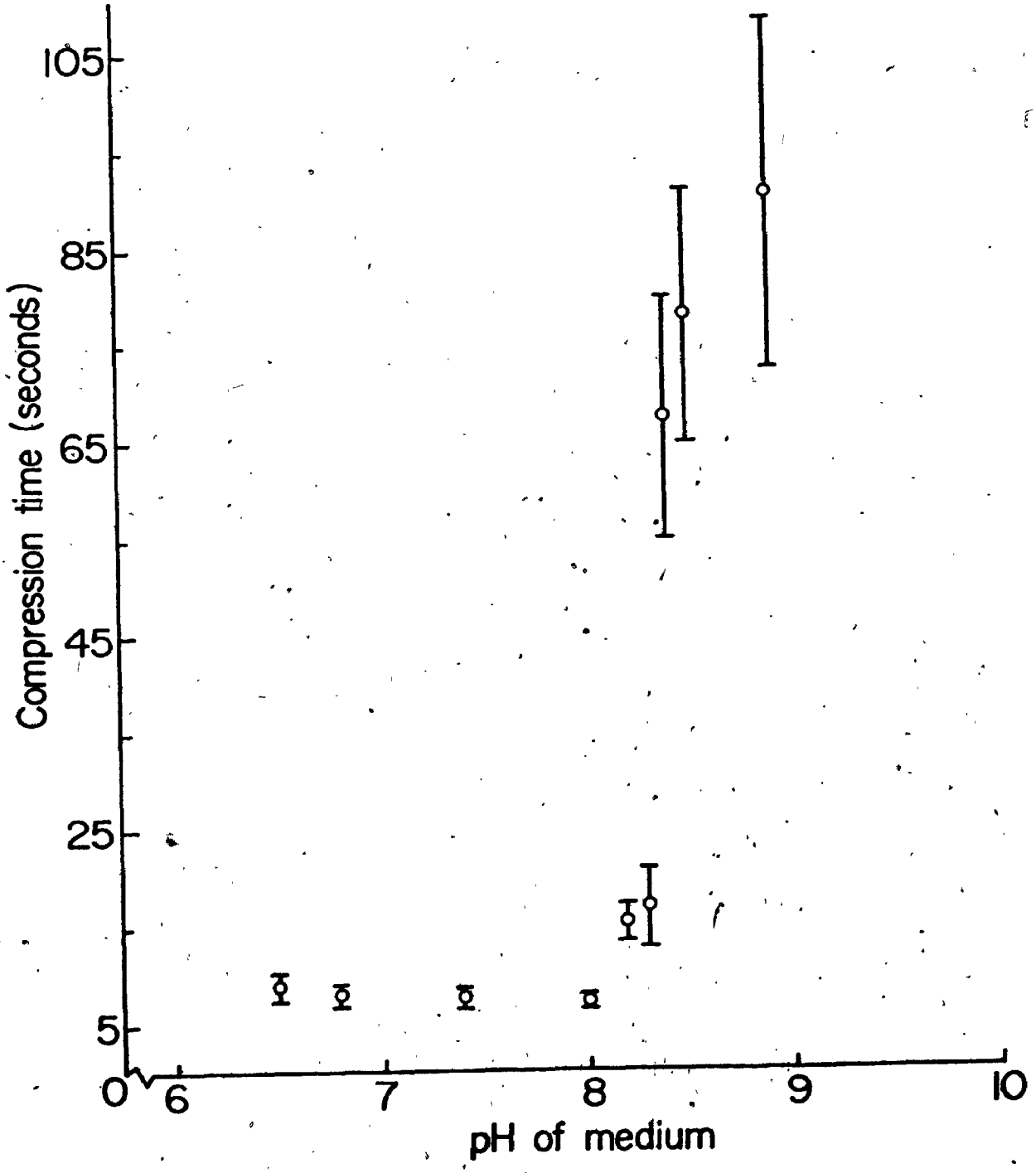
Table VI. Measurements on Human Red Cells Forming Doublets at Various pH's.

pH of Medium	Number of Observations	Percentage of Sliding Events	Adhesion Length Ratio (± S.E.M.)
6.5	21	76%	0.56±0.01
6.8	16	81%	0.56±0.01
7.4	21	96%	0.56±0.01
8.0	10	90%	0.56±0.01
8.2	29	79%	0.56±0.01
8.3	16	75%	0.56±0.01
8.4	24	75%	0.55±0.01
8.5	17	77%	0.56±0.01
8.9	12	75%	0.56±0.01

FIGURE 43

Variation of compression time with the pH of the medium.

(The error bars are S.E.M.).


105
85
65
45
25
5
0
Compression time (seconds)
6
7
8
9
10
pH of medium

of the findings in this study will be purely speculative.

