

RICE UNIVERSITY

ERYTHROCYTE DEFORMABILITY AS
ASSESSED BY NUCLEPORE FILTERS

by

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

Thesis Director's Signature:

J. C. McIntire

August, 1977

Abstract

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Erythrocyte deformability characteristics were assessed by passing red cell suspensions through Nuclepore polycarbonate filters having pore diameters of 3 μm . with pore lengths of 12 μm . During flow through these filters, at a constant flow rate of 2.17 cc/min., the pressure drop across the filter was monitored as a function of time. This measurement was the primary index of altered deformability. These filters serve as a reproducible technique for measuring erythrocyte deformability, and are a physical model of the splenic microcirculation.

Shear stresses of 1500 dynes/cm² or greater applied to erythrocytes for two minutes in a concentric cylinder viscometer result in red cell fragmentation and hemolysis. In this study the effects of lower shear stresses on cell deformability were measured by observing the relative ability of erythrocytes to traverse Nuclepore filters. Cell morphology was examined immediately after shear stress, and after subsequent passage through the filter to assess cell damage. Shear stresses from 250-1400 dynes/cm², applied

with a concentric cylinder viscometer, were studied. A good correlation was found between the magnitude of the applied shear stress, and both the pressure drop produced at constant flow rate and the extent of morphologic damage due to filter passage. Behavior indistinguishable from unsheared cells was observed only when the applied shear stress was at or below a lower threshold value of approximately 250 dynes/cm². This indicates that altered erythrocyte deformability results from application of shear stresses considerably less than those required to produce red cell fragmentation and overt hemolysis.

The shear stress induced cell alterations in deformability were determined not to be time recoverable. Incubations with glucose, dibutyryl cyclic AMP and cyclic GMP, and adenosine triphosphate following shear were made in an attempt to reverse the effects of shear stress. These had little or no effect. Incubation with adenosine, however, was capable of returning sheared cells to their behavior before shear stress. The effects of adenosine incubation were so complete that the deformability of the sheared and incubated cells was virtually the same as fresh unsheared cells as assessed by Nuclepore filter passage.

Aging erythrocytes for periods of up to 24 hours, produced cells that caused much higher pressure drops during filtration. A cell, however, retained most of its deformability characteristics if the incubation time was less than six hours. Loss of deformability was rapid after ten hours.

The effects of incubation with the protein denaturing compounds colchicine, vinblastine and cytochalasin B to produce deformability alterations were tested using the filtration technique. No consistently measurable differences in deformability were seen between the incubated cells and the control cells.

The results imply that cell ATP levels play an important role in maintenance of deformability. Application of shear stress appears to induce a membrane defect similar to that seen in metabolically depleted cells. Presumably the shear stress forces cause a drastically increased energy demand within the cell, which depletes the intracellular ATP, resulting in a more rigid, less deformable cell. The in vivo survival of such a cell may be reduced, due to greater likelihood of sequestering in the spleen.

To the memory of my father:

Harry E. Velker

ACKNOWLEDGEMENTS

My sincere gratitude to all those people who contributed their time and effort to this work:

Dr. L.V. McIntire for his guidance of the research, and for his encouragement throughout.

Dr. E.C. Lynch for his advice and the interest he showed in the work, which was especially appreciated during my advisor's absence, and for serving on my oral examination committee.

Dr. J.D. Hellums for serving on my oral examination committee.

Ms. Patsy McPherson for her "painless" venipuncture.

Miss Marcella Estrella for the time she spent in assisting with the experiments.

Mrs. Nancy Wolff and Mrs. Helen Hekemian for their speedy typing.

The many donors who gave their blood, and NIH Grant HL18672.

A special appreciation goes to all the many people who made the stay at Rice the enjoyable experience it was, but especially to Fitzpatrick, M.G., Schulkenoids, Lee, Nasir, Shirine, Heardie, Barb Italiano, and a girl named "The Gomp".

And finally, I thank my mother for her continued strength and support of my goals.

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Introduction

Red cell deformability is crucial to the survival of the erythrocyte as it traverses the tortuous route through the human microcirculation. In fact, increasing cellular rigidity as red cells age in vivo is believed to be a major cause of eventual cell destruction and capture in the spleen and liver (1).

This study examined factors which could potentially alter red cell deformability. The existence and extent of alterations were measured by passing suspensions of erythrocytes at constant flow rate through Nuclepore filters. During flow, the pressure drop across the filter was measured as a function of time; the magnitude of the pressures generated was an indication of ease of filterability, and hence cell deformability. The filters used were of the polycarbonate type, all having right cylindrical pores, with diameters of 3 μm , and lengths of 12 μm . It is believed that these filters are a reproducible method for measuring the bulk deformability characteristics of a cell population. At the same time, the filter pores are an adequate physical model of the microcirculation, specifically that of the spleen.

Blood damage problems have been known to limit the usefulness of blood processing devices, such as artificial cardiac valves, artificial hearts, and extra-corporeal

circulation system. With the increased use of such prosthetic devices, there has been interest in the interactions of erythrocytes and mechanical forces which may cause hemolysis and morphologic change. Physical forces of potential importance are pressure changes, crushing of erythrocytes between solid surfaces, simple surface adhesion and removal, and bulk shear stress.

In this study, red blood cells were subjected to known shear stress levels below the threshold value required for hemolysis. A constant shear stress cylindrical viscometer was used for this purpose. Cells subjected to shear stress were tested for altered deformability by using the Nuclepore filtration technique. Damage incurred by flow through the filter was observed by light microscopy studies.

If an alteration in erythrocyte deformability was indeed a result of shear stress application, this study would attempt to correct or reverse this change by incubating sheared cells in substances that might return cells to their normal deformability. These compounds were glucose, dibutyryl adenosine 3':5' cyclic monophosphoric acid (DBCAMP), dibutyryl guanosine 3':5' cyclic monophosphoric acid (DBC GMP), adenosine triphosphate (ATP) and adenosine.

Previous evidence of increased cell rigidity as cells are aged in vitro was reexamined by metabolically depleting normal erythrocytes for up to 24 hours, followed by passage of these cells through Nuclepore filters.

Evidence suggests the possibility that fibrous proteins imbedded in the red cell membrane, called microtubules and microfilaments, could be influential in determining cell shape and deformability. This was examined by subjecting red cells to incubating solutions of protein denaturants such as colchicine, vinblastine and cytochalasin B. These substances are believed to disrupt the structure and function of the membrane fibrous proteins.

The study was carried out with the supposition that erythrocytes showing markedly decreased flow through 3 μ m pores, might exhibit decreased in vivo life spans, due to increased susceptibility of splenic capture. Therefore the in vitro model is believed to be important for testing potential clinical abnormalities.

General Remarks on the Erythrocyte

The earliest known reference to the red blood cell was by Swammerdam, when in 1682 he observed that the intestine of the louse was perfused by "liquid containing corpuscles" (2). Antony van Leeuwenhoek was the first to attribute the red color of the blood to the erythrocyte. He also made an amazingly accurate estimate of the red cells' size at 10 μm (3).

Erythrocytes are anuclear blood cells whose primary role is the transport of oxygen from the lungs to the body's organs and tissues. The cells oxygen carrying capacity is due to its contained hemoglobin. Hemoglobin, in one of its forms, has a high affinity for oxygen, combines with oxygen while passing through the pulmonary circulation, and releases oxygen in the tissue capillaries.

The normal, unstressed shape of the red blood cell is that of a biconcave disk. The exact reason for this shape is still an active area of research. Human erythrocytes have a diameter from 7.5 to 8.3 microns, which decreases slightly with age. They are about 1.7 microns thick, have a volume of approximately 83 cubic microns, and a surface area of 145 m^2 . The size variations in erythrocytes are normally within 5 percent (4).

The red cell is by far the most numerous of the circulating blood cells. Normal male red cell count is 5.11 $\pm$

0.38×10^6 per mm^3 of blood. In contrast, there are only $248,000 \pm 50,000$ platelets per mm^3 of blood, and 7250 ± 1690 white cells per mm^3 of whole blood. Nearly one-half of total whole blood volume is occupied by red cells.

The intracellular contents of the erythrocyte is contained within a membrane. Electron microscopy measurements reveal a membrane between $60\text{-}100 \text{ \AA}$ thick that appears sheet-like, having cylindrical structures which in some cases extend through the entire membrane (5). It is believed that these may serve as sites for some of the cells transport mechanisms. A dry weight analysis of the membrane indicates a composition as follows: 50 percent protein, 43 percent lipid, and 7 percent carbohydrate (6). The membrane model that has evolved as the most widely held is the so-called "bi-lipid layer", in which a lipid interior is sandwiched between protein layers. Recently, the fluid mosaic model of Singer (7) has gained acceptance. In this model the proteins that are integral to the membrane are a heterogeneous set of globular molecules, with the ionic and polar groups protruding from the membrane into the aqueous phase, and the non-polar groups contained within the hydrophobic membrane interior. The phospholipids are believed to form a discontinuous fluid bilayer, although a small amount may interact with the membrane proteins.

In their normal whole blood milieu, erythrocytes exist in osmotic equilibrium with the surrounding plasma. The cell membrane is semi-permeable, allowing certain species such as

water and glucose to pass through freely, while being totally impermeable to others such as hemoglobin. Since hemoglobin, in relatively high concentration is trapped within the cell, it exerts a substantial osmotic pressure. If this pressure was not in some manner balanced, there would be a rapid influx of water into the cell, and subsequent hemolysis. To counteract this, the cell actively pumps sodium ions out against a concentration gradient. This active transport, which is essential to the cell's survival, requires an energy input. This is supplied by the cell's principal energy mechanism, the anaerobic glycolytic pathway, which metabolizes glucose to lactic acid. Although two moles of high energy phosphate in the form of adenosine triphosphate (ATP) are required in the initial preparation of glucose for its further metabolism, the full pathway can phosphorylate four moles of adenosine diphosphate to ATP. Hence, a net yield of 2 moles of ATP are yielded per mole of glucose metabolized. It is in the high energy phosphate bonds of this excess ATP that the cell stores its energy requirements for the active transport mechanisms. In addition to the sodium pump, the erythrocyte actively transports potassium ions into the cell. A dynamic equilibrium is established, while simulatenously, sodium passively diffuses into the cell, and potassium diffuses out.

A person's erythrocytes form a hetergeneous population with respect to their age. Average life span of an erythrocyte is 120 days. Hence about 0.8% of the erythrocyte

population is replaced on a daily basis. The older cells are different in several known aspects from the balance of the population. They can be separated by centrifugation due to their higher density, and are also slightly smaller. The ATP level of the oldest cells is significantly lower than the overall average. Simultaneous to the depletion of the cells ATP content, is an increasing rigidity of the cell membrane, but whether this is a cause and effect relationship is not known. The cell's increased rigidity has been suspected to be due to high levels of calcium in the older cell's membrane, but is still a widely debated phenomena (8). The cells decreased deformability is the probably cause of the cells eventual in vivo demise.

The erythrocyte is a highly deformable entity. Indeed, flowing through the microcirculation, the red cell is dependent for its very survival on its ability to undergo moment-to-moment shape changes. These deformations are required when cells are packed to a concentration of greater than 60 percent, or clearly when forced to negotiate orifices smaller than itself (9,10). From in vitro studies (11), the erythrocyte is deformed by quite small forces. These observations suggest that small changes in mechanical properties of the cell could markedly change its flexibility. The survival of such an altered cell, particularly in passage through the spleen, where the severest test of erythrocyte deformability (sinuses of 3 μ m or less) exists, might be severely reduced.

It appears that the cell shape, the physical properties of the membrane, and the condition of the cell contents all could play a role in determining overall erythrocyte deformability. The biconcave shape of the normal erythrocyte makes it ideally suited for shape change, due to the fact that the normal discoid shape supplies the cell with 60-70 percent excess surface area over that required to contain its contents (2). This allows the membrane to bend to accommodate deformation, without requiring an increase in surface area, while the cell membrane is easily bent, stretching the membrane enough to produce surface area increases more than 15% results in rupture and hemolysis (12). The membrane has been shown to be viscoelastic in nature (13), with the possibility of a plastic component (14). The cellular contents are widely believed to be liquid (15,16) and this does not contribute significantly to cell flexibility, except in cells with abnormal hemoglobin disorders, as those of sickle cell disease.

Related Investigations

One of the earliest and most successful techniques for measuring red cell deformability, and specifically the mechanical properties of red cells, was the micropipette method. The original work, using this technique with success, was done by Rand and Burton (12,13) and involved measuring the pressure and time required to suck an individual cell into a micropipette. They concluded that hemolysis occurred at a critical strain, rather than stress, of the cell membrane. Their work confirmed that of Katchalsky (17) that the membrane was visco-elastic in nature, with no yield stress. Using a simple viscoelastic model, they determined a "Young's modulus" of from 10^6 to 10^8 dynes/cm², and a viscosity of 10^7 to 10^{10} poise. The high degree of membrane flexibility was also noted by Rand and Burton, who at the same time claimed a strain capability of the membrane of about 20%.

Several workers have attempted to ascertain the smallest diameter capillary that a red cell could successfully negotiate. Canham and Burton (18) originated the term "minimum cylindrical diameter". They proposed that if a vessel becomes sufficiently small, a limiting diameter will be reached through which the cell may no longer pass. This geometrical parameter, or minimum cylindrical diameter, is described as the smallest, right cylindrical channel through

which the flexible cell might pass without increasing its membrane area. Canham and Burton have postulated this dimension to be in excess of 3 μm . Jay (19) calculated a minimum of 3.33 μm , again assuming the membrane cannot survive any stretch. Subsequent studies have shown a slightly smaller minimum cylindrical diameter. Using the micropipette technique Weed and LaCelle (20,21) observed an MCD of 2.80-2.85 μm . Gregerson, et al. (22) demonstrated by using thin polycarbonate filters, similar to those used in the present study, that erythrocytes could assume a cylindrical shape with a minimum diameter of 2.8 μm . The length of the channels he used were approximately 12 μm . These minimum cylindrical diameters imply a slight degree of membrane stretch, however even the existence of membrane distensibility is still an area of research (23). In negotiating diameters on the order of 2.8 μm , these studies have shown that after assuming the cylindrical shape the erythrocyte is capable, due to its elastic nature, of returning to its biconcave disk configuration.

In channels with diameters less than 2.6 μm , a critical parameter becomes the length of the channel (24). If channels this small are longer than 8 to 12 μm they are prohibitive to erythrocyte passage. The cell, due to the hydrostatic force of the circulation, enters partially into the pore, assuming the typical cylindrical shape. However, that portion of the cell outside the channel becomes spherical in shape, and incapable of further deformation.

If additional force is present, cell fragmentation will occur. However, if the passage is short (approximately 4 to 5 μm) the erythrocyte can flow through due to its extreme deformability. This is believed to be the case in the membrane separating the splenic cords from sinuses. These openings are anywhere from 0.5 to 5.0 μm in diameter, but are short enough to allow erythrocyte passage. It appears that the minimum cylindrical diameter required of an erythrocyte increases to 3.6 to 4.0 μm after splenectomy.

Another technique used for the determination of the membrane modulus of elasticity is the fluid shear deformation of attached cells (25,26). In addition to making other observations of red cells under the influence of shear stress, Hochmuth and Mohan Das reported for small strains (approximately 10-20%) a modulus of elasticity of 10^4 dynes/cm². Their experiments indicated that the modulus increased as the amount of strain increased. The shear stress levels used were less than 180 dynes/cm², and in one set of experiments between only 1 and 10 dynes/cm².

Evans (27) observed the fluid shear deformation of point attached cells, and aspirated erythrocytes into calibrated micro-pipettes to measure physical properties of the cell membrane. He defined an elastic constant as being the shear modulus multiplied by the assumed membrane thickness. By both techniques the measured elastic constant was on the order of 10^{-2} dynes/cm, corresponding to a shear modulus of 10^4 dynes/cm².

Richardson (28) examined the question of whether there is membrane slip at the pipette mouth, concluding that the real situation is somewhere between the conditions of no slip and perfect slip. His experiments also resulted in a value for the product of the elastic modulus and the membrane thickness of from 0.002 to 0.016 dynes/cm².

Braasch (29) showed by using the micropipette technique that sphered cells were less deformable than normals, by measuring comparatively increased passage times for the sphered cells.

The effects of the application of various levels of shear stress on erythrocyte structure and function have been studied by numerous workers. To a large extent, this work was stimulated by the greatly increased use of prosthetic circulatory devices. Blood damage problems have sometimes limited the utility of devices such as artificial cardiac valves, total artificial hearts and extracorporeal circulation systems (30,31). Hemolytic anemia due to mechanical causes became apparent after the use of prosthetic materials to repair cardiac defects (32,33). After development and use of a totally cloth-covered prosthetic valve, markedly increased hemolysis and incidences of anemia were noted (34,35). An excellent, recent review of the incidence and severity of hemolysis in the aortic prosthesis is given by Santinga et al. (36).

Sutera, et al. (37) clearly demonstrated the transformation in red cell shape from the normal biconcave disk

to an ellipsoid under the influence of a shear field. The ellipsoid shaped cells aligned their major axis with the direction of shear stress.

Hochmuth and Mohandas (25) measured strain in the direction of flow of human erythrocytes adhering to a glass surface for wall shear stresses between 1 and 10 dynes/cm². Depending on one or two point attachment, they measured strains of from 40-60% at a shear stress of 10 dynes/cm². Hochmuth, Mohandas, and Blackshear (26) observed the phenomena of "tethering". Tethers are extremely long extensions of the membrane. These tethers have been observed to be as long as 200 μ m and are caused by the application of shear stresses greater than 1.5 dynes/cm², which was called the threshold shear stress for tether formation. They concluded that the tether was representative of the whole cell membrane and not a component of the membrane such as the phospholipid. The measured modulus of elasticity for the tether was comparable to that of whole cell membranes. Secondly, the measured thickness of the tether (about 0.2 μ m) was relatively thick compared to, for example, the double thickness of a lipid film which is approximately 0.02 μ m. The tether of a red cell also appears to have character of both a fluid and a solid. Above the threshold shear stress of 1.5 dynes/cm², a tether formed and grew slowly with time. However, for a tether of given length and at zero stress, stress-strain experiments were performed with a resulting modulus of elasticity being measured. Hochmuth and Mohandas

(25) also observed the pronounced change in shape of fluid shear-deformed cells to that of a tear drop.

Usami and Chien (38) performed shear deformation studies to test the hypothesis that overall cell deformability was in part determined by the ratio in viscosities of the external medium and the internal medium. Their experiments investigated the role of the internal fluid viscosity, by using hemoglobin-poor red cell ghosts. They concluded that the apparent viscosity of cell ghost suspensions was lower than that of normal erythrocyte suspensions at the same cell concentration. It was not ruled out, however, that the membrane flexibility might have been altered during preparation of the ghosts, which was by osmotic lysis.

Chevalier, et al. (39) used the technique of freeze etching to study shear deformed red cells. Upon application of shear stress, freeze etched cells showed a decrease in membrane associated particles. For example, at an applied shear stress of 3000 dynes/cm^2 the number of particles per square micron decreased from 2630 ± 37 to 2050 ± 26 on one face of the cell.

Many workers have addressed the problem of measuring the levels of shear stress that could cause irreversible erythrocyte damage. Clearly, one of the manifestations of severe cell damage that is easy to measure is hemolysis. Not coincidentally, several studies have been made measuring the degree of hemolysis in response to various shear stress levels.

Some of the earliest work on shear induced hemolysis was by Blackshear, et al. (40) using dog erythrocytes. The primary conclusion of this work was that the hemolysis observed was due to cell-wall interactions more than anything else. Somewhat later, the free jet tests of Blackshear, et al. (41) and Indeglia, et al. (42) indicated that unless shear stresses approach the level of 10^4 dynes/cm², hemolysis away from surfaces was insignificant. The jet device does not afford a good measure of the maximum shear stress, and results are not directly comparable to those of other techniques. The exposure time to the shear stress is quite low, and is in part the cause for the high threshold value (approximately 40,000 dynes/cm²).

Blackshear (43), using canine blood and capillary viscometry, observed hemolysis levels. With these flows the maximum shear stress can easily be calculated. For ordinary capillaries a threshold value of shear stress for hemolysis was about 4,500 dynes/cm², and for smooth, tapered capillaries about 7000 dynes/cm².

Two ingenious sets of experiments involved application of the shear stress in very different ways. Rooney (44) subjected a small volume of human and canine erythrocyte suspensions to the action of a manipulated gas bubble, oscillating at 20 kilohertz. The release of hemoglobin was observed when the oscillation amplitude exceeded a critical threshold. From this, Rooney determined a threshold level of shear stress of about $4500 \text{ dynes/cm}^2 \pm 1500 \text{ dynes/cm}^2$. The

exposure to shear stress time was on the order of 10^{-3} seconds. Williams, et al. exposed human and dog erythrocyte suspensions to the hydrodynamic forces generated by a partially submerged tungsten wire set into oscillation at 20 kilohertz (45). Near the wire, eddy motions are established, and high velocity gradients exist in the boundary layer very near to the oscillating surface. At a threshold value for the amplitude of oscillations, hemoglobin release was noted. This critical amplitude corresponded to a shear stress of about 5600 dynes/cm^2 , again with a fairly large degree of uncertainty. Williams' exposure time was about 10^{-4} seconds.

Three more studies seemed to further cloud the issue. Bacher and Williams (46) ran capillary flow experiments with steer blood and indicated a very approximate threshold shear stress for damage of 5000 dynes/cm^2 . The authors' results were difficult to interpret since they used aged blood, that they claimed might be more easily damaged than fresh blood. They espoused that surface interactions were more important than the applied shear stress. Shapiro and Williams (47) performed concentric cylinder flow experiments, with maximum shear stresses of 600 dynes/cm^2 . In this range, they were of the opinion that surface effects dominated over shear stress effects. Their results were also with old blood (5-6 days) which makes interpretation difficult.

The work of previous researchers in the Rice Biomedical Laboratory are particularly pertinent since their equipment

used and experimental procedures followed were quite similar to those of the present study. Nevaril, et al. (48) subjected red cells to relatively high shear stress levels in concentric cylinder apparatus for two minutes. He observed little overt hemolysis below 3000 dynes/cm^2 . However, morphologic alterations were evident at shear stresses as low as 1500 dynes/cm^2 . Above 2500 dynes/cm^2 many types of alterations were visible, including some cell fragmentation. Although the surface to volume ratio was not varied in his experiments, the viscosity of the sheared suspensions was varied, so that the shear rate could be varied independently. Leverett et al. (49) using very similar equipment and two minute exposure times, observed a threshold level of shear stress for hemolysis of 1500 dynes/cm^2 . This work indicated clearly that at these levels, shear stress was indeed the dominant traumatizing factor. The surface to volume ratio of the viscometer was varied in this shear stress dominated regime without effect. However, their work confirmed that of others, that in a low shear stress regime, hemolysis was directly proportional to the surface to volume ratio. The authors claimed that with application of low shear stresses, surface induced hemolysis could result if exposure time was long enough. MacCallum et al. (50) corroborated these findings in his work with normal cells, and also examined the fragility of abnormal erythrocytes. Using an exposure time of approximately two minutes, he found de-oxygenated erythrocytes containing Hb SS (Sickle cell) to be extremely

fragile, by observing a threshold shear stress of less than 400 dynes/cm². Pathologic erythrocytes differed widely in their shear resistance, and could be ordered in increasing resistance: sickle cells, iron deficient, thalassemia minor, pyruvate kinase deficient, normals, and hereditary spherocytes.

A novel study by Sutura and Mehrjardi (37) gave important understanding to the question of shear vs. surface induced erythrocyte hemolysis. By means of gluteraldehyde fixation, human red cells were "frozen" during suspension in turbulent shear flow. Between 100-2500 dynes/cm², the shear stress deformed the cells into smooth ellipsoids of increasing length. But at stresses above 2500 dynes/cm², fragmentation of cells occurred, along with an increase in free hemoglobin levels in the suspending medium. The photographs taken indicated that cells rupture in the stress field of the bulk flow.

An excellent review of all the work that has determined a threshold shear stress level for red cell destruction is given by Hellums and Brown (51). The available data is presented in a coherent manner in which the two controlling parameters, stress level and exposure time are combined to delineate regimes where stress effects dominate, and where surface effects dominate.

The effects of sub-hemolytic shear stress to cause irreversible cell alterations has been a less studied problem. Nevaril et al. (48) and Champion, et al. (52)

showed that erythrocyte fragmentation could occur without overt hemolysis. Sandza, et al. (53) demonstrated that rabbit red cells, subjected to low levels of shear stress, are recognized and sequestered at increased levels by the spleen. It would appear that his shear stress levels were so low (maximum of 120 dynes/cm^2) and exposure times sufficiently long (1 hour) that the effects may be surface dominated. Nanjappa, et al. (54) performed experiments with canine red cells subjected to very low shear stresses (90 dynes/cm^2) for up to 60 minutes in a Weissenberg rheogoniometer having a high surface to volume ratio (450 cm^{-1}). These cells exhibited decreased in vivo survival, increased osmotic fragility, higher sodium permeability, and lower membrane acetylcholinesterase activity. Lubowitz, et al. (55) addressed only the question of increased sodium permeability in human erythrocytes exposed to sub-hemolytic shear stress. At shear stresses no higher than 500 dynes/cm^2 (exposure time of 3-4 hours) an increased passive sodium influx to the cell was observed. The active sodium pump was undamaged, and in fact, was stimulated to partially compensate for the increased inward diffusion of sodium ions. The net result, however, was a build-up of internal sodium. Indeglia and Bernstein (56) detected a release in cell lipids and phospholipids after exposure to mechanical trauma.

The resistance to mechanical hemolysis of erythrocytes was measured by Chien et al. (57) by observing filtration of

cell suspensions through polycarbonate filters. Jandl et al (58) successfully used filtration techniques to separate suspended mixtures of normal and pathogenic cells.

The factors which maintain erythrocyte shape and deformability, and conversely those which may alter red cell shape and deformability leading to decreased in vivo survival are still being determined in active research. Adenosine triphosphate is widely believed to be the source of high energy phosphate necessary to the maintenance of normal cellular cation composition. Almost 20 years ago Nakao, et al. (59, 60), reported evidence and suggested that ATP might also play a role in preservation of the normal biconcave disk shape. Nakao (61), slightly later, showed that survival of transfused cells' was a direct function of the cells ATP levels.

Experiments involving the effects of metabolic depletion of red cells were carried out by Weed et al. (62). Cells were incubated up to 28 hours. An early loss of membrane deformability, as assessed by viscosity measurements and the micropipette technique, was observed after 4 to 6 hours, when ATP levels were about 70% of initial levels. At about 10 hours, when ATP content had fallen to less than 15%, simultaneous increases in cell calcium levels were observed. After 24 hours the calcium concentrations increased by 400% and the viscosity of cell suspensions had risen over 500%. At this point, the ATP levels were essentially zero, and it required a 10 fold increase in negative pressure to deform the cell into a micropipette. Weed and his workers suggest the following sequence of events during metabolic

depletion: an early decrease in membrane deformability, followed by some swelling. After ATP levels are less than 15% and calcium levels greatly increased, a reversible ATP dependent transformation of a membrane protein from a sol to a gel occurs at the membrane interior. He concludes that a delicate balance between ATP, Ca^{++} and Mg^{++} is required for deformability maintenance: ATP and Mg^{++} aiding deformability, and Ca^{++} enhancing rigidity.

Another suspected regulator of cell shape and deformability are filamentous proteins in the membrane referred to, among other things, as microfilaments and microtubules (63). These have been noted in the platelet (64). The existence of such microfilaments and microtubules in red cells is in doubt, but evidence of some type of fibrous protein, commonly called spectrin, exists (65). Experiments have been performed by several workers (66) in which a group of known fibrous protein denaturants and depolymerizers (vinblastine, colchicine, cytochalasin B) were shown to induce erythrocyte morphologic alterations, specifically spherizing. Jacob and Yawata and co-workers (67) believe this is due to the direct action of the denaturant on the membrane spectrin, causing rigidity, cation leakiness, and splenic sequestration of these cells.

A number of workers have presented evidence suggesting that these fibrous proteins are regulated by the cyclic nucleotides 3' .5' cyclic AMP and 3'5' cyclic GMP (66,68-70). Most of this work has been with cells other than red cells,

and indicates a maintenance of cell shape and permeability by these two compounds.

Jacob, et al. (67) performed experiments whereby the denaturing effect of vinblastine and colchicine was inhibited by cyclic AMP and cyclic GMP. His conclusion was that the action of the protein spectrin requires phosphorylation by ATP that is catalyzed by cyclic nucleotides, stabilizing the spectrin against denaturing.

Altered Erythrocyte Deformability Due to
Sub-Hemolytic Shear Stress Exposure

Procedures

Donor Selection

In the collection of blood, certain precautions were taken in order to assure a degree of standardization among samples. These guidelines had been observed by previous workers in the Rice Biomedical Engineering Laboratory. All donors were males between age 20 and 30, who were in apparent good health. They had no known cardiovascular disorders, and were not under medication. Also, all donors did not smoke, were not heavy drinkers, and had fasted for approximately ten hours before blood donation. Most of the blood used for experimentation was taken from only a few donors, but all donors were restricted to giving no more than 450 cc during any three-month period. They also were fully informed and had given their written consent, in accord with the declaration of Helsinki.

Blood Collection

Blood was obtained by venipuncture performed at the Hematology Section of the Methodist Hospital, Houston, Texas by their own medical technologists. Typically, the blood was drawn through 19 gauge butterfly needles, and into 20 cc plastic disposable syringes. Nine cc of the blood was delivered into glass vacutainers containing 1 cc of 3.8 percent tri-sodium citrate for anticoagulant. The tubes were stoppered, and the blood and anticoagulant thoroughly

mixed in preparation for transportation to the Rice Bio-medical Engineering Laboratory.

Preparation of Washed Red Cells

Immediately upon return to Rice University, the blood samples were centrifuged for 15 minutes. The supernatant plasma and buffy coat, containing the white cells, were removed by using Pasteur disposable pipettes. The remaining packed red cells were washed with an equal volume of isotonic (0.9 percent) saline solution by gently mixing of the erythrocytes with the saline. This suspension was centrifuged for five minutes, and the resulting supernatant removed. The erythrocytes received two subsequent washes with equal volumes of Travenol Ringers injection solution. After these three washes, the packed cells were resuspended to approximately original volume in Ringers solution to which 50 mg/liter of human albumin had been added to aid the preservation of cell shape.

The red cell count of this suspension was determined electronically, using a Model B Coulter Counter (Coulter Electronics, Inc.) fitted with a 100 μ m aperture tube. Erythrocyte dilutions were made using standard Isoton (Coulter Diagnostics, Inc., Hialeah, Fla.) as diluent in the ratios given by the manufacturer. The erythrocyte suspension was now ready for shearing.

The Viscometer

The viscometer used in all the shear stress experiments

of this study was the ROM-8 rotational viscometer designed and developed at Rice University by W. O'Bannon and R.N. MacCallum (71). The raison d'etre of this original "Rice Viscometer" was the need to study shear stress destruction of red cells. With this in mind, the designers incorporated many features that make this viscometer ideally suited for this type of study.

Rotational viscometers have important characteristics, making them useful for studying shear degradation of any biological fluid. Most important of these is that the applied shear stress is easily known, and nearly constant for a particular sample volume. However, standard viscometers are primarily designed for rheological studies, and, hence, have some drawbacks when used in shear stress degradation experiments. A cross sectional view of the head of the Rice Viscometer is shown in Figure 1, and a schematic drawing is shown in Figure 2. The most important aspect of the design is the existence of three distinct shearing zones. The cone-and-cone section at the top alleviates several problems typical of conventional viscometers. This configuration eliminates the large, unsheared reservoir that exists in this area of most viscometers. In addition, there is a relatively small specimen-air interface in this region, compared to most viscometers. This interface is in very rapid motion when high shear rates are used. The cell hemolysis resulting solely from the interface and mixing effects has been shown to be significant by Leverett et al.,

Figure 1. Cross section of the "Rice Viscometer".

