Biochem. J. (1984) **218**, 531–537
Printed in Great Britain

Decreased enzymic protection and increased sensitivity to oxidative damage in erythrocytes as a function of cell and donor aging

G. Allison GLASS and David GERSHON
Department of Biology, Technion–Israel Institute of Technology, Haifa, Israel

(*Received 23 June 1983/Accepted 14 November 1983*)

Erythrocytes from young and old rats were separated into four age fractions by density-gradient centrifugation. The specific activities per cell were determined for glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutathione peroxidase (EC 1.11.1.9), glutathione reductase (EC 1.6.4.2) and catalase (EC 1.11.1.6). Decreased specific activities were observed with increasing cell age for all four enzymes in both young and old animals. In addition, significant differences in the activities of these enzymes were observed between cells of the same age fraction from young and old donors. Susceptibility of fractionated erythrocytes to oxidative attack *in vitro* generated by incubation with xanthine/xanthine oxidase increased with both cell and animal age. The amount of membrane-lipid peroxidation also increased with cell and animal aging, as measured by both thiobarbituric acid and fluorescent chromolipid assays. Increases of 2–3-fold in the contents of lipid peroxides were observed between the youngest and oldest age fractions of young rats. Lipid peroxide contents in young cells of old animals were equal to those in old erythrocytes from young rats and increased by 30% with cell aging in the old donors. These results suggest that the extent of enzymic protection against oxidative and peroxidative damage decreases with erythrocyte aging. More importantly, enzymic protection in cells of old rats is considerably decreased already in the early stages of their lifespan.

One of the major aims of experimental gerontology has been to observe and compile the physiological and biochemical changes that occur during the aging process. Measuring the changes in the specific enzyme activities in various tissues with increasing age has been an important approach in gerontological research. The erythrocyte has proved to be a valuable model system for observing the effects of cell aging in higher species (Fornaini, 1967), This is due to the fact that the erythrocyte has a fixed lifespan, is incapable of protein synthesis *de novo* and has the capacity for only low extents of proteolysis (Rifkind *et al.*, 1974). In addition, the biochemistry of the erythrocyte is relatively simple, with its metabolism dependent primarily on glycolysis and the pentose phosphate shunt. The cell has a fixed lifespan of 55–60 days in rats and 110–120 days in humans. As the cell ages its density increases, so that cells of different age classes can be separated by density-gradient centrifugation (Borun *et al.*, 1957). We have previously demonstrated by ^{59}Fe pulse-labelling that rat erythrocytes could be fractionated by density-gradient centrifugation into age groups. The youngest fraction was the least dense, and the oldest fraction had the highest density (Glass, 1983; Glass *et al.*, 1983).

It has been shown that the specific activities of several enzymes decline as the cell ages (Bunn, 1972). The activity of free glutathione and glutathione reductase (Abraham *et al.*, 1978) and superoxide dismutase (Glass & Gershon, 1981) decrease with respect to cell age and, more interestingly, with respect to donor age between erythrocytes of the same age class. These enzymes are important in protecting the cell against oxidative and peroxidative damage (Chance *et al.*, 1979). It has been reported that the activities of other enzymes involved in protection against such damage, e.g. catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase, decline with increasing cell age (Bunn, 1972). Very little information is available on the effect of donor age on the activity of these enzymes in the erythrocyte.

The present study was thus undertaken to determine if these enzymic components of the erythrocyte system of protection against oxidative and peroxidative damage are affected during

animal aging. Furthermore, sensitivity of erythrocytes of different cell age classes from young and old donors to oxidative damage *in vitro* and the extent of lipid peroxidation *in vivo* were investigated.

Materials and methods

Animals

Inbred WF rats, bred and maintained at the Weizmann Institute of Science, were used in all experiments. The colony has a mean life expectancy of approx. 27 months. Young rats were 6–8 months old and old rats were 27–31 months old.

Erythrocyte preparation and fractionation

Fresh blood was obtained by cardiac puncture. The blood was withdrawn into phosphate-buffered saline (60mM-potassium phosphate/81mM-NaCl, pH7.4) containing 150 units of sodium heparin (Calbiochem)/ml. The amount of phosphate-buffered saline/heparin was 0.1ml/ml of blood withdrawn (15 units of heparin/ml of blood). The blood was washed and the packed cells were separated into age classes by density-gradient centrifugation on an iso-osmotic Stractan gradient as previously described (Glass & Gershon, 1981). Fraction I comprised the youngest and Fraction IV the oldest cells. Erythrocyte concentrations were determined by counting the cells in a haemocytometer under 250× magnification. Haemolysates were prepared by suspending packed cells in 100vol. of 10mM-potassium phosphate buffer. The pH varied according to the enzyme to be measured and is given for each enzyme.