Some relevant work of Leblond (1972) suggests an increased elastic stiffness and increased membrane viscosity at pH 8.5. Echinocytes produced at pH 8.5 could be drawn into a micropipette with a smaller sucking pressure (interpreted in this study to be due to reduced cell volume) but the pressure required to produce a 1μm bulge of membrane was twice that required for normal cells at pH 7.4. It is acknowledged that echinocytes are very different in shape with several large surface spicules, and that Leblond has not reported on the rate at which the pipette-induced changes occurred. Also of interest is the finding of an increased affinity of the membrane for calcium at high pH's (Forstner and Manery, 1971). Weed and Chailley (1972) postulated that the increased affinity for calcium might cause a redistribution of calcium already present within the membrane, for example, to proteins that could polymerize or change their conformation. Weed and his associates used this explanation in an attempt to account for the shape changes of the red cell that occur at high pH's. Their explanation might also suffice to account for the increased membrane rigidity at high pH's (Leblond, 1972). Weed and Chailley (1972) however conceded that their model was speculative and subject to modifications.

LaCelle (1969), using blood stored for three weeks, reported a nearly three-fold decrease in cellular deform-

ability after lowering the pH of the medium from 7.6 to 6.6. Deformability was evaluated as the negative pressure required to produce a standard hemispherical deformation of the cell membrane into the tip of a pipette of diameter 2.85μm. The validity of the results of LaCelle, however, could be questioned on the premise that only 79% of the three-week-old cells were viable (DeVerdier, Garby, Hjelm and Högman, 1964).

As discussed above, the findings and interpretation of the work of several investigators suggest, however inconclusive they might be, that there is a decrease in cellular deformability at both high and low pH's. As discussed in chapter 7 the adhesion length ratio depends on the adhesive force between the cells (resultant of the bridging force of the macromolecules and the opposing electrostatic repulsive force) and the deformability of the cells. Since the deformability of the cells has been reported to decrease at both high and low pH's then the constancy of the adhesion length ratio suggests that the adhesive force between the cells is increased at both high and low pH's. Neither surface charge density (Heard and Seaman, 1960) nor the electrophoretic mobility of the cells (Tenforde, 1970) is altered over the range of pH used in these experiments, therefore it could be assumed here that the electrostatic repulsive force (F_e) is constant between pH 6.5 and 9.0. An increase in the adhesive force then implies an increase in the bridging

force of the macromolecules (F_b). Chien (1973), in discussing the force balance at the surfaces of aggregating cells at the 7th European Conference on Microcirculation in Aberdeen, suggested that the bridging force (F_b) depends on a) the adsorption force (f) per adsorption bond, b) the number (n) of adsorption bonds per molecule, and c) the number (n_m) of bridging macromolecules per cell pair. He assumed that, because of the difference in the nature of adsorption, polybases have a stronger (f) than neutral polymers. The neutral polymers probably have larger values of (n), which increase with molecular size. The value of (n_m) depends on the concentration of the macromolecules in the bulk medium (Hummel, 1963, 1969); in this study the PVP concentration was kept constant at 4g/l. In view of the hypothesis of Chien (1973) it is conceivable that an increase and a decrease of pH might change the conformation of the PVP molecules in such a way that the bridging force (F_b) is increased. However, it must be kept in mind that this is just a speculation; there is still a need to understand the effect of pH on the molecular properties of PVP.

The red cell membrane is viscoelastic, resisting stretch as an elastic structure, and resisting deformation as a viscous structure. The resistance to deformation is time-dependent. The increased membrane viscosity at high pH's (Leblond, 1973) probably accounts for the moderate increases in compression time at pH 8.2 and 8.3,

but it is doubtful whether the membrane viscosity could fully account for the ten-fold increase in compression time at pH's greater than 8.3

A satisfactory explanation for the variation of the average sliding velocity with pH will necessitate a knowledge of the effect of pH on the flexibility of the PVP molecules. As mentioned earlier, such information is lacking. The variation in sliding velocity could not be explained by possible changes in the viscosity of the bulk medium. The influence of altered pH on the viscosity of the bulk solution of PVP-Ringer was thought to be negligible from the work of Kassem and Maltha (1970) who demonstrated only an 8% increase in viscosity from pH 6.0 to pH 9.0 for a PVP solution of 70g/l. A possibly reduced flexibility of the PVP molecules could however increase drastically the viscosity of the medium between the adhering or sliding cells. This increased viscosity would increase the resistance to sliding between the cells. The behaviour of a suspension is expected to be very different at or near interfaces compared to "in bulk" suspension.

Although most red cells remain biconcave over the pH range of 6.5 to 9.0, they do change in volume. Increasing the pH of the medium from 6.5 to 9.0 decreases the erythrocyte volume approximately 25% in a linear fashion (Murphy, 1967). The change in volume would certainly alter the hydrodynamic drag. It is possible that these

changes in geometry could partly account for the variation of the average rate of sliding. However, since the volume changes monotonically with pH, it is unlikely that this effect alone could account for the maximum in the relation between pH and rate of sliding.

E. Cell Age

The results of the cell age study are shown in Table VII. The adhesion length ratio of old cells was found to be significantly less ($p < 0.01$) than that for young cells. There was however no significant difference between the values obtained for the compression time.

