TRAUMA OF THE ERYTHROCYTE MEMBRANE ASSOCIATED WITH LOW SHEAR STRESS

B. N. NANJAPPA, HSIN-KANG CHANG, *and* CHESTER A. GLOMSKI

From the Departments of Civil Engineering and Anatomy, State University of New York at Buffalo, Buffalo, New York 14214

ABSTRACT Studies have been performed on erythrocytes that have been subjected to a low shear stress of less than 100 dyn/cm² in a cone-and-plate viscometer. Alterations that were observed included decreased red cell survival, increased osmotic fragility, changes in the cation permeability of the red cell membrane, and a reduction in membrane-associated acetylcholinesterase activity. Some of these alterations are similar to those accompanying aging. The observed data suggest that one segment of the erythrocyte population is more susceptible to shear-induced damage than the rest of the cells.

INTRODUCTION

When blood comes in contact with implanted cardiovascular prostheses or passes through an extracorporeal apparatus (Williams, 1971) several modifications of the cells and other constituents of the blood are known to occur. One of the effects is an alteration of the structure of the erythrocyte membrane which can result in irreversible damage and premature destruction (Marsh, 1964). There is evidence that various levels of shear stress can cause varying degrees of cellular damage. Thus, complete hemolysis of human erythrocytes is associated with high shear stresses (greater than 4,000 dyn/cm²) while little hemolysis is observable below the threshold level of 1,500 dyn/cm² (Blackshear et al., 1965; Nevaril et al., 1968, 1969; Sutera et al., 1972; Leverett et al., 1972).

In order to study further the nature of the damage imposed upon erythrocytes by mechanical forces, the effects of low level shear stress upon these cells were investigated. It has been demonstrated that shortened life spans are observed in aged cells and in erythrocytes with certain known abnormalities. Such cells also show (*a*) increased susceptibility to osmotic lysis (Young et al., 1951), (*b*) altered membrane permeability (Hoffman, 1966; Oski and Naiman, 1969), and (*c*) in aged cells, decreased membrane-associated acetylcholinesterase activity (Pritchard, 1949). In light of these factors, the present investigation included an evaluation of the survival rates, osmotic fragility, cation permeability, and acetylcholinesterase activity of sheared and unsheared cells.

APPARATUS AND LABORATORY METHODS

Viscometer

A model 17 Weissenberg rheogoniometer (Sangamo Controls Ltd., North Bersted, Sussex, England) with a cone-and-plate system was used in this investigation. The platen has a diameter of 7.9 cm with a half-cone angle of 1°1'34" and a minimum gap width of 44 μm at the apex of the cone. The ratio of cone-plate surface area to the volume of the sample contained in the apparatus is 450 cm^{-1}. The motor and gap settings permit variations of shear rate from 0 to 2,110 s^{-1}. The latter shear rate (equivalent to approximately 90 dyn/cm^2) was used throughout the study. The surfaces of the cone and plate were siliconized using Siliclad (Clay Adams, Div. of Becton, Dickinson and Co., Parsippany, N.J.); they were washed with detergent, rinsed several times with distilled water, and dried before the placement of the samples. All the experiments were performed at room temperature.

Blood Sampling

Blood samples were obtained from healthy mongrel dogs that were maintained on a standard laboratory animal diet and water ad libitum. Blood was collected per venipuncture into sterile plastic syringes containing acid citrate dextrose (A.C.D.) solution (Strumia formula, Abbott Scientific Products Div., Abbot Laboratories, South Pasadena, Calif.).

Red Cell Survival Studies

Survival of canine erythrocytes was determined by the use of radiochromium labeling (Rachromate-51, Abbott Scientific Products Div.). A 10-ml aliquot of whole blood was incubated with 3 ml of A.C.D. solution and 60 μCi of $Na_2{}^{51}CrO_4$ at room temperature for 30 min. At the end of this period, labeling was stopped by the addition of 1 ml of ascorbic acid solution (100 mg) to convert any unbound hexavalent chromium to the trivalent state. The blood was then sheared for varying periods and reinjected into the donor animal through a foreleg vein. Control experiments using unsheared blood were performed on the same dogs. The first sample was withdrawn 30 min postinjection from a vein in the alternate foreleg. Subsequent samples were withdrawn 24 h after injection and at intervals of 1–3 days for a period of about 30 days. The gamma activity of the blood samples was determined with a scintillation spectrometer. All the samples were counted at the same time to obviate the need for correction for physical decay of the isotope. The half-life of the red blood cells was determined graphically.