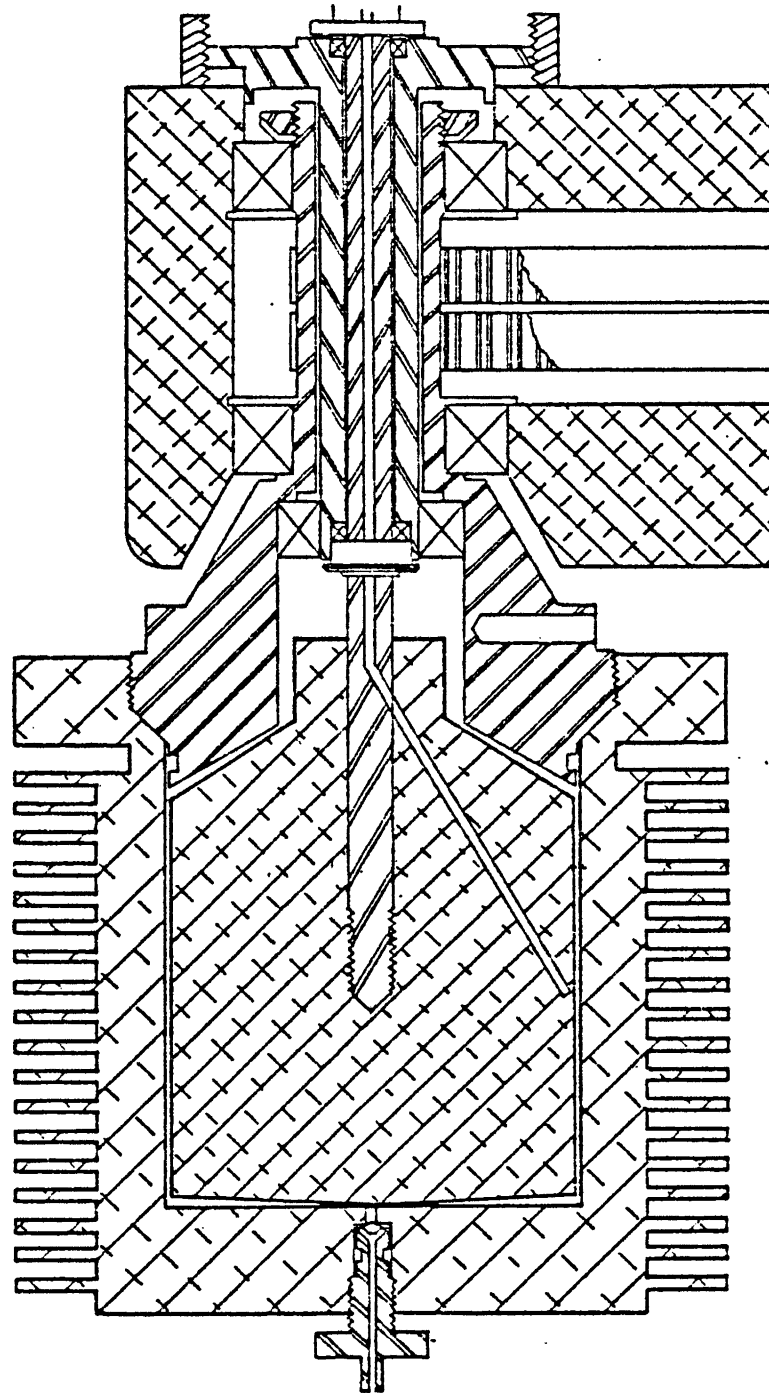
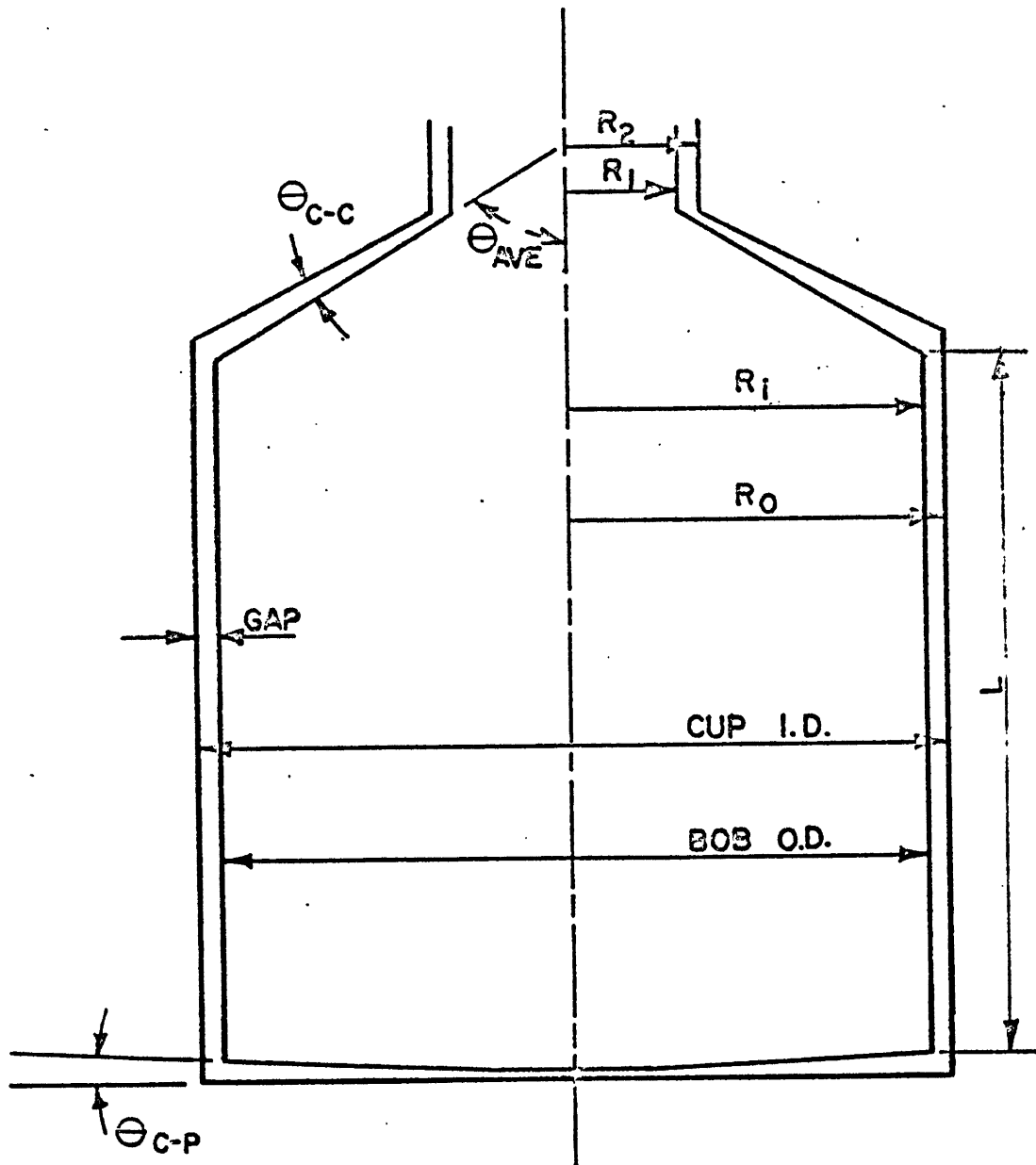


Figure 2. Schematic diagram of the "Rice Viscometer".



(49), and hence, efforts to minimize the interfacial area are clearly desirable. The (upper) cone-and-cone section, the (middle) concentric cylinder section, and the (bottom) cone-and-plate section were all designed so that the entire specimen is exposed to the same shear rate.

In shear degradation studies, higher stress levels than can be attained with most viscometers are necessary. The Rice Viscometer is extremely flexible and can produce shear rates up to $200,000 \text{ sec}^{-1}$ in laminar flow. The sample subjected to shear is of adequate quantity for subsequent study, and is easily removable from the viscometer. The viscometer is of the type in which the outer cup rotates, while the inner bob remains stationary. This design is by theory more stable to laminar flow than with a rotating bob and stationary cup (72).

During operation, as the outer cup rotates, a shearing force is transmitted through the sheared sample fluid to the surface of the inner stationary bob. The bob is suspended from a torsion spring of known spring constant. Responding to the shearing force, the bob rotates to the extent that the resistance of the spring will allow. The amount of spring deflection was measured by an angular-displacement transducer (Brush Instruments, Cleveland, Ohio, #761803993). The output of the transducer is displayed on a digital voltmeter. With the knowledge of the spring's elastic constant, and the extent of its deflection upon shearing, the shear stress exerted upon the fluid can be calculated.

The specifications of the particular cup and bob configuration used in all the shear stress experiments are given in Table 1. The cup and bob were both made of aluminum (series 6065 and 7075) and had a thin, hard anodized coating. An overall view of the Rice Viscometer and adjoining control console is shown in Figure 3.

Shearing Procedure

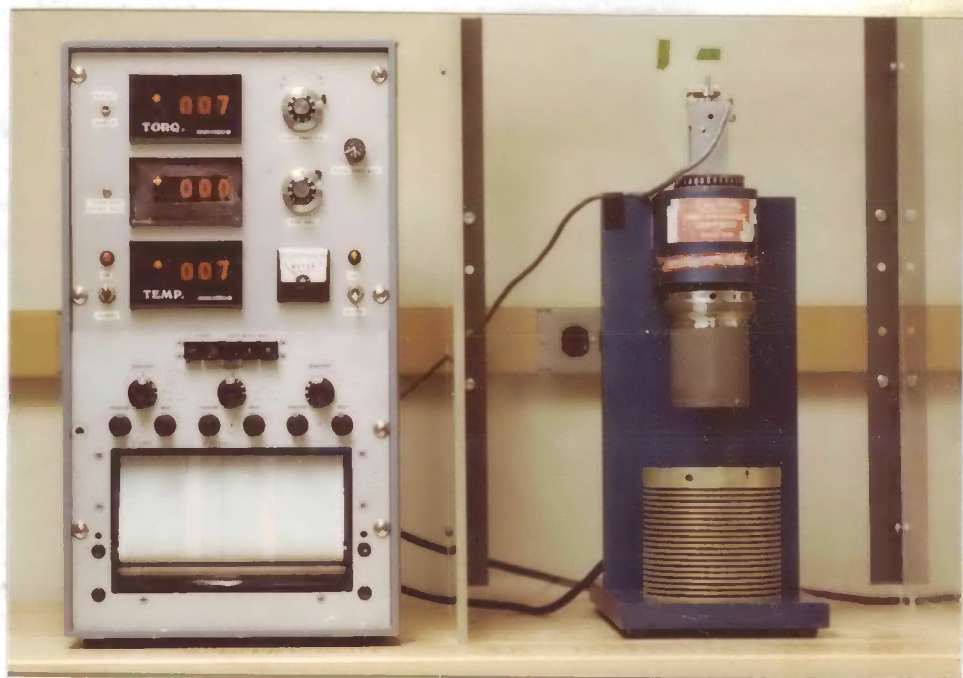
In immediate preparation to shearing a red cell suspension, the viscometer control unit was turned on 20-30 minutes prior to a run, to allow warm up of the electronic components. The cup and bob, which had previously been thoroughly scrubbed and rinsed in isotonic saline, were removed from their drying oven (maintained at 37°C). The bob was then screwed onto the torque shaft, and the torque reading on the control panel set to zero with the torque zero adjustment control. The cup was carefully fitted over the bob, and screwed onto the motor drive. The torque reading was again checked to read zero, and adjusted if necessary. Approximately 6.5 cc of erythrocyte suspension was injected, by syringe, into the viscometer through the filler port valve at the bottom of the cup. This volume was a few tenths of a cc above the theoretical sheared volume. This excess resided primarily in the filler valve itself, and of course was not sheared. A final readjustment was made to zero the torque reading.

The drive motor was then turned on, and the cup was brought to its desired rotational speed. The correct shear

Table 1
Viscometer Configuration Data

Cup I.D.	7.0010 cm
Bob O.D.	6.9416 cm
Gap width (cylindrical region)	0.0297 cm
$K = \text{Bob O.D.}/\text{Cup I.D.}$	0.991528
θ_{ave} (cone-plate)	0.487°
Volume (cone-plate)	0.75 cc
Volume (cylindrical)	4.56 cc
Volume (cone-cone)	0.79 cc
Total sheared volume	6.1 cc
Surface/Volume	81 cm^{-1}

Figure 3. "Rice Viscometer" and control console.



stress could be obtained in less than 10 seconds, and after this period a stop watch was started in order to time the run. All shear stress exposures in this study were for a two minute duration.

During the two minutes, the torque reading was monitored, and the rotational speed adjusted if necessary. Usually, this meant slight increases in cup speed. This was due to a slight amount of viscous heating, which raised the temperature of the suspension and hence lowered its viscosity. At the end of the shearing period the motor was turned off, with the cup coming to rest in about 3 seconds. The cup was immediately unscrewed from the motor drive. From the bottom of the cup the 6 cc of sheared sample was removed by syringe and transferred to a glass vacutainer. Both the cup and bob were rinsed and cleaned in tap water, then distilled water, then 0.9 percent isotonic saline. If another shear stress run was to be made, the cup and bob were dried and replaced on the viscometer, if not they were put in the incubator.

The sheared suspensions were returned to the Rice Biomedical Engineering Laboratory, for flow characteristic studies using Nuclepore filters.

Filtration Procedure

Preparation

Upon return to Rice, that portion of the original cell suspension that was not sheared was prepared for filtration. These cells were run of course as a control, for comparison

with the subsequent runs with sheared cells. All suspensions, whether of sheared cells or normal control cells, were run in exactly the same manner. A particular suspension was diluted to ten fold volume with the Ringers-albumin solution previously used. The final suspension volume was typically 30 cc, and had a red cell count of from 3.0 to 3.5×10^5 RBC per cubic millimeter. This suspension was drawn into a 50 cc plastic disposable syringe and was ready for filtration.

The entire filtration scheme, along with supporting electronic and recording devices, is shown in Figure 4. Before a run, the amplifier and chart recorder were turned on and given a sufficient warm-up period. During this time, a filter was chosen and fitted into its holder.

Polycarbonate Filters

Polycarbonate sieves were developed by Fleischer (73). All filters used were manufactured by the Nuclepore Corporation, Pleasanton, California. A list of the pertinent specifications for the type of filter used is given in Table 2. A picture taken by light microscopy of a typical filter face is shown in Figure 5.

The Nuclepore filter, due to its unique characteristics, was chosen over other filter types. Made by an atomic etching process, the technique produces a filter with very well controlled pore diameters. More importantly, the pores are cylindrical in nature and essentially normal to the filter face. This configuration is highly advantageous to

Figure 4. Nuclepore filtration apparatus.

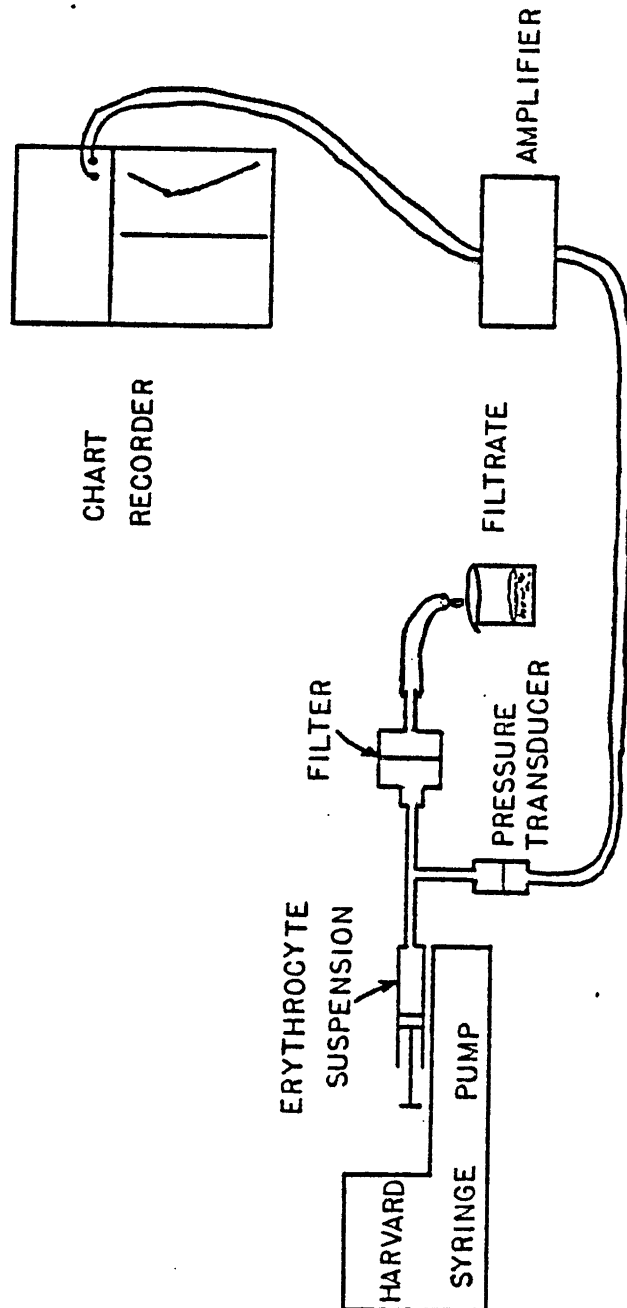
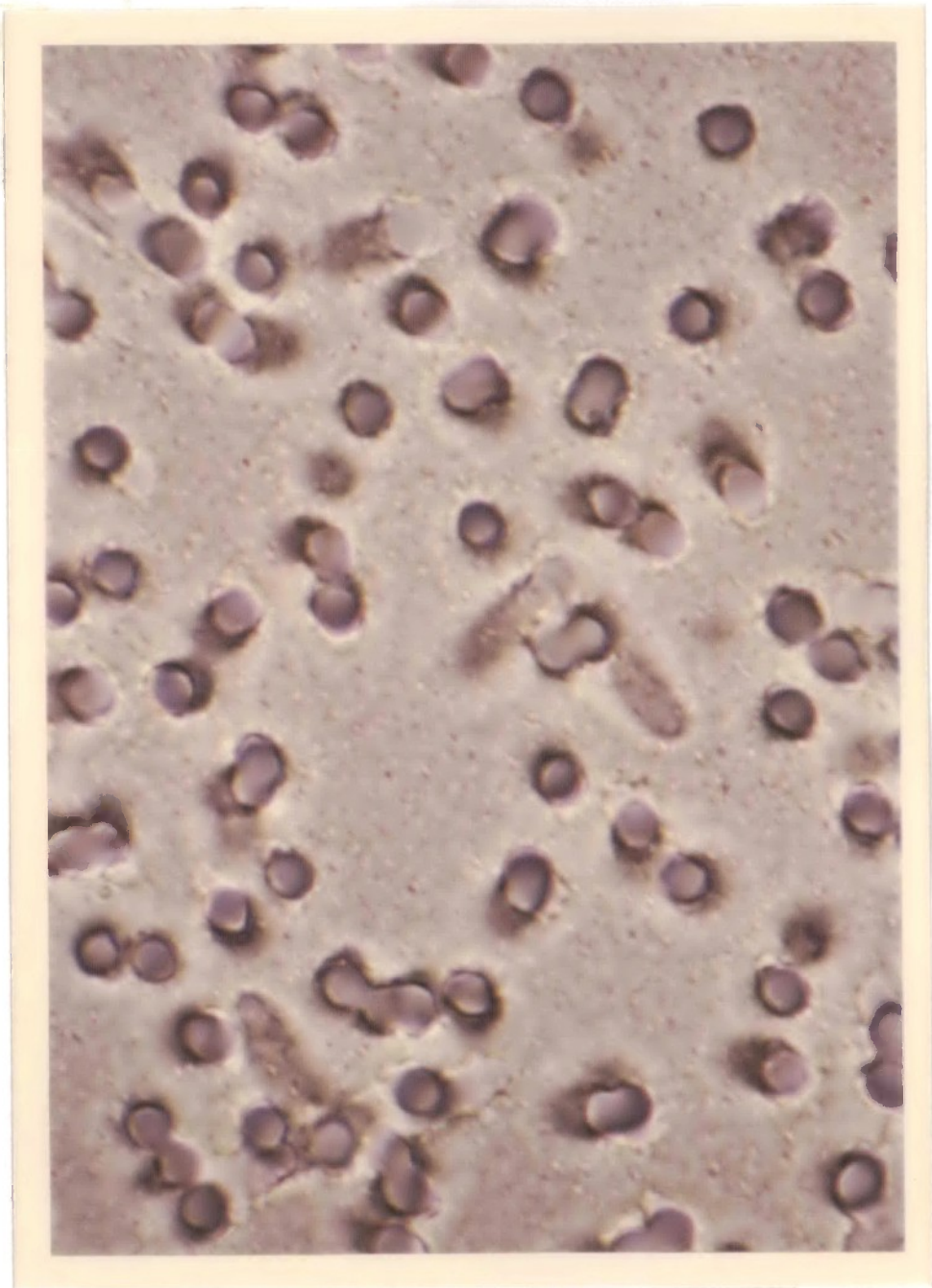


Table 2
Nuclepore Filter Specifications

Pore diameter	3.0 μm
Pore length	12.0 μm
Filter diameter	13.0 μm
Filter diameter available for filtration	9.0 μm
Pore density	2.0×10^6 pores/cm ²

Figure 5. Face of the 3 μ m Nuclepore filter (1250 x).



that of the Millipore filter, for example, for erythrocyte deformability studies. Being a cellulose type filter, the overall pore structure is of the network variety with a much wider pore size distribution. It is believed that the Nuclepore filters used provide both a reproducible method for testing red cell deformability, and a physical model of the microcirculation, specifically the splenic microcirculation where typical capillary diameters are as small as 3 μm .

Filtration

The Nuclepore filter was placed in its holder and locked into place. The filter holder was also manufactured by Nuclepore, and was rinsed before using with isotonic saline solution. The filter itself was never handled manually. Instead, the filters were moved by mouth suction applied by a Pasteur disposable pipette.

Before each filtration run, the pressure measuring equipment was calibrated using a pressure bottle with a Tycos pressure gauge.

The pressure measuring equipment is shown in Figure 4. Pressure was measured directly by a P23 pressure transducer (Statham Inc., Hato Rey, Puerto Rico) in conjunction with a P23 pressure amplifier. The output from the amplifier was fed to a two channel strip chart recorder (Brush Instruments, Cleveland, Ohio).

After sufficient warm-up and calibration, the syringe

containing the red cell suspension was placed in the pumping apparatus, a Harvard infusion pump (Harvard Apparatus, Cambridge, Mass.). Attached directly to the end of the syringe by Luer lock fitting was a T-type fitting. At the other end of the straight section from the syringe, the Nuclepore filter holder was connected. The pressure transducer was securely connected to the third arm of the T connector. A section of rubber tubing was connected to the downstream side of the filter holder. This served to deliver suspension filtrate to a collection beaker for subsequent cell counting and morphology studies.

A final adjustment was made to zero the pressure reading, and the infusion pump was turned on. The flow rate for all filtration runs was 2.17 cc per minute. This flow rate, in conjunction with the range of cell concentrations used, produced pressures of a magnitude easily measured, within the limits of the P23 transducer.

During the course of a run, the gauge pressure on the upstream side of the Nuclepore filter was monitored as a function of time. Since the pressure on the downstream side of the filter was atmospheric, the gauge pressure measured on the upstream side of the filter was equivalent to the pressure drop across the filter.

After a run, which typically lasted approximately 10-12 minutes, the infusion pump was first turned off. After 20-30 seconds most of the pressure within the system had subsided, so that the transducer could be removed without it

experiencing any sudden, extreme pressure drops. The syringe was lifted from the pump attached to the T connection and the filter holder. The holder was disconnected, and taken apart. The used filter was discarded (filters were only used once), the two sections of the filter holder were thoroughly rinsed in tap water, and then in isotonic saline. The syringe was discarded and the T connection thoroughly washed. All parts were now ready for a new run. If another run was to be made, the entire procedure was repeated exactly as given.

Filtrate Cell Counts

Immediately after a day's run, red cell counts were made of the collected filtrate suspensions. These cell counts were made on the same Coulter Particle counter as that used for the original count made before shearing.

Morphology Studies

To determine any damage or alteration of the erythrocytes that had traversed the Nuclepore filter, smears were made and examined microscopically. Smears were also made of cells subjected to shear stress but prior to passage through the filter. Slides were made by centrifuging the suspensions to concentrate the red cells. The final volume of red cells was diluted with an equal volume of the donor's plasma. The plasma had been saved from the initial centrifugation of the donor's whole blood. The film's made from this suspension were stained with Wright's stain.

The slides were examined at the Hematology Section of

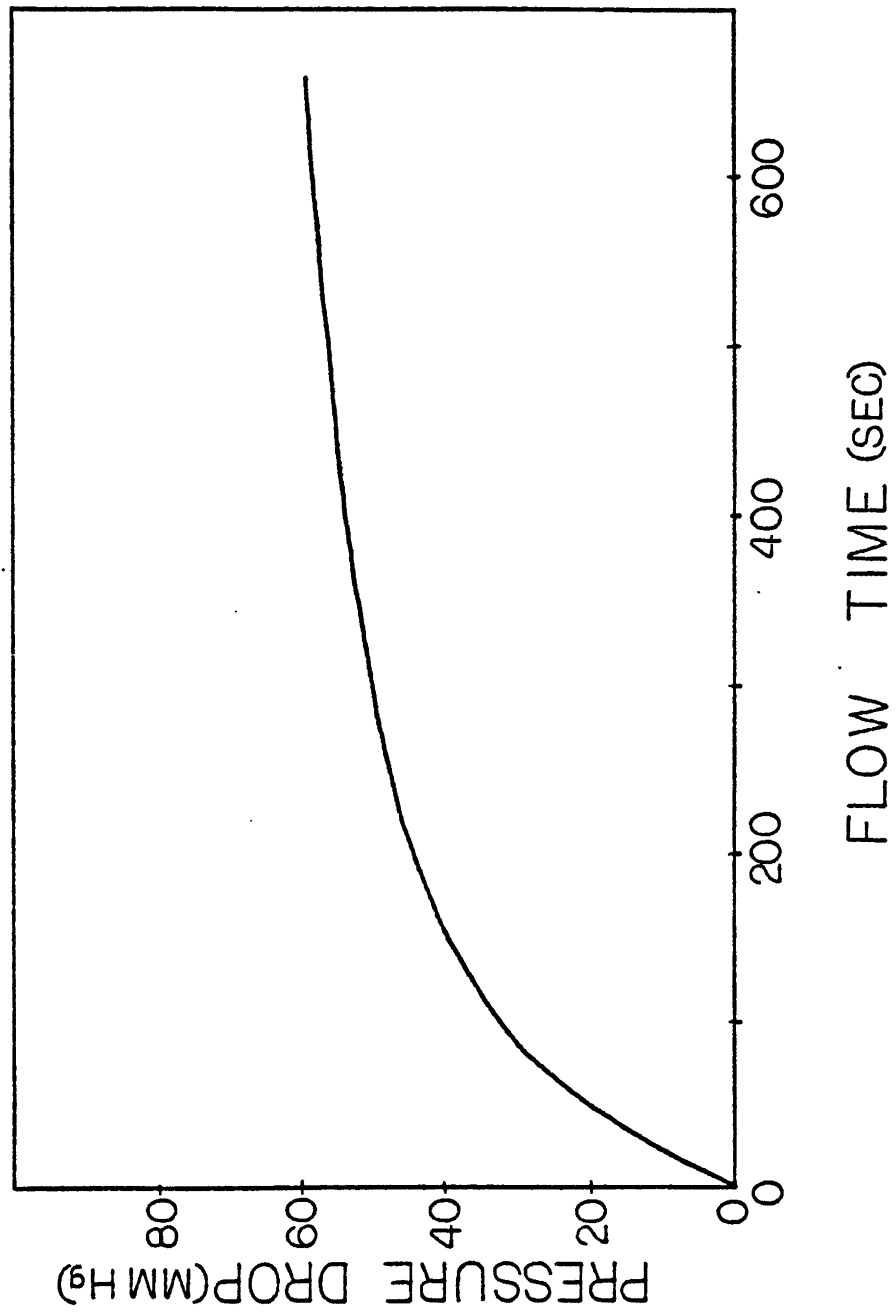
Methodist Hospital using a Carl Zeiss microscope. Color photographs were taken at 1250 x magnification.

Results

This study repeatedly showed that normal erythrocytes, not exposed to shear stress, were able to traverse the 3 μ m diameter pores of a Nuclepore filter with little difficulty. Both methods of assessing the cells filterability demonstrated this clearly. Figure 6 is a typical plot of the pressure drop across the Nuclepore filter generated during flow of normal unsheared red cells. The shape of this curve is quite similar to all those curves generated by suspensions of normal unsheared erythrocytes. Typically, the pressure drop rapidly increased to a given limiting level with only a slight further increase throughout the duration of the run. Maximum pressures incurred by unsheared suspensions were typically about 30-70 mm Hg and rarely exceeded 100 mm Hg under the flow conditions employed.

The pressure drops produced during filtration of normal erythrocytes were directly attributable to the presence of the cells alone. Under the same flow conditions, filtering only the suspending medium in the absence of cells, no measurable pressure drop could be recorded. If the flow rate of a pure fluid through the filter was increased so that a pressure could be sensed, an expected pressure vs. time curve was obtained. Almost instantaneously the pressure reached a value which then remained entirely constant thereafter. The effect of different erythrocyte flow rates on the resulting pressure drops generated was

Figure 6. Pressure drops vs. flow time curve for normal erythrocytes.



observed by varying the suspension red cell concentration and the suspension flow rate independently. This is shown in Figure 7 which plots limiting pressure drop (that produced after approximately 10 minutes of flow) versus red blood cell flow rate. The relationship is linear and such that an increase in erythrocyte flow rate results in a proportionate increase in limiting pressure drop. The ease with which normal cells traversed the Nuclepore filter was further evidenced by the morphology of the cells examined from the filtrate suspension. Upon centrifuging the filtrate to concentrate the erythrocytes, very low hemolysis was indicated by the presence of a colorless supernatant liquid. The cells, as they appear after filtration, are shown in Figure 8 under light microscopy, 1250 x. This is a normal cell population in appearance. There are no cell fragments present, the cell surfaces have remained smooth, and the biconcave disk shape has been preserved, as evidenced by the central pallor in each cell.

This study also verified that cells exposed to shear stress levels below 1500 dynes/cm^2 for 2 minutes do not exhibit hemolysis or morphologic alteration. Cells exposed to 1400 dynes/cm^2 for 2 minutes are shown in Figure 9. In physical appearance this is also a normal erythrocyte population. The application of the shear stress has not resulted in detectable morphologic alteration. Fragments are not evidenced, the surface appears smooth, and the biconcave shape remains despite the shear stress exposure.

Figure 7. The effect of erythrocyte flow rate on "limiting" pressure drop.