Table VII. Measurements on Young and Old Cells.

Type	Number of measurements	Adhesion Length Ratio (± S.E.M.)	Compression Time (± S.E.M.)
Young	12	0.58±0.01	8.4±2.0 seconds
Old	9	0.55±0.01	10.2±2.4 seconds

Dixon (1975, Chap. 5) calculated the average rates of sliding for the two types of cells and found no significant difference between them. Also, the percentage of sliding events was almost the same (~80%) for both the young and old cells. These experiments were all carried out for the red cells suspended in PVP-Ringer at a PVP concentra-

tion of 4g/l.

The decrease in the adhesion length ratio for the old cells suggests that either there was a decrease in the adhesive force (F_a) between the cells or a decrease in the deformability of the cells. The work of Marikovsky, Danon and Katchalsky (1966) and Marikovsky and Danon (1969) has shown that the net surface charge on old cells is significantly less than that on young cells. However, Knox, Nordt, Regan and Seaman (1977) recently examined the relationship between cell surface sialic acid and cell surface charge density of both young and old cells, and found that while there were significant decreases in the level of membrane-bound sialic acid in the old cells the surface charge density remained unaltered; that is, there was no change in the electrophoretic mobility of the two types of cells. (Sialic acid is the major contributor to red cell surface charge (Cook et al., 1961; Eylar et al., 1962)). Knox et al. (1977) attributed the loss of membrane-associated sialic acid without change in the electrophoretic mobility of the cells to a decrease in surface membrane area which accompanies the loss of portions of the membrane during its long journey through the circulation. As a result of these recent findings of Knox et al. (1977) it would not be unreasonable to assume in this study that cell age has no effect on the electrostatic repulsive force between the cells, that is, F_e remains constant. There is also no documented evidence to

suggest that cell age has any effect on the adsorption of the PVP molecules. It could therefore be assumed that the adhesive force (F_a) remained constant for both young and old cells.

The significant decrease in the adhesion length ratio in the old cells could therefore be explained by a possible decrease in the deformability of the cells. The micropipette studies of LaCelle, Kirkpatrick, Udkow and Arkin (1972) and Smith, LaCelle and LaCelle (1977) have shown that old cells are indeed less deformable than young cells. The decreased deformability is not only due to the increased sphericity (Canham, 1969) but also to a decreased membrane flexibility. Smith et al. (1977) demonstrated the increased membrane stiffness by comparing the results of old cells with those obtained for glutaraldehyde-fixed cells. Glutaraldehyde-fixed cells do not exhibit any changes in geometry (Jay and Canham, 1972) but their membranes exhibit reduced flexibility.

8.3. Discussion

The four principal factors which bear on doublet formation are adhesive force (resultant of the bridging force (F_b) of the macromolecules and the electrostatic repulsive force (F_e) between the cells), viscous drag between the cells, cell geometry and membrane deformability. Two additional factors which have been interpreted to be of lesser significance in this study are the coverslip

surface and the viscosity of the suspending medium. It is thought that the early phases of doublet formation would be sensitive to the adhesive force, geometry and deformability but not viscous drag because there is no apparent intermembranous shear; the sliding velocity would be influenced by the viscous drag. The effect of geometry has been reduced by using the dimensionless parameter of adhesion length ratio. Also the height of each cell on the coverslip is relatively constant because the thickness of indivual cells in a population is one of the least variable measurements (Jay, 1975).

The present study assesses the effects of surface charge, bridging force of macromolecules, and cellular deformability on red cell adhesion at the very cellular level. Increased concentration of the rouleaux-inducing agent (PVP in this case) increases rouleaux formation by resulting in an increase in the number of adsorbed macromolecules and thus an increase in the bridging force, F_b. A reduction in the surface charge density also increases rouleaux formation by resulting in a decrease in the electrostatic repulsive force (F_e) between the cells. The cell age study illustrates how the reduced deformability of the old cells causes a reduction in cell adhesion. The pH study is not conclusive but suggestive. It suggests a possible change in the conformation of the PVP molecules in solutions of both elevated and reduced hydrogen ion concentration away from neutral pH. It also

suggests that variations in the pH of the medium result in rearrangements of the cell membrane components which affect the binding of the PVP molecules to the cell membrane. The marked increase in the presliding time in doublet formation at high pH's could be partly explained by increased membrane viscosity, but the difficulty in finding a complete explanation suggests the need for much investigative work to completely understand the effect of pH on cell membrane. The results of the study of red cells in a no-calcium medium do not conclusively support or disprove the hypothesis of Brooks (1973c) and Jan and Chien (1973b) that the lack of divalent ions in the medium increases the electrostatic repulsive force between the cells; however, the general evidence from this study is more in support of their hypothesis.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