Osmotic Fragility Determination

The method used is based on the procedure presented by Emerson et al. (1956). Sheared and unsheared cells were placed as a 50% suspension into a series of solutions containing sodium chloride in concentrations ranging from 0.9 to 0.2% in 0.1% decrements or less. The suspensions were incubated at room temperature for 30 min. The resultant hemoglobin concentration of each supernatant fluid was measured by the cyanmethemoglobin technique.

Cation Permeability Studies

Sodium and potassium transport studies on sheared and unsheared cells were performed by methods of Bertles (1957) and of Jacob and Jandl (1964). Human erythrocytes were used in these studies. Influx and efflux of sodium in sheared (at a rate of 2,110 s^{-1} for 60 min) and

control red cells were measured by using tracer ^{24}Na (Iso-Serve, Inc.). Sodium influx was measured in a 30% washed cell suspension incubated in an isoosmolal solution containing 5% dialyzed human serum albumin, glucose, and the radioactive isotope. The other constituents of the medium were K, 6.5 meq/liter; Na, 165 meq/liter; PO_4, 40 mmol/liter; HCO_3, 25 meq/liter; and sufficient Cl to make up the balance of anions. After a 2-h incubation period, a fractional portion of the cells and suspending medium was removed and individually analyzed for stable and radioactive sodium by flame photometry (Berry et al., 1946) and scintillation spectrometry. Subsequent samples were also analyzed at various intervals during the total 24-h incubation.

For sodium efflux studies, a sample was obtained after 2 h of incubation. The cells were washed three times in isotonic $MgCl_2$ and resuspended in the isoosmolal medium used previously but without added radioactive sodium. Aliquots of cells and medium were subsequently withdrawn from this suspension at several intervals during the following 2-h period. The intracellular and extracellular concentrations of sodium were assayed as described above.

Potassium efflux from the erythrocytes was also determined by flame photometry on the same samples of cells analyzed for sodium influx.

Acetylcholinesterase Assay

The assay method utilized was that of Herz and Kaplan (1970). Blood from five dogs was centrifuged, and the plasma and buffy coat were removed by aspiration. The red cells were washed three times with ice-cold phosphate-buffered saline, pH 7.4. After the last washing and centrifugation 50% suspensions of these cells were prepared in the same buffer. An aliquot of each suspension was taken for the preparation of hemoglobin-free membranes to be used as controls. The membranes were prepared by osmotically induced hemolysis and stored at 4°C as a 50% suspension. The remainder of the suspensions of erythrocytes were sheared for various durations, after which time the cells were washed and suspensions of erythrocyte membranes were prepared as detailed above. The acetylcholinesterase activity of membranes from sheared and control cells was determined. In addition, the supernatant fluid from the sheared erythrocytes along with that from postshearing washings were assayed. Suspensions of erythrocytes were also placed in the viscometer for periods of 5–90 min without shearing; the membranes of these cells were similarly analyzed for enzymatic activity. The acetylcholinesterase activity was measured spectrophotometerically at 412 nm using acetylthiocholine as substrate and 5:5′-dithiobis-(2-nitrobenzoic acid) as color reagent. The specific activity was expressed as ΔOD per minute per milligram of hemoglobin measured at 540 nm as cyanmethemoglobin.

RESULTS

The results of a study of five dogs indicate that the half-life of erythrocytes decreases with the length of exposure to a shear rate of 2,110 s^{-1}. The decrease in survival ranged from 22% after 5 min shear to 60% after 1 h (Table I). The rate of percent decrease in red cell life span with respect to the duration of shear was greater after shorter periods of shear, while shear durations exceeding 45 min were associated with little additional decrease in survival. The half-life of unsheared chromium-labeled canine erythrocytes seen here is in the range of that observed for the mongrel dog in other studies, i.e., approximately 21–30 days (Stohlman and Schneiderman,

TABLE I

SURVIVAL OF SHEARED AND UNSHEARED CHROMIUM-51-LABELED ERYTHROCYTES

Dog	Half-life		Duration of shear*	Decrease in half-life
	Unsheared	Sheared		
	days		*min*	%
1	27.5	21.4	5	22
2	25.0	13.0	15	48
3	17.4	8.4	30	52
4	24.4	10.0	45	59
5	27.6	10.9	60	60

* Shear rate employed: 2,110 s^{-1}.

