The present experiments were undertaken to show the presence of the Ca-binding sites in *Paramecium* by the use of a fixative which did not contain Ca ions, i.e. the pyroantimonate fixation method.

Specimens of *P. caudatum* grown in hay infusion were washed with the adaptation solution, 2 mM $CaCl_2$, 1 mM Tris-HCl pH 7.2, and were adapted for more than 30 minutes before fixation. They were fixed for one hour at room temperature in 1% OsO_4 solution, pH 6.8 adjusted by 0.01 N acetic acid, containing 2% potassium pyroantimonate ($K_2H_2Sb_2O_7 \cdot 4H_2O$). In fixation, special care had to be taken to make the volume of the adaptation solution as small as possible, 0.02 ml or less, and a large volume of fixative, 10 ml or more, was added to the adaptation solution which contained the specimen, otherwise no deposits could be observed within the cell. The specimens were dehydrated in ethanol, and embedded in epon 812. Ultrathin sections, cut on a Porter-Blum MT 2 microtome, unstained or stained weakly with uranyl acetate and lead citrate, were examined with a Hitachi HS-9 electron microscope. Carbon was evaporated on the section for X-ray microanalysis, which was made with an energy dispersive X-ray microanalyzer (EDAX 707B) attached to a Hitachi HHS-2R scanning electron microscope.

Highly electron-dense deposits were found on the inner surface of the ciliary membrane above the axosome, in almost all the cilia, and this region is the same one as observed in the glutaraldehyde method[6]. However, there were some differences in results between the 2 methods. In the present method, a lot of irregular shaped deposits were observed in the extracellular space (fig. 1). Intracellular deposits at the bases of cilia appeared nearly round and their diameter was commonly 30–50 nm, which was similar to their size when observed using the glutaraldehyde method. No deposits were present in the cilia of the specimens treated with a Ca-free solution before fixation. Since pyroantimonate is known to produce electron-opaque precipitates not only with Ca but also with other cations, the presence of Ca in the deposit was examined by means of the electron probe X-ray microanalysis. A typical result of the spot analysis performed on the deposit at the bases of the cilia is shown in figure 2. The X-ray spectrum shows a distinct peak at 3620-40 eV, which is a combination of Sb-Lα emission (3600 eV) and Ca-Kα emission (3690 eV). This fact indicates that the deposit contains Ca[9,10]. The peak of Sb-Lβ, emission (3840 eV) is also seen.

In view of the fact that the intracellular deposits could be observed only when a large volume of the fixative was used, it is considered that most of the extracellular Ca ions in the adaptation solution combined with $H_2Sb_2O_7^{--}$ to form $CaH_2Sb_2O_7$ which could not penetrate the cell membrane, and only $H_2Sb_2O_7^{--}$ could penetrate and bind to intracellular Ca.

Although the role of these binding sites is not clear, the localization of the deposits just above the ciliary necklace[11] linking the ciliary membrane and the outer doublet microtubules suggests the important role of these binding sites in the control of the direction of ciliary beating. Recent results show that Ca ions do not change the direction and the velocity of the sliding of the microtubules obtained from *Paramecium* cilia[12]. This fact indicates that Ca ions do not act directly on dynein but on other structures which are destroyed by the trypsin treatment. Therefore, what we have to find is the sites of action of Ca ions in a cilium and after that the function of the Ca-binding sites, shown in the present and preceding work[6,7], will be clear.

1 Y. Naitoh and H. Kaneko, Science *176*, 523 (1972).
2 T. Tsuchiya, Experientia *32*, 1439 (1976).
3 T. Tsuchiya, Comp. Biochem. Physiol. *56A*, 353 (1977).
4 A. Ogura and K. Takahashi, Nature *264*, 170 (1976).
5 Y. Naitoh, J. gen. Physiol. *51*, 85 (1968).
6 T. Tsuchiya and K. Takahashi, J. Protozool. *23*, 523 (1976).
7 H. Plattner, J. Cell Sci. *18*, 257 (1975).
8 T. Tsuchiya, Experientia *32*, 1176 (1976).
9 V. Mizuhira, Acta histochem. cytochem. *9*, 69 (1976).
10 S. Suzuki and H. Sugi, J. Cell Biol. *79*, 467 (1978).
11 B. Satir, C. Schooley and P. Satir, Acta protozool. *11*, 291 (1972).
12 Y. Mogami and K. Takahashi, Zool. Mag. *88*, 513 (1979).