Rouleaux formation results from bridging between adjacent red cells (Brooks, 1973b; Chien and Jan, 1973) by the plasma proteins (fibrinogen and serum globulins) or other macromolecules of comparable molecular size (Fåhraeus, 1921) adsorbed to the cell surfaces. The use of radioactive-iodine labelled plasma proteins has shown that the plasma protein fractions can be adsorbed to the red cell surface (Traber and Kolman, 1963; Müller and Grämlich, 1965). With the use of tritium-labelled dextran having a mean molecular weight of 77,600, Brooks (1973b) has shown that the adsorption of this polysaccharide to the red cell surface is linearly related to its concentration in the suspending medium over a wide concentration range. The force of aggregation due to macromolecular adsorption and bridging is counteracted by the forces of disaggregation due to mechanical shearing and electrostatic repulsion between the charged surfaces of the adjacent cells (Chien, 1973). Another consideration in the formation of rouleaux is the deformability of the cells.

Decreased cellular deformability reduces the probability of the cells aligning themselves in rouleaux formation. As an example, the red cells of goat have a very low membrane flexibility (compared with human, Chien et al., 1971) and they hardly form rouleaux. Also, glutaraldehyde-fixed cells (greatly reduced deformability) do not form rouleaux (Chien et al., 1967). Therefore, the degree of red cell aggregation depends upon the properties of the red cells as well as their external environment.

Because of the incredible amount of effort devoted to the study of the interactions between red blood cells the process by which erythrocytes adhere reversibly is becoming less obscure. However, a full understanding of the phenomenon of rouleaux formation still remains a challenging study. This study was initiated with the hope that it would help in this understanding. As with the many other studies in this field of research, this work, in addition to providing several answers, has added more unanswered questions. In any event, the mere fact that this study has led to more questions about the mechanism of rouleaux formation suggests that we are increasingly understanding the phenomenon. An increased awareness of what need be known becomes more apparent.

In this study a non-flowing system was used for studying rouleaux formation. The reason for this is that the shearing forces which increase the probability of cell contact and which can also disrupt rouleaux formation

would not have to be considered. A non-flowing environment would allow for better controlled conditions. It is acknowledged however that this experimental system is not directly analogous to the in vivo situation.

9.1. Studies of the Mechanics of Rouleaux Formation

Attempts to formulate or describe the process of rouleaux formation can be broadly divided into two sections: (i) the statistical approaches which consider a whole population of red cells and the resultant interactions among them; and (ii) the individual-cell approaches which depend on microscopic observation of small numbers of cells while the process is underway.

The sedimentation method, "syllectometry" method, viscosity method, and MAI method described in section 1.2E (ii) are all statistical methods. They are unable to reveal precise information about the process at the cellular level during rouleaux formation. These methods are used only to measure quantitatively the degree of aggregation, and even so, they have their limitations. As an example, the sedimentation phenomenon, while it has the advantage in that determinations can be made at hematocrit levels similar to that existing in the normal circulation, is complicated by the formation and dispersion of the rouleaux, the bending and rotation of the sedimentary particles, and the return flow of the suspending medium (Thorsén and Hint, 1950; Charm, McComis and Kurland, 1964;

Canham, Jay and Tilsworth, 1971).

The individual-cell approaches permit direct observation of the cells as they are participating in the adhesion process. Fåhraeus (1929) who reported that erythrocytes would sometimes "glide" on top of one another, was perhaps the first to observe the process of rouleaux formation. Since then several investigators have speculated on the manner in which two red cells, after initial contact, align themselves in rouleau. Hummel (1969) expected the cells to move within their respective polymer coatings into alignment. He postulated that the polymer coatings would adhere to each other and remain fixed at their point of contact. Then, by performing a tank-tread motion around the cells, the polymer coatings would increase their area of contact with each other. As this happens, the red cells would become aligned broadside against broadside. Chien and Jan (1973), using the MAI, viscosity and sedimentation methods, expected that there would be no slippage at all between the cells. The contact area would enlarge, accompanied by deformations and rotation of the cell membranes until they were in rouleaux formation.

Rowlands and Skibo (1972) observed the manner in which two sedimenting rouleaux in plasma join together. First, the cells at the end of each rouleau may slide over one another broadside against broadside. Second, one rouleau may rotate about an axis through the point of contact bringing the end cells into broadside to broadside contact.

This latter process they termed "penknife closure". Besides observing rouleaux formation among sedimenting cells, Rowlands and Skibo (1972) observed the formation of rouleaux doublets. One cell hung in plasma from an inverted coverslip by its edge and a second cell was brought into contact with it using a micropipette. A rouleaux doublet formed invariably by the latter cell sliding up against the hanging cell, broadside against broadside.

Fung (1971) and Dixon (1975) also studied the process of doublet formation. The cells were initially observed lying broadside down on a coverslip. Initial contact between the cells was consequently rim to rim. Because of this consistent pattern of approach, doublet formation was found to occur in one of three distinct and reproducible modes. These three modes, sliding, cresting and flipping were described in section 6.3.