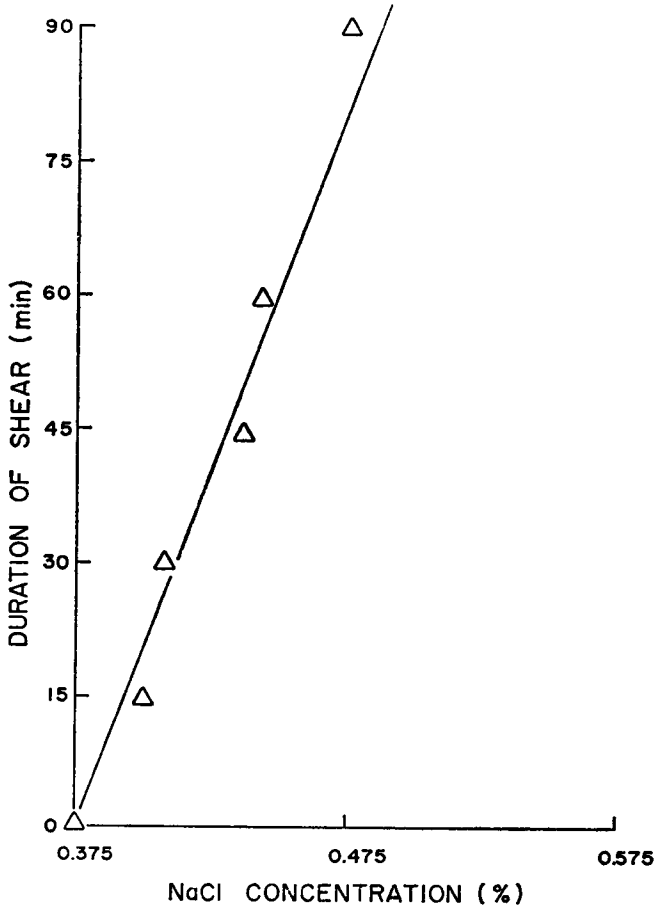

FIGURE 1 Concentrations of sodium chloride required for 50% hemolysis of canine erythrocytes at various durations of shear at a rate of 2,110 s^{-1}.

1956). The value of 17.4 days obtained for dog no. 3 is somewhat low; however, half-lives as low as 15 days have been reported for the occasional healthy dog.

Exposing erythrocytes to the low level of shear used in the study also rendered them more susceptible to osmotic lysis. Fig. 1 depicts the concentration of sodium chloride that will produce 50% hemolysis after given durations of shearing. Within the limits of the experiment, a nearly linear relationship is seen to exist. From a comparative study of all hemolysis curves (Fig. 2), however, it is seen that the greatest alterations of osmotic fragility occur in the region of the onset of hemolysis. Initial hemolysis of sheared cells is seen at near isotonic concentrations of sodium chloride, and as a group, these curves appear flattened and extended.

The results of the cation permeability studies (Fig. 3) indicate that upon incubation in a glucose-containing medium, intracellular sodium accumulated more rapidly and reached higher levels in sheared erythrocytes than in the simultaneously incubated normal red cells. The slopes of the sodium efflux curves of sheared and normal erythrocytes also differ considerably in that the rate of tracer ^{24}Na extrusion was greater in the sheared than in control cells (Fig. 4).