Enzyme determinations

For measurements of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity, the lysis buffer contained 20μM-$NADP^+$ (Sigma) and 1mM-EDTA. Glucose-6-phosphate dehydrogenase activity was measured spectrophotometrically as described by Kahn & Dreyfus (1974) by observing the conversion of $NADP^+$ into NADPH after addition of glucose 6-phosphate (Sigma). One unit of activity is defined as the amount of enzyme required to reduce 1μmol of $NADP^+$ to NADPH/min at 30°C. Catalase (EC 1.11.1.6) activity was measured spectrophotometrically by the method of Beers & Sizer (1952). One unit of activity is defined as the amount of enzyme required to clear 1μmol of H_2O_2/min at 25°C.

Glutathione peroxidase (EC 1.11.1.9) and glutathione reductase (EC 1.6.4.2) activities were measured by the method of Ohrloff *et al.* (1980). One unit of glutathione peroxidase activity is defined as the amount of enzyme required to clear 1μmol of t-butyl hydroperoxide/min (equivalent to 2μmol of NADPH oxidized/min at 25°C). One unit of glutathione reductase activity is defined as the amount of enzyme required to oxidize 1μmol of NADPH/min after the addition of substrate (oxidized glutathione; Sigma) at 25°C.

Susceptibility of cells to oxidative damage in vitro

Age-fractionated washed erythrocytes were incubated for up to 2h at 37°C with a xanthine/xanthine oxidase $O_2^{-\bullet}$-generating system as described by Lynch & Fridovich (1978). Samples (10μl) were removed every 30min and incubated at room temperature for 10min with an equal volume of 0.5% Trypan Blue (Fluka A.G.) in phosphate-buffered saline. The cells were then examined by light microscopy at 400× magnification. Damaged cells were judged as those cells that did not exclude the dye. In a separate experiment 25 units of catalase (Sigma; bovine liver)/ml and/or 30 units of superoxide dismutase (Sigma; bovine blood)/ml were added to the incubation media. In this manner the role of these enzymes in protecting the erythrocytes against the oxidative damage caused by xanthine/xanthine oxidase was determined.

Membrane peroxidation

The amount of lipid peroxide in the membranes of fractionated erythrocyte ghosts, washed free of haemoglobin, was determined by incubating the membranes with 0.75% thiobarbituric acid (Aldrich Chemical Co.) and measuring at 532nm the amount of malonaldehyde produced by the method of Trotta *et al.* (1981).

Since haemaglobin can influence the amount of malonaldehyde produced (Kellogg & Fridovich, 1975), lipid peroxidase was also measured by the procedure of Jain & Hochstein (1980). Fractionated washed ghosts were incubated overnight at 37°C with malonaldehyde produced by the acid hydrolysis of malonaldehyde bis(dimethylacetal) (Aldrich Chemical Co.). The resulting fluorescent chromolipid was extracted with propan-2-ol/chloroform (3:2, v/v). The amount of chromolipid in the organic layer was determined by measuring the fluorescence at 390 ± 2nm caused by excitation at 460 ± 5nm. Positive controls were provided by determining the amount of lipid peroxide in ghosts from young erythrocytes obtained from young animals, which were preincubated in a xanthine/xanthine oxidase $O_2^{-\bullet}$-generating system for 2 and 4h.