The studies of doublet formation commenced by Fung (Fung, 1971; Fung and Canham, 1974) and continued by Dixon (1975) were extended in this study to include the formation of longer rouleaux. The reasons or the various points of interest to this study were discussed in section 6.1. In addition to repeating in part the study of Fung (1971) and Dixon (1975) where the modes of doublet formation were examined for red cells in PVP-Ringer, this study also looked at the modes of rouleaux formation for red cells suspended in Dx-Ringer.

The general evidence from the results of this study

is in agreement with that of Fung (1971) and Dixon (1975, Chap. 7) that the "tank-tread" models of Hummel (1969) and Chien and Jan (1973) are unlikely. A model was also proposed in this work for explaining the lack of complete overlap observed at the end of the sliding mode in doublet formation; and the adhesive force between the cells was proposed to be the main determinant in explaining the transition from sliding to non-sliding. The observations in the study of the formation of longer rouleaux, in both PVP-Ringer and Dx-Ringer, are in agreement with the finding of Rowlands and Skibo (1972) that the process of rouleaux formation involves a surface energy with the general tendency to minimize this energy by reducing the amount of red cell surface area in contact with the plasma-like environment.

9.2. Measurements in Doublet Formation

Doublets, the simplest of rouleaux, represent perhaps the best system for studying and understanding the mechanism of rouleaux formation. In this study three parameters in doublet formation were measured to assess the effect of cellular surface charge, macromolecular adsorption and cellular deformability on red cell adhesion.

Increased PVP concentration resulted in increased cellular interaction as was evident in the measurements of ahesion length ratio. Also, the decrease in the compression times at high PVP concentrations was not indicative

of an increased membrane flexibility as Rampling and Sirs (1976) suggested but rather the increased interaction between the cells resulting from increased macromolecular adsorption (Hummel and Szczepanski, 1963).

The reduced surface charge of the cells, after Neuraminidase-treatment, demonstrated the role of the electrostatic repulsive force in red cell adhesion. The reduction in the surface charge density resulted in an increase in the adhesive force (F_a) between the cells which was indicated by the significantly elevated values of adhesion length ratio and average rates of sliding. Neuraminidase-treatment has no effect on cellular deformability (LaCelle, 1969; Durocher et al., 1975) and the dimensions of the cells, so the results on adhesion length ratio and average sliding velocity were interpreted to be entirely due to the decrease in the repulsive force (F_e) between the cells.

The results of the study on red cells in a no-calcium medium are not conclusive because of the lack of knowledge of what effect the absence of extracellular calcium has on the deformability of the cells. The general evidence, however, seems to support the hypothesis of Brooks (1973c) and Jan and Chien (1973b) that the reduction in the divalent-ion concentration results in an increase in the electrostatic repulsive force (F_e) between the cells.

The findings in the pH study resulted in more questions than answers. It emphasized the need for much more investigation to find out what effects the variation in

pH of the medium has on the possible rearrangement of the constituents of the red-cell membrane. The results of this study suggest that such possible rearrangements of the intra-membrane components have resulted in increased binding of the PVP molecules at both high and low pH's. The decrease in the average rates of sliding also suggests that the flexibility of the PVP molecules is reduced at both high and low pH's. What is conclusive in this study though is that the membrane viscosity is increased at high pH's (Leblond, 1972). This is indicated by the significant increases in the compression-time values.

The question of whether young cells are more aggregable than old cells had been investigated. Heyd (1972) used a sedimentation method to determine an aggregability index for both young cells and old cells. This index was 33% larger for young cells than for old cells. However, his index was dependent on the number of collisions between the sedimenting cells, and the adhesiveness between the cells after collision. Dixon (1975) used the average sliding velocity as an index of the aggregability of the cells but found no difference between the two types of cells. His finding, nevertheless, did not mean necessarily that there was no difference in the aggregability of the cells but suggests that the slightly but significantly elevated sphericity-index value (Canham, 1969) has facilitated sliding in the old cells. The dimple at the centre of the cells is less pronounced in the old cells and this

probably makes it easier for one cell to slide over the other. (The dimple no longer exists for cells in rouleaux (Rowlands and Skibo, 1972; Chien and Jan, 1973).

The results of this study indicated that young cells are more aggregable than old cells. This is shown by the significantly higher value obtained for the adhesion length ratio of young cells. The increased aggregability might be due to the greater deformability of young cells (LaCelle et al., 1972) and not to an increased adsorption of the rouleaux-inducing molecules.