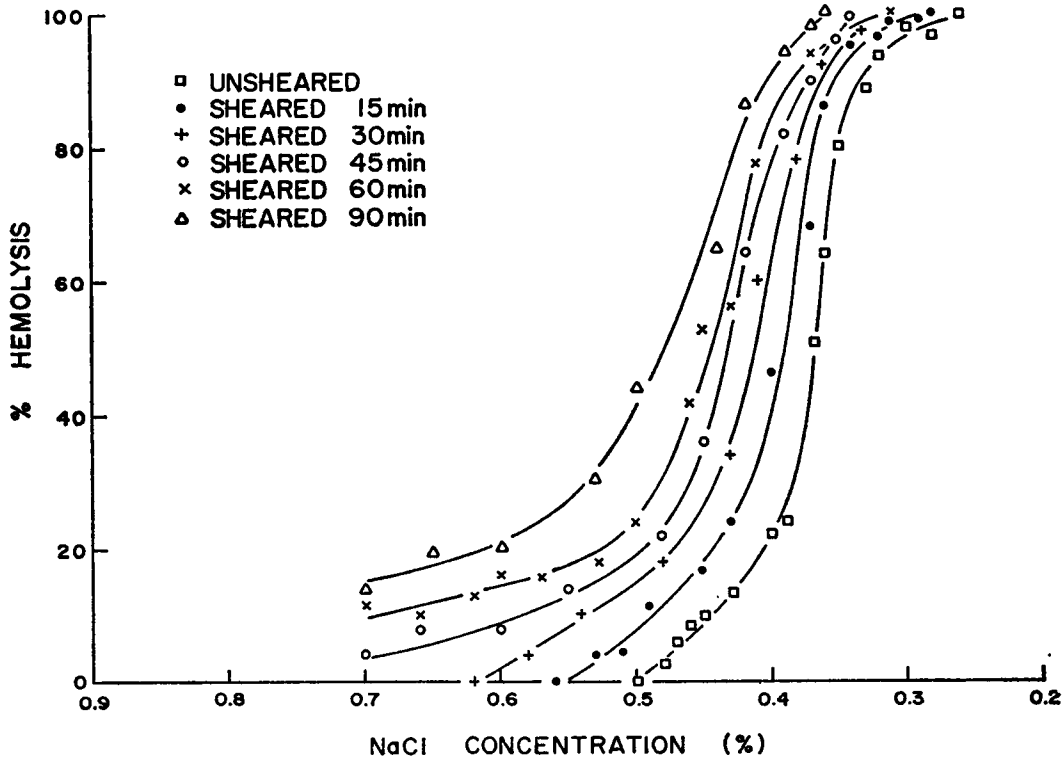

FIGURE 2 Osmotic fragility of sheared and unsheared canine erythrocytes. Shear rate 2,110 s^{-1}.

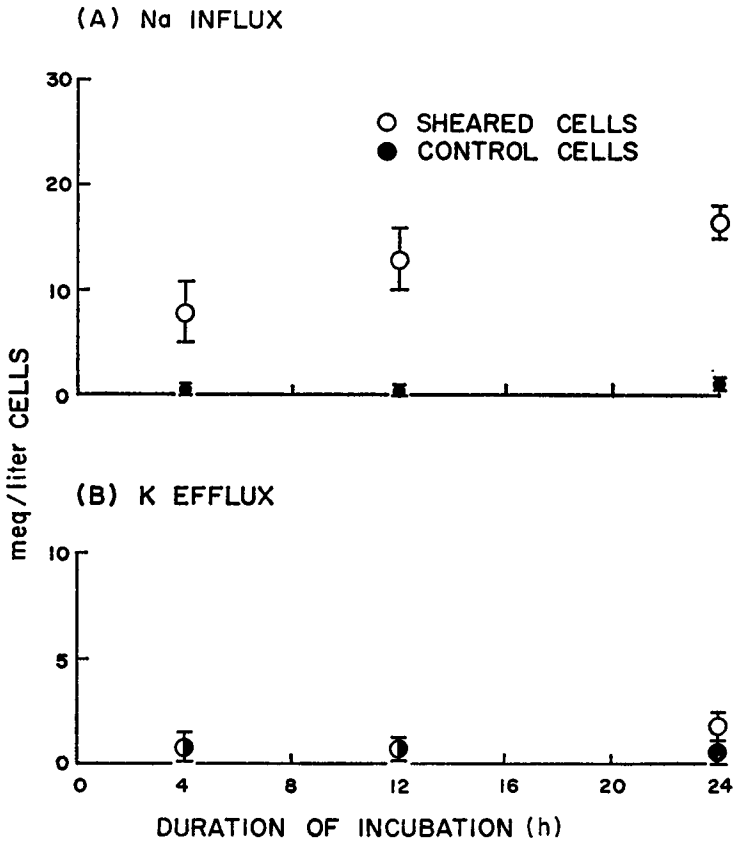

FIGURE 3 (A) Sodium influx into sheared and unsheared human erythrocytes. (B) Potassium efflux from sheared and unsheared human erythrocytes. Each point represents the mean value of five experiments; the bars represent 1 SD. Open circles identify erythrocytes sheared for 60 min at 2,110 s^{-1}; solid circles identify unsheared erythrocytes.