Results

Erythrocytes from young and old rats were separated into four age classes by density-gradient centrifugation. The activities of glucose-6-phosphate dehydrogenase, catalase, glutathione reduc-

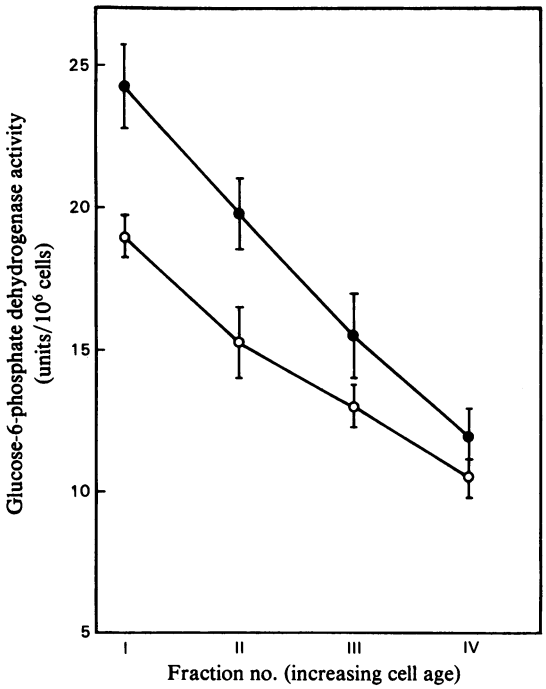

Fig. 1. *Glucose-6-phosphate dehydrogenase activities in various age classes of erythrocytes from young and old rats*
Erythrocytes were separated according to cell age by density-gradient centrifugation and were lysed in 100 vol. of packed cells in 10 mM-potassium phosphate buffer, pH 7.8, containing 20 μM-$NADP^+$ and 1 mM-EDTA. Haemolysates were centrifuged at 12000*g* for 30 min to remove the stroma. Enzyme activity was determined by addition of 15 or 30 μl of haemolysate to a reaction mixture containing 810 μl of 0.055 M-Tris/HCl buffer, pH 7.8, 30 μl of 0.006 M-$NADP^+$ and 30 μl of 0.1 M-glucose 6-phosphate. The conversion of $NADP^+$ into NADPH was measured spectrophotometrically at 340 nm. One unit of enzyme activity is defined as the amount of enzyme required to reduce 1 μmol of $NADP^+$ to NADP/min at 30°C. Activities are presented in units per 10^6 cells for young (●) and old (○) rats.

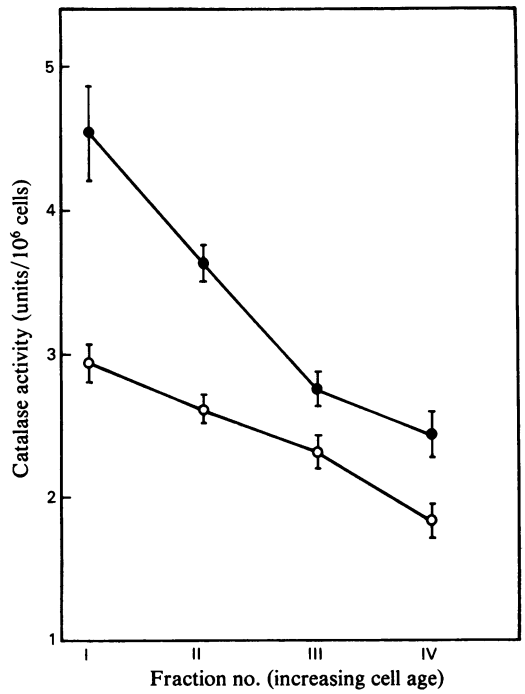

Fig. 2. *Catalase activities in various age classes of erythrocytes from young and old rats*
Erythrocytes were separated according to cell age by density-gradient centrifugation and were lysed in 100 vol. of packed cells of 10 mM-potassium phosphate buffer, pH 7.0. The stroma was removed by centrifugation at 12000*g* for 30 min. Enzyme activity was determined by the addition of 10 μl of haemolysate to 1.0 ml of 50 mM-potassium phosphate buffer, pH 7.0, containing 2 μl of 30% (v/v) H_2O_2. The resulting disappearance of H_2O_2 was measured spectrophotometrically at 240 nm. One unit of activity is defined as the amount of enzyme required to clear 1 μmol of H_2O_2/min at 25°C. Activities are presented in units per 10^6 cells for young (●) and old (○) rats.

tase and glutathione peroxidase were measured for each cell fraction, and are presented in Figs. 1–4 respectively. The activities of all four enzymes decrease with increasing cell age, as previously reported (Bunn, 1972; Fornaini, 1967), and also significant donor age differences exist between cell fractions of the same age. For all the enzymes measured, the activities in young cells from old rats were considerably lower than the activities measured in young cells from young donors, and the activities continued to decline with increasing cell age.