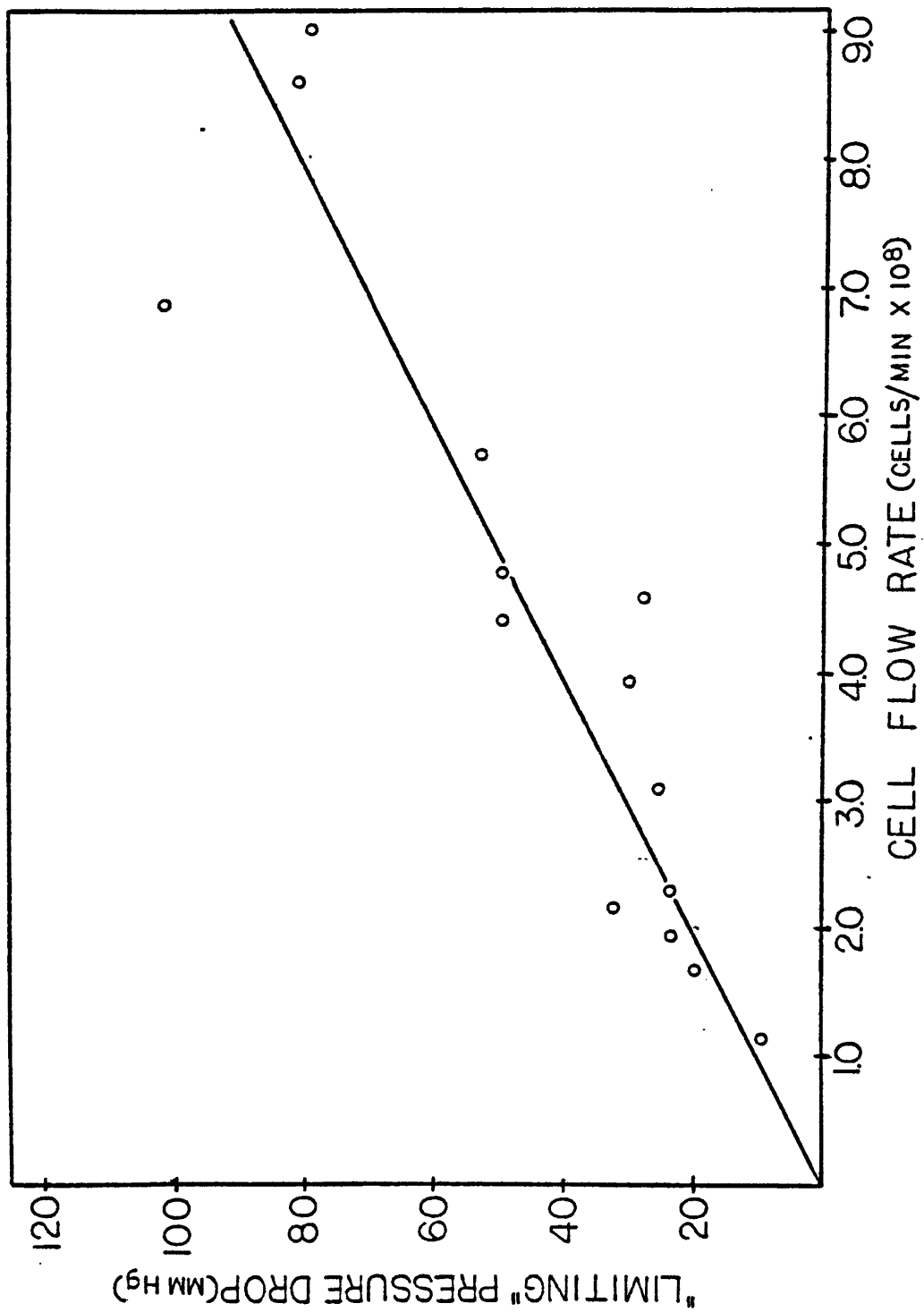


Figure 8. Normal erythrocytes after flow through 3 μ m Nuclepore filter (1250 x).

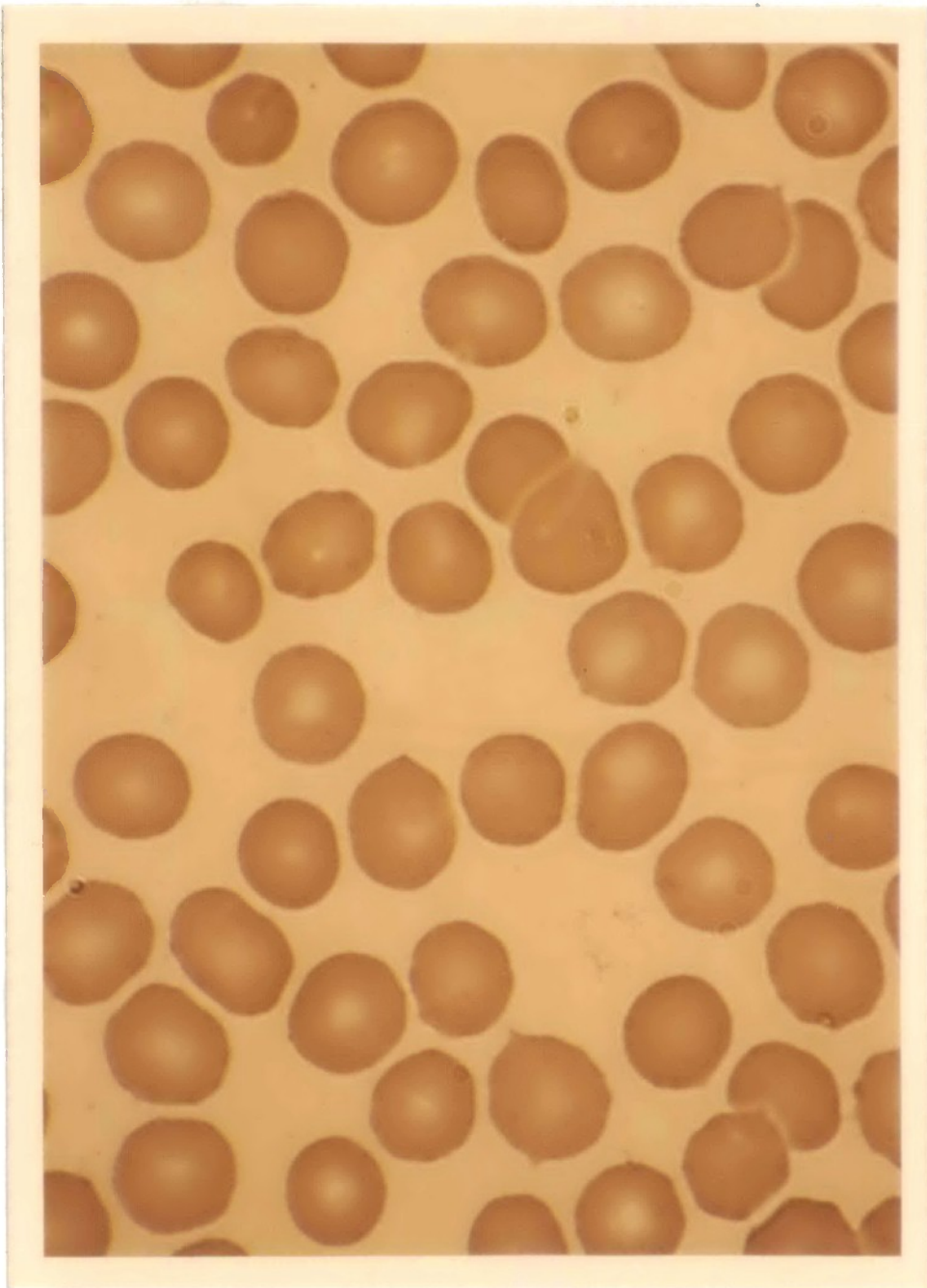


Figure 9. Erythrocytes immediately after exposure to 1400 dynes/cm² shear stress (1250 x).

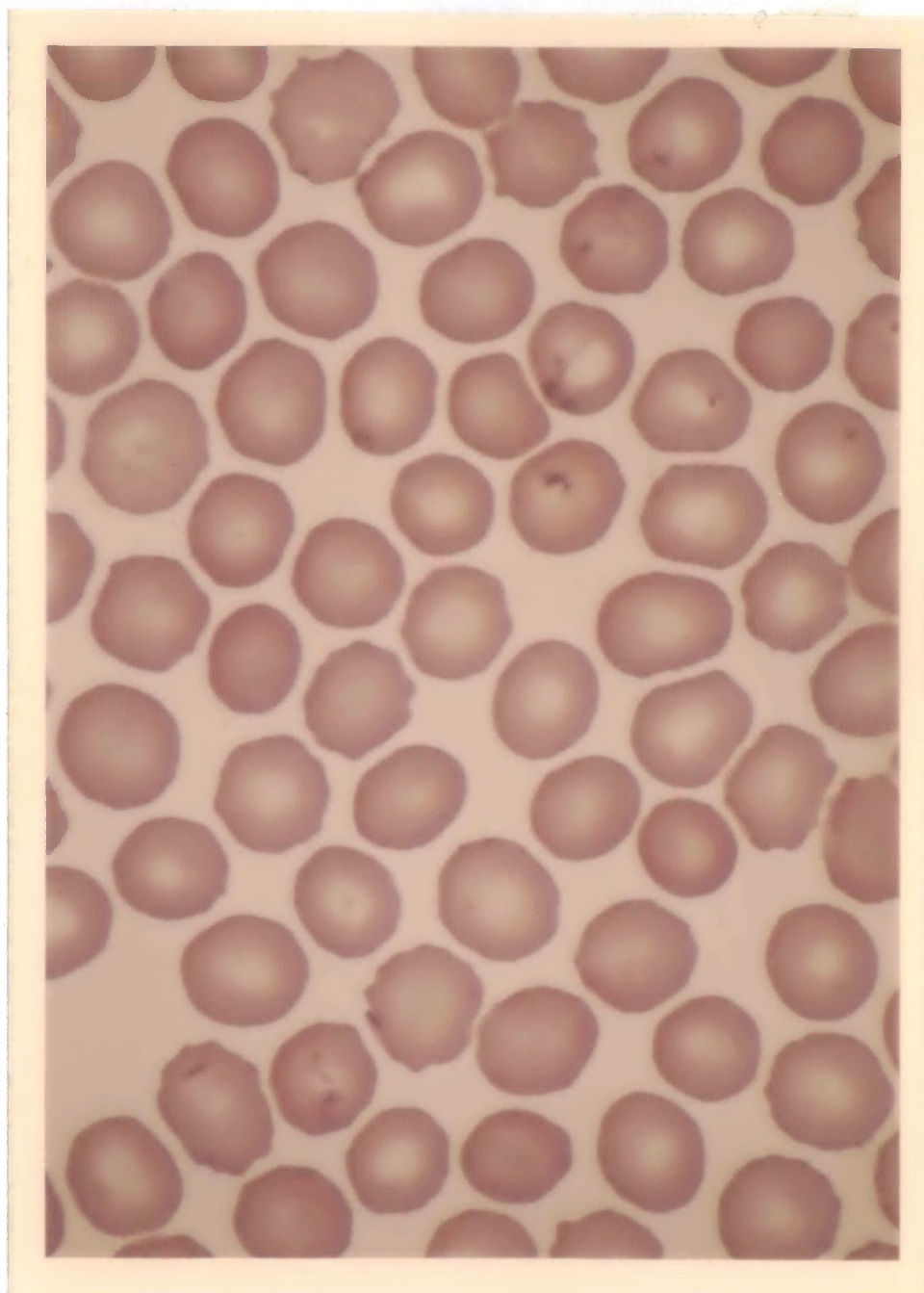


Figure 10 shows a plot of the pressure produced during flow through a Nuclepore filter having 3 μm diameter pores of erythrocytes exposed for 2 minutes to 1400 dynes/cm² shear stress. For comparison, the graph also shows the pressure produced during flow of unsheared cells through the filter. The two curves were generated with cells from the same donor on the same day. The flow conditions for the shear and unsheared cells were identical. In each case, suspension flow rate and cell count were the same.

Clearly, the behavior of the cells exposed to shear stress is extremely different, as shown by the markedly increased pressure drop produced during their flow through the filter. The shape of the higher pressure curve is typical of those produced by cells exposed to the higher shear stresses of this study. At these shear stresses (above 1000 dynes/cm²) the cells exposed cause a rapid and steady increase in pressure across the filter. Not only does the pressure increase, but also characteristic to these cells is that the slope of the pressure vs. time curve increases with time.

When cells subjected to 1400 dynes/cm² are passed through the filter, extreme fragmentation and hemolysis of erythrocytes occurred as shown in Figure 11. As well as fragmentation, numerous shape altered cells are evident, along with a large number of ghost cells. These are cells which at some point have ruptured, released some or all their hemoglobin, and subsequently resealed.

Cells exposed to 950 dynes/cm² shear stress alone are,

Figure 10. Pressure drop vs. flow time curves for erythrocytes exposed to 1400 dynes/cm², and normal unsheared erythrocytes.

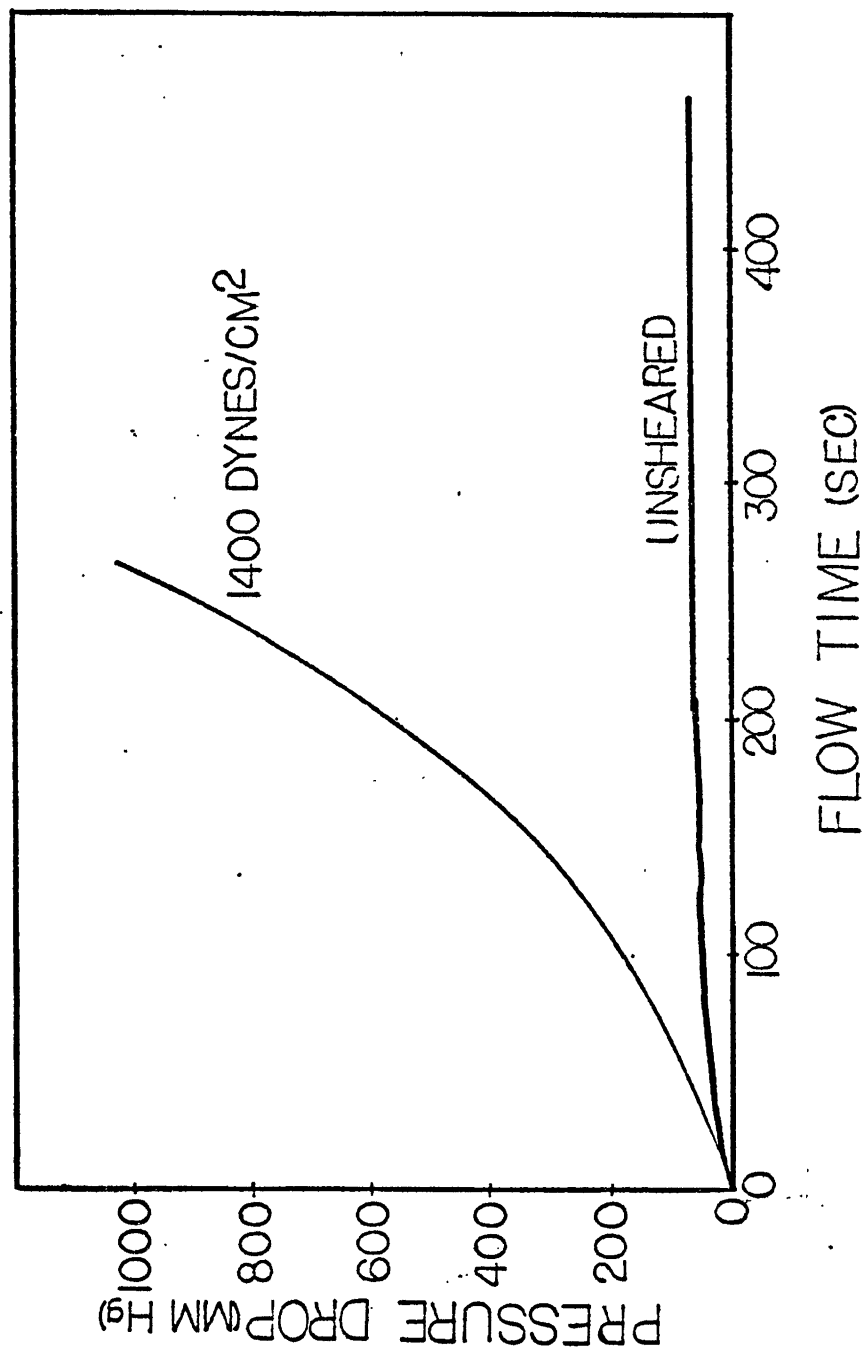
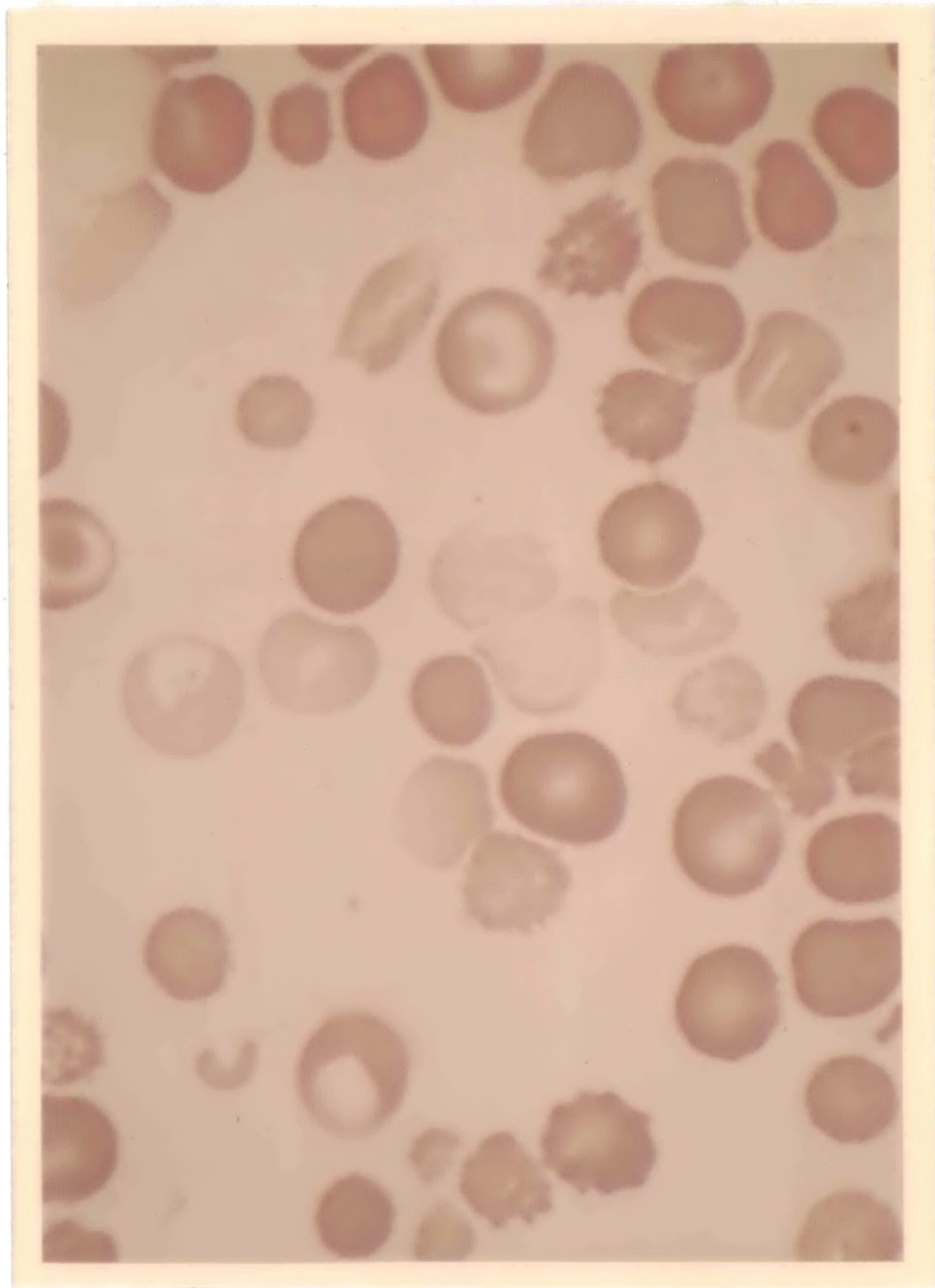


Figure 11. Erythrocytes exposed to 1400 dynes/cm^2 and after flow through the Nuclepore filter.



like those exposed to 1400 dynes/cm^2 , undamaged and morphologically unaltered (Figure 12) before passage through the $3 \mu\text{m}$ pores. After Nuclepore filter passage the cells are highly damaged with a large amount of fragmentation and cell ghosts as seen in Figure 13.

The pressure drop generated during the flow of these cells is plotted in Figure 14. Compared to the unsheared normal cells the sheared cells' pressure drop is much higher, and not appreciably lower than those of cells sheared at 1400 dynes/cm^2 .

The pressure vs. time curves observed for passage through the Nuclepore filter of cells exposed to 800 and 600 dynes/cm^2 are shown in Figure 15. It is evident from this plot that these cells traversed the Nuclepore filter with much greater ease than those exposed to 1400 dynes/cm^2 . Comparing Figure 10 and 15 shows that the pressure curves generated by cells sheared at 800 and 600 dynes/cm^2 are much closer to duplicating the corresponding curve for unsheared cells than the curve produced by cells sheared at 1400 dynes/cm^2 . While closer to the behavior of normal, unsheared cells the erythrocytes sheared at 800 and 600 dynes/cm^2 are still distinctly different.

Cells exposed to 600 dynes/cm^2 shear stress are shown in Figure 16. The same erythrocytes after subsequent passage through the Nuclepore filter show less extreme damage than those exposed to higher levels of shear stress upon filtration (Figure 17). Cell fragmentation is evident, but to a much

Figure 12. Erythrocytes exposed to 950 dynes/cm^2 shear stress (1250 x).

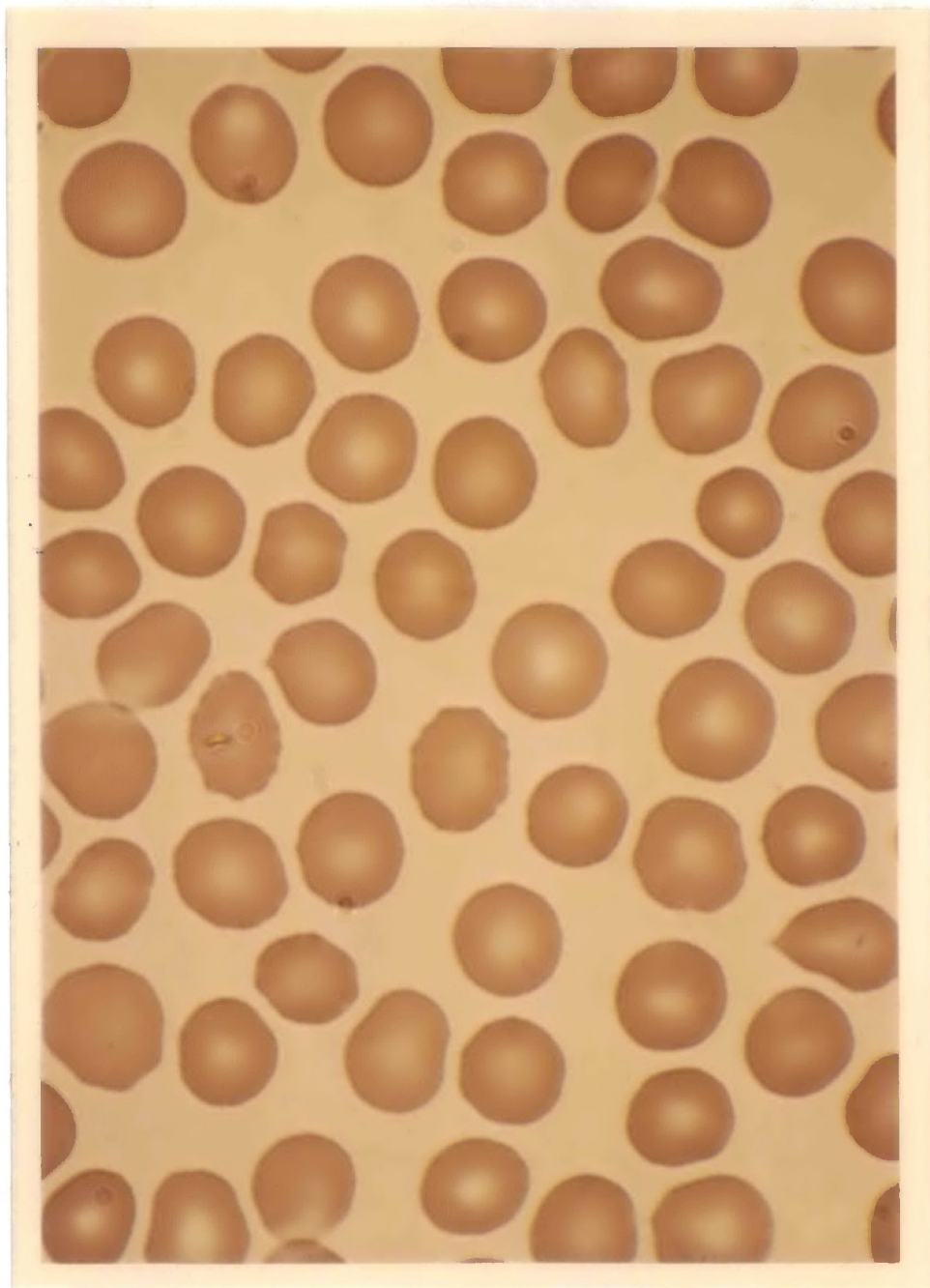


Figure 13. Erythrocytes exposed to 950 dynes/cm^2 shear stress after passage through the Nuclepore filter (1250 x).

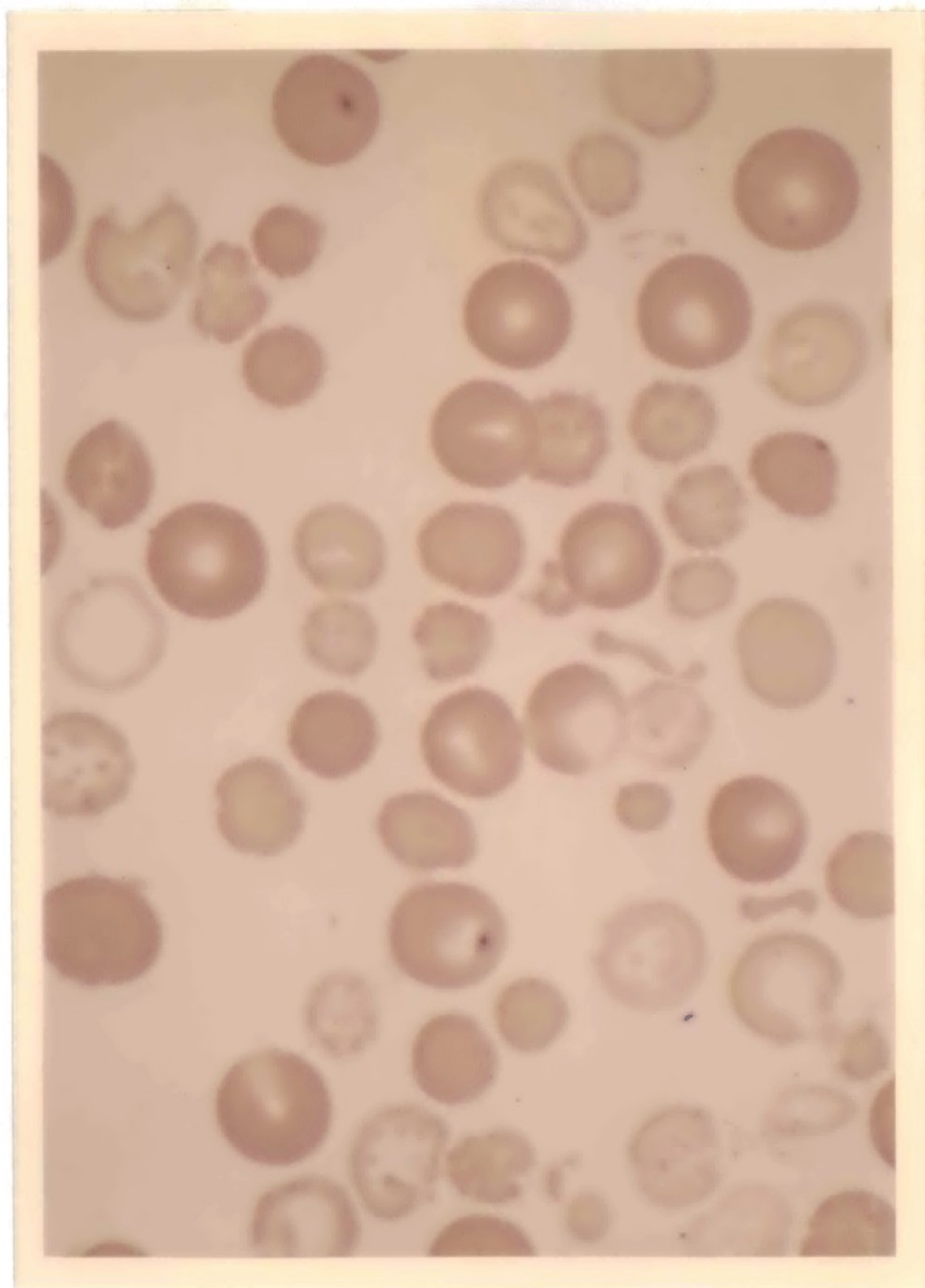


Figure 14. Pressure drop vs. flow time curves for erythrocytes exposed to 950 dynes/cm², and normal erythrocytes.

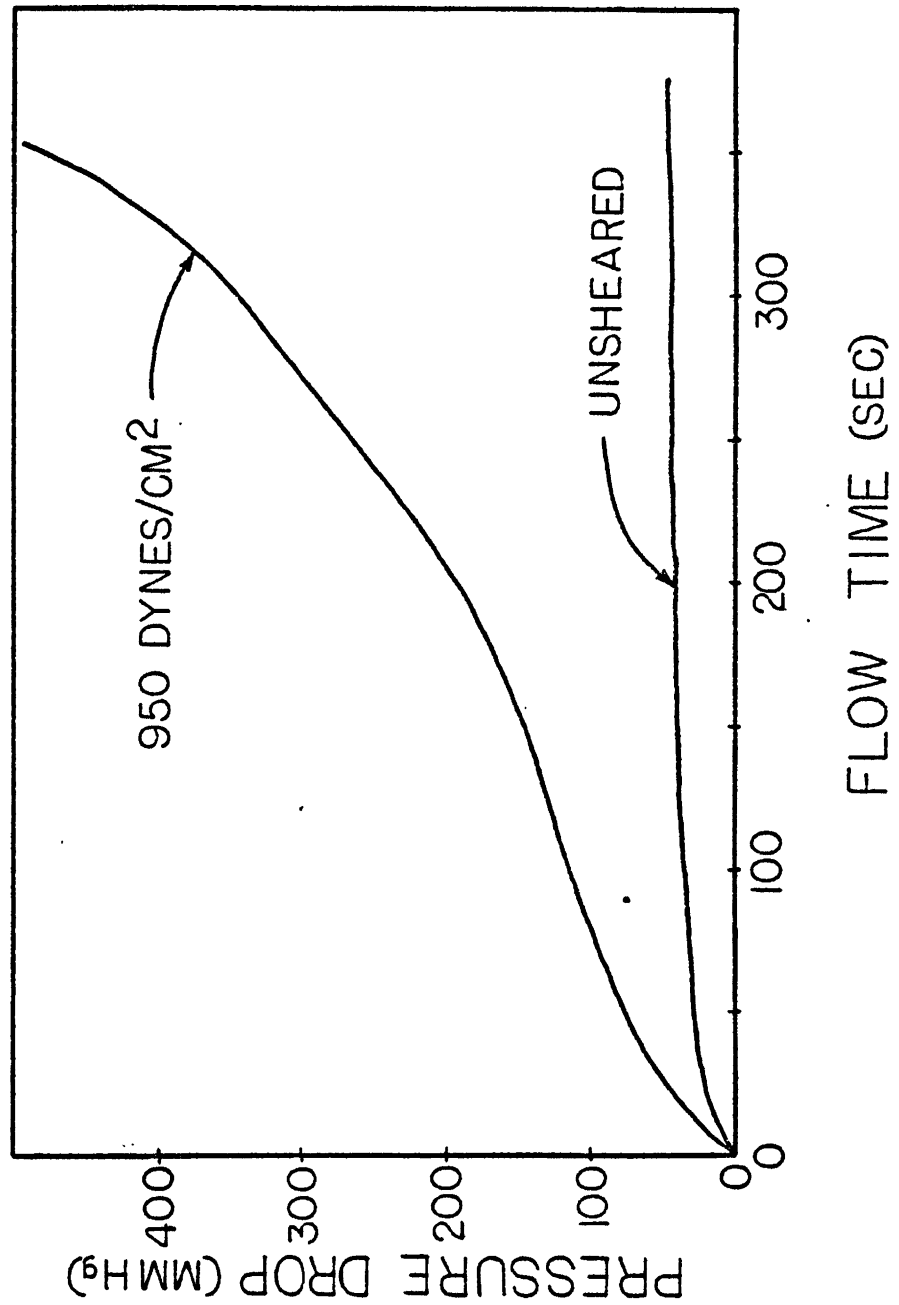


Figure 15. Pressure drop vs. flow time curves for erythrocytes exposed to 800 and 600 dynes/cm² and normal erythrocytes.