9.3. Observed Differences in the Rouleaux-Inducing Tendencies of PVP and Dx

The red cell surface is negatively charged, mainly as a result of the presence of N-acetylneuraminic acid (sialic acid) (Seaman and Uhlenbruck, 1963). The electrostatic repulsive force between the cell surfaces can be estimated from the theoretical treatment developed in colloidal chemistry (Overbeek, 1952) for parallel plates with like surface charges. The electrostatic repulsive pressure (Π) between two plates with a distance of separation (d) can be calculated as:

$$\Pi = 0.064cRT\tanh^2(ze\chi_s/4kT)\exp(-d/x) \qquad (1)$$

where c and z are respectively the molal concentration and the valency of the ions, R is the gas constant, T is the

absolute temperature, e is the electronic charge, χ_s is the surface potential, k is the Boltzmann constant, and x is the thickness of the electric double layer. χ_s can be taken as the zeta potential (ζ) determined by electrophoresis of red cells (Bangham, Pethica and Seaman, 1958) and x can be calculated as:

$$x = (1000DkT/8\pi e^2 Ncz^2)^{1/2} \qquad (2)$$

where D is the dielectric constant of the suspending medium, and N is Avogadro's number. An increase in ionic strength in the suspending medium causes a decrease in x (eqn. (2)) and hence a reduction in Π (eqn. (1)). For red cells with a normal surface potential of approximately -15mV (Seaman, 1971) and suspended in an isotonic medium composed only of monovalent ions, and at a temperature of 25°C, eqn. (2) gives a value of approximately 0.8nm for x (Jan and Chien, 1973a).

Eqn. (1), however, does not take into account the influence of the macromolecule on the effective ionic strength by volume exclusion (Brooks, 1973a and 1973b). The macromolecules, adsorbed on the red cell surface are reported (Brooks, 1973b) to cause a reduction in the effective ionic strength near the cell surface, an expansion of the double layer, and an increase in the surface potential of the cells. These alterations are interrelated and can be expressed in terms of the parameter Z, the relative zeta

potential (Brooks, 1973a and 1973b):

$$Z = \zeta_\beta/\zeta_o = x_\beta/x_o = (c_o/c_\beta)^{1/2} \qquad (3)$$

where the subscripts 0 and β refer to the absence and presence of the macromolecules respectively. If ζ_β and ζ_o are determined, c_o is known and x_o is calculated, then c_β and x_β can be calculated from eqn. (3). Substitution of c_β, ζ_β, and x_β for c, χ_s, and x respectively in eqn. (I) yields a higher Π_β value, that is,

$$\Pi_\beta = 0.064 c_\beta RT \tanh^2(ze\zeta_\beta/4kT)\exp(-d/xZ) \qquad (4)$$

Since the value for $[0.064 c_\beta RT \tanh^2(ze\zeta_\beta/4kT)]$ usually changes by less than 10% from 5×10^6 (Jan and Chien, 1973a) even with high Dx concentrations, Π_β for normal red cells suspended in an isotonic solution of monovalent ions can be approximated as:

$$\Pi_\beta \simeq 5\times10^6 \exp(-1.29d/Z) \qquad (4)$$

where Π_β is in dynes/cm^2 and d in nm.

Eqn. (4) shows that the electrostatic repulsive force increases exponentially with increases in Z. This is the basis of the model of Brooks (1973b, 1973c) and Jan and Chien (1973a, 1973b) for explaining why high concentrations of Dx's fail to induce aggregation of human red cells.

The fact that Dx's resulted in an increase in the zeta potential of the cells was observed some time before Brooks proposed his model. Ross and Ebert (1959), in an attempt to explain the increase in the zeta potential, proposed that the interaction of Dx with cell surface proteins causes a rearrangement of the interfacial region. Such a rearrangement might expose previously undetected charged groups at the shear plane, thus increasing the surface charge density and consequently the zeta potential. Brooks (1973a), however, discounted this hypothesis on the grounds that glutaraldehyde-fixed cells also exhibit a zeta-potential increase in Dx solutions. Glutaraldehyde-fixed cells are rigid and would not be expected to undergo interfacial rearrangement. Brooks however acknowledged that the greater increase in zeta potential for the normal red blood cell suspensions might be due to an interfacial rearrangement.

A second hypothesis was proposed by Pollack, Hager, Rockel, Toren and Singher (1965) who interpreted their results in terms of a change of dielectric constant. These workers found a four-fold increase in the bulk dielectric constant of a 50g/l Dx-250 solution. Brooks (1973a) also discounted this hypothesis in view of his estimation that the dielectric constant near the cell surface would have to be increased 1000-fold to produce the measured effect, a number that is inexplicably large.

Brooks (1973b) proposed that his model would be valid

for all neutral polymers, and Jan and Chien (1973a, 1973b) presented evidence in support of the model of Brooks when they found that high concentrations of Dx's failed to produce aggregation of human red cells. It was therefore the main purpose of this study to test the model of Brooks using PVP, a neutral polymer, to induce rouleaux formation. PVP was found in this study to induce aggregation not only of human cells but also red cells of other species, at all concentrations above 1.0g/l. That red cell aggregation does not occur at high Dx concentrations was also confirmed in this study for human cells, but not for red cells of other species.

The results of this study did not question the validity of Brook's hypothesis, but merely suggested that it be confined to the effect of Dx's on human red cells only. No one has reported any studies on the effect of Dx's or PVP on the zeta potential of red cells of other species. It is difficult from these studies to offer an explanation of the interaction of the macromolecules with the various types of red cell surfaces. There is still a need to know and understand the conformation of the adsorbed macromolecules, and the areas of the red cell surfaces to which they are adsorbed.