Fractionated erythrocytes from young and old rats were incubated in a xanthine/xanthine oxidase superoxide-generating system to determine their sensitivity to this oxidative species. Samples were removed, incubated with 0.5% Trypan Blue and then examined by light microscopy to determine the number of damaged (stained) cells. The results are given in Fig. 5 of four such experiments. Control cells were incubated with xanthine and buffer without xanthine oxidase. No damaged cells were observed in controls throughout the time course of the experiment. Old cells from old animals are the most sensitive to superoxide damage, and young cells from young animals are the least sensitive. Old cells from young animals and young cells from old animals were somewhat variable in their susceptibility to $O_2^{-\bullet}$ from experiment to experiment, but always demonstrated an intermediate susceptibility, in between that of

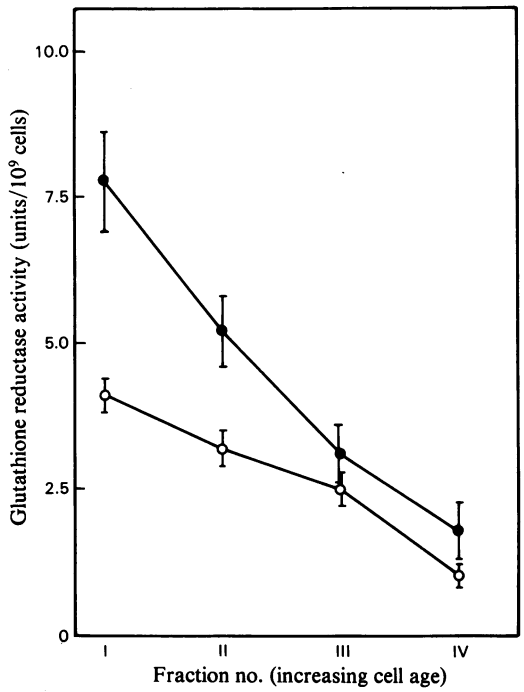

Fig. 3. *Glutathione reductase activities in various age classes of erythrocytes from young and old rats*
Erythrocytes were separated according to cell age by density-gradient centrifugation and were lysed in 100 vol. of packed cells in 10 mM-potassium phosphate buffer, pH 7.4. The stroma was removed by centrifugation at 12000*g* for 30 min. Haemolysates were adjusted to a haemoglobin concentration of 20 g/litre, and enzyme activities were measured by addition of 100 μl of haemolysate to 2.4 ml of 0.1 M-potassium phosphate buffer, pH 7.4, containing 80 mM-EDTA, 0.5% $NaHCO_3$, 4 μM-NADPH and 50 μM-oxidized glutathione. The subsequent oxidation of NADPH to $NADP^+$ was measured spectrophotometrically at 340 nm for a minimum period of 5 min. Control values, obtained by adding 100 μl of haemolysate to the reaction mixture in the absence of oxidized glutathione, were subtracted from the experimental values. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH/min at 25°C. Activities are presented in units per 10^9 cells for young (●) and old (○) rats.