The acetylcholinesterase activity of canine erythrocytes is seen to decrease with shear stress. Fig. 5 shows that the loss of activity is dependent on shear duration, with 20% less after 15 min of shearing and approximately 60% decrease after 1 h. No additional change was produced with longer shearing periods. Concomitant with the reduction of this membrane-associated activity, measurable levels of enzymatic activity could be observed in the suspending medium and post-shearing washings (Fig. 5). No alterations were observed in control cells that were not sheared but analyzed after having been placed in the viscometer for periods ranging from 5 to 90 min.

DISCUSSION

The intent of the present study was to investigate alterations of the erythrocyte produced by mechanical forces of such magnitude that do not result in overt hemoly-

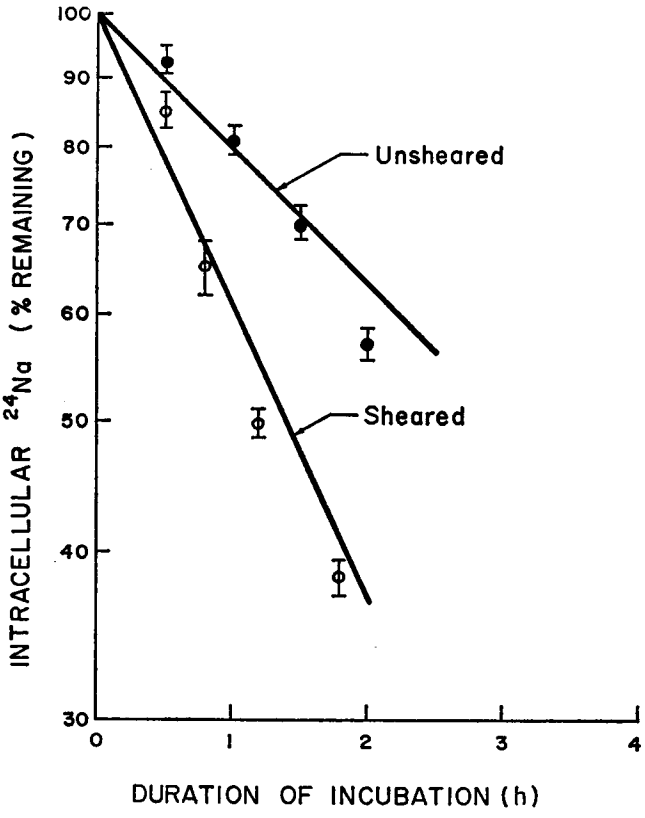

FIGURE 4 Efflux of sodium-24 from sheared and unsheared human erythrocytes. Each point represents the mean value of five experiments; the bars represent 1 SD. Open circles identify erythrocytes sheared for 60 min at 2,110 s^{-1}; solid circles identify unsheared erythrocytes.

sis of the cell. The rheogoniometer utilized in this study satisfied this requirement in that it can generate a nearly uniform shear stress less than 100 dyn/cm^2 throughout the sample. It has been shown (Weed et al., 1969) that erythrocytes exposed to shear rates much lower than those utilized here demonstrate increased rigidity associated with decreased ATP levels, thus suggesting that even minimal shear stress can alter the physical as well as biochemical properties of the erythrocyte. Several groups of workers, however, have stated that at low shear stresses the damage is predominantly caused by surface-to-cell interaction rather than by direct shear (Knapp and Yarborough, 1969; Shapiro and Williams, 1970; Leverett et al., 1972). Since the cone-and plate viscometer utilized here has a surface area-to-volume ratio of 450 cm^{-1}, a value considerably higher than those reported in most other studies, it can be assumed that surface effects played a significant role in causing the alterations reported here.