Aging of the erythrocyte. II. Activities of peroxide-detoxifying enzymes

G. Bartosz and A. Bartkowiak[1]

Department of Biophysics, Institute of Biochemistry and Biophysics, University of Łódź, 90–237 Łódź (Poland), 28 April 1980

Summary. The activity of glutathione peroxidase decreases and that of catalase does not change during erythrocyte aging, as judged from a comparison of bovine red cell fractions separated by density.

A general phenomenon associated with red cell aging in vivo is a decrease in the activities of a majority of erythrocyte enzymes[2–4]. In some cases these changes seem to be of diagnostic value only but others may have serious consequences for the further functioning of the cell[3,5]. Alterations in the activities of defensive enzymes of oxygen metabolism might belong to the 2nd group, as these enzymes seem to be indispensable for the protection of the cell against deleterious reactions of active oxygen intermediates[6]. We have demonstrated that erythrocyte aging involves a decrease in superoxide dismutase activity[7]. The present report deals with the behavior of 2 other enzymes of this protective pathway; catalase (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, EC 1.11.1.6) and glutathione peroxidase (glutathione: hydrogen-peroxide oxidoreductase, EC 1.11.1.9), during the aging of bovine erythrocyte.

Material and methods. Bovine erythrocytes were separated according to age by the method of Murphy[8]. The ultracentrifuged suspension was separated into 6 equal-volume fractions. Glutathione peroxidase activity was estimated according to Sinet et al.[9]. Catalase activity was determined by the method of Beers and Sizer[10]. Hemoglobin was assayed according to Drabkin[11]. Erythrocyte density was estimated by the dropping time method[12].

Results and discussion. Mean density of erythrocytes in successive fractions withdrawn from the top of centrifuge tubes increased progressively from 1.081 (fraction 1) to 1.090 (fraction 6) on the average. The density of bovine red cells has been shown to correlate with their physiological age[13]. Therefore separation by density apparently produces fractionation of erythrocytes according to age. This conclusion is confirmed by distribution of ^{59}Fe-labeled cells of different age in the stratified column of bovine red cells

separated by the method of Murphy[8] (to be published). No reticulocytes were found in any fraction, in agreement with the previous report on the absence of reticulocytes from the blood of adult cows[13].

Mean activities of glutathione peroxidase and catalase in the fraction of lightest (youngest) erythrocytes were 68.0±26.4 e.u./mg hemoglobin (n=8) and 13.5±3.5 Bergmeyer units/mg hemoglobin (n=7), respectively. When comparing enzyme activities in cell fractions of various ages, activity in the lightest fraction was assumed as 100% in each separation to eliminate differences in absolute values between different animals. Results shown in the table indicate a decrease in the activity of glutathione peroxidase and no definite changes in the activity of catalase during erythrocyte aging.

Although the catalase activity was reported to decrease in older human erythrocytes[14,15], the more recent study of Sass et al.[4] did not confirm this, revealing even a slight negative correlation between the activity of this enzyme and the activity of aspartate aminotransferase, an enzyme especially sensitive to erythrocyte age. The present data on bovine red blood cells would be in line with those of Sass et al.[4], demonstrating no significant decrease in the activity of catalase during erythrocyte aging in vivo. Results of the present study indicate a diminution in the capacity of aging red cells for dealing with endogenously produced and exogenous hydrogen peroxide; the more so because glutathione peroxidase has been demonstrated to have a lower K_m for H_2O_2 than catalase and to be more relevant in protection against physiological concentrations of this agent[16]. The results offer at least a partial explanation for the increased sensitivity of aging erythrocytes to oxidative stress mediated by hydrogen peroxide[17].