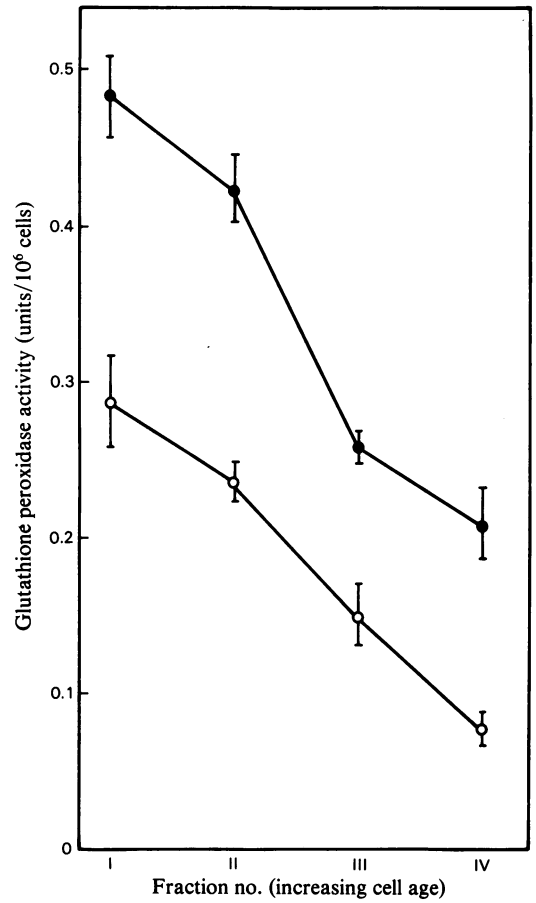

Fig. 4. *Glutathione peroxidase in various age classes of erythrocytes from young and old rats*
Haemolysates were prepared from fractionated erythrocytes as described in Fig. 3. Glutathione peroxidase activity was determined by measuring the oxidation of NADPH to $NADP^+$ at 340 nm after addition of 100 μl of haemolysate to 2.4 ml of 0.1 M-potassium phosphate buffer containing 3 mM-EDTA, 0.33 mM-NADPH, 2 mM-NaN_3, 8 units of glutathione reductase (Sigma; yeast), 8 mM-reduced glutathione and 0.4 mM-t-butyl hydroperoxide. The reaction was monitored for a minimum of 5 min. Control values, obtained by adding 100 μl of lysate to the reaction mixture in the absence of t-butyl hydroperoxide, were subtracted from the experimental values. One unit of enzyme activity is defined as the amount of enzyme required to clear 1 μmol of t-butyl hydroperoxide (equivalent to 2 μmol of NADPH oxidized)/min at 25°C. Activities are presented in units per 10^6 cells for young (●) and old (○) rats.

young cells from young rats and old cells from old rats. Part of the variability from experiment to experiment may be a result of procedures, since some cell preparations were stored at 4°C for periods of up to 3½ h before exposure to the xanthine/xanthine oxidase system. Other preparations were used after much shorter intervals. In all cases, however, the addition of exogenous catalase, but not superoxide dismutase, to the incubation media provided complete protection against oxidative attack in all cell fractions. This indicates that H_2O_2 and not $O_2^{-\bullet}$ is the major species responsible for the cell damage observed in these experiments.

The extents of lipid peroxidation *in vivo* in different erythrocyte age classes from young and old rats are presented in Table 1. The amounts of lipid peroxide per 10^9 cells were measured by deter-

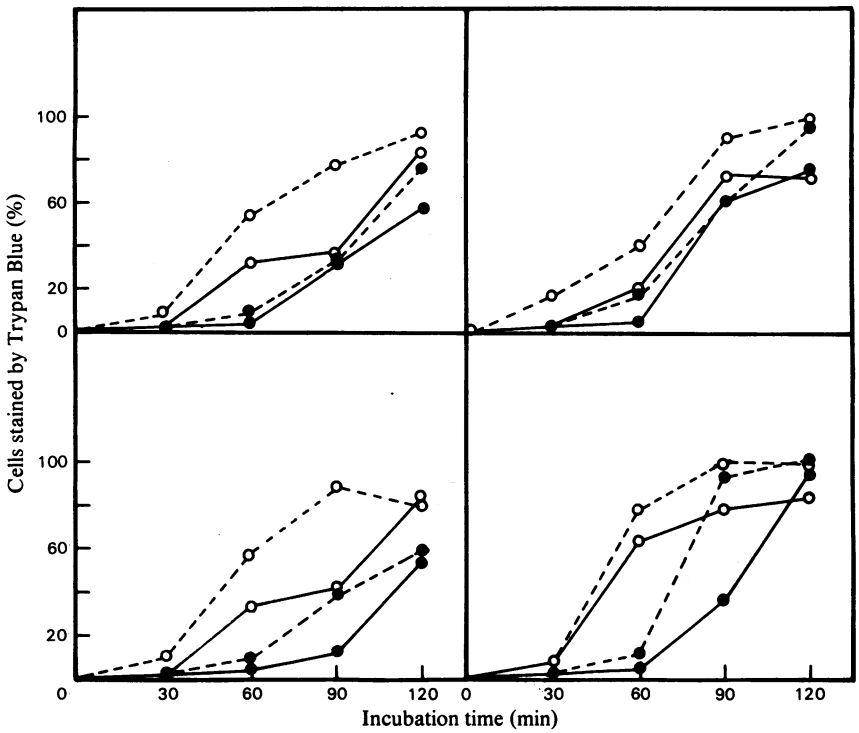

Fig. 5. *Susceptibility of age-separated erythrocytes from young and old rats to oxidative damage in vitro*
The procedure followed was as described in the text. Oxidative damage caused by xanthine/xanthine oxidase-generated $O_2^{-\bullet}$ is shown as percentage of killing of the cells, as judged by their inability to exclude Trypan Blue. Values are presented for the youngest (Fraction I, ●——●) and the oldest (Fraction IV, ●--●) erythrocytes from young rats and the youngest (Fraction I, ○——○) and the oldest (Fraction IV. ○--○) erythrocytes from old rats. Each of the four panels depicts the results of the incubation of fractionated cells from one young and one old rat.