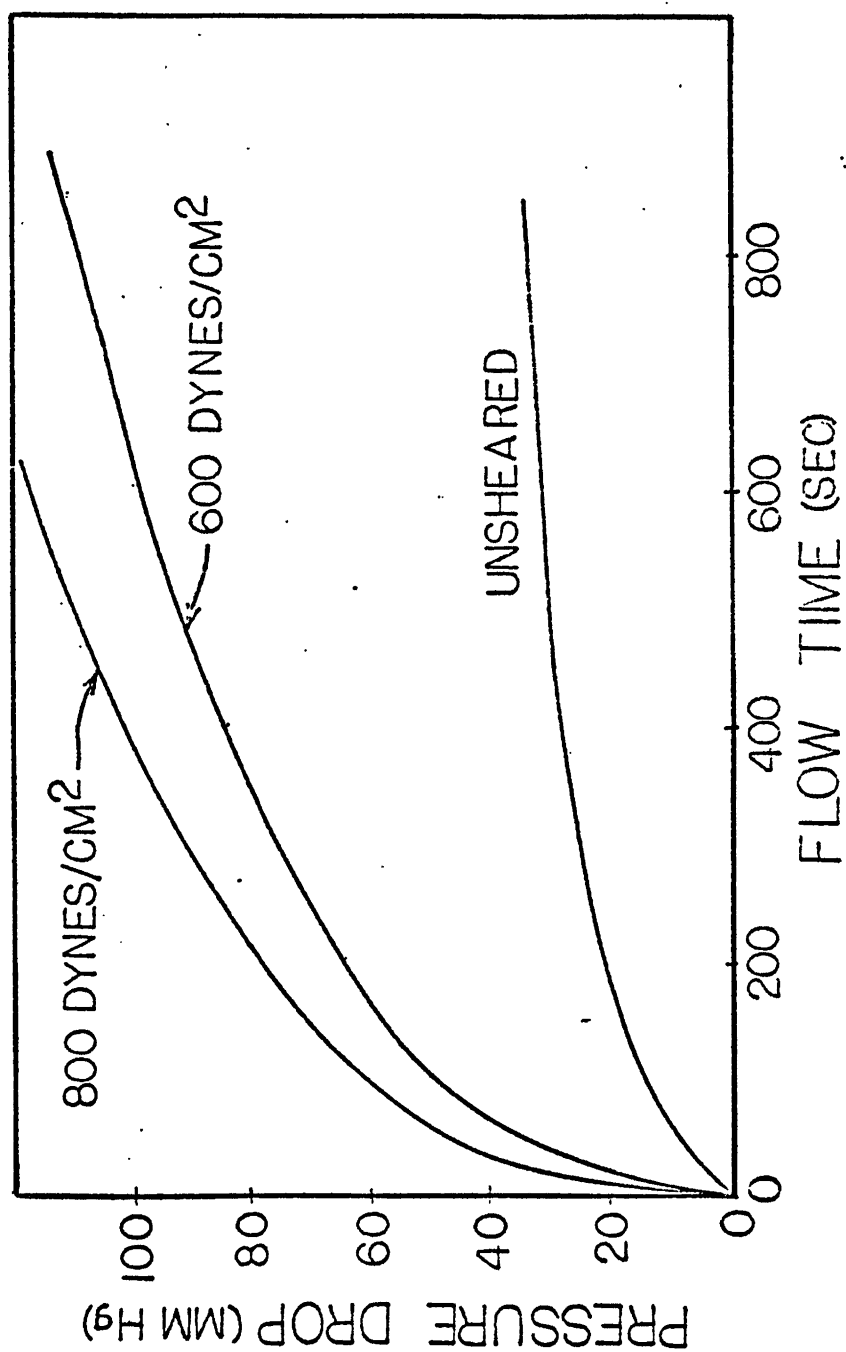


Figure 16. Erythrocytes exposed to 600 dynes/cm^2 shear stress (1250 x).

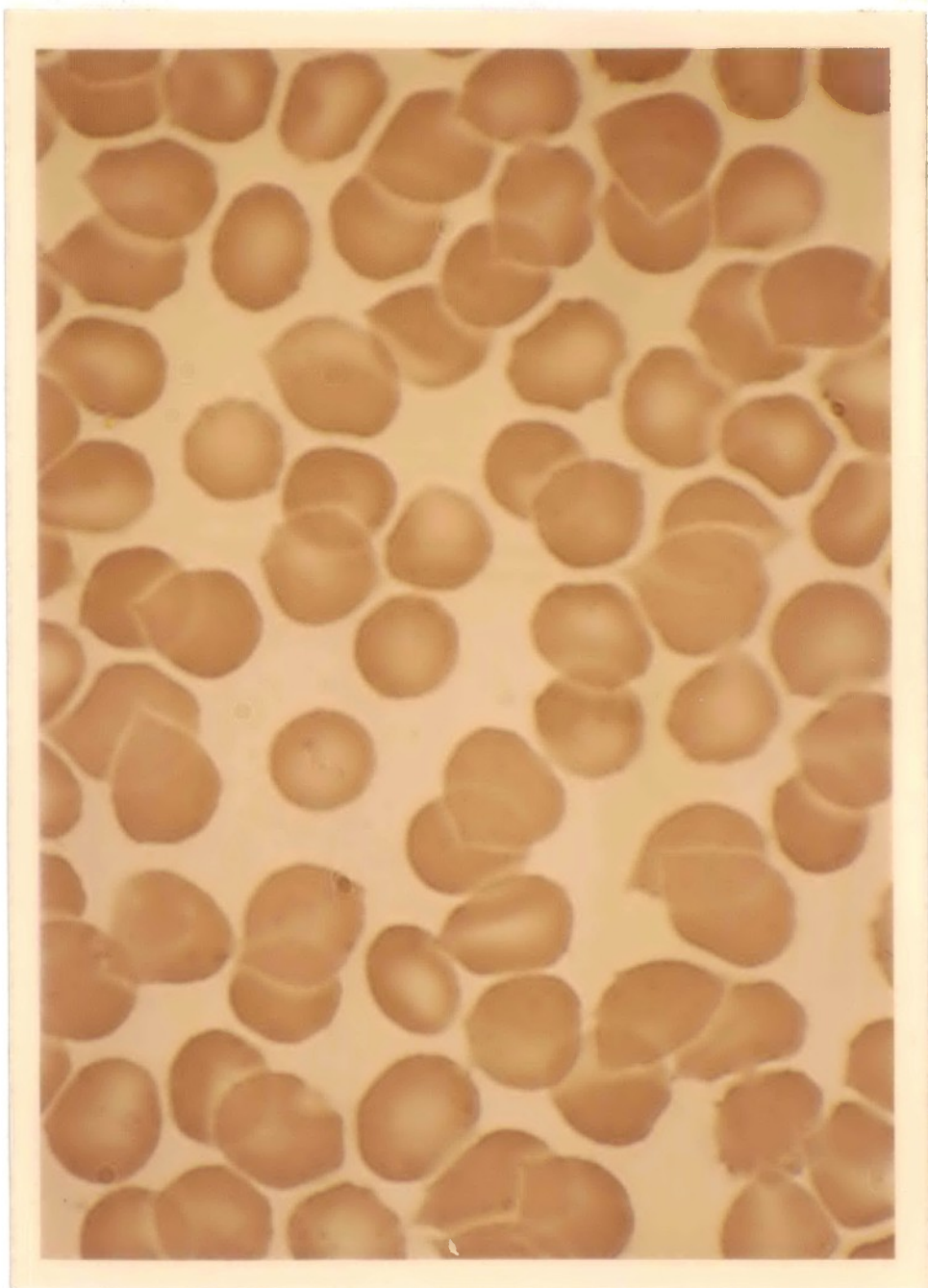
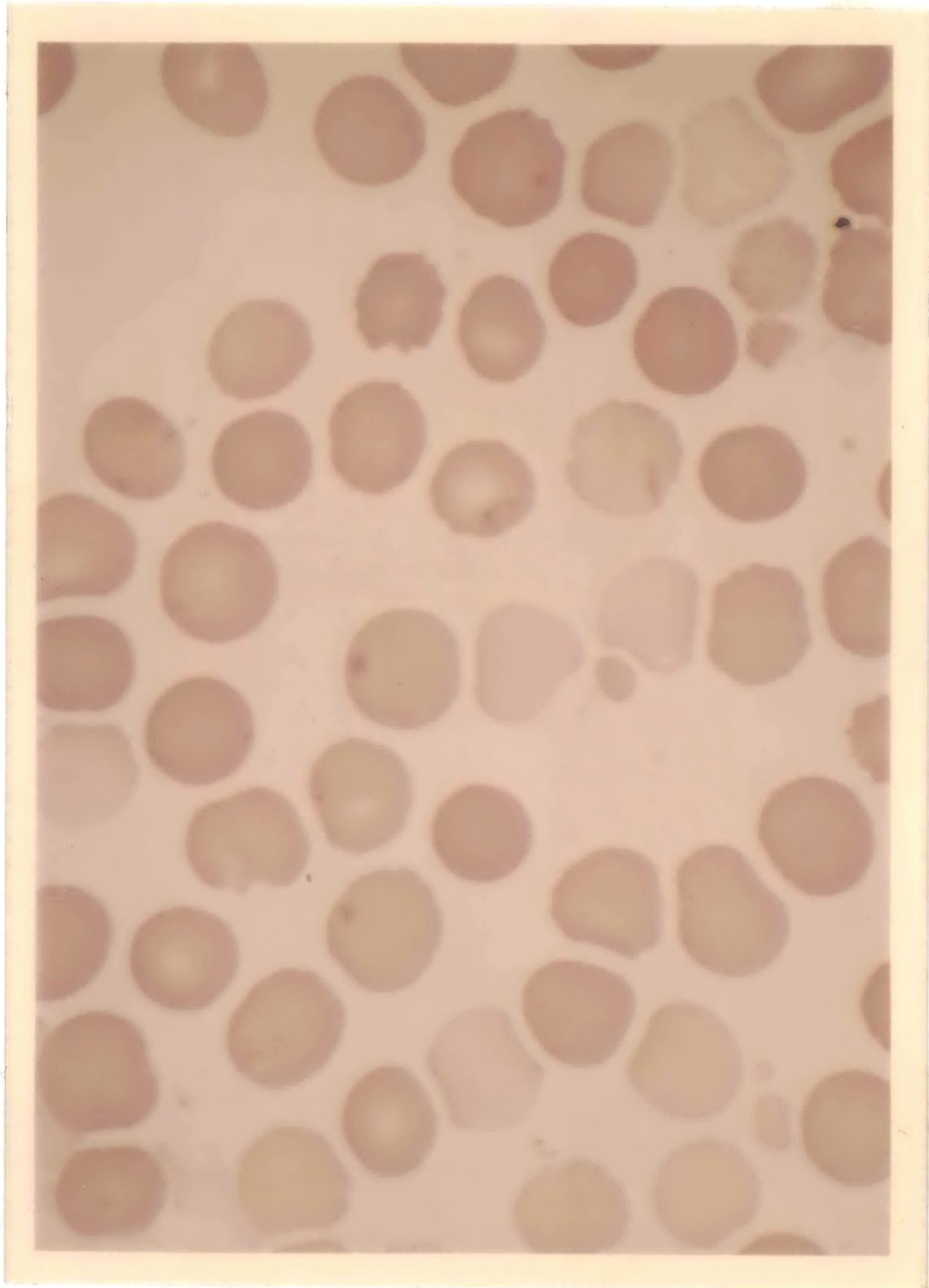


Figure 17. Erythrocytes exposed to 600 dynes/cm^2 shear stress after passage through the Nuclepore filter (1250 x).



lesser degree than seen before. A number of cell ghosts are apparent, but at the same time a larger percent of essentially normal appearing cells are present.

Figure 18 shows the pressure vs. flow time curves for suspensions of erythrocytes subjected to 700 and 500 dynes/cm² shear stress. As before, these two curves are obtained with the same donors blood on a single day along with the control curve generated with the donor's fresh unsheared red cells. Continuing the trend seen earlier, the two curves representing behavior of the two sheared samples are closer to approximating the control curve for unsheared cells. This would clearly indicate that the behavior of the cells sheared at decreasing levels is approaching that of unsheared cells.

The pressure drops across the Nuclepore filter generated by cells sheared at 450 and 350 dynes/cm² are seen to be even closer to the control pressures shown in Figure 19. Correspondingly, Figure 20 shows erythrocytes after exposure to 350 dynes/cm² and passage through the filter. This cell population is essentially normal, with at most, half a dozen cells showing minimally altered morphology.

The results of experiments involving red cells subjected to the lowest shear stress examined in this study are shown in Figure 21. Suspensions of erythrocytes exposed to 250 dynes/cm² shear stress when passed through the Nuclepore filter produce pressures during flow virtually identical to those of the unsheared control sample. This indicates that

Figure 18. Pressure drop vs. flow time curves for erythrocytes exposed to 700 and 500 dynes/cm², and normal erythrocytes.

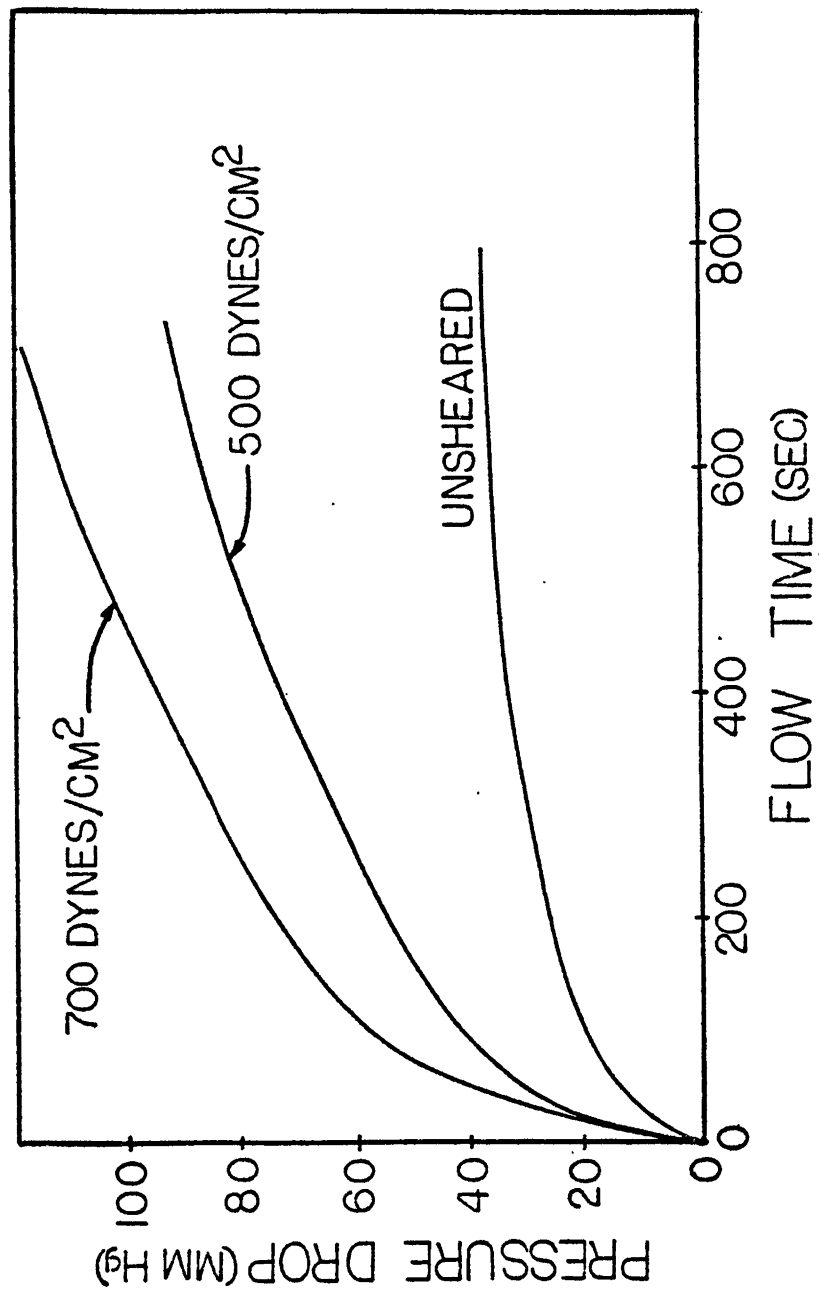


Figure 19. Pressure drop vs. flow time curves for erythrocytes exposed to 450 and 350 dynes/cm² and normal erythrocytes.

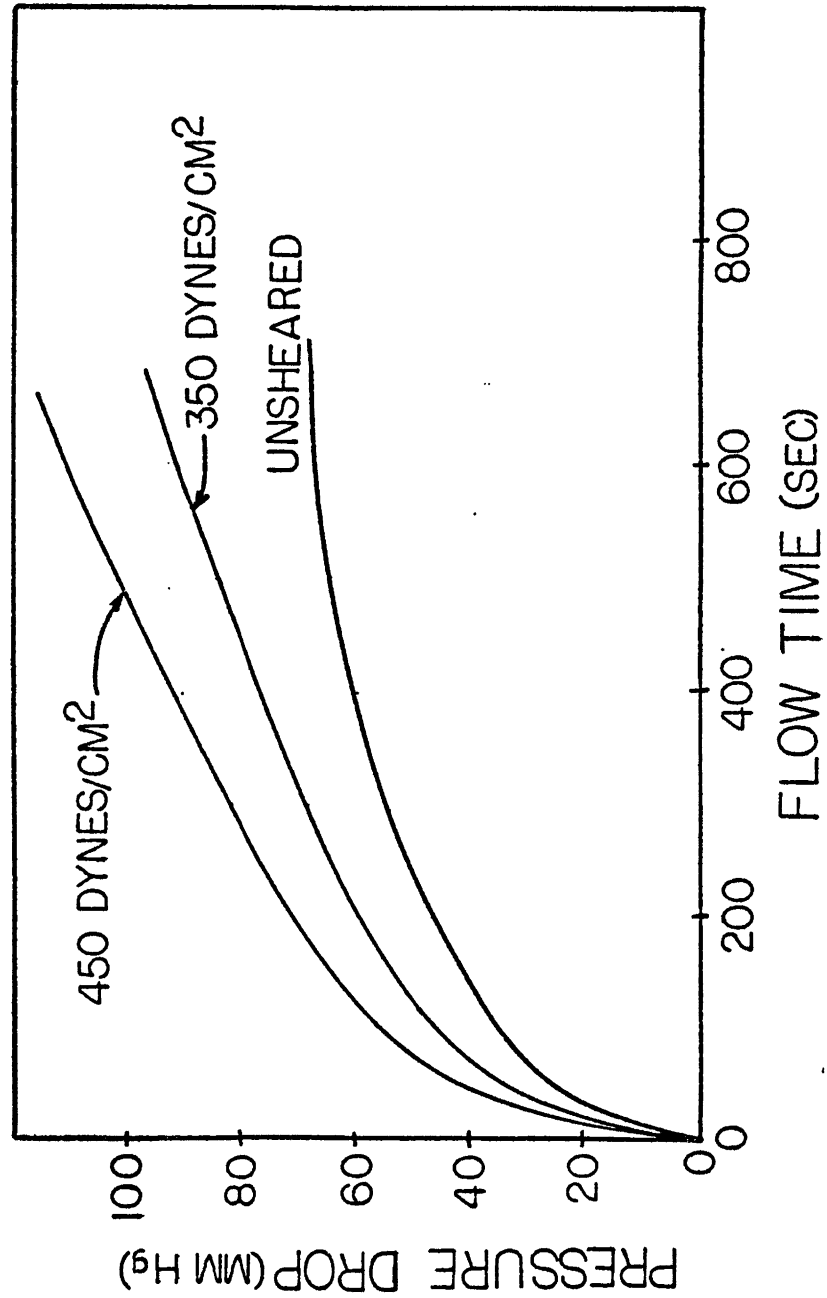


Figure 20. Erythrocytes exposed to 350 dynes/cm^2 after passage through the Nuclepore filter.

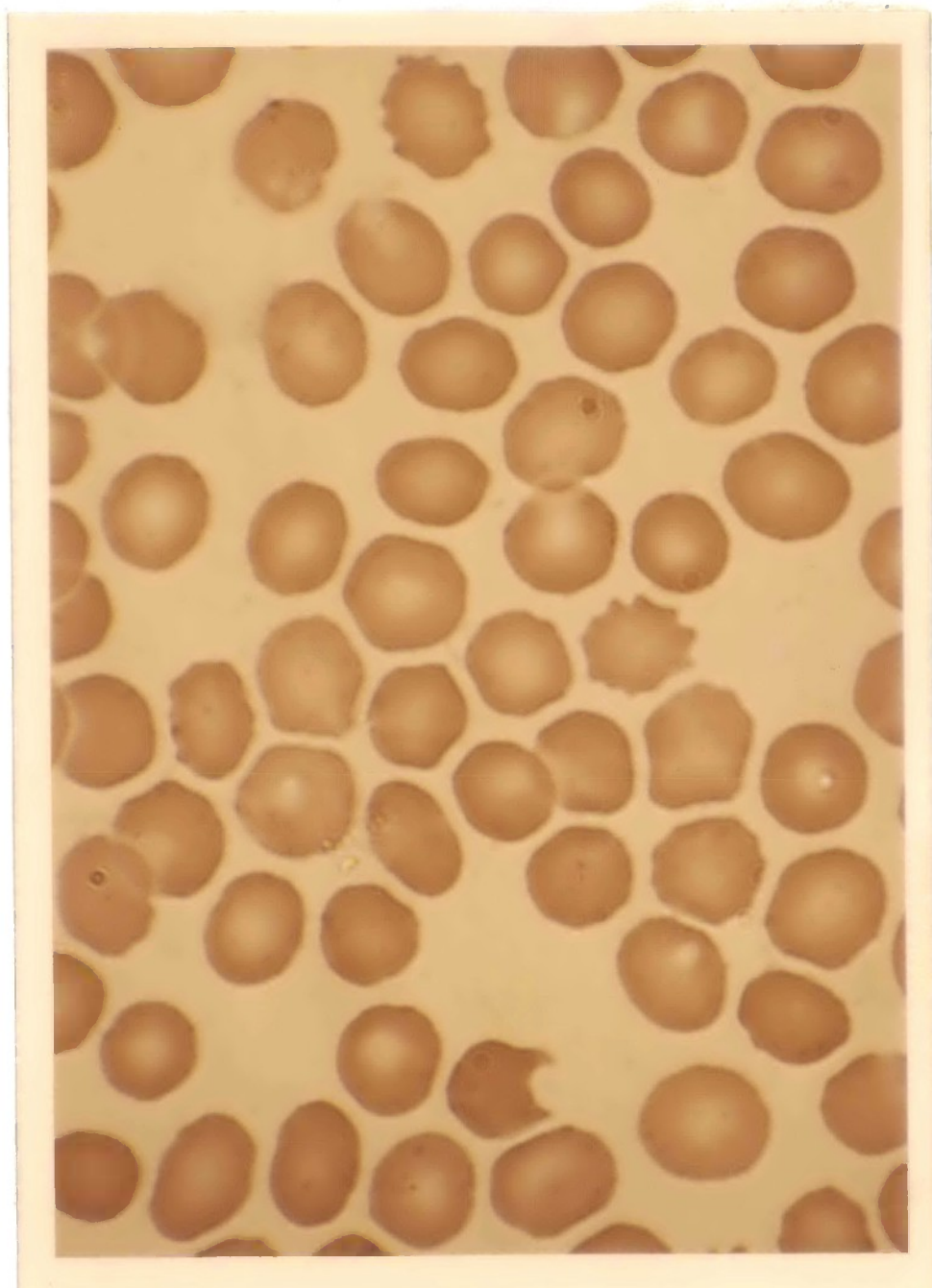
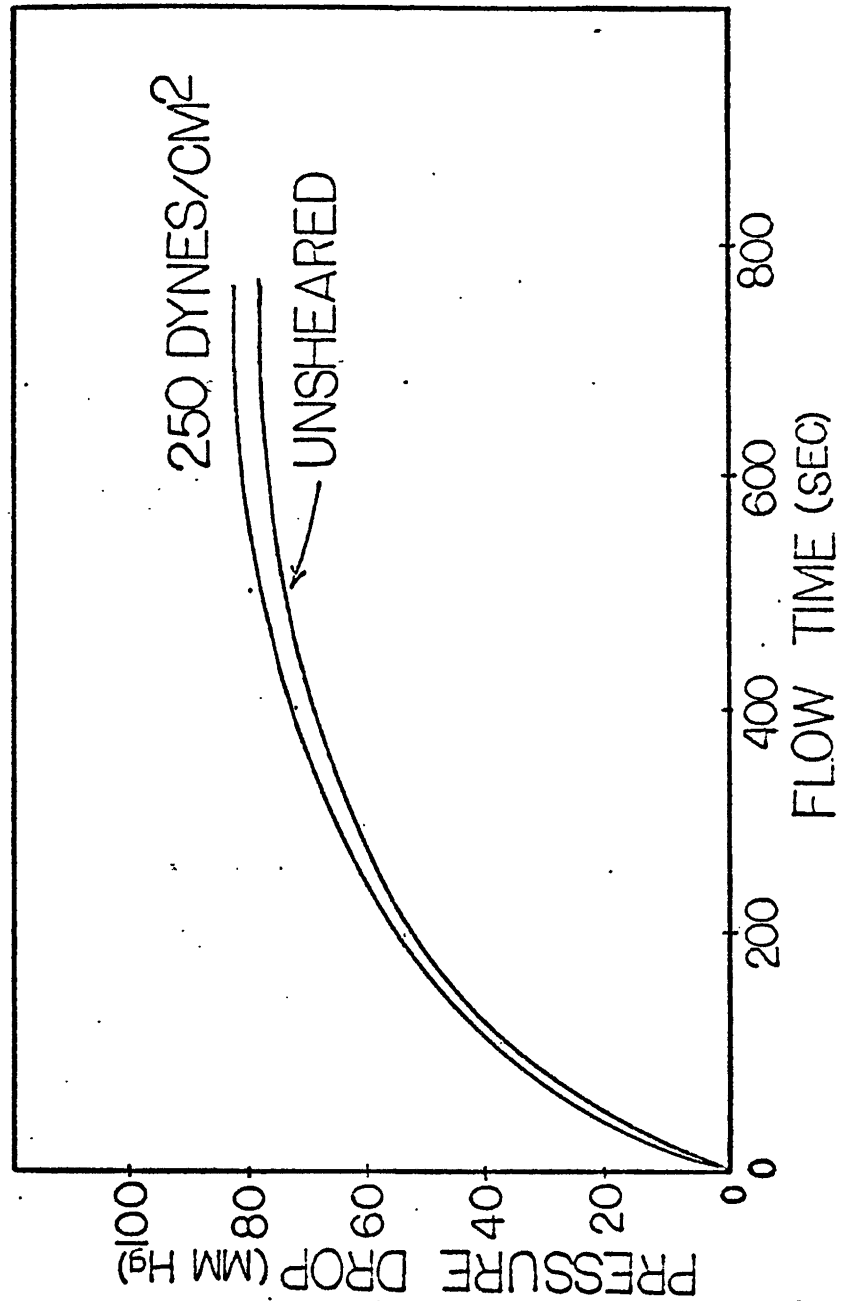


Figure 21. Pressure drop vs. time curves for erythrocytes exposed to 250 dynes/cm², and normal erythrocytes.



application of this low level of shear stress has not induced the cell alteration that is detectable by the filtration test in cells exposed to shear stresses above 250 dynes/cm².

Subsequent Procedures

After it became evident that a fairly severe membrane defect could be induced by the application of sub-hemolytic shear stress, attempts were made to reverse or repair the abnormality through incubation with the following substances:

- a. The normal Ringers-albumin solution (to determine if the alteration was simply reversible with time).
- b. Glucose.
- c. Dibutyryl adenosine 3':5'-cyclic monophosphoric acid (DB C AMP) and dibutyryl guanosine 3':5'-cyclic monophosphoric acid (DB C GMP) (Sigma Chemical Company).
- d. Adenosine triphosphate (ATP) (Sigma Chemical Company).
- e. Adenosine (Sigma Chemical Company).

Solutions b-e were prepared by dissolving the solid directly into the Ringers-albumin solution. Solutions c-e were stored below 0° C, while the solids for these solutions were stored dessicated below 0° C.

For these experiments a standard applied shear stress was chosen for all runs. A value of 1000 dynes/cm² for an exposure time of 2 minutes was used throughout. This level produces clearly measurable results, and at the same time is at least 500 dynes/cm² below the threshold level for hemolysis.

After application of the shear stress, the total sheared volume (slightly over 6 cc) was divided into either three 2 cc sections or two 3 cc sections. The filtration through 3 μ m pore diameter Nuclepore filters of the freshly sheared cells used one of these volumes. Depending on whether 4 or 3 cc of the total sheared volume remained, two or one incubation studies could be made, as at least 2 cc of suspension was required for a filtration run.

Incubations were carried out with equal volumes of the sheared cell suspension and the incubation solution being thoroughly mixed in a glass vacutainer. All incubations were done at 37°C for periods between 60-180 minutes. Approximately every 30 minutes the suspensions were gently mixed. Three hours was considered the maximum incubation time, since after this period the cells were approximately six or seven hours old.

After incubation, the cell suspensions were centrifuged to separate the cells, and the supernatant liquid removed. The cells were washed twice in Ringers-albumin and resuspended in Ringers-albumin. A final dilution was made to produce a cell count that matched that of the final suspension of the freshly sheared cells. The sheared and incubated erythrocyte suspension was filtered through 3 μ m Nuclepore filters in precisely the same manner as the control cells that were filtered immediately after shearing.

Repair of Shear Stress Defect Results

Experiments indicate clearly that the alterations in deformability caused by exposure to sub-hemolytic shear stress were not reversible during an incubation in only the Ringers-albumin solution. Figure 22 shows the pressure vs. flow time curves generated by one donor's red cells on the same day. The three curves represent the behavior of fresh un-sheared cells, cells sheared at 1000 dynes/cm^2 for two minutes and filtered immediately, and cells that had been sheared and then incubated 120 minutes at 37°C . The behavior of the cells after incubation is virtually the same as that of the cells filtered soon after shear stress exposure. Figure 23 shows the same three curves for a different donor and an incubation time after shear of 90 minutes. Here there is a slight decrease in pressure generated by the incubated cells. This is not, however, a significant return to normal un-sheared behavior.