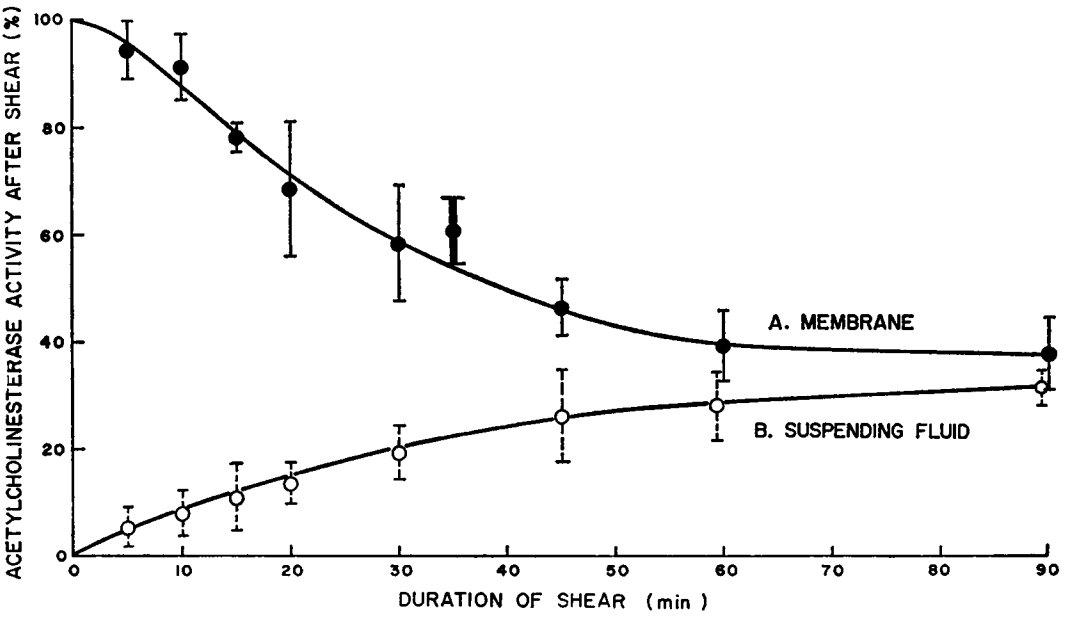

FIGURE 5 (A) Level of acetylcholinesterase activity of the canine erythrocytic membrane after various durations of shear at a rate of 2,110 s^{-1}. (B) The recovery of acetylcholinesterase activity from the suspending fluid and cell washings after shearing. Each circle represents the mean of five experiments on different animals; bars represent 1 SD. The thick solid bar (curve A) represents the data of five experiments on the same dog.

Under the conditions of the present experiments, there is a strong possibility of secondary flow in the form of vortices. These vortices may lead to increased surface-cell interaction as well as increased shear stress. The increased surface-cell interaction will most likely produce more membrane alterations which could be significant. The shear stress increase, on the other hand, is not expected to be substantial since the velocities of the secondary (vertical and radial) flow are generally no more than a few percent of that of the primary (circumferential) flow.

The present studies reveal that low levels of shear stress are associated with changes in the characteristics of the erythrocytic membrane. The presence of these alterations is indicated by shortened red cell life spans, increased susceptibility to osmotic forces, changes in cation permeability, and decreased enzymatic activity. The red cell survival data (as well as the results of the osmotic and enzymatic studies) suggest the presence of a selective factor in the induction of damage to the erythrocytes. It is conceivable that primarily one population of susceptible cells is affected to the point of producing a shortened life span. A likely group of cells could be the older ones since it can be assumed that they would be least capable of maintaining and/or repairing their membranes. The shear rate employed here may not be sufficient to injure young cells, while the altered senescent cells are