Table 1. *Erythrocyte membrane lipid peroxidation*
Thiobarbituric acid measurements are given in nmol of malonaldehyde per 10^6 cells reacting with thiobarbituric acid. Fluorescence measurements were done by the procedure of Jain & Hochstein (1980) and are given in arbitrary units of fluorescence per 10^6 cells, at 390nm after excitation at 460nm. For details see the Materials and methods section.

Cells	Amounts of malonaldehyde (nmol/10^6 cells)	Chromolipid fluorescence (units)
Fraction I, young animals	10.8±3.2	19.3±3.0
Fraction IV, young animals	32.5±3.2	44.0±3.0
Fraction I, old animals	37.8±1.3	44.2±3.0
Fraction IV, old animals	48.6±2.6	57.8±4.0
Erythrocytes incubated with xanthine/xanthine oxidase for 2 h	21.8±1.3	20.7±0.6
Erythrocytes incubated with xanthine/xanthine oxidase for 4 h	48.7±5.1	55.7±4.0

mining the amount of malonaldehyde produced by the incubation of washed ghosts with thiobarbituric acid and by measuring the amount of fluorescent chromolipid produced by the reaction of washed ghosts with malonaldehyde. As the presence of haemoglobin can affect the production of malonaldehyde, the cell membranes were disrupted by sonication and washed in 20-fold excess of lysing buffer at least three times before incubation with thiobarbituric acid. In addition, the production of chromolipid after incubation of the membranes with malonaldehyde, which is unaffected by haemoglobin (Jain & Hochstein, 1980), correlated well with results obtained from the thiobarbituric acid determinations. Both measurements show that the amount of lipid peroxidation increases with both cell and donor aging. Positive controls consisted of young cells from young donors, which were incubated for 2 or 4 h with xanthine/xanthine oxidase. After incubation, the cells were lysed, ghosts prepared, and the degree of lipid peroxidation was determined. These controls show increases in both malonaldehyde and fluorescent chromolipid with respect to incubation time.

Ghosts from cells incubated for up to 4h with only xanthine showed no increase in either malonaldehyde or fluorescent chromolipid.

Discussion

Although numerous researchers have observed biochemical changes in erythrocytes with increasing cell age (Fornaini, 1967; Bunn, 1972), most have either not detected or not looked for effects of donor age on the erythrocyte. The possible reasons for the inability of previous studies to find donor-age-related differences in erythrocytes have been discussed in some detail by Glass & Gershon (1981). Abraham *et al.* (1978), however, have described significant decreases in the contents of total glutathione and reduced glutathione and in glutathione reductase activity in erythrocytes from C57/Bl mice of 30 months of age and older. In addition, Glass & Gershon (1981) have shown that there is a decrease in the activity of superoxide dismutase with respect to both cell and donor age in erythrocytes from WF rats and that the amounts of measurable haemoglobin decrease with respect to cell age in the old rats, but not in young animals. Both groups noted that the half-life of erythrocytes in the old animals was markedly shortened from that in the young animals. Other authors have described differences with respect to donor age in the lipid content and the susceptibility of erythrocytes to osmotic shock (Detranglia *et al.*, 1974).

In the present investigation, it is shown that the specific activity per cell of those enzymes responsible for protecting the erythrocyte against oxidative and peroxidative damage, such as catalase, glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione peroxidase, decline as a function of cell aging *in vivo*. More importantly, the activities of these enzymes are lower in erythrocytes of old WF rats when compared with the same age-class cells from young donors. Thus even young cells from an old animal contain lower activities of these enzymes, in some cases similar to those of the oldest cells from young donors.