The effects of glucose incubation in repairing shear induced cell deformability alterations are shown in Figures 24 and 25. The first figure shows a family of four curves representative of filtration of fresh cells, cells sheared at 1000 dynes/cm^2 , and cells filtered after 120 and 180 minutes incubation following shear at 37°C in 1.0 mg/ml glucose solution. Clearly the glucose exposure has not in any way served to reverse the shear stress damage. In fact the longer incubation time only produced cells giving a

Figure 22. Effects of incubation in Ringers solution (120 min) following shear stress.

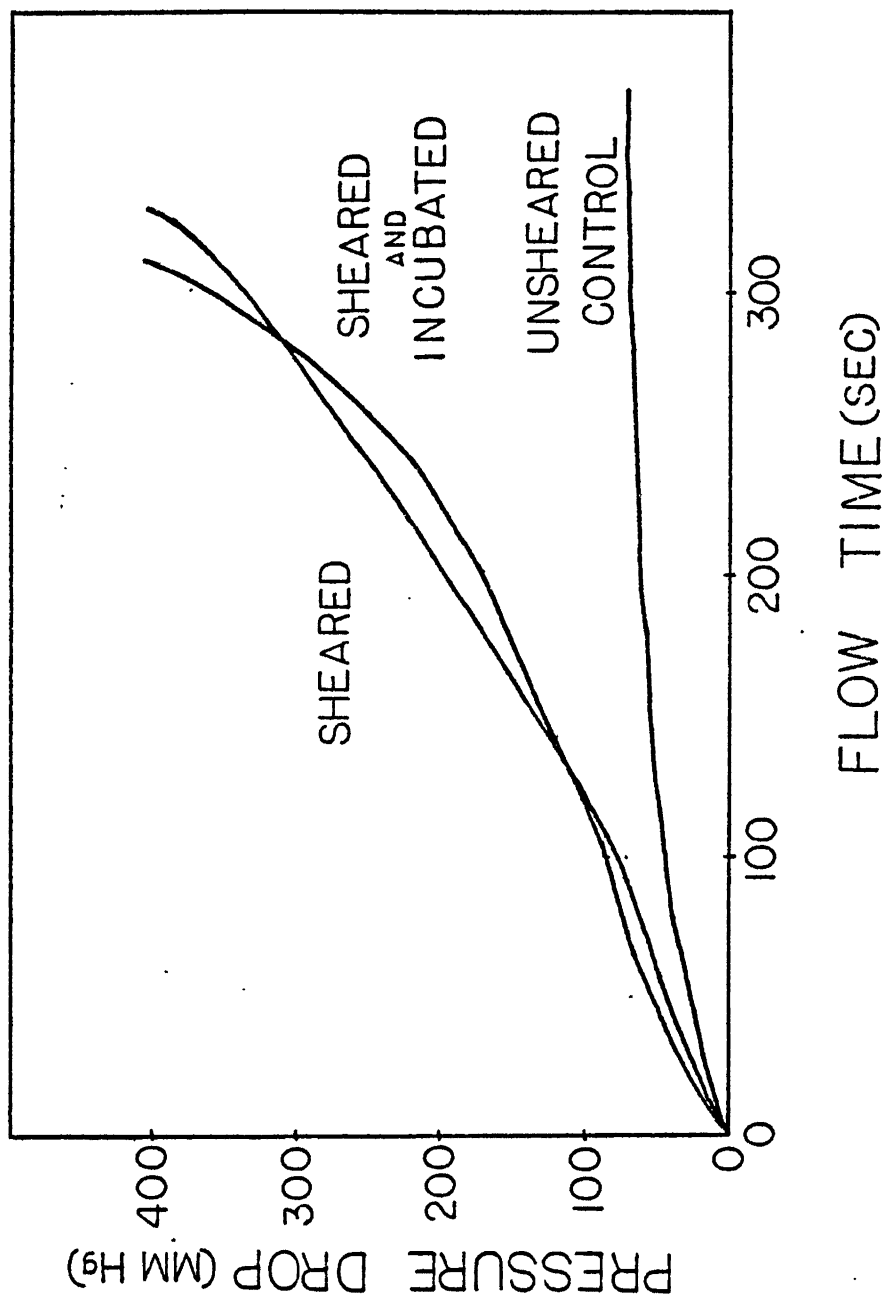


Figure 23. Effects of incubation in Ringers solution (90 min) following shear stress.

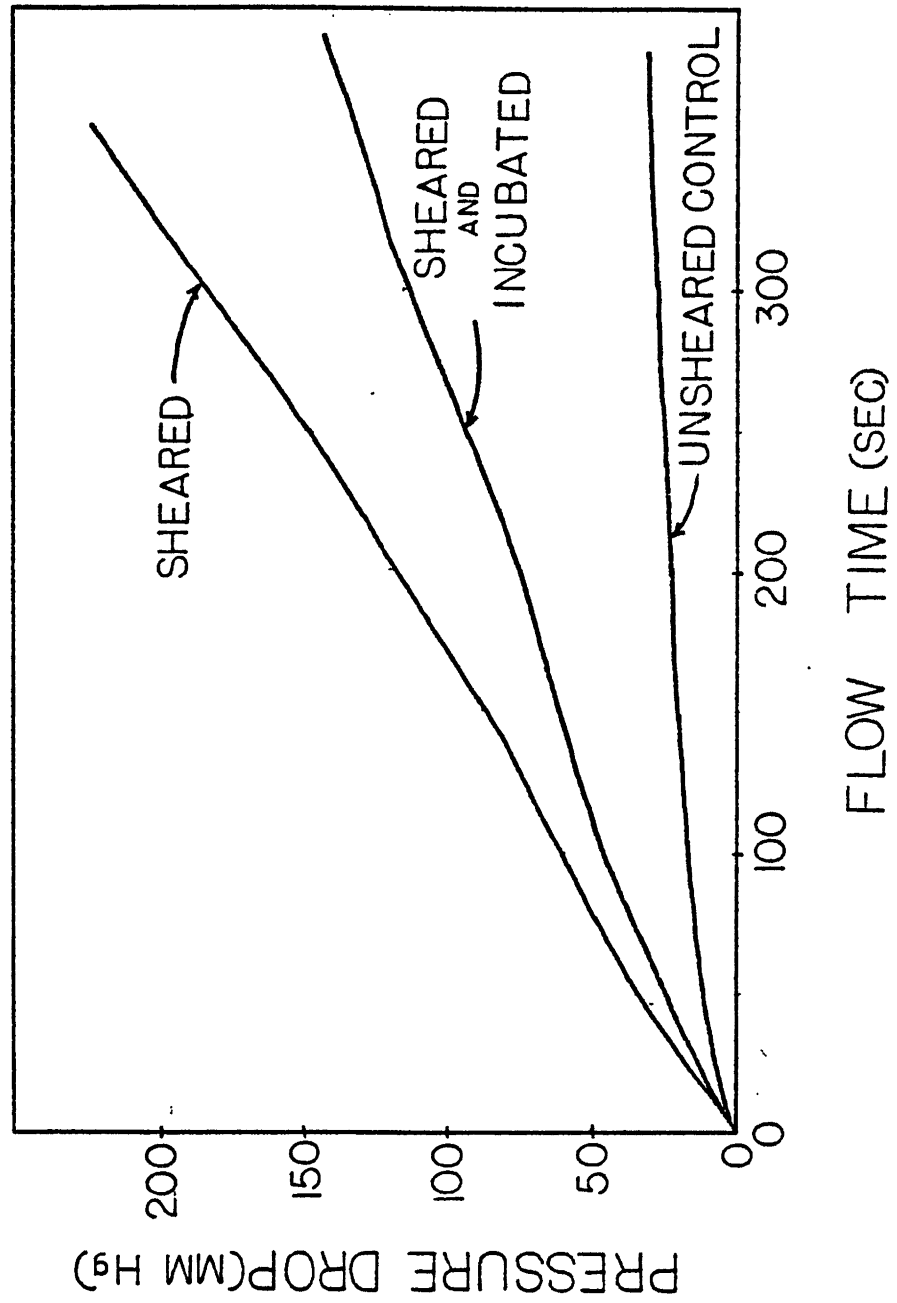


Figure 24. Effects of incubation in glucose solution (1.0 mg/ml) following shear stress.

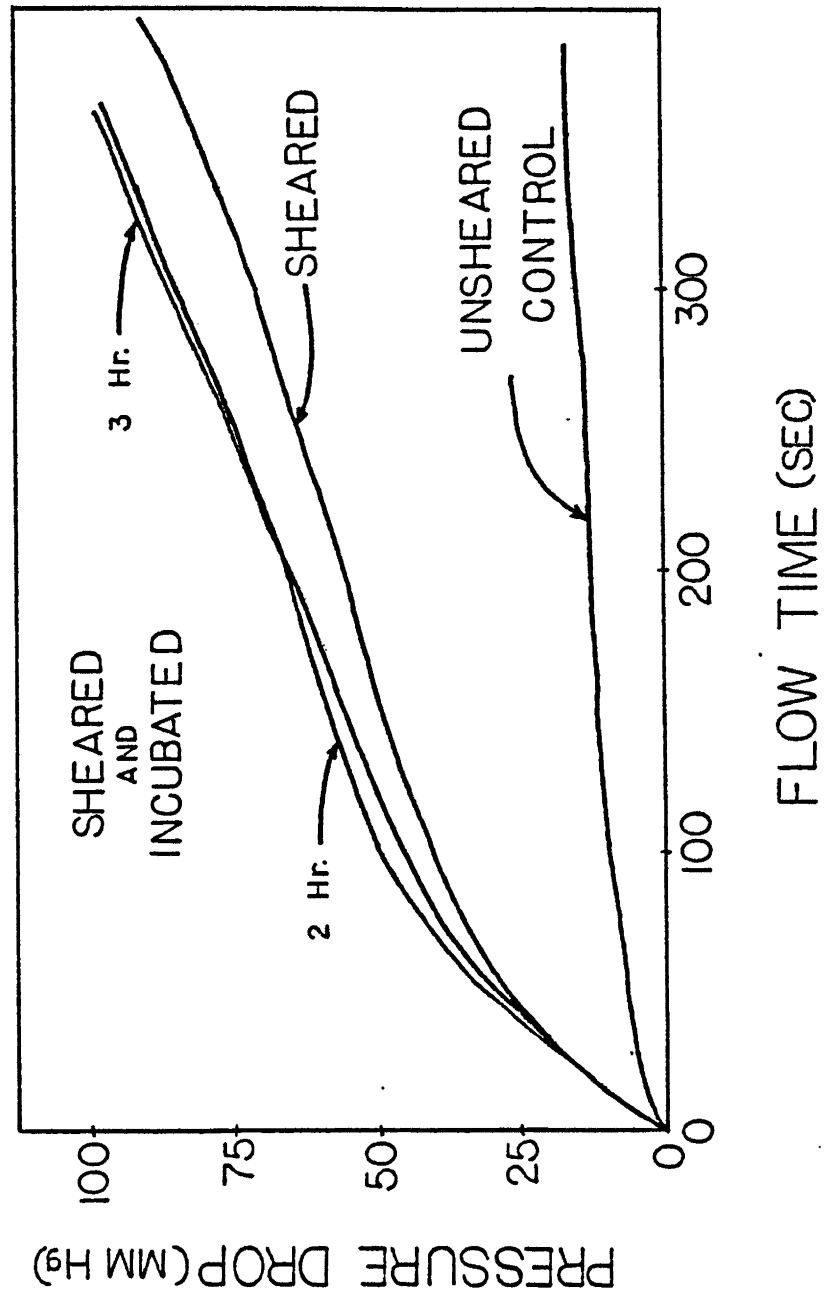


Figure 25. Effects of incubation in glucose solution (10.0 mg/ml) following shear stress.

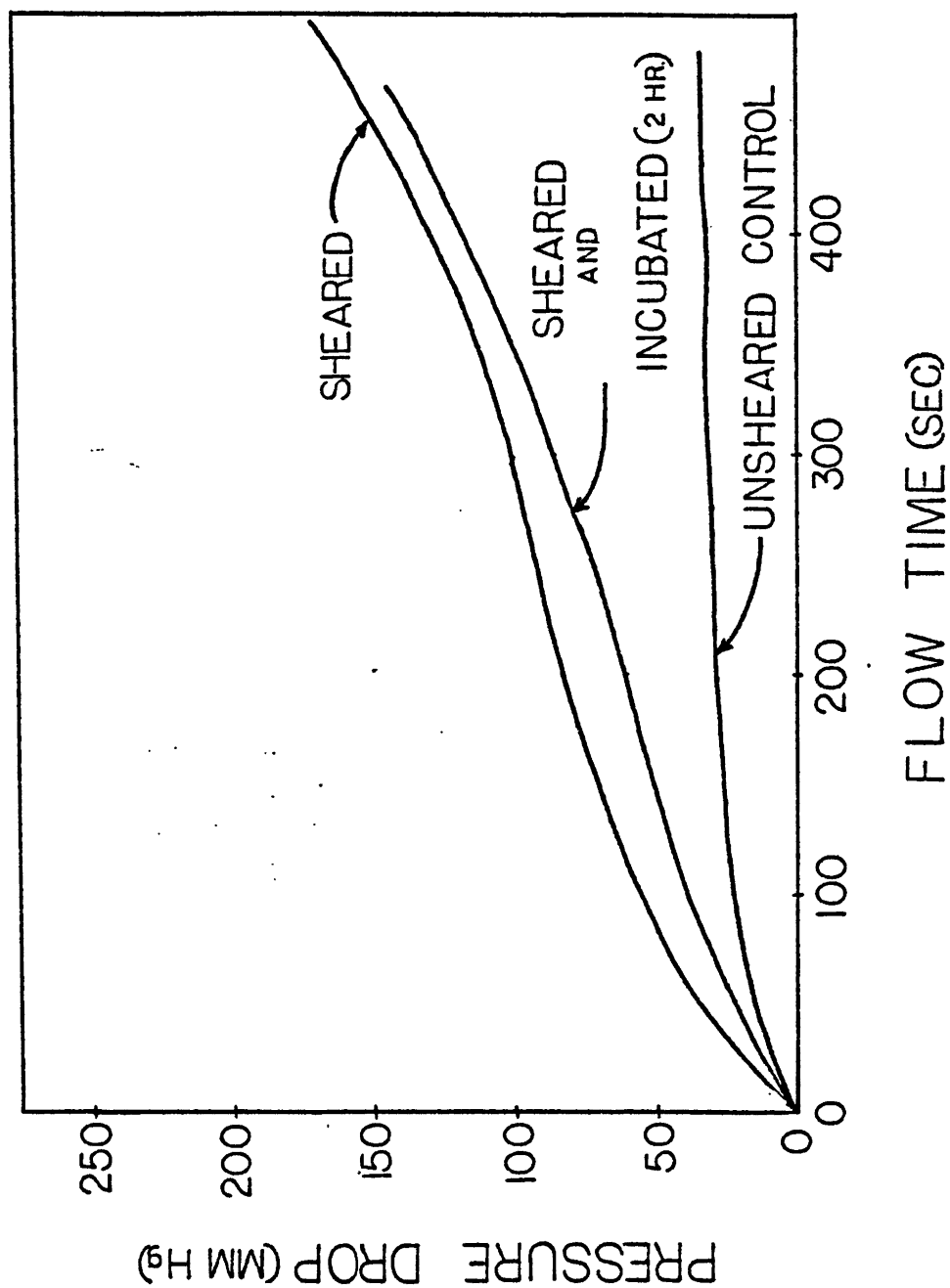
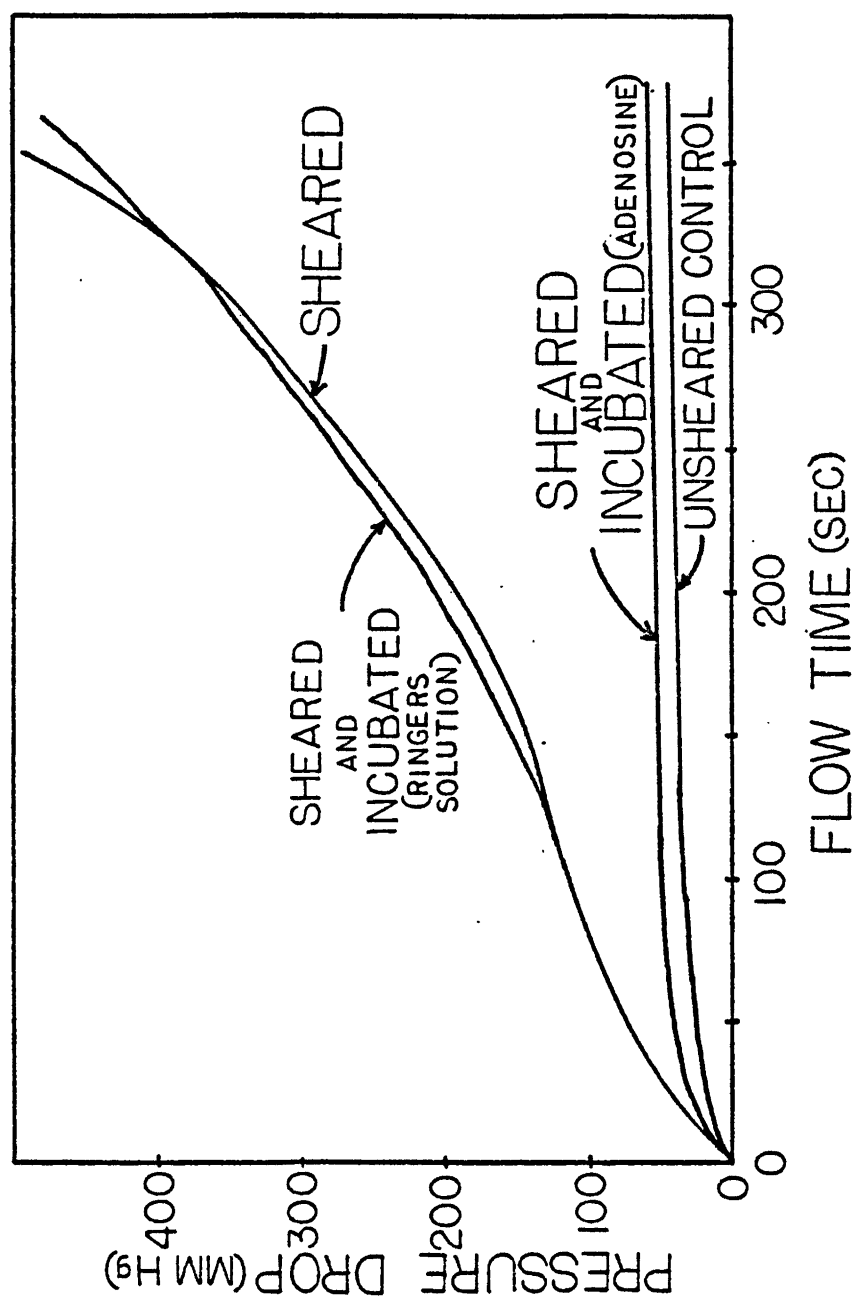


Figure 30. Effects of incubation in adenosine (2.0×10^{-2} M, 120 min.) following shear stress.



slightly higher pressure drop during flow. Figure 25 shows a slight decrease in the pressure generated during flow of cells incubated with 10 mg/ml glucose solution for 120 minutes after being sheared. The behavior of these cells still do not bear any resemblance to that of the unsheared control cells.

Figures 26 and 27 show the failure of incubation with 10^{-2} molar ATP for 90 and 120 minutes respectively to cause a significant reversal of the cell rigidity caused by shear stress. Figure 26 indicates a slight decrease in pressures during flow, meaning a marginal increase in deformability. This is contradicted by Figure 27 which shows somewhat increased pressures caused by flow of cells incubated with ATP following application of shear stress. In no case was ATP incubation successful in returning sheared cells to their normal behavior.

The typical effects of a 120 minute incubation with 5.0×10^{-2} molar dibutyryl CGMP and CAMP are shown in Figure 28. On this day, a donor's erythrocytes were sheared and then incubated in both DB CGMP and DB CAMP, and subsequently filtered. This run is indicative of others (in Appendix I) that together indicate DBCAMP or DBGBMP may bring about small but real decreases in the cell rigidity brought on by shear stress trauma. As evidenced by Figure 28, this small effect seems to be manifested equally by either CGMP or CAMP.

In contrast to the ineffectiveness of the previously

Figure 26. Effects of incubation in ATP (10^{-2} M, 90 min) following shear stress.

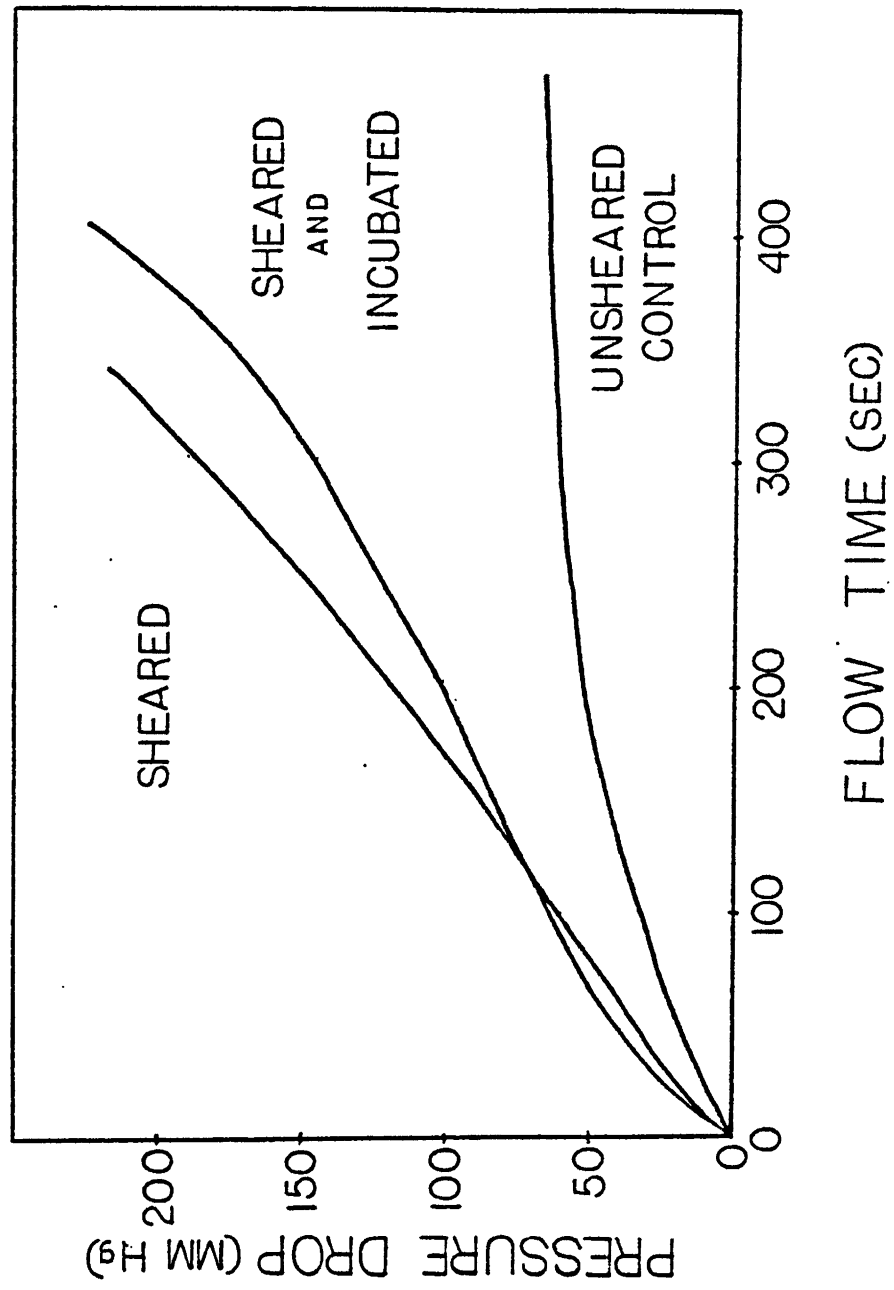


Figure 27. Effects of incubation in ATP (10^{-2} M, 120 min) following shear stress.

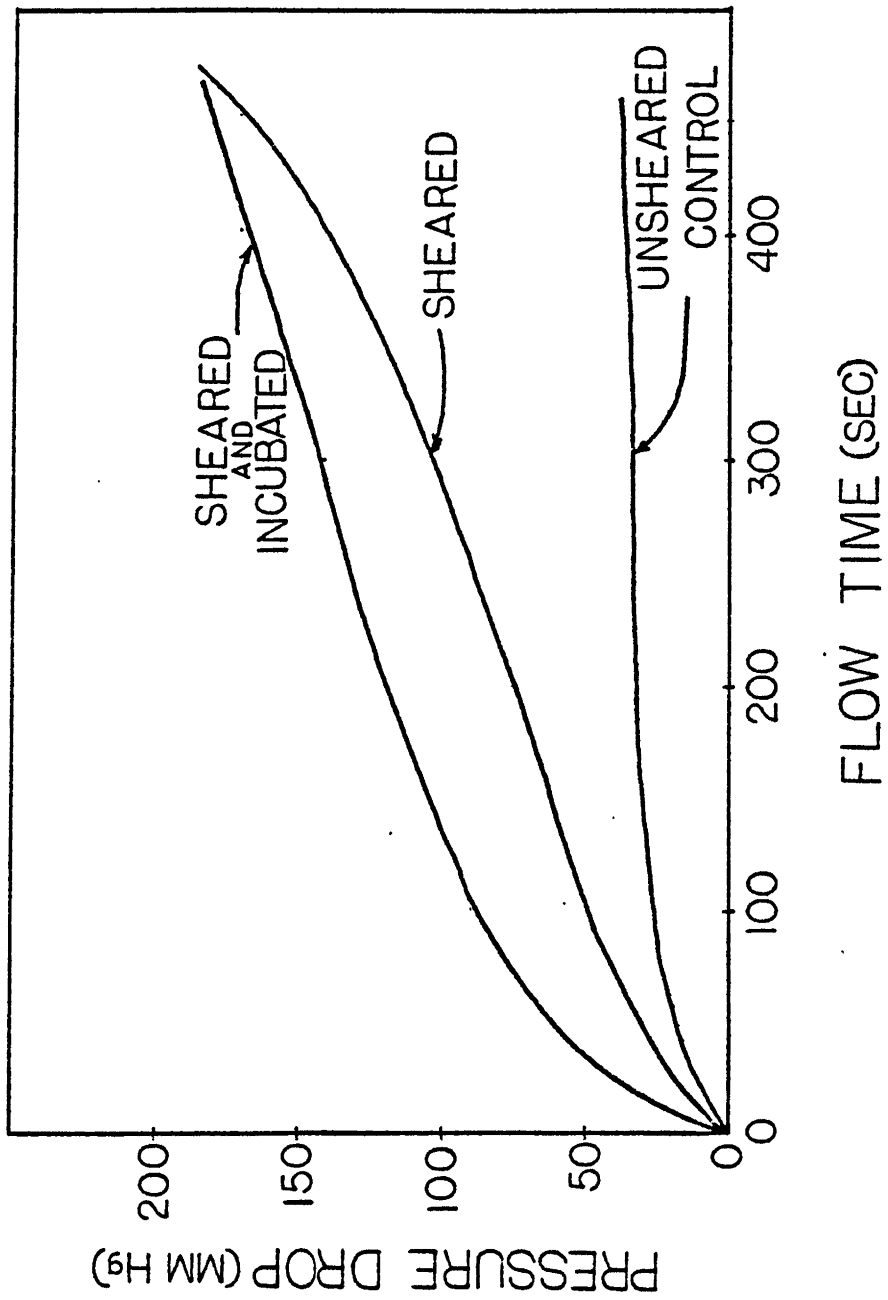
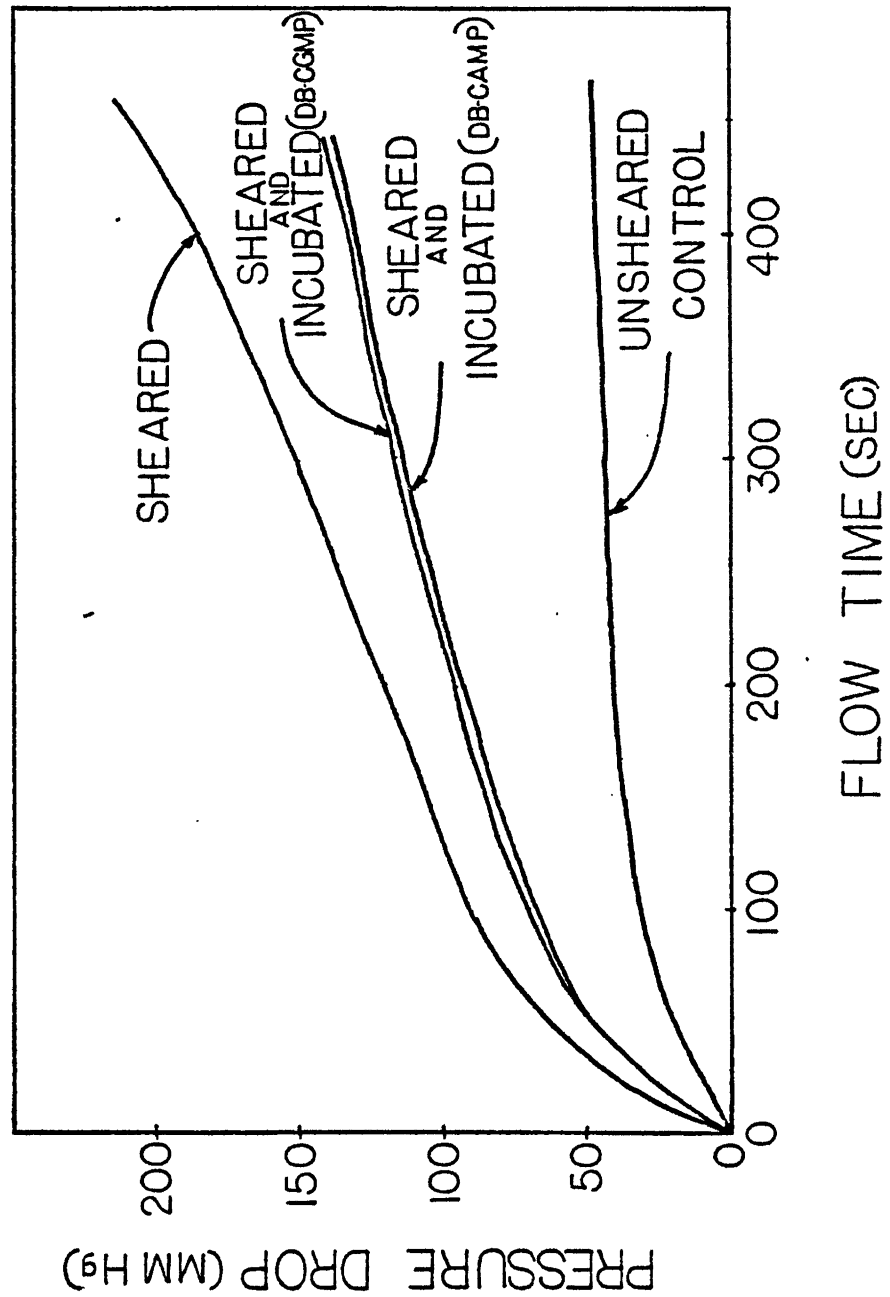
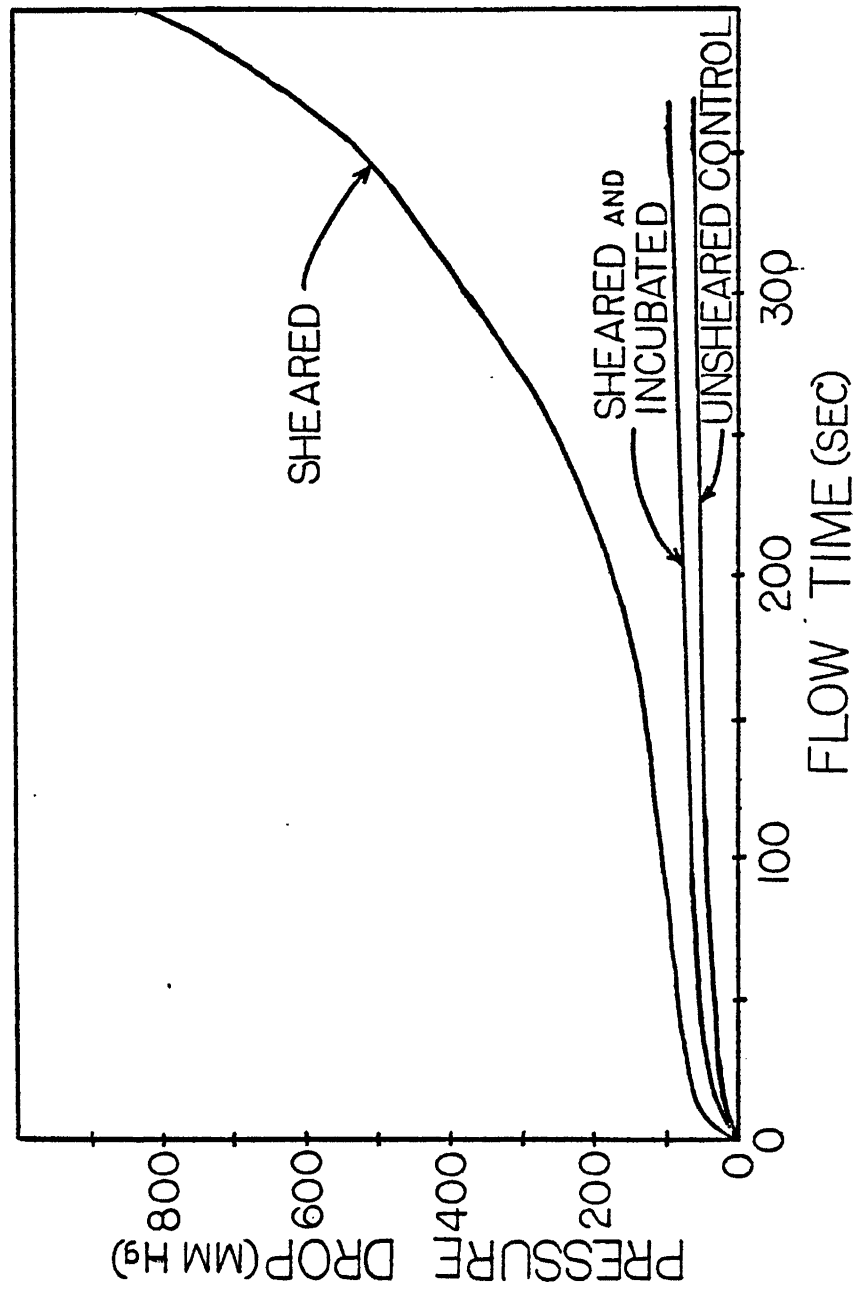


Figure 28. Effects of incubation in DBCAMP and DBCGMP (5.0×10^{-2} M, 120 min.) following shear stress.



discussed incubations to repair the cell abnormalities due to shear stress, Figures 29 and 30 clearly show the striking ability of adenosine to return a cell's deformability to essentially normal, following shear stress exposure. Figure 30, for completeness, shows the full family of pressure vs. time curves for a donor's fresh, unsheared cells, his cells filtered immediately after shear stress, after shear stress and subsequent 120 minute incubation at 37°C in 2.0×10^{-2} molar adenosine, and finally cells sheared and incubated in only Ringers-albumin solution for 120 minutes. As opposed to almost an order of magnitude increase in pressure drop generated by the sheared cells, the adenosine incubation has produced a curve essentially identical to that of the unsheared, control cells.

