# Oxidative Damage to the Membrane of Canine Erythrocytes with Inherited High Na, K-ATPase Activity

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(Received 26 June 1991/Accepted 14 October 1991)

ABSTRACT. Oxidative damage to the membrane in canine erythrocytes with inherited high Na, K-ATPase activity (HK cells) was compared with that in normal canine cells (LK cells). When 30 mM β-acetylphenylhydrazine (APH) was applied to HK and LK cells, lipid peroxidation and hemoglobin denaturation occurred. Lipid peroxidation determined from malondialdehyde (MDA) formation was significantly lower in HK than in LK cells so far as endogenous glutathione (GSH) concentration was maintained at appropriate levels. With the depletion of GSH, MDA formation was accelerated and difference between HK and LK cells was not significant. Denatured hemoglobin bound to the membrane protein was less in HK than in LK cells. During incubation with APH, osmotic fragility increased markedly in LK cells, while HK cells showed very little change. The amounts of total lipid, total and free cholesterol, glycolipid, phospholipid and fatty acids were essentially the same in both cell types. Fatty acid compositions showed very small differences. The membrane of HK cells thus appear to have greater protection against oxidative damage induced by APH, owing to the presence of excess GSH in HK cells. The capability of HK cells to withstand oxidative damage would not be due to differences in membrane lipid compositions.—KEY WORDS: canine HK cell, glutathione, lipid peroxidation, malondialdehyde, β-acetylphenylhydrazine.

*J. Vet. Med. Sci.* 54(1): 57–62, 1992

Hemoglobin of canine erythrocytes with inherited high Na, K-ATPase activity (HK cells) was previously shown to be more resistant against oxidative damage induced by sodium nitrite [18]. In HK cells, nitrite-induced methemoglobin formation occurred more slowly and its reduction in a glucose medium occurred more rapidly than in normal canine cells (LK cells), owing to excess amount of reduced glutathione (GSH) and increased glycolytic rate. Of various oxidizing agents, sodium nitrite and β-acetylphenylhydrazine (APH) have been studied to clarify the mechanisms for their action on erythrocytes [5, 26, 27]. Both generate superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) which cause methemoglobin formation and peroxidative damage to cell lipids unless sufficiently decomposed by defence systems in red blood cells. The extent of lipid peroxidation may depend on the efficiency of defence systems as well as membrane lipid composition. The present study was conducted to examine oxidative damage to the membrane with APH and biochemical properties of membrane lipids in HK cells.

## MATERIALS AND METHODS

Erythrocytes were obtained from three mongrel dogs of one family possessing HK cells, and three mongrel dogs with normal LK cells as control. Venous blood was drawn into heparinized syringes, centrifuged at 1,500 × g for 10 min and the plasma and buffy coat were removed by aspiration. The red blood cells were then washed three times at 4°C with 0.9% NaCl solution. The packed cells obtained were resuspended in a medium consisting of 135 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 2 mM $Na_2HPO_4$, 20 mM Hepes/Tris (pH 7.4), 10 mM inosine, 10 mM lithium pyruvate with or without 10 mM glucose, and hematocrit value was adjusted to about 50%.

*Incubation of erythrocytes with APH*: The red cell suspensions were diluted to 10% hematocrit with the above medium and incubated with or without APH in a shaker water bath at 37°C. Aliquots of each suspension were withdrawn for analysis at specified times. Lipid peroxidation was determined from thiobarbituric acid (TBA) reactant malondialdehyde (MDA) content according to the method of Yagi [29] modified by Yoshimitu [30]. Reduced glutathione (GSH) was assayed as described by Beutler *et al.* [2]. Osmotic fragility test was conducted according to Parpart *et al.* [21]. Denatured hemoglobin bound to the membrane was estimated from absorption spectrum of hemoglobin in the washed ghost. Two m*l* of a 50% red cell suspension

was hemolyzed with 28 m*l* of 5 mM potassium phosphate buffer (pH 7.4). The ghost was sedimented by centrifugation at 12,000 × g for 10 min at 4°C, and was washed with the same buffer until the supernatant became colorless. The washed ghost was dissolved in 5% SDS solution and transferred to a 50 m*l* volumetric flask. The absorption spectrum of the solution was measured between 250 and 700 nm with a double beam spectrophotometer (Hitachi model 220) following appropriate dilution.