Although the full effects of the lower activities of these enzymes in old cells from young rats and all ages of cells from senescent animals are not fully known at present, our work suggests that the lowered activities correlate with increased sensitivity of the cells to peroxidative damage. This conclusion is based on our observation that old erythrocytes from old donors are much more susceptible to oxidative damage *in vitro* than are young cells from young donors. Young cells from old donors and old cells from young donors demonstrated intermediate susceptibility to oxidative attack *in vitro* produced by incubation with xanthine/xanthine oxidase. Our data also indicate that the membranes of circulating erythrocytes contain increasing amounts of lipid peroxides as a function of cell age and that the membranes of young erythrocytes from old donors contain as much lipid peroxide as the membranes of senescent erythrocytes in young animals. Glass & Gershon (1981) have reported that the amount of haemoglobin measured as cyanomethaemoglobin declines with erythrocyte aging in old rats. It is possible that peroxidative damage to haemoglobin, brought about by diminished cellular protection against this damage, may result in partial denaturation of the globin molecule and the subsequent loss of the haem moiety (Jacob, 1974) in the aging cells from old rats. No significant loss of globin is observed.

It has been shown that the modification of an existing outer-membrane protein (band 3), and its subsequent recognition and binding by normally circulating immunoglobulin-G autoantibodies, are responsible for the sequestration by phagocytosis of senescent erythrocytes (Kay *et al.*, 1983). Glass *et al.* (1983) have shown that such an antigen is found on the cell surface of all age fractions in old rats, but primarily on the senescent cell fractions in young animals. It is possible that increased oxidative and peroxidative damage to the membrane and membrane components of erythrocytes from old rats may contribute to this premature exposure of surface antigen. This process may play a role in the early sequestration of erythrocytes in old animals. Experiments are required to determine the extent to which oxidative and peroxidative damage contributes to this premature modification of surface antigen, rendering it accessible to antibody and resulting in the early removal of the erythrocyte from circulation.

This work was supported by U.S. Public Health Service Grant 5 R01 AG-00459.

References

Abraham, E. C., Taylor, T. F. & Lang, C. A. (1978) *Biochem. J.* **174**, 819–825

Beers, R. F. & Sizer, I. W. (1952) *J. Biol. Chem.* **195**, 133–140

Borun, E. R., Figueroa, W. G. & Perry, S. M. (1957) *J. Clin. Invest.* **36**, 676–679

Bunn, H. F. (1972) *Semin. Hematol.* **9**, 3–17

Chance, B., Sies, H. & Boveris, A. (1979) *Physiol. Rev.* **59**, 527–605

Detranglia, M., Cook, F. B., Stasiw, D. M. & Corney, L. C. (1974) *Biochim. Biophys. Acta* **345**, 213–219

Fornaini, G. (1967) *Ital. J. Biochem.* **16**, 258–330

Glass, G. A. (1983) D.Sc. Thesis, Israel Institute of Technology

Glass, G. A. & Gershon, D. (1981) *Biochem. Biophys. Res. Commun.* **103**, 1245–1253

Glass, G. A., Gershon, H. & Gershon, D. (1983) *Exp. Hematol.* in the press

Jacob, H. S. (1974) in *The Red Blood Cell*, 2nd edn. (Surgenor, D. MacN., ed.), vol. 1, pp. 387–435, Academic Press, New York

Jain, S. K. & Hochstein, P. (1980) *Biochem. Biophys. Res. Commun.* **92**, 247–254

Kahn, A. & Dreyfus, J. C. (1974) *Biochim. Biophys. Acta* **334**, 257–265

Kay, M. M. B., Goodman, S. R., Sorensen, K., Whitfield, C. F., Wong, P., Zaki, L. & Rudloff, V. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1631–1633

Kellogg, E. W., III & Fridovich, I. (1975) *J. Biol. Chem.* **250**, 8812–8817

Lynch, R. E. & Fridovich, I. (1978) *J. Biol. Chem.* **253**, 1838–1845

Ohrloff, C., Lange, G. & Hockwin, O. (1980) *Mech. Ageing Dev.* **14**, 453–458

Rifkind, R. A., Bank, A. & Marks, P. A. (1974) in *The Red Blood Cell*, 2nd edn. (Surgenor, D. MacN., ed.), vol. 1, pp. 387–435, Academic Press, New York

Trotta, R. J., Sullivan, S. G. & Stern, A. (1981) *Biochim. Biophys. Acta* **678**, 230–237