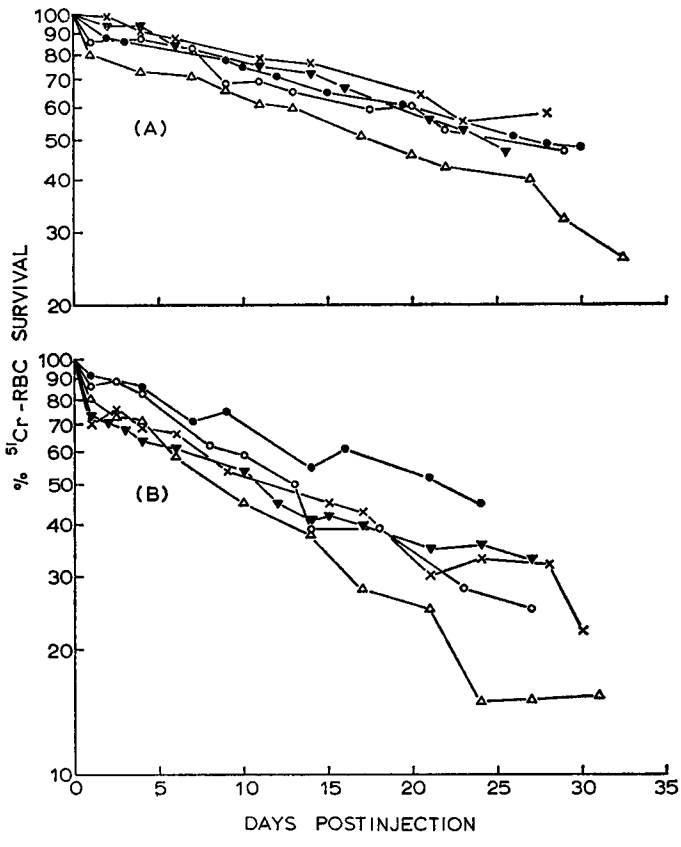

FIGURE 6 Disappearance of nonsheared (A) and sheared (B) chromium-51-labeled erythrocytes (RBC) from circulation. Dog 1 (solid circle), 5 min shear; dog 2 (open circle), 15 min shear; dog 3 (open triangle) 30 min shear; dog 4 (solid inverted triangle), 45 min shear; dog 5 (X), 60 min shear.

detected in vivo and lysed by the phagocytic reticuloendothelial cells of the spleen and liver. The supposition of the existence of a group of highly susceptible erythrocytes also receives some support from the observation that shearing for extended periods did not additively affect the red cell survival. Nevaril and his co-workers (1968) in their study of rabbit erythrocytes sheared at 4,000 dyn/cm^2 have shown two-component ^{51}Cr survival curves. The curves resulting from the present study involving shear stresses of approximately 100 dyn/cm^2 have similar though correspondingly less pronounced features (Fig. 6).

As indicated in the results, the configurations of the hemolysis curves suggest that the level of shear stress used here tended to affect one population of cells. It is generally accepted that the cells hemolyzed at the point of "initial hemolysis" in a sample of normal unsheared blood are the oldest, senescent cells. These cells are represented by the initial segment of the sigmoid hemolysis curve. Because the

greatest alterations in the hemolysis curves of the sheared cells were localized in this region, this supposition appears to have merit. It should be realized, however, that since the hemolysis curves also progressively shift to the left with increased durations of shear, other cells are also affected but to a lesser extent.

The decreased survival of sheared red cells was associated with a propensity to accumulate intracellular sodium as compared with the normal controls. In contrast, potassium losses from both cell types are similar (Fig. 3). As seen in Fig. 4, in spite of the tendency to accumulate sodium, the sheared red cells were also found to transfer this ion from the intracellular to extracellular compartment at a rate more rapid than normal. Such a phenomenon has been reported to occur in red cells of persons having hereditary spherocytosis (Jacob and Jandl, 1964; Prankard, 1960). The ionic alterations after shear stress appear to be expressions of cell damage and of physiologic attempts to maintain a viable state. Although the entire spectrum of physical and biochemical alterations produced by shearing are as yet not well defined, they do include a greater than normal passive influx of sodium into the cell.

Since it is known that acetylcholinesterase is a membrane-associated enzyme that projects its active sites into the surrounding plasma (Herz et al., 1963), it is possible that shearing forces imposed upon the erythrocytes could affect its activity. The results of the present study support this hypothesis. Fig. 5 illustrates that the shear-associated reduction in the specific activity of this enzyme is followed by a corresponding appearance and rise of activity in the suspending fluid, suggesting that low shear stresses can strip the enzymatic moiety off the red cell membrane. This graph also shows that shearing beyond 1 h induces little additional loss of enzymatic activity. Virtually identical information is seen in the human erythrocytes (Nanjappa et al., 1973). The configuration of the enzyme activity reduction curve may be accounted for by the partial loss of activity via one or more mechanisms. First, since acetylcholinesterase present in the red cell membrane is known to exist in different molecular weights with varying number of active sites, it could be postulated that at the energy levels used in this study only the high molecular weight moieties are affected. Secondly, there is a strong possibility that due to cell rotation and folding during shearing the enzyme recoils upon itself concealing some of the active sites thereby preventing them from entering into reaction with the substrate. Thirdly, there is also a likelihood of configurational rearrangement of the enzymatic molecules causing changes in the forces of interaction between enzyme and substrate such as (*a*) coulombic attraction between the positively charged substrate and the negatively charged anionic site, (*b*) van der Waals attraction between the methyl and methylene groups of the substrate and nonpolar portions of the active site, and (*c*) weak covalent bonding between the basic group of the esteratic site and the electrophilic carbon atom of the ester. The existence of one or more of these mechanisms or the denaturation of some of the enzyme molecules could also help account for the fact that the acetylcholinesterase activity recovered from the super-

natant fluid of sheared erythrocytes never equaled the activity lost from the membranes.