Figure 29. Effects of incubation in adenosine (10^{-2} M, 90 min.) following shear stress.



ATP Depletion by Aging and Internal
Protein Alterations

Procedures

The same procedures and precautions used in earlier sections for the collection of blood were followed in the studies of ATP depletion. Immediately after drawing the whole blood, however, it was defibrinated using the technique of Tadano (74). With this method, 25 ml quantities of whole blood, not in the presence of anti-coagulant, were swirled in an erlenmyer flask containing 25 glass beads. In two to four minutes a clot formed around the beads, thus removing the vital clotting factor, fibrinogen, from the plasma. After full clot formation the remainder of the blood was decanted from the clot into small plastic tubes, sealed quickly, and put into a 37°C water bath. Enough blood, after being defibrinated, was set aside to take to Rice to run as the fresh control sample.

This defibrinated blood was treated precisely as though it were anticoagulated whole blood. As before, the cells were concentrated by centrifugation, washed once in isotonic saline, and twice in Ringer's solution. After resuspension in Ringers-albumin solution a Coulter cell count was made. The final dilution was made with Ringers-albumin solution.

The cell suspension was run through the Nuclepore filters in exactly the same manner as described in the earlier section.

Subsequent samples from the same donor's blood were centrifuged to obtain red cells, and then washed as usual.

A Coulter cell count was taken and a final dilution made to match the count of the fresh, control suspension.

Aged samples were run at four, six, or eight hour intervals, up to 24 hours total incubation time. At the end of each incubation time, the cells were passed through the Nuclepore filter in the usual manner, measuring the pressure drop generated during flow. Flow rate (2.17 cc/min) was the same in all of these filtration experiments.

Along with cell aging, the effects of incubation with the protein denaturants, colchicine, vinblastine, and cytochalasin B, were investigated to assess any possible deformability alterations. The procedures followed for these experiments closely paralleled those already outlined. Blood was collected in exactly the same manner as for all other filtration experiments. After the usual practice of centrifugation to separate the red cells, washing the cells once in saline and twice in Ringer's solution, a Coulter cell count was made of the final erythrocyte suspension. After a ten fold dilution in Ringer's albumin solution, the resulting suspension was filtered through 3 μ m pore diameter Nuclepore filters, at a flow rate of 2.17 cc/min. The pressure drop across the filter was recorded during the flow of these control cells, to compare to that of the donor's incubated cells.

Colchicine, vinblastine, and cytochalasin B were all obtained from Sigma Chemical Company. Solutions of colchicine and vinblastine were made by dissolving the crystals

directly in the Ringers-albumin solution. Both solutions were frozen between usages. Cytochalasin B solutions were prepared by dissolving the desired quantity of the solid in a small amount of di-methyl sulfoxide. A solution of the desired concentration was then made by diluting with the Ringers-albumin solution.

An incubation, regardless of whether with colchicine, vinblastine, or cytochalasin B, always followed the same general procedure. Approximately 2 cc of the suspension of washed erythrocytes, and an equal volume of the incubating solution, were thoroughly mixed in a glass vacutainer. The tube or tubes were then placed in a 37° C thermostated water bath for the duration of the incubation period, which was between 1 and 3 hours. Every thirty minutes the suspension was mixed by gently inverting the tube. At the end of the incubation the tube(s) was centrifuged to pack the red cells. The supernatant liquid was removed, and the cells were washed twice in Ringers solution. The cells were resuspended in Ringers-albumin solution and a Coulter cell count was made. Based on this count a final dilution on the order of 10 fold was made with Ringers-albumin solution so as to match the count of the original control suspension, that had previously been filtered.

The filtration of the incubated cell suspensions followed an identical procedure to all other filtrations, with the flow rate remaining constant at 2.17 cc/min. As usual,

during flow through 3 μm pore diameter Nuclepore filters, the pressure drop across the filter was monitored to compare to that of the normal control cells.

Results

Figures 31-34 show the results of four incubation runs in which the effects of cell aging (ATP depletion) are clearly seen in the cells diminished ability to traverse the Nuclepore filter. In all four runs it is seen that prior to 6 or 8 hours a cell's behavior is only marginally different from that of the donor's fresh cells. This is assessed by the magnitude of the pressure drops generated during flow through the filter of erythrocytes aged for varying times. After twelve hours there appears to be a rapid decline in the cells' ease of filterability as ATP levels drop more quickly. The effect of almost complete depletion of ATP after 24 hours is seen in Figures 33 and 34. These cells show a marked increase in rigidity which is manifested by the 4 to 5 fold increase in resultant pressure drop incurred, compared to the same donor's fresh control cells.

The degree to which cell deformability was altered by the exposure to colchicine, vinblastine, and cytochalasin B, was much less evident. Figures 35-37 show the pressure drop vs. time curves for three representative runs involving colchicine incubation. Others are included in Appendix I. On each figure two curves are presented: one of the donor's incubated cells, and the other that of the donor's fresh normal cells. Using a concentration of 1 mg/ml of colchicine, Figures 35 and 36 show the results of two incubation times: 90 and 180 minutes. Within the sensitivity of the method,

Figure 31. Effects of cell aging on erythrocyte filterability.

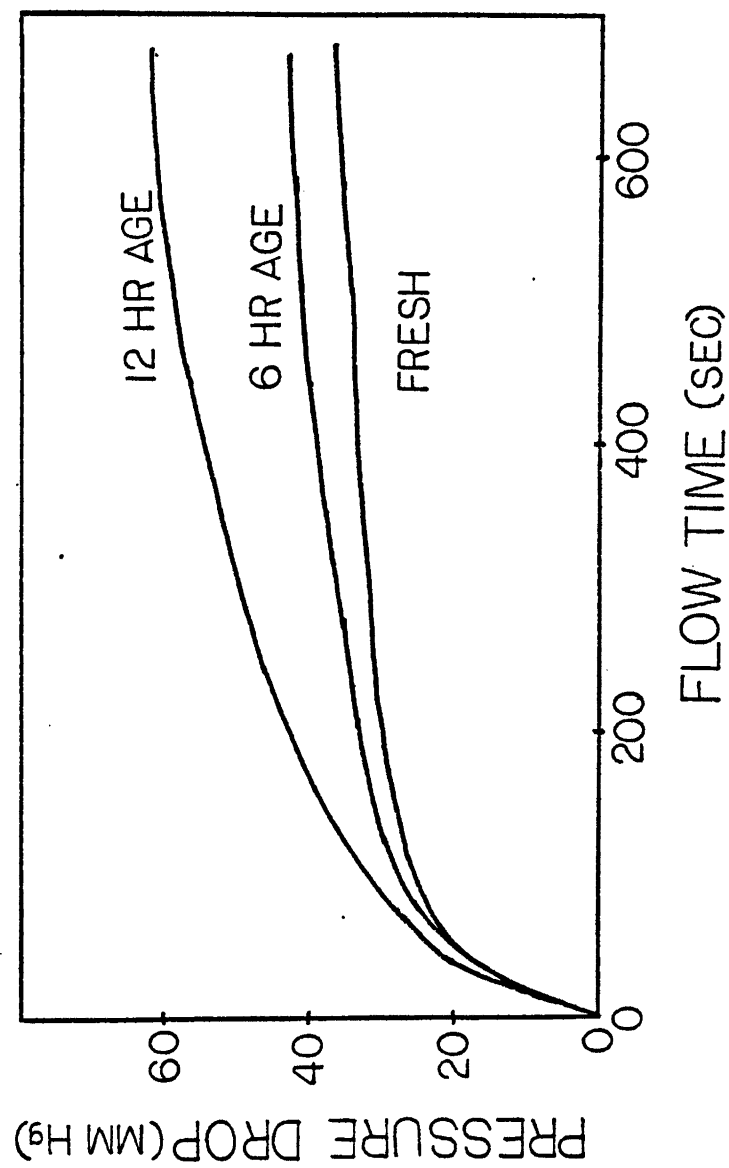


Figure 32. Effects of cell aging on erythrocyte filterability.

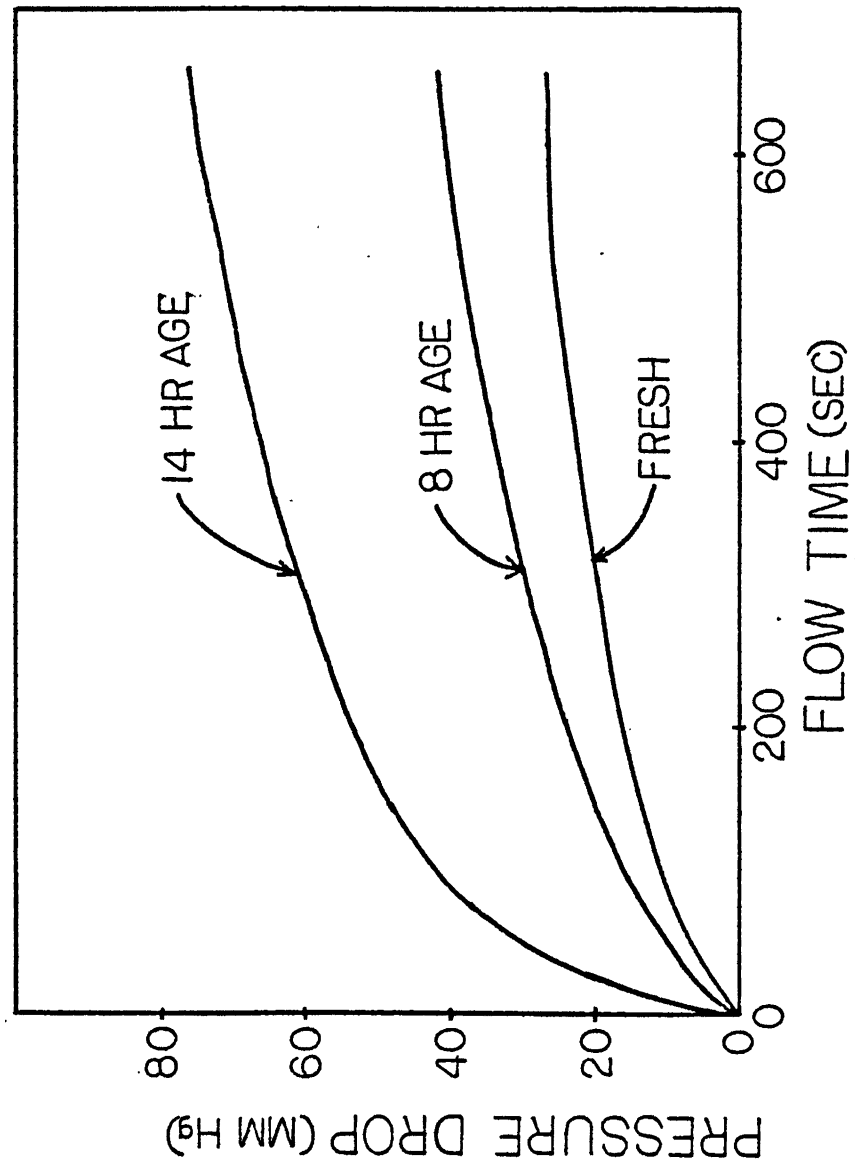


Figure 33. Effects of cell aging on erythrocyte filterability.

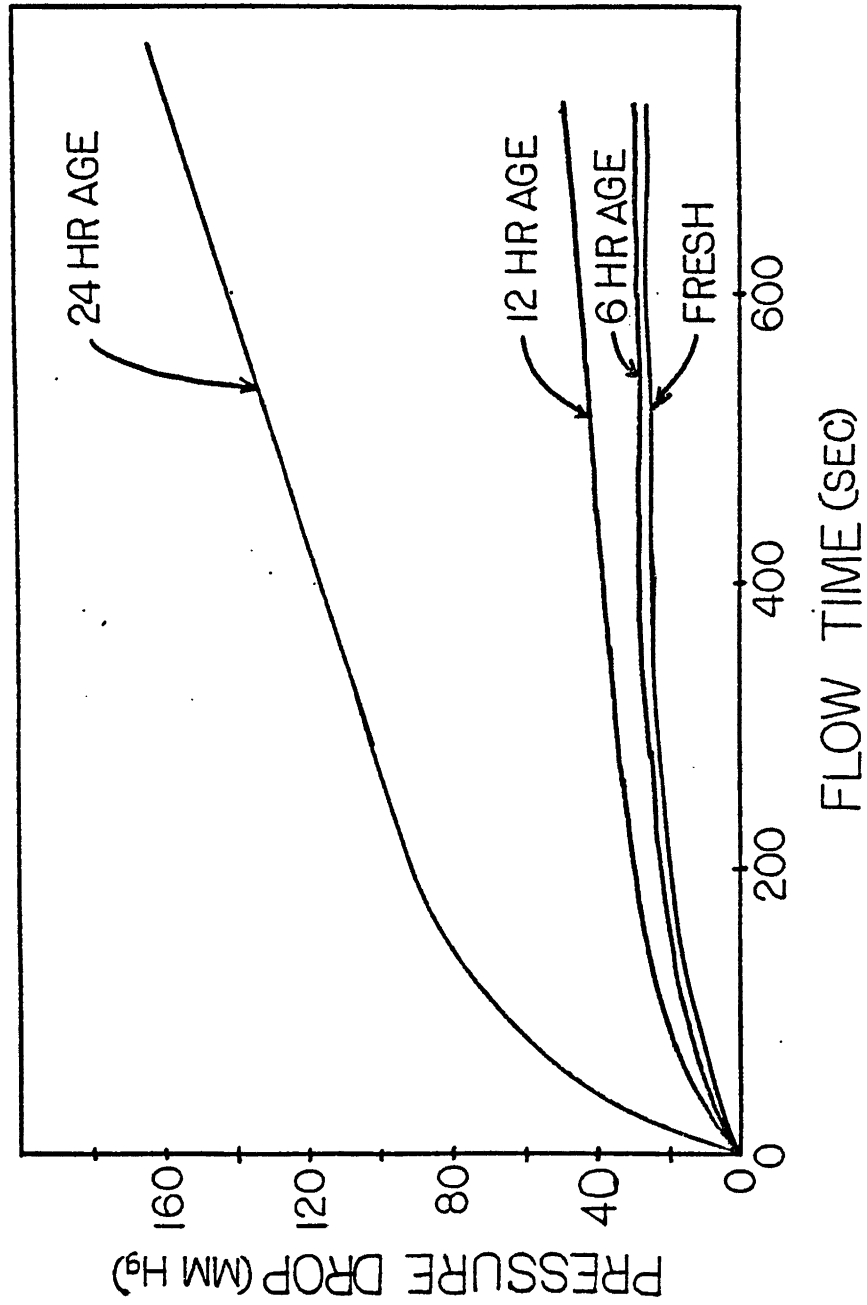


Figure 34. Effects of cell aging on erythrocyte filterability.

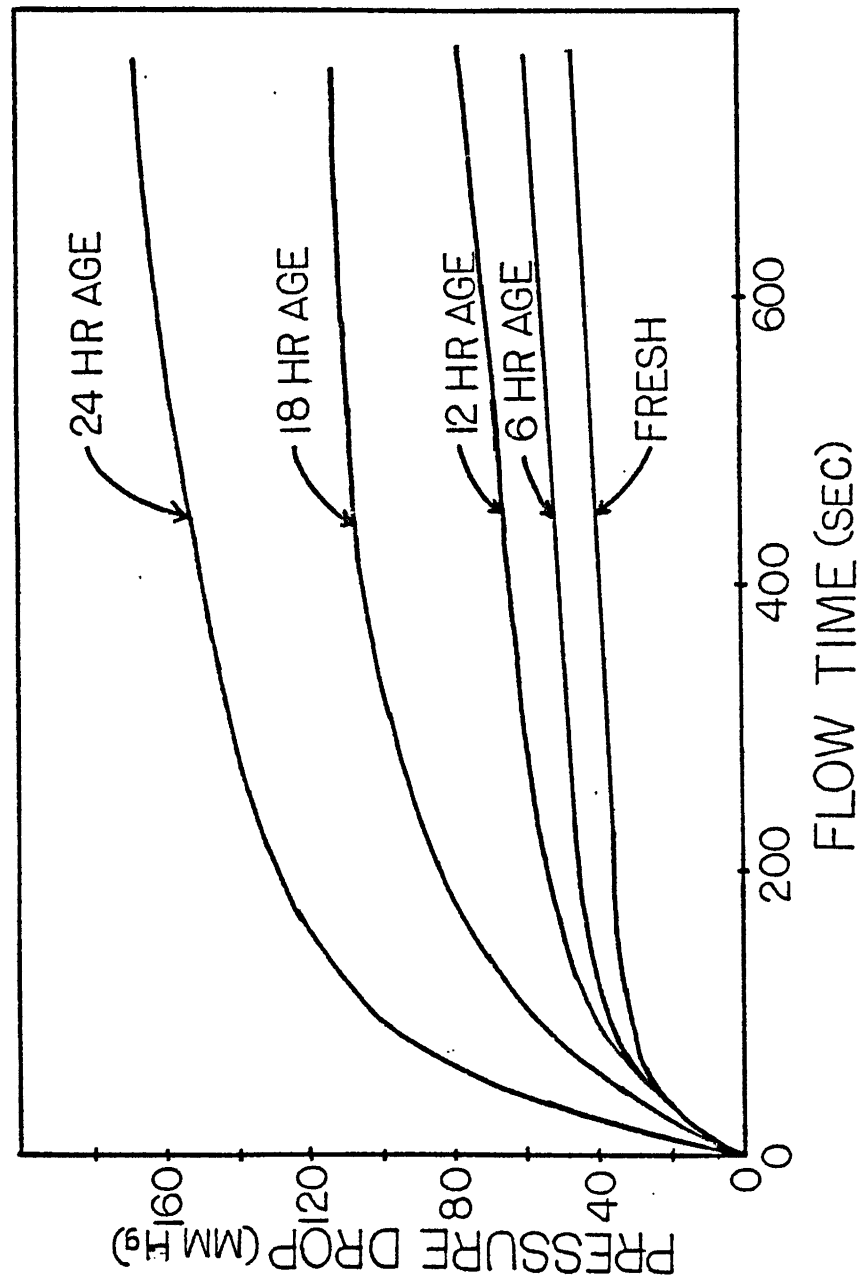


Figure 35. Effects of incubation in colchicine (1 mg/ml, 90 min.) on erythrocyte filterability.

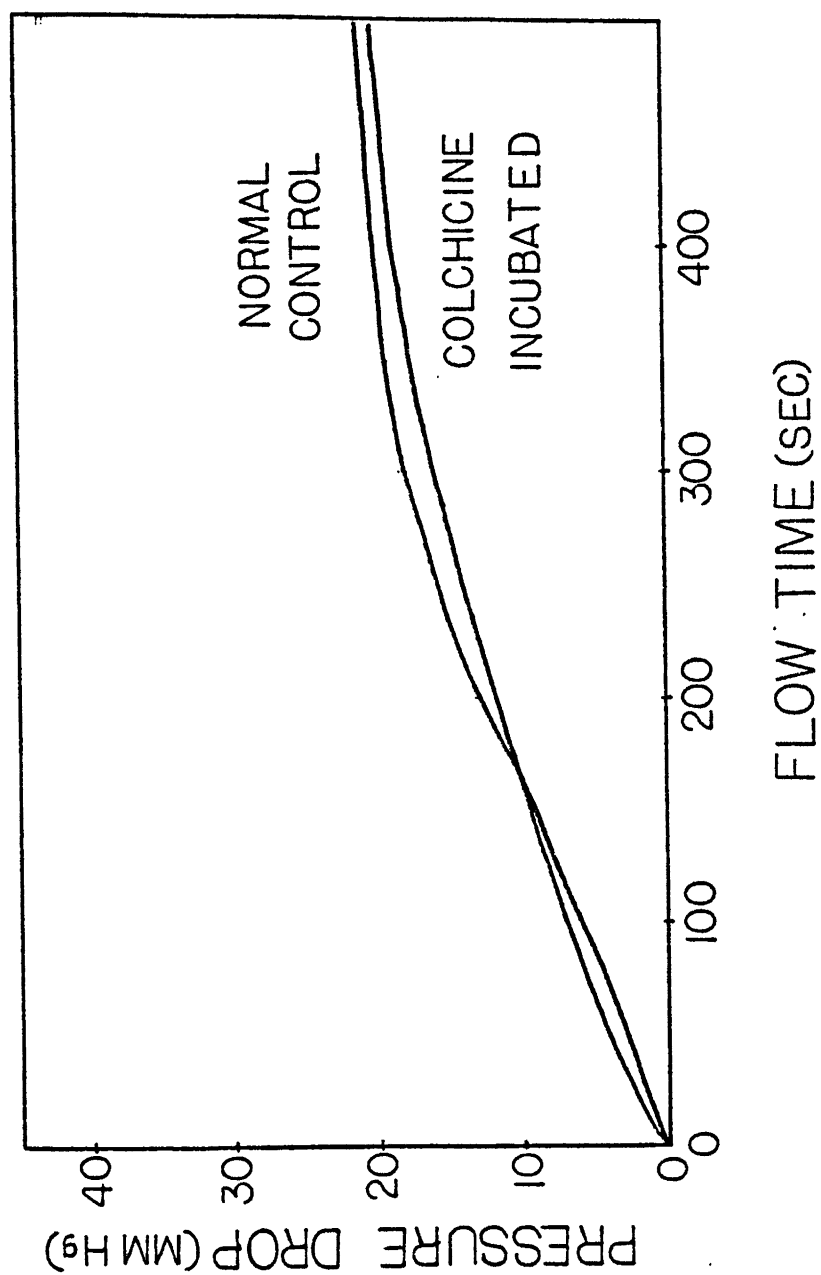


Figure 36. Effects of incubation in colchicine (1 mg/ml, 180 min.) on erythrocyte filterability.

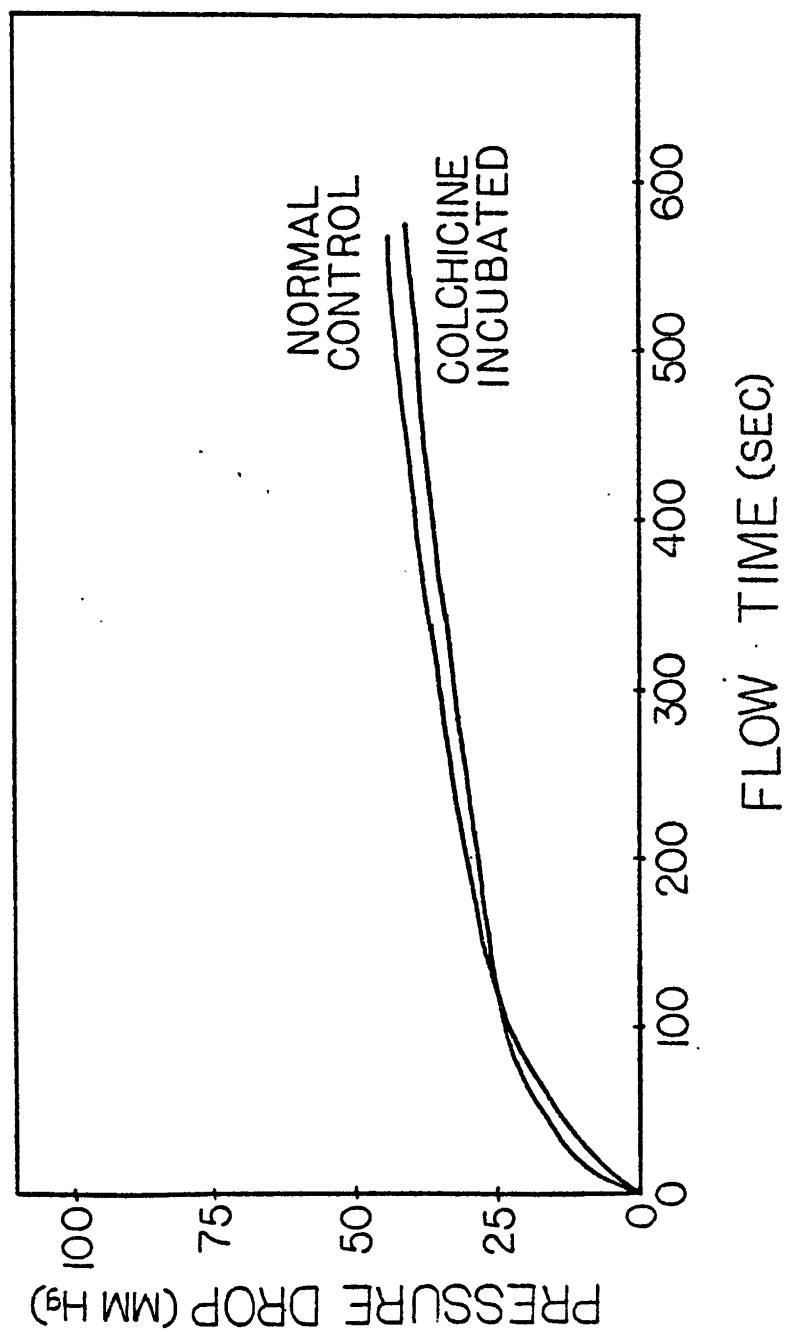
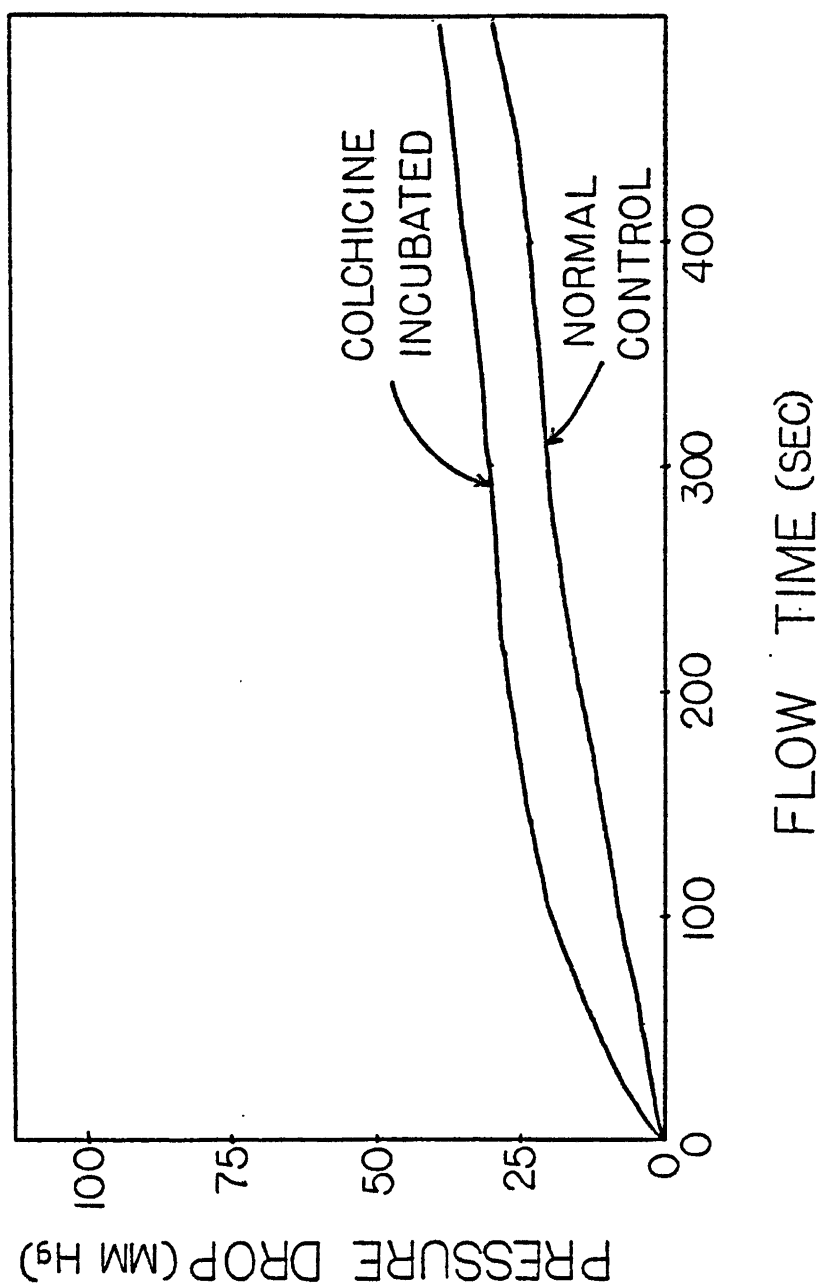


Figure 37. Effects of incubation in colchicine (10 mg/ml, 180 min.) on erythrocyte filterability.



the colchicine incubated cells exhibit virtually identical deformability characteristics assessed by their ability to traverse 3 μ m Nuclepore filters. Increasing the concentration of the colchicine incubation solution to an extremely high 10 mg/ml had little effect, as shown in Figure 37. Even at this clinically impractical concentration and an incubation of three hours, the exposed cells did not exhibit significantly different behavior from unexposed, fresh cells.