It is perhaps of interest to mention that Brooks (1973a, 1973b) did not consider in his model the results of Castaneda et al. (1965) who reported that the surface of the human red cell becomes more electropositive in PVP

solutions of concentrations above 7.5g/l.

Finally, the results of this work also served as a warning for any new substance that might be introduced as a plasma expander. The warning is that the results obtained from tests on blood from animal species might not be valid for human blood.

9.4. Species-Specificity in Rouleaux Formation

In this study, red cells from any one species were found to adhere in rouleaux formation to red cells from any other species. A statistical analysis of the inter-species' cells in rouleaux, however, showed that the cells preferred to adhere to cells of their own kind (species). The rat-mouse combination was the only one that showed no preference, perhaps because these two species belong to the same family. Here again, it was difficult to explain the general species-specificity in rouleaux formation. The surface structures of the red cells of the different species are still not known, even though indirect studies have indicated them to be different (Seaman and Uhlenbruck, 1963).

CHAPTER 10

PROPOSALS FOR FUTURE WORK

1) In the study of Dx-induced red cell aggregation it was found that high concentrations of Dx solutions failed to induce aggregation of human cells but continued to induce aggregation of red cells of other species. The increase in the relative zeta potential of the human cells in solutions of high Dx concentrations is probably large enough to lead to the absence of aggregation. It is not known however how the zeta potential of red cells of other species varies with the Dx concentration. It will therefore be of interest to carry out electrophoresis studies similar to those of Brooks (1973c) on red cells of other species. The results of such studies will have two-fold significance: if the zeta potential increases in the same proportion (as for human cells) then this will suggest that the high zeta potential of human cells is not a sufficient explanation for the lack of aggregation of human cells in solutions of high Dx concentrations; on the other hand, if the zeta potential does not increase significantly then this will provide evidence

against the hypothesis of Brooks (1973b) that the adsorption of neutral molecules increases the surface potential of the cells by reducing the counterion concentration next to the cell surface.

2) The question of species specificity in rouleaux formation can be extended to human cells of different blood groups. The red cells of the mixed rouleaux could be identified by using fluorescent labelling.

3) The pH study of this thesis work showed that an altered pH of the medium might result in an increased binding of the PVP molecules to the red cell membranes. This possibility could be investigated by using radioactively-labelled PVP and measuring the amount of PVP molecules adsorbed to the cell membranes as a function of pH.

Young and old red cells could also be studied similarly. The greater aggregability of young cells has been partly attributed in this study to their greater deformability. However, it is still not known whether young cells adsorb more rouleaux-inducing molecules per unit area than old cells. The study involving the use of radioactively-labelled PVP will provide the solution to this problem.

APPENDIX A

DERIVATION OF FORMULA FOR CELLULAR VOLUME, V, OF RED BLOOD CELL IN ROULEAU

Consider figure 44 which shows the cell to be flat-sided and with parabolic rims.

Eqn. of parabola:

$$z^2 = b - ay \tag{1}$$

where a and b are constants.