It has been shown that aging of red cells is accompanied by decreased acetylcholinesterase activity (Allison and Burn, 1955). Thus, it appears that shearing causes certain changes that are similarly observed in the normal process of aging. Since aged erythrocytes are known to be less flexible than their younger counterparts (Weed et al., 1969), it is also possible that the physically projecting acetylcholinesterase molecules of the former cells are more likely to be damaged than those of the younger population.

Finally, it is of interest to note that in almost all the described experiments the greatest rate of alteration was produced during the initial periods of shear. This suggests an interrelationship of these changes as well as supporting the concept of a population of cells more susceptible to shear damage than others.

The authors are greatly indebted to Dr. J. T. Hoogeveen and Dr. Kenneth M. Kiser for their generous help and encouragement in this study.

Received for publication 13 March 1973 and in revised form 12 June 1973.

REFERENCES

ALLISON, A. C., and G. P. BURN. 1955. *Brit. J. Haematol.* **1**:291.
BERRY, J. W., D. G. CHAPPELL, and R. B. BARNES. 1946. *Ind. Eng. Chem. Anal. Ed.* **18**:19.
BERTLES, J. G. 1957. *J. Clin. Invest.* **36**:816.
BLACKSHEAR, P. L., JR., F. D. DORMAN, and J. H. STEINBACH. 1965. *Trans. Am. Soc. Artif. Intern. Org.* **11**:112.
EMERSON, C. P., JR., S. C. SHEN, T. H. HAM, E. M. FLEMING, and W. B. CASTLE. 1956. *Arch. Intern. Med.* **97**:1.
HERZ, F., and E. KAPLAN. 1970. *Proc. Soc. Exp. Biol. Med.* **134**:437.
HERZ, F., E. KAPLAN, and J. H. STEVENSEN. 1963. *Nature (Lond.).* **200**:901.
HOFFMAN, J. F. 1966. *Am. J. Med.* **41**:666.
JACOB, H. S., and H. S. JANDL. 1964. *J. Clin. Invest.* **43**:1704.
KNAPP, C. F., and K. A. YARBOROUGH. 1969. Proceedings of the 8th International Conference on Medical Biological Engineering. **18**:10.
LEVERETT, L. B., J. D. HELLUMS, C. P. ALFREY, and E. C. LYNCH. 1972. *Biophys. J.* **12**:257.
MARSH, G. W. 1964. *Lancet.* **2**:986.
NANJAPPA, B. N., H. K. CHANG, and C. A. GLOMSKI. 1973. *Anat. Rec.* **175**:510.
NEVARIL, C. G., C. P. ALFREY, JR., and E. C. LYNCH. 1969. *Am. Inst. Chem. Eng. J.* **15**:707.
NEVARIL, C. G., E. C. LYNCH, C. P. ALFREY, JR., and J. D. HELLUMS. 1968. *J. Lab. Clin. Med.* **71**:784.
OSKI, F. A., and J. L. NAIMAN. 1969. *N. Engl. J. Med.* **280**:909.
PRANKARD, T. A. J. 1960. *Q. J. Med.* **29**:199.
PRITCHARD, J. A. 1949. *Am. J. Physiol.* **72**:158.
SHAPIRO, S. I., and M. C. WILLIAMS. 1970. *Am. Inst. Chem. Eng. J.* **16**:575.
STOHLMAN, F., JR., and M. A. SCHNEIDERMAN. 1956. *Ind. Eng. Chem. Anal. Ed.* **18**:19.
SUTERA, S. P., P. A. CROCE, and M. MEHRJARDI. 1972. *Trans. Am. Soc. Artif. Int. Org.* **18**:335.
WEED, R. I., P. L. LACELLE, and E. W. MERRILL. 1969. *J. Clin. Invest.* **48**:795.
WILLIAMS, D. F. 1971. *Biomed. Eng.* **5**:197.
YOUNG, L. E., M. J. IZZO, and R. F. PLATZER. 1951. *Blood.* **6**:1073.