The effects of cell incubation with solutions of vinblastine on producing a cell defect showing decreased deformability were no more visible than with colchicine. Figures 38-40 show the results of three different incubation times with 0.1 mg/ml vinblastine. As evidenced by the strong similarity in the pressure drop curves generated by the incubated cells and the control cells, a measurable alteration in cell deformability has not been produced by vinblastine exposure.

Three experiments with incubation of erythrocytes with cytochalasin B failed also to reveal a measurable change in cell deformability as seen in Figures 41-42. Incubation with 0.1 mg/ml cytochalasin B for 120 and 180 minutes, followed by passage of these cells through 3 μ m Nuclepore filters, produced pressure vs. time curves very similar to those produced by unincubated cells (Figures 41 and 42). Increasing the cytochalasin B concentration to 1.0 mg/ml and incubating for 120 minutes also did not produce a deformability change as shown in Figure 43.

Figure 38. Effects of incubation in vinblastine (0.1 mg/ml 120 min.) on erythrocyte filterability.

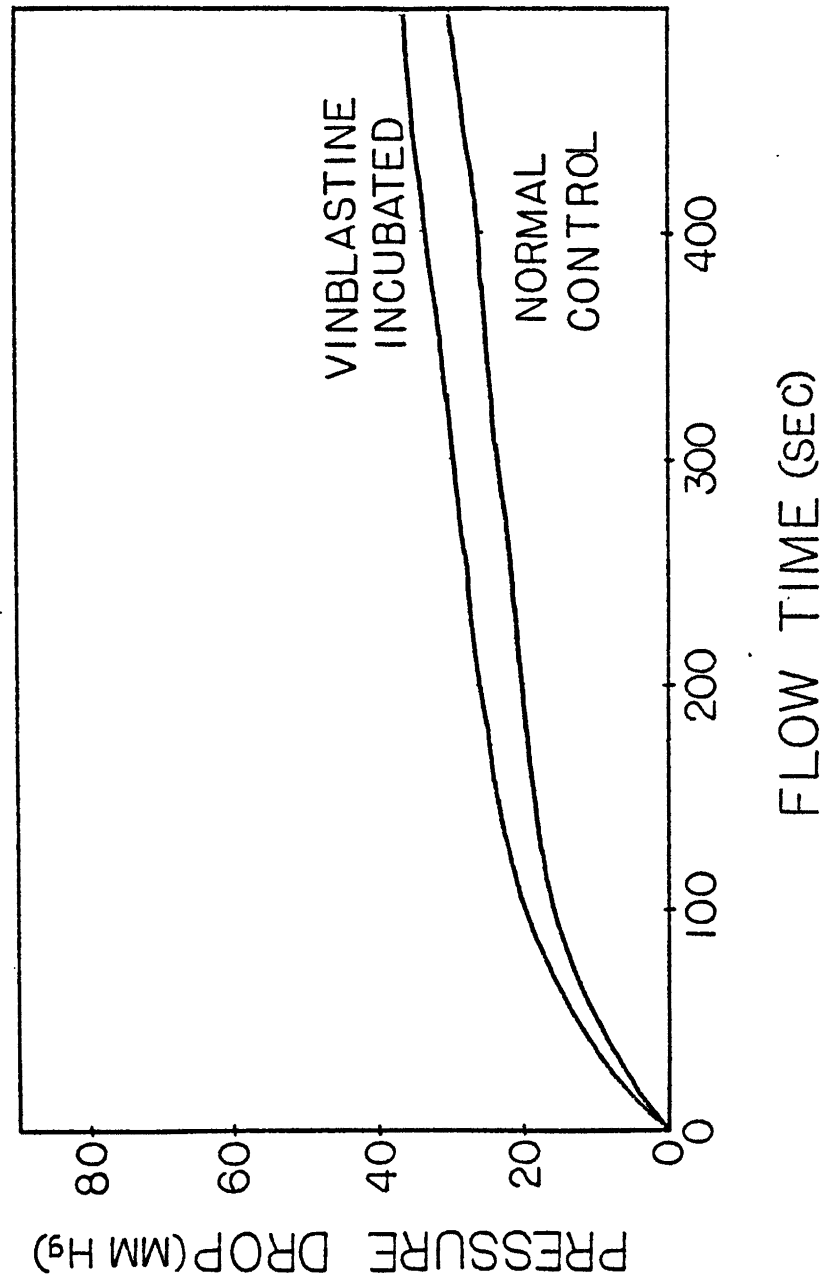


Figure 39. Effects of incubation in vinblastine (0.1 ml/ml, 150 min.) on erythrocyte filterability.

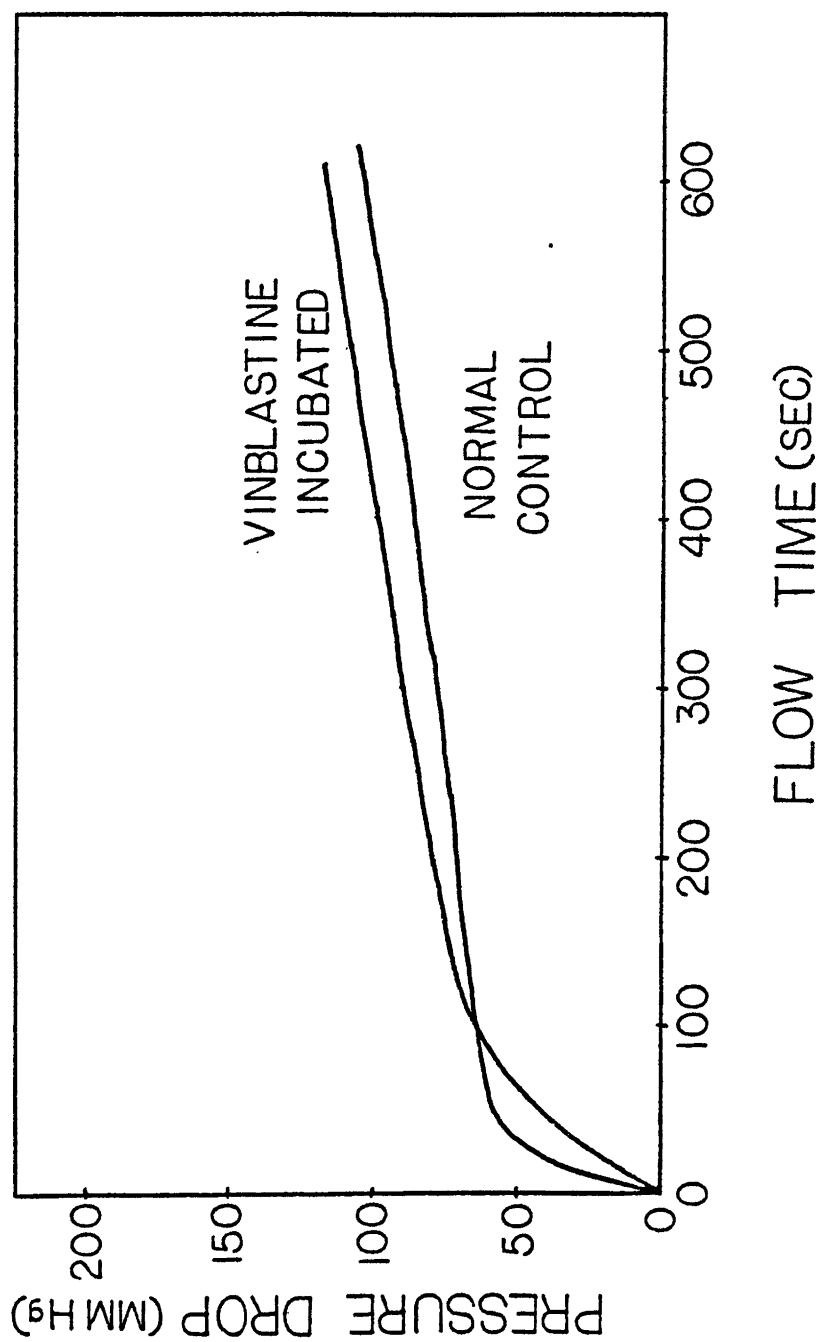


Figure 40. Effects of incubation in vinblastine (0.1 mg/ml, 180 min.) on erythrocyte filterability.

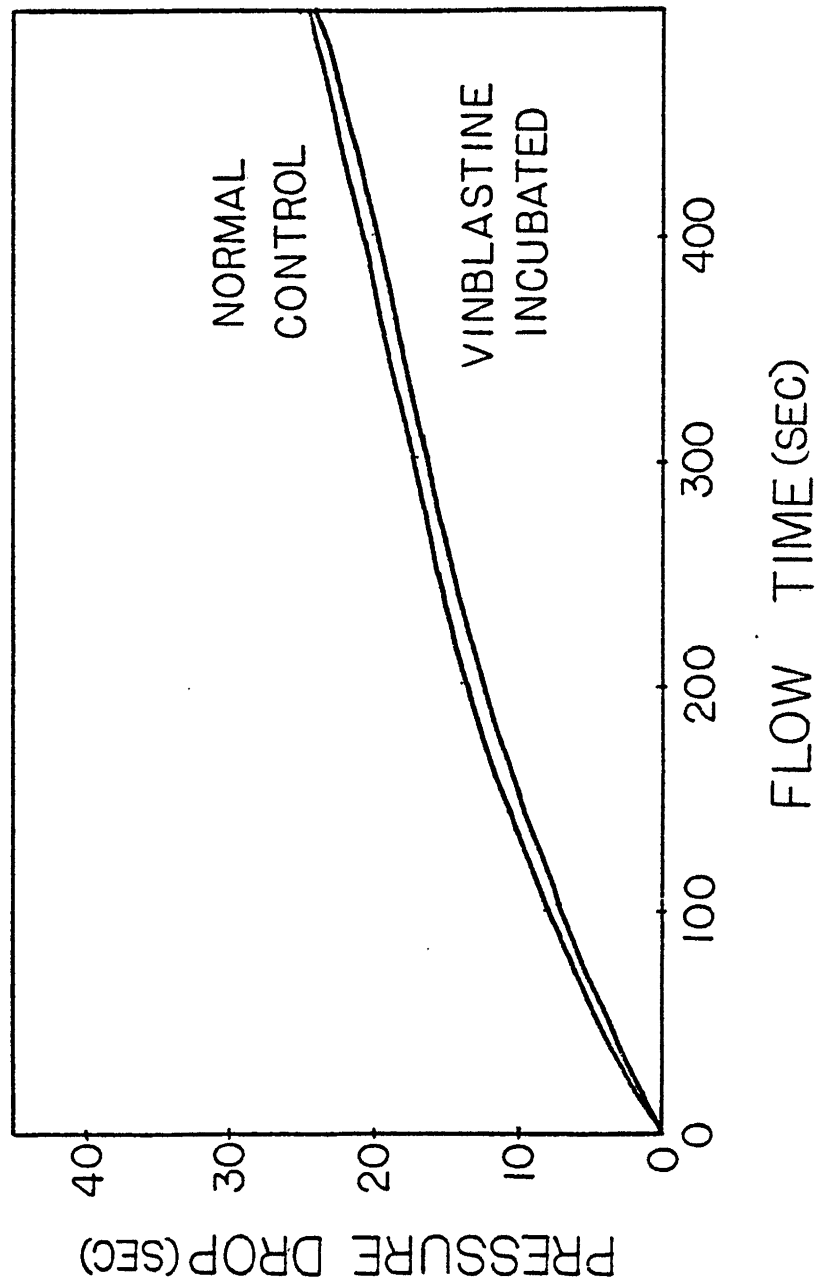


Figure 41. Effects of incubation in cytochalasin B (0.1 mg/ml, 120 min.) on cell filterability.

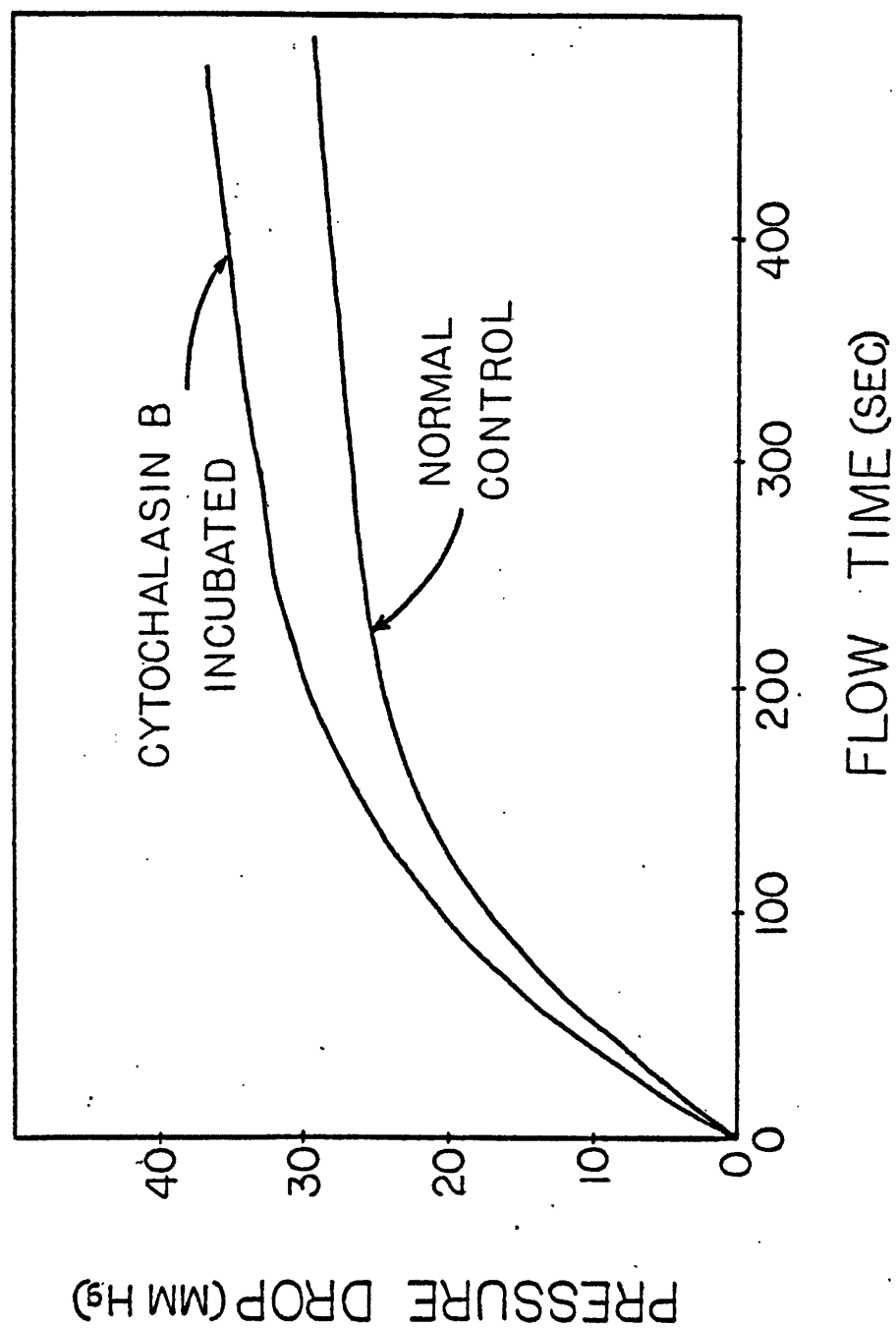


Figure 42. Effects of incubation in cytochalasin B (0.1 mg/ml, 180 min.) on cell filterability.

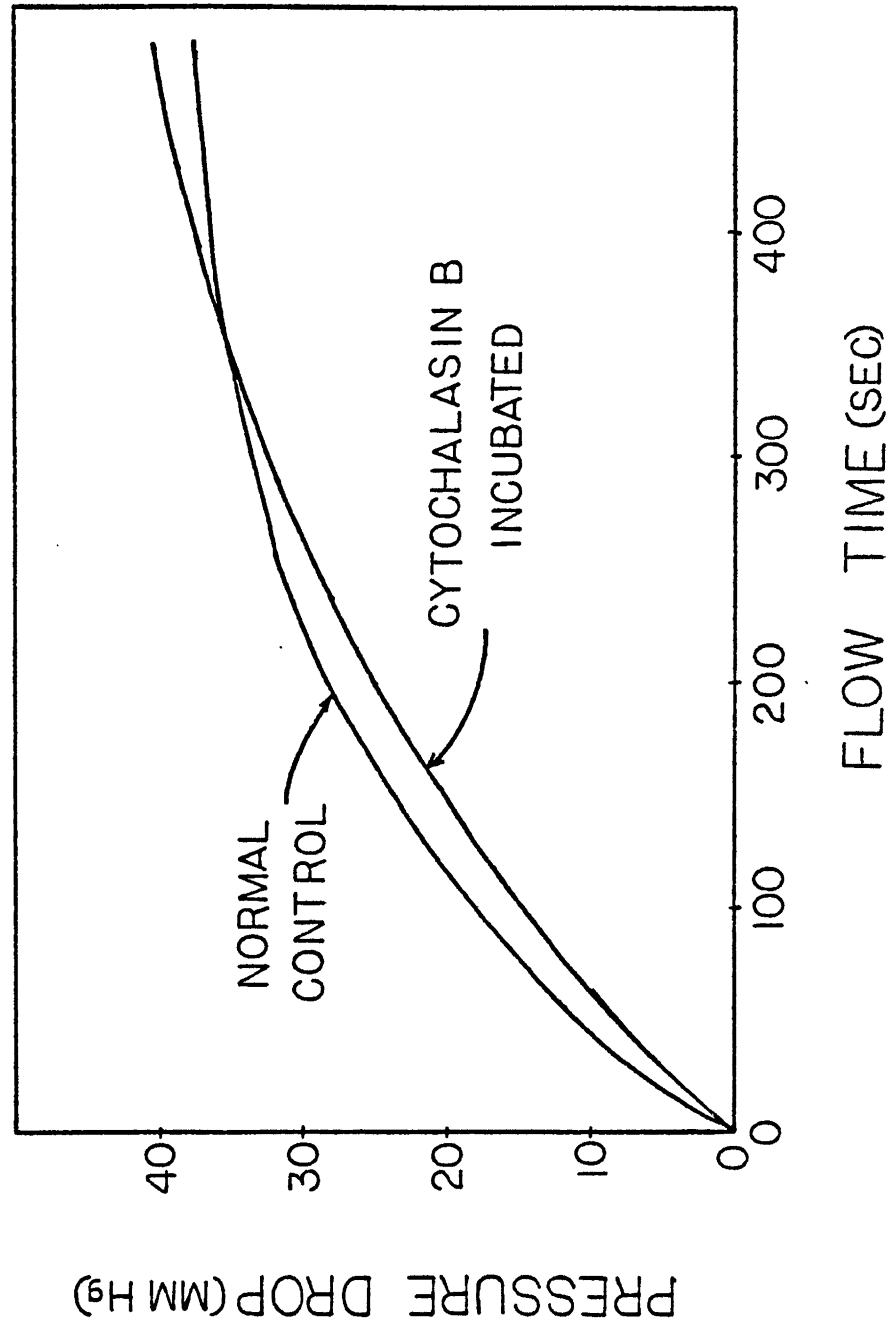
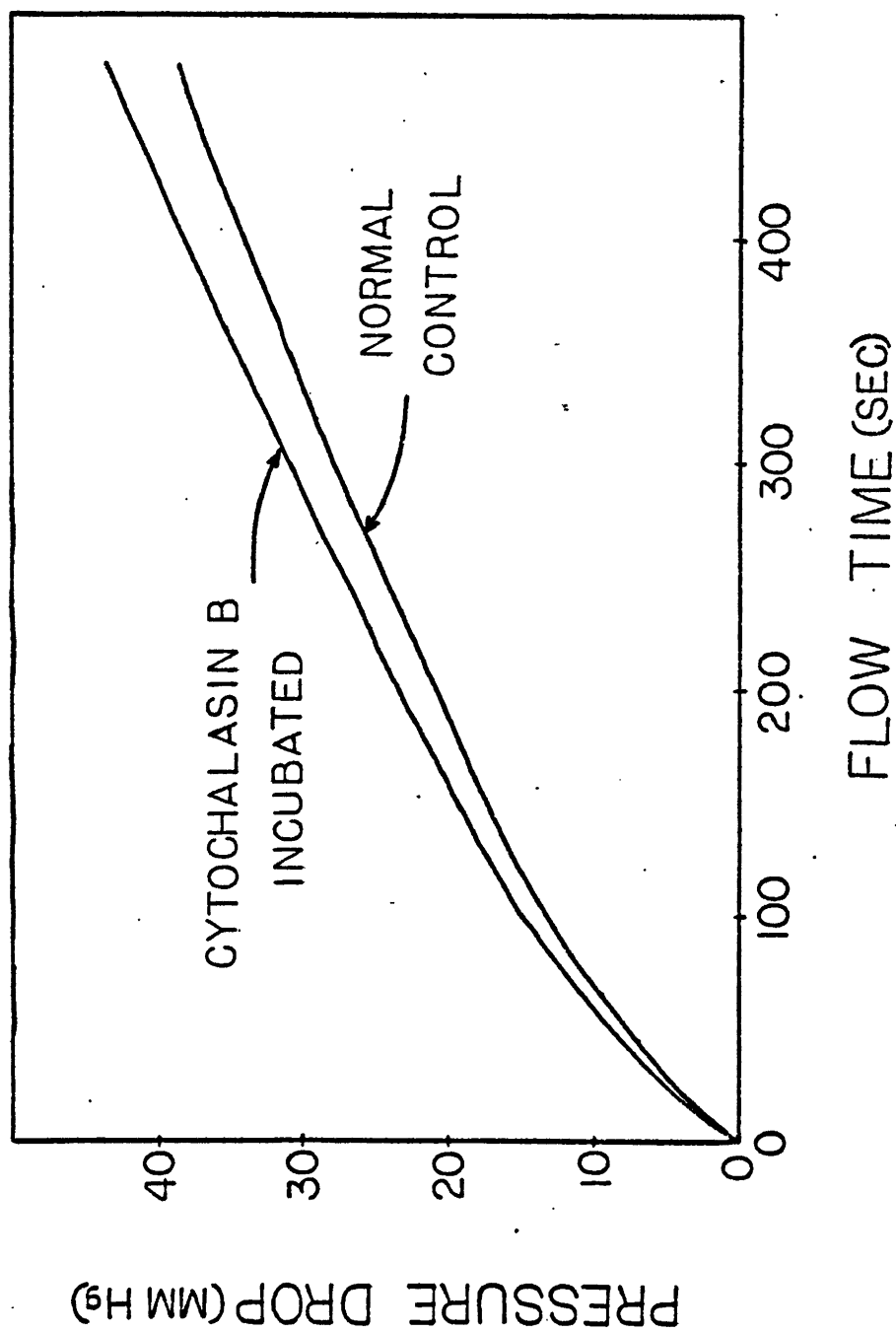


Figure 43. Effects of incubation in cytochalasin B (1.0 mg/ml, 120 min.) on cell filterability.



Discussion and Conclusions

As assessed by altered Nuclepore filter passage, this study clearly indicates that application of sub-hemolytic shear stresses directly results in decreased erythrocyte deformability. The shear stresses studied, (from 250 to 1400 dynes/cm² at exposure times of 2 minutes) in addition to being insufficient to cause overt hemolysis, do not result in any morphologic alterations seen by light microscopy. However, depending on the level of applied shear stress there is a commensurate increase in the difficulty of the traumatized cells to pass through Nuclepore filters having pores of 3 μ m diameter. This is determined by both the pressures generated during constant flow rate, and the extent of morphologic alterations, including fragmentation incurred by flow through the filter. Both manifestations of altered erythrocyte deformability are discernable at all shear stresses above 250 dynes/cm². Only at or below shear stresses of 250 dynes/cm² is the deformability behavior of stressed cells essentially the same as unstressed cells, as judged by their ability to traverse the filter. This evidence would indicate the existence of a threshold value of shear stress required for significant deformability alterations: namely 250 dynes/cm² for exposure time of 2 minutes.

The studies involving incubation of erythrocytes with various substances after application of shear stress were undertaken in an attempt to shed light on the nature of the

alteration induced. In general, these experiments were guided by a hypothesis that a metabolic alteration had occurred in the traumatized erythrocytes. That the observed cellular alterations were not significantly reversible after an incubation in the standard suspending solution would tend to support the argument that mechanical trauma induced a defect which is not corrected by simple removal of the stress.

The experiments with glucose incubation, in which glucose was unable to repair the increased rigidity, would indicate that the cells are not glucose deficient. If indeed the cells were in need of glucose, it could be readily absorbed during incubation through the cell membrane which is quite permeable to glucose.

The ineffectiveness of DBCGMP and DBCAMP is not terribly surprising. If the theories of Yawata et al. (66) are to be believed, then presumably the application of shear stress might have altered microfilament and microtubule structure and function. This might be concluded if, in fact, these fibrous proteins are instrumental in maintaining membrane deformability. If the cyclic nucleotides are important in regulating these proteins' function, then one might have expected to see an effect due to incubation with DBCGMP and DBCAMP. This was not the case. It should be pointed out also that a circulating red cell contains no measurable quantities of CGMP or CAMP.

Incubation with ATP was not effective in curing the

cellular defect. This is not surprising since a drastic and irreversible change in membrane permeability would be required to allow passage of ATP into the cell. Normal red cells are almost completely impermeable to ATP.

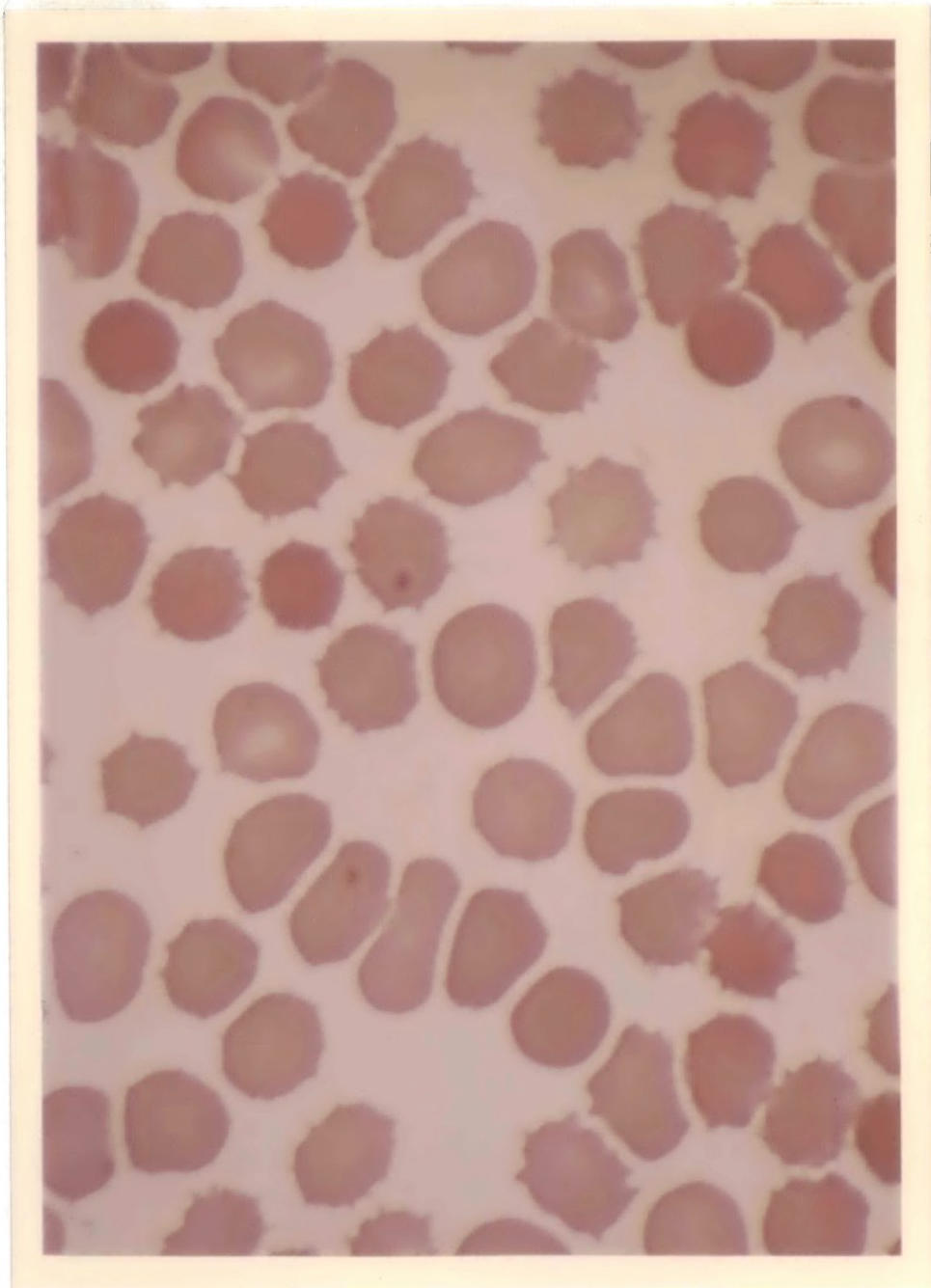
Only adenosine had the ability to return the deformability of shear stressed cells to that observed prior to application of the shear. It does so to such a complete degree that the sheared and incubated cells are virtually identical to fresh unsheared cells in terms of deformability characteristics. This overall behavior closely parallels that seen by Weed, et al. (8) of erythrocytes incubated 24 hours, at which time measured cellular ATP is essentially zero. These cells, after incubation with adenosine for 2 hours, had ATP, Ca^{++} , viscosity, and membrane deformability values very close to those measured before incubation. Thus, there is strong evidence that the defect induced by sub-hemolytic shear stress falls generally into the category of one that is a result of ATP depletion. The present study lends evidence to the argument that intracellular ATP levels are important to membrane deformability and hence to overall cell deformability.

It is important to observe that the cells subjected to moderate levels of shear stress for 2 minutes are not identical to cells metabolically depleted for 24 hours, and certainly have not gone through the precise same sequence of events. Firstly, cells shear stressed at 1000 dynes/cm^2

for 2 minutes appear to be even less deformable than those that have been aged for 24 hours. This is evidenced by comparing pressure drops during flow of cells sheared at moderate to high shear stress to those incurred by 24 hour old cells. Secondly, the shear stressed cells do not exhibit the characteristic morphology changes of aged cells (sphering and surface spicules) shown in Figur 44.