Glutathione peroxidase and catalase activities in different age fractions of bovine erythrocytes

Fraction No.	Relative activity (%) Glutathione peroxidase Mean	SD	Catalase Mean	SD
1	100		100	
2	92.5	6.5	110.1	13.4
3	87.7	8.1	106.9	15.3
4	88.6	7.0	107.5	8.4
5	79.4	9.3	98.1	16.3
6	77.8	9.7	102.2	11.3

Activities (per mg hemoglobin in hemolysates) are expressed as percent of activities found in the youngest cell fraction. Combined data from measurements on blood from 8 and 7 animals, respectively.

1 We are indebted to Prof. W. Leyko for many stimulating discussions.

2 A.M. Ganzoni, J.P. Barras and H.R. Marti, Vox sang. *30*, 161 (1976).
3 R.G. Chapman and L. Schaumburg, Br. J. Haemat. *13*, 665 (1967).
4 M.D. Sass, E. Vorsanger and P.W. Spear, Clin. chim. Acta *10*, 21 (1964).
5 R.E. Bernstein, J. clin. Invest. *38*, 1572 (1959).
6 R.W. Carrell, C.C. Winterbourn and E.A. Rachmilewitz, Br. J. Haemat. *30*, 259 (1975).
7 G. Bartosz, Ch. Tannert, R. Fried and W. Leyko, Experientia *34*, 1464 (1978).
8 J.R. Murphy, J. Lab. clin. Med. *82*, 334 (1973).
9 P.M. Sinet, A.M. Michelson, A. Bazin, J. Lejeune and H. Jerome, Biochem. biophys. Res. Commun. *67*, 910 (1975).
10 R.F. Beers and I. Sizer, J. biol. Chem. *159*, 133 (1952).
11 W.G. Zijlstra and E. van Kampen, Clin. chim. Acta *5*, 719 (1960).
12 K. Narozny, in: Practical course in biophysics and physics (in Polish), p. 155. Ed. B. Kedzia.
13 Y. Israel, A. MacDonald, J. Bernstein and E. Rosenmann, J. gen. Physiol. *59*, 270 (1972).
14 A.C. Allison and G.P. Burn, Br. J. Haemat. *1*, 291 (1955).
15 T.A.J. Prankerd, Folia haemat. *78*, 382 (1961).
16 B. Halliwell, New Phytol. *73*, 1075 (1974).
17 G. Bartosz, Acta biol. med. germ., in press (1981).

Aging of the erythrocyte. III. Cation content

G. Bartosz, B. Świerczyński and R. Gondko

Department of Biophysics and Laboratory of Biological Sciences, University of Łódź, PL-90-237 Łódź (Poland), 28 April 1980

Summary. Studies on the main cation content of density-separated bovine erythrocytes showed a progressive decrease in the levels of K^+ and Mg^{2+} with increasing cell density (and age) accompanied by an increase in the level of Na^+. The magnitude of net cation loss corresponded to that of red cell volume decrease, but could not account for the total increase in the microviscosity of the erythrocyte interior.

Changes in intracellular cation concentrations are a known characteristic of in vivo erythrocyte aging in several mammalian species[1-6]. These changes result in a net cation loss and this could be the main mechanism responsible for the shrinking of senescent red blood cells. In a previous report[7] we demonstrated that the increase in density accompanying the intravascular aging of bovine erythrocytes is related to an increase in the microviscosity of their interior. The aim of this study was to examine whether the red cell microviscosity increase during in vivo aging can be accounted for by electrolyte loss. With this goal in mind, cell density- (and age-)related alterations in main cellular cations were measured in bovine erythrocytes.

Methods. Separation of erythrocytes according to density (and age), and estimation of hemoglobin, were carried out as described in the accompanying paper. Sodium and potassium were estimated by atomic emission photometry, and magnesium by atomic absorption photometry in an AAS-2 atomic absorption spectrophotometer (Karl Zeiss, GDR).

Results and discussion. As in the case of human red cells[8], the method of Murphy[9] permits an excellent separation of bovine erythrocytes according to density and a reasonable separation according to age (to be published). Although it is hardly possible to extract exact quantitative data on rates of in vivo change from studies of various fractions of red