When $z = 0$, then $y = d/2$

$$\therefore b = ad/2 \tag{2}$$

It is assumed that $t = \ell = h/2$

$\therefore$ when $z = h/2$ then $y = d/2 - h/2$

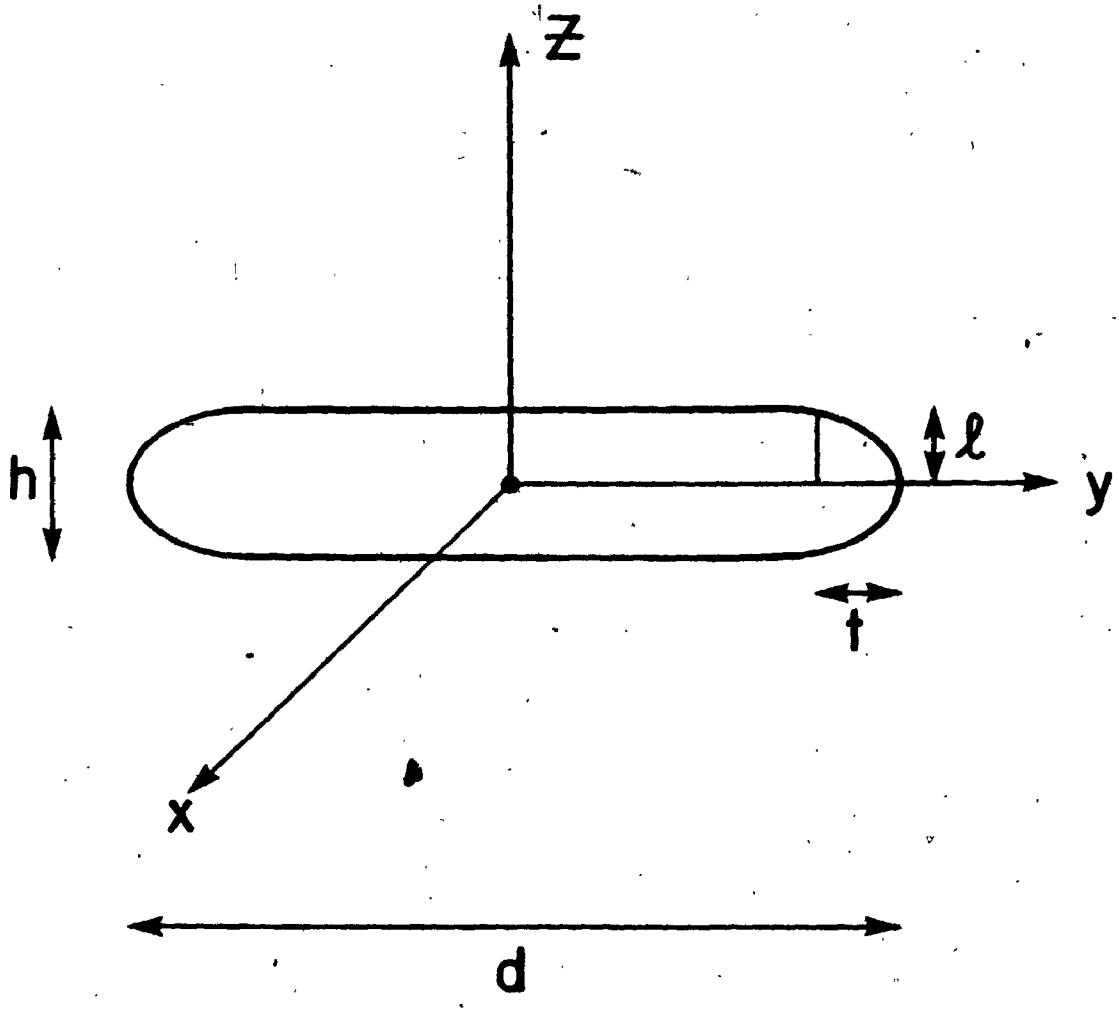
$$\text{i.e.} \quad a = h/2 \tag{3}$$

$$\text{and} \quad b = hd/4 \tag{4}$$

$$\text{Cellular volume, } V, = 2\pi \int_0^{h/2} y^2 dz \tag{5}$$

FIGURE 44

Derivation of the formula for cellular volume, V, and surface area, A, of the red blood cell in rouleau.


z
h
ℓ
y
t
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d

From eqn. (1), $y^2 = \left(\frac{b-z^2}{a}\right)^2$

$$\text{i.e. } y^2 = \frac{z^4-2bz^2+b^2}{a^2} \qquad (6)$$

∴ in eqn. (5),

$$V = \frac{2\pi}{a^2} \int_0^{h/2} (z^2-2bz^2+b^2)\,dz$$

$$\text{i.e. } V = \frac{\pi h}{a^2} \left[\frac{h^4}{80} - \frac{bh^2}{6} + b^2\right] \qquad (7)$$

Substituting for a and b from (3) and (4) and simplifying,

$$V = \pi h[0.25d^2-0.17hd+0.05h^2]$$

APPENDIX B

DERIVATION OF FORMULA FOR SURFACE AREA, A, OF RED BLOOD CELL IN ROULEAU

Consider figure 44 again.

$$\text{Surface area, } A, = 4\pi \int_0^{h/2} y \frac{ds}{dz}\, dz \qquad (8)$$

where $ds^2 = dy^2 + dz^2$

$$\text{i.e. } \frac{ds}{dz} = \left(\frac{dy^2}{dz^2} + 1\right)^{1/2} \qquad (9)$$

From eqn. (1)

$$2zdz = -ady$$

$$\text{i.e. } \left(\frac{dy}{dz}\right)^2 = \frac{4z^2}{a^2} \qquad (10)$$

in eqn. 9

$$\frac{ds}{dz} = \left(\frac{4z^2}{a^2} + 1\right)^{1/2}$$

eqn. (8) becomes

$$A = \frac{4\pi}{a} \int_0^{h/2} (b - z^2) \left(\frac{4z^2}{a^2} + 1\right)^{1/2} dz$$

$$= \frac{8\pi b}{a^2} \int_0^{h/2} (z^2 + a^2/4)^{1/2}\, dz -$$

$$- \frac{8\pi}{a^2} \int_0^{h/2} z^2 (z^2 + a^2/4)^{1/2}\, dz$$

$$\text{i.e. } A = \frac{\pi}{16}(a^2+16b)\left\{\frac{h}{a^2}(h^2+a^2)^{1/2}+\ln[h+(h^2+a^2)^{1/2}]/a\right\}$$

$$- \frac{\pi}{8a^2}[(a^2+h^2)^{3/2}h-(2ad-h^2)^2] \qquad (11)$$

Substituting for a and b from (3) and (4) and simplifying,

$$A = 0.5\pi[d^2+0.96hd-0.22h^2]$$

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