The mechanism by which the sheared cells have become ATP depleted, assuming they have been, is open to educated speculation. Two general causes for loss of internal ATP might be given: one that in the stressed state the membrane permeability has been drastically changed so as to cause passive ATP leak from the cell, and second that a shear stress induced cell alteration greatly stimulated the use of internal ATP, so as to cause almost complete depletion. The former proposition seems unlikely since the high rate of ATP loss necessary would be accompanied by large hemoglobin loss. This is not observed. If indeed membrane permeability is changed so drastically during shear stress exposure, so as to cause ATP leakage, then this alteration must rapidly return to near normal very soon after shear stress removal. Evidence of this is the observation that ATP incubation after shear stress does not return a cell to its pre-shear stress deformability behavior. This is presumably due to the normal impermeability of the red cell to ATP. A more plausible cause of ATP depletion is through a mechanism whereby an

Figure 44. Erythrocytes aged for 24 hours (1250 x).



increased demand for ATP greatly speeded its internal usage. The most obvious usage of ATP is in maintaining the cell's cationic balance through the active transport system. If, during the shear stress application, the cells permability changed enough to become very leaky to sodium influx and/or potassium efflux, this could conceivably put a severe drain on ATP stores. The active transport mechanisms would attempt to maintain the necessary ionic balance by working much harder, requiring a greater energy input. This type of defect is thought to exist in hereditary spherocytes which are leaky to sodium ions (75). It is believed that these cells might not be ATP deficient, but have greatly increased ATP usage in order to compensate for the altered permeability. The studies of Lubowitz et al. (55) and Najappa et al. (54) both reported increased sodium permeability of normal erythrocytes following application of sub-hemolytic shear stress levels. These results lend further support to the hypothesis of increased ATP usage to compensate for the ionic imbalances caused by shear stress.

The basic question as to whether the shear stressed cells have lost ATP to their environment, or have used it internally can be settled by making ATP measurements in the suspending fluid before and after shear stress application.

The results of the present study corroborate other work regarding decreased cell deformability as a result of cell aging. The experiments also served as a control for other

investigations involving Nuclepore filtration, by showing that for cell ages of about six hours or less, almost no decrease in deformability was observable.

The experiments of this study did not support the theory that the protein denaturants, colchicine, vinblastine, and cytochalasin B, can be used to alter cell shape and deformability. Although morphologic studies were not done, incubations with these compounds did not produce a significant alteration in cell deformability as assessed by Nuclepore filtration. This is in conflict with Yawata, et al. (66), and their theory that microtubules and microfilaments are primary to cell deformability maintenance and that compounds such as colchicine can disturb the function of these proteins.

This study has demonstrated the utility of the Nuclepore filtration technique in detecting and measuring relative deformability changes in erythrocyte populations. It was not used in this work as an absolute method, for example to measure a bulk modulus of an individual cell's membrane. The method is clearly not suited for such measurements. Although the Nuclepore filtration technique of this study seems clearly capable of measuring deformability alterations, it is not, of itself, able to discern what factor has led to the changed deformability. For example, there is no way in which the technique alone might be used to pinpoint whether an observed decrease in deformability was due to a shape change,

an increase in overall cell size, or simply a more rigid membrane. Other techniques, used in conjunction with polycarbonate sieve filtration, would be necessary.

The present study suggests immediate experiments measuring free ATP levels after application of shear stress to erythrocytes. This would be a conclusive answer to whether ATP is leaked from the cell as a result of shear stress, or is used internally at a greatly accelerated rate.

More generally, if the filtration of erythrocytes through Nuclepore filters is accepted as a reliable method of measuring cell deformability, and as a good model of the microcirculation, then the procedure can be used for testing almost any potential clinical abnormality in cell deformability.

APPENDIX:
Additional Filtration Runs

Figure 45

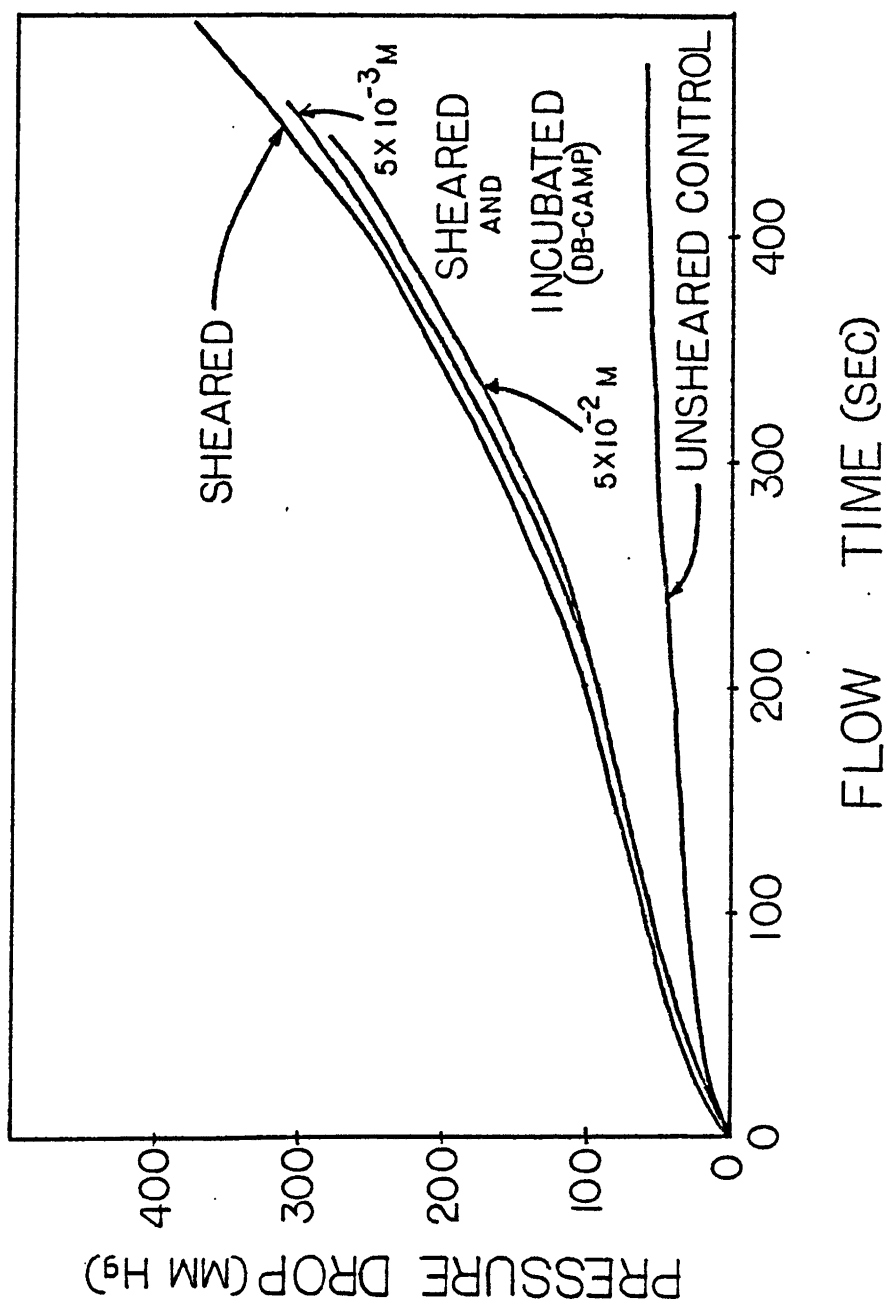


Figure 45a

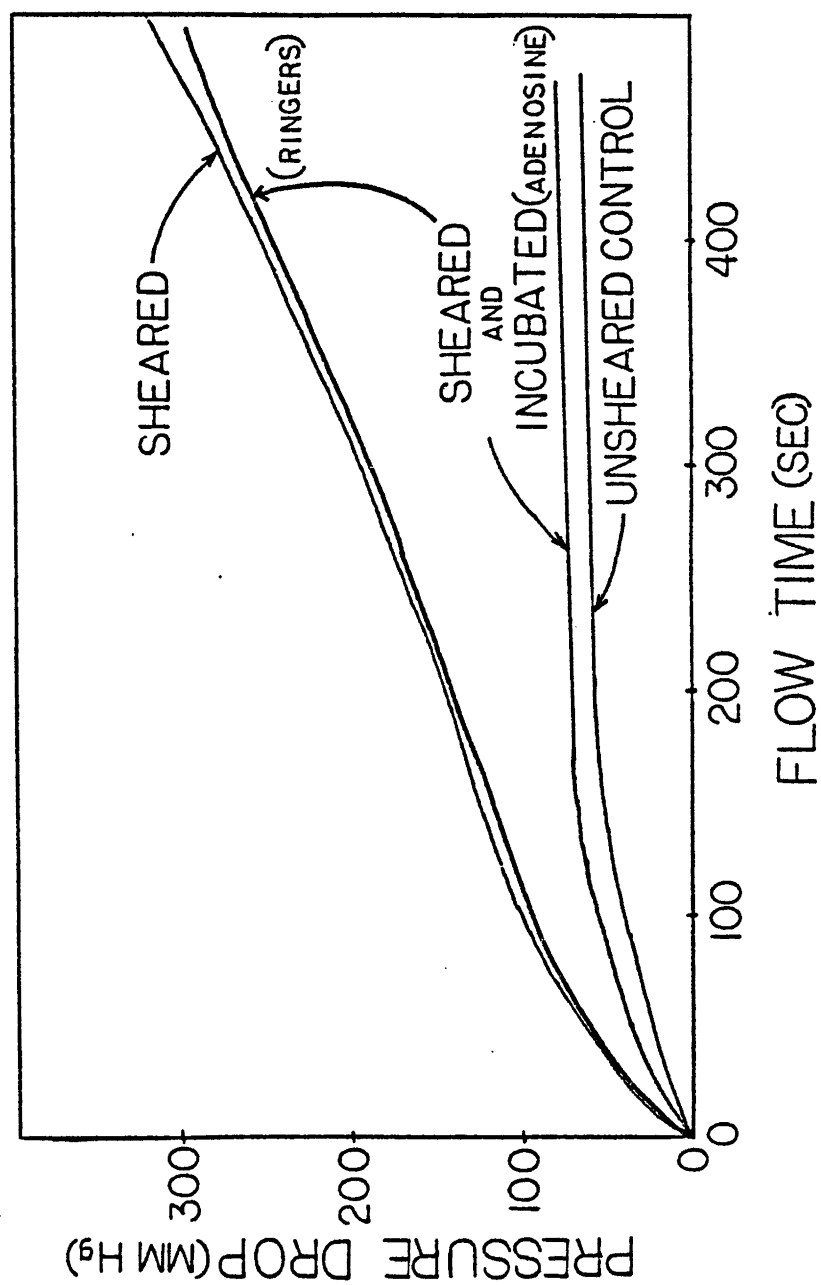


Figure 46

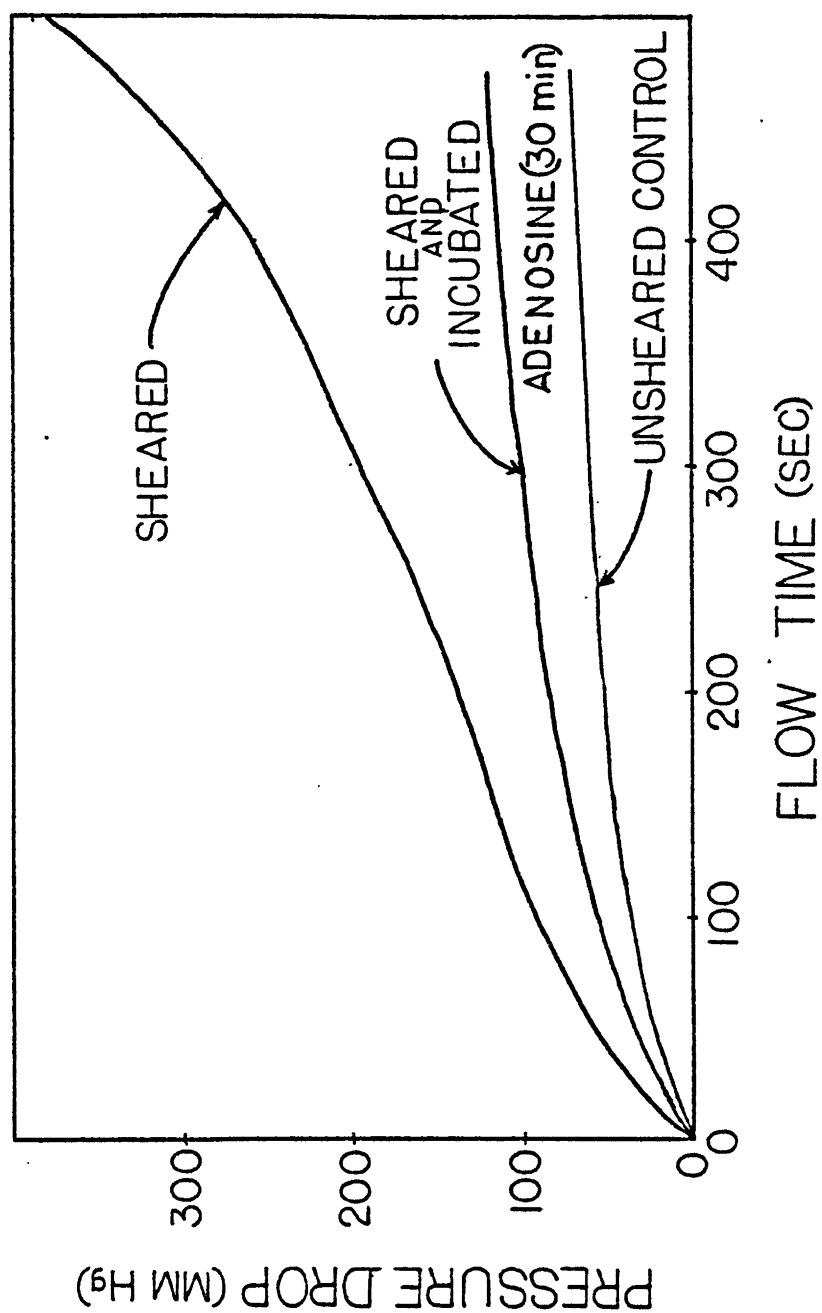


Figure 47

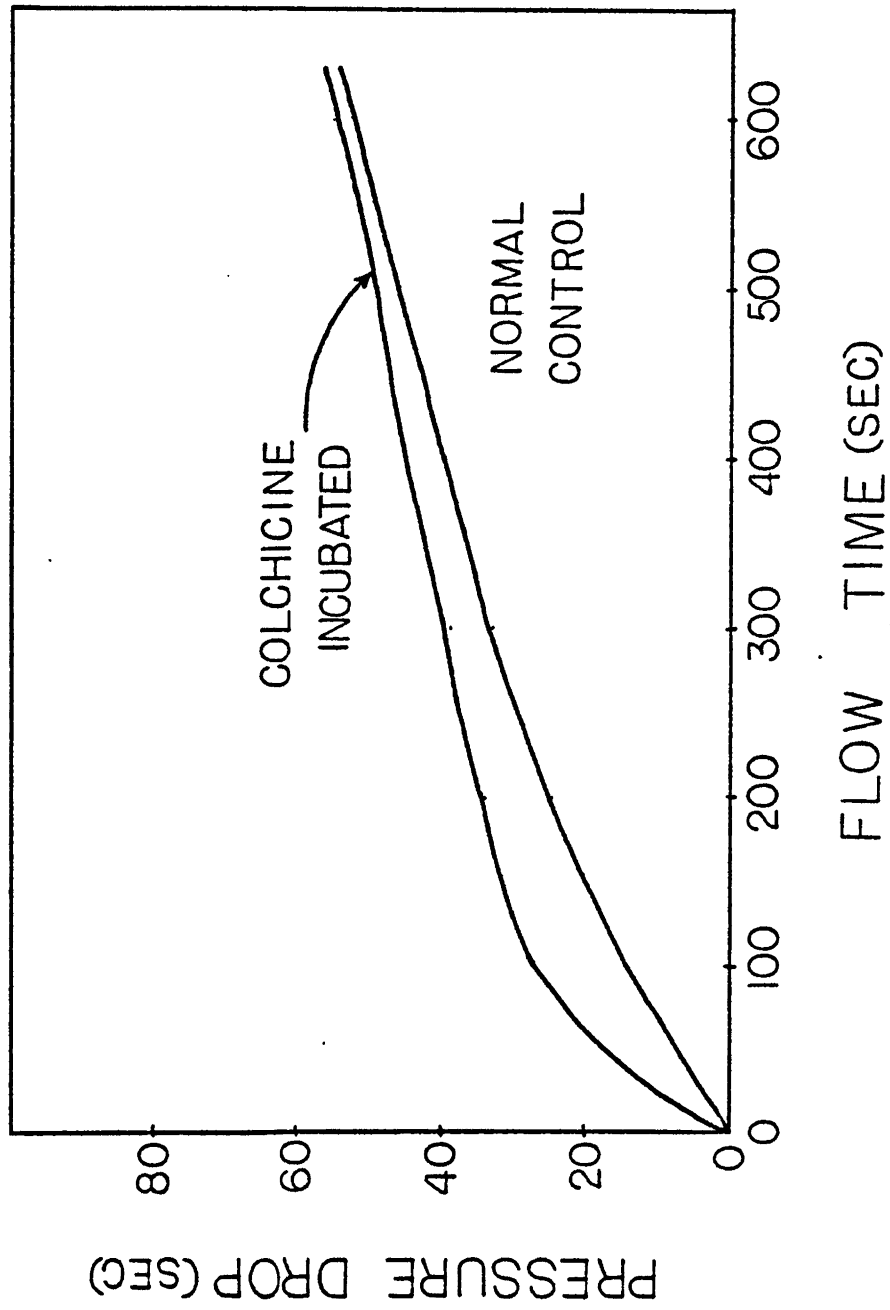


Figure 48

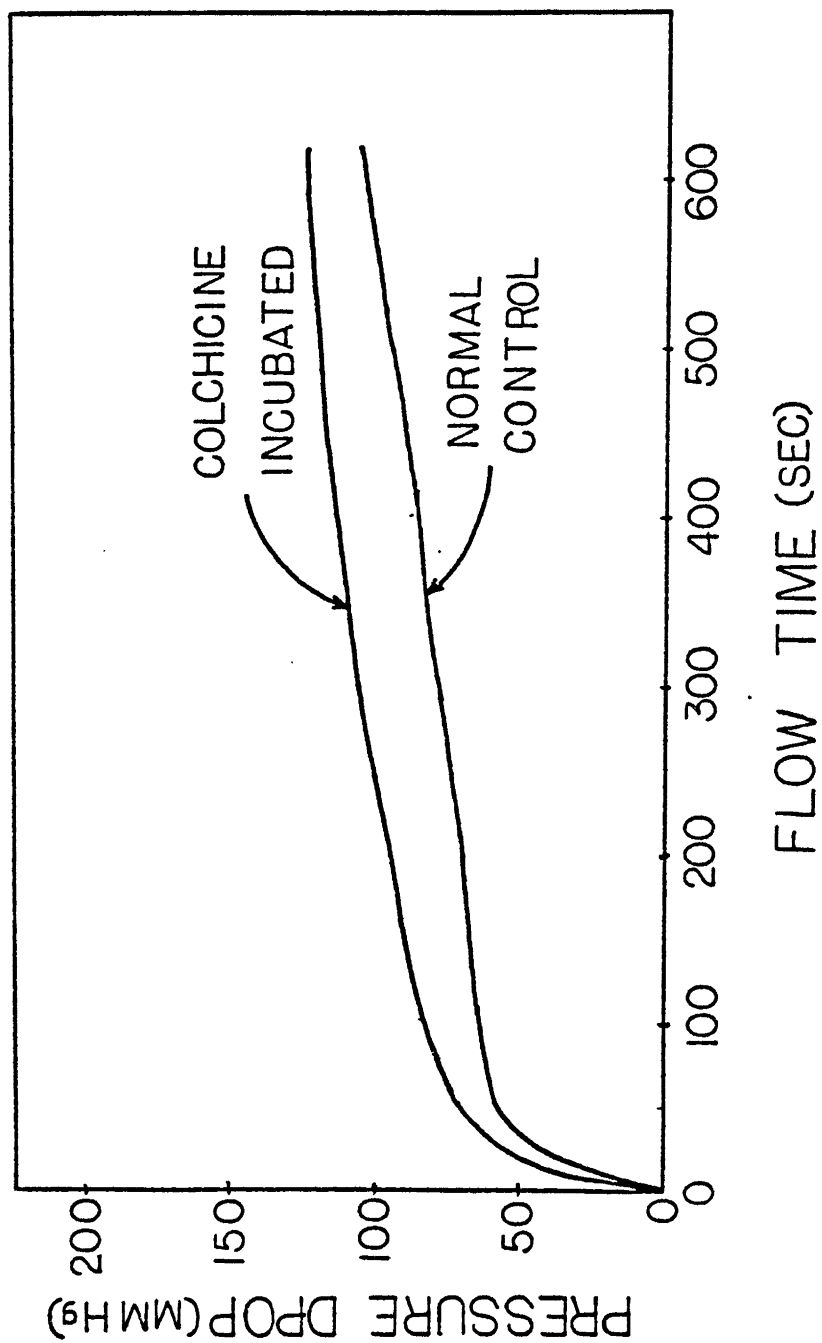


Figure 49

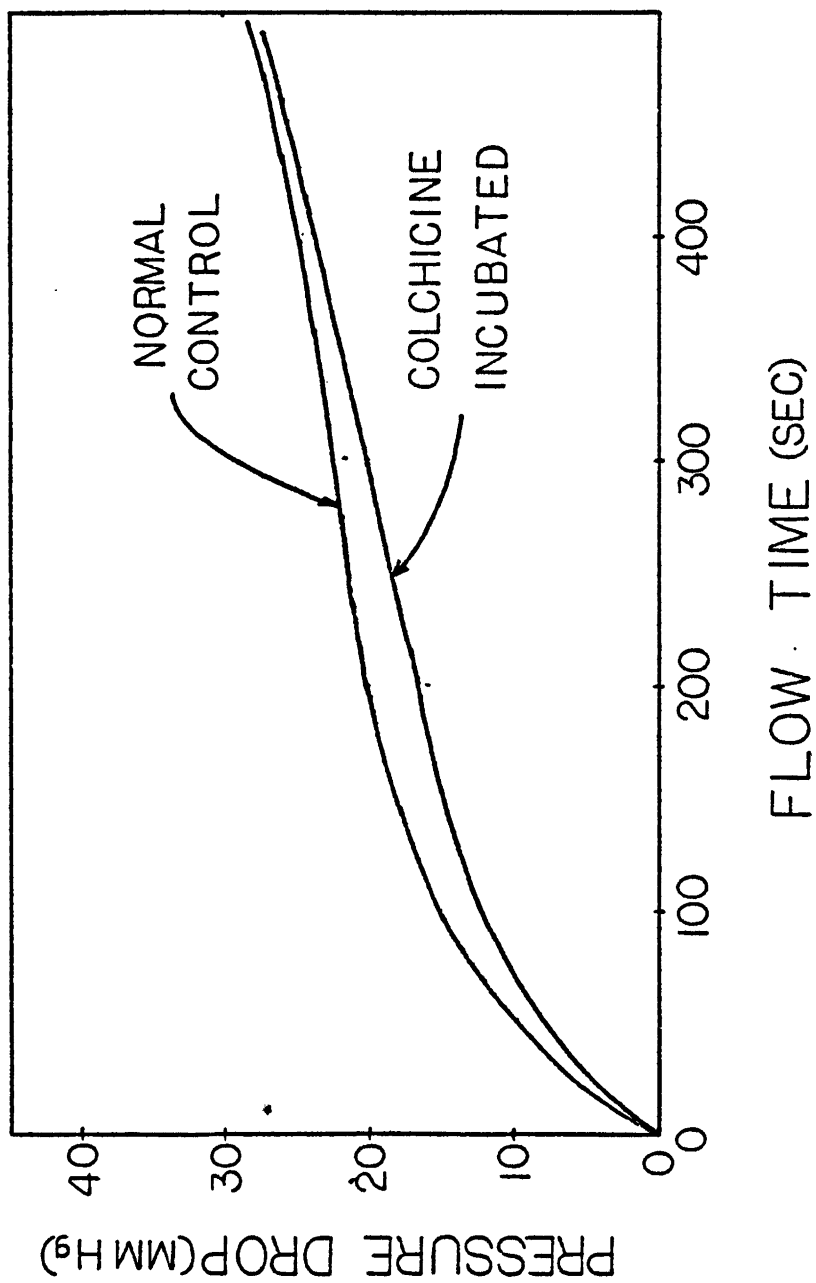


Figure 50

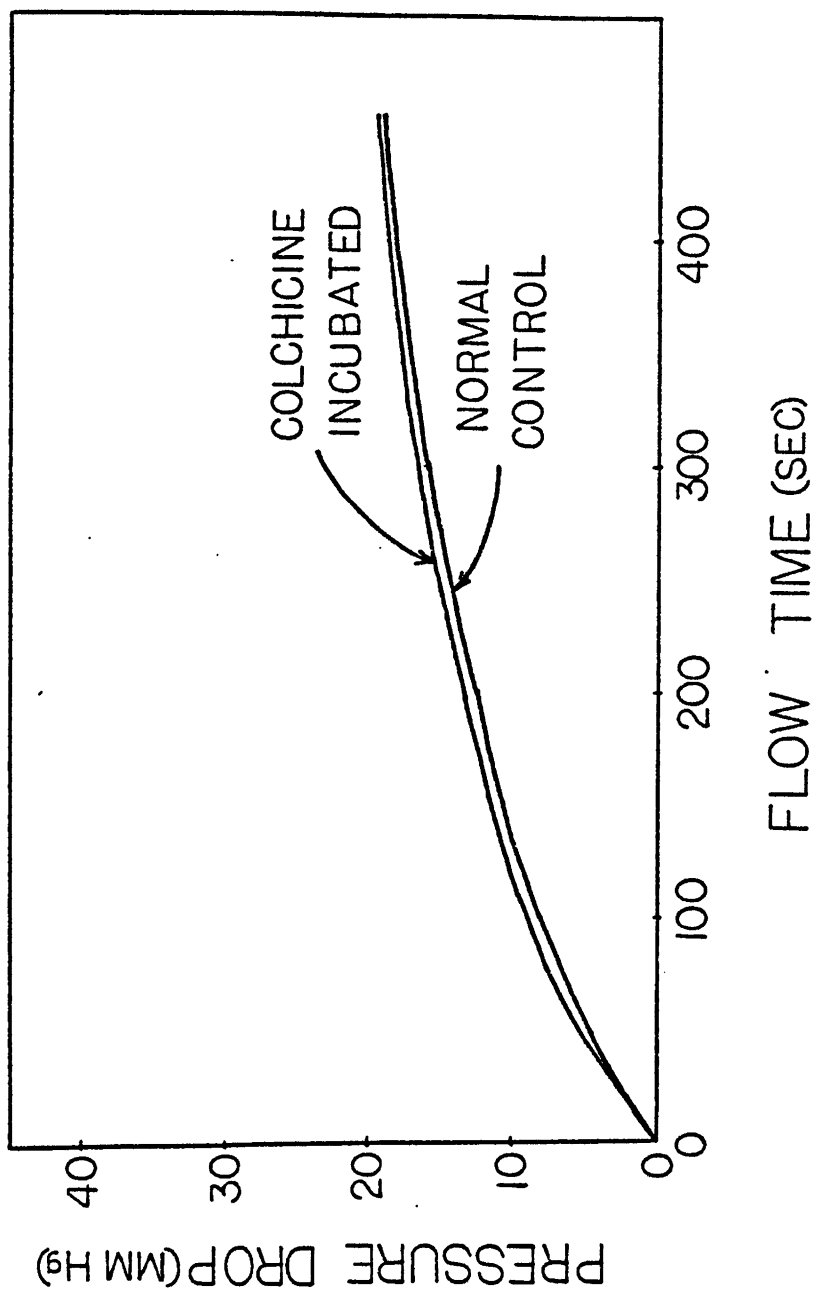
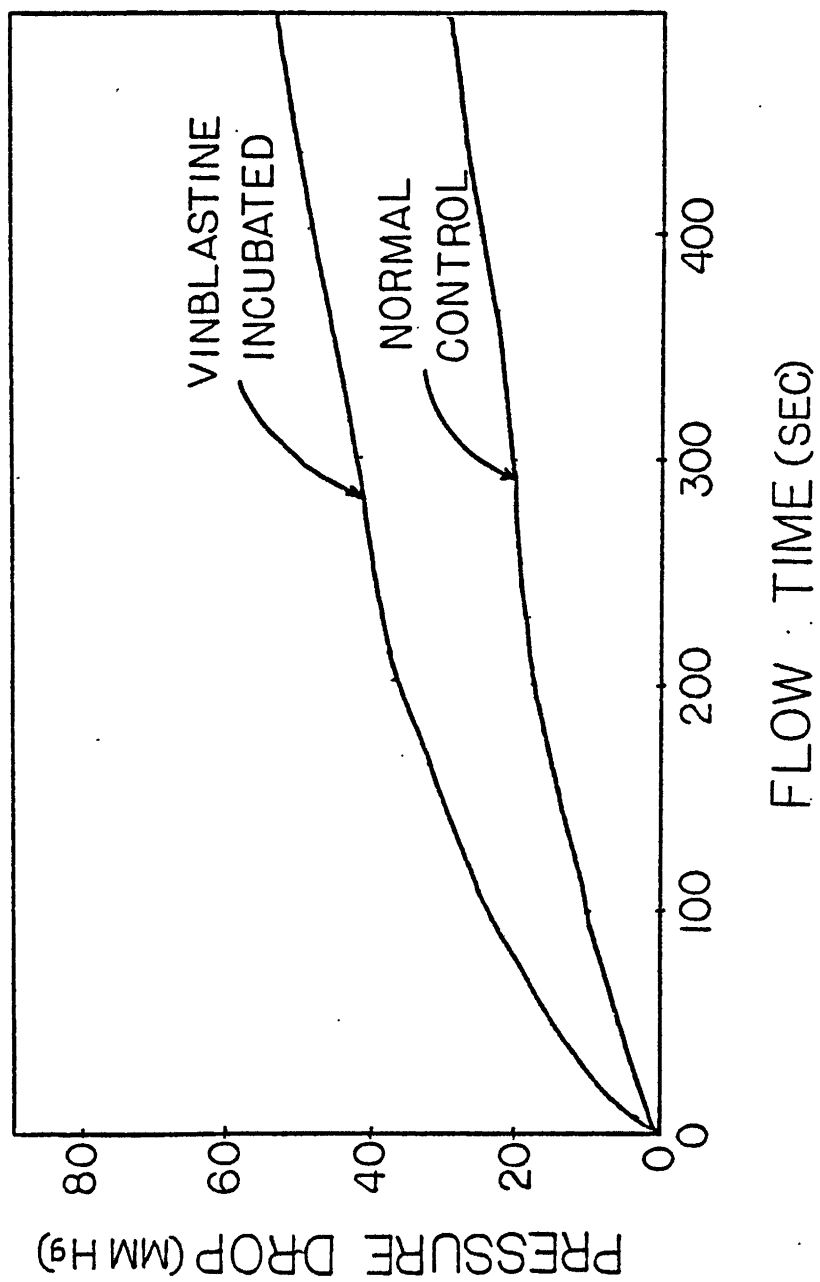


Figure 51



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