*Analysis of lipid composition*: Two m*l* of packed cells was suspended in an equal volume of 0.9% NaCl solution and lipid was extracted with 120 m*l* of chloroform-methanol (2:1, v/v) according to the method of Folch *et al.* [4]. The supernatant solution was evaporated to dryness under reduced pressure and the residue transferred to a glass-stopped graduated tube with 4 m*l* of chloroform and stored at −20°C. Total phospholipid was determined by the method of Bartlett [1] and total and free cholesterol, by the method of Zak [31]. Total sugar from glycolipid was assayed by an anthrone procedure [22]. The phospholipid class composition was determined by silica gel thin layer chromatography according to Skipski *et al.* [23]. Free fatty acids were obtained by refluxing the lipid extract with 5% KOH in propyleneglycol for 30 min, and total fatty acid content was assayed with NEFA Test Wako kit (Wako Pure Chemicals Ltd.). Fatty acid methyl esters were obtained from the lipid extract by transmethylation with sodium methoxide, and fatty acid composition was analyzed with gas-liquid chromatography using a column packed with 10% diethyleneglycol succinate on chromosorb WAW (80–100 mesh) [24, 25].

## RESULTS

When 30 mM APH was applied to HK and LK cells in the absence of glucose, GSH gradually decreased to about 45% of the initial value in HK cells and 30% in LK cells at 2 hr, and it had become virtually depleted at 4 hr (Fig. 1). MDA formation, which represents lipid peroxidation in the membrane, increased markedly during incubation with APH (Fig. 2A). Without APH, MDA formation in either cells remained constant at the basal or initial value throughout a 6 hr period, and basal MDA was noted to be higher in LK than in HK cells. Net increase in MDA from the basal value was calculated and shown in Fig. 2B. It was significantly higher in LK than in HK cells at 2 and 4 hr, but the difference was not significant at 6 hr. In HK cells, MDA formation was accelerated from 4 to 6 hr with GSH being essentially depleted. During incubation

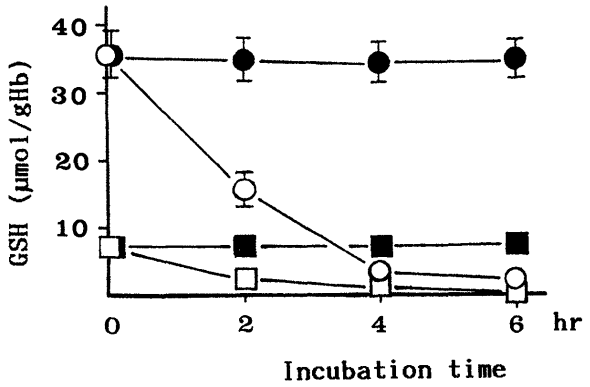


Fig. 1. Change of GSH concentration in HK cells (○) and LK cells (□) incubated with APH in the absence of glucose. Filled symbols indicate control experiments without APH in the medium. Data represent the mean ± SD of 3 animals.

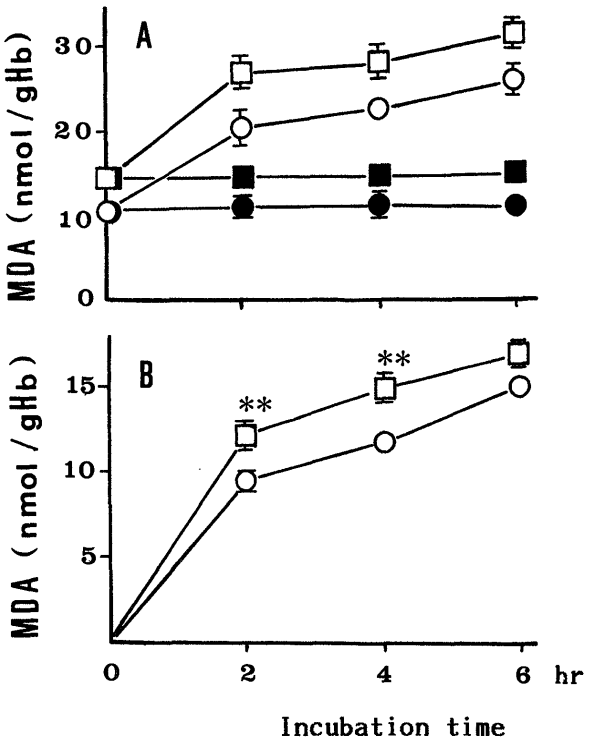


Fig. 2. (A) Change of MDA concentration in HK cells (○) and LK cells (□) incubated with APH in the absence of glucose. Filled symbols indicate control experiments without APH in the medium. Data are the mean ± SD of 3 animals. (B) Net increase in MDA concentration from initial values in HK cells (○) and LK cells (□). Values were calculated from data A. Significant difference between groups is indicated as **P<0.01. (Student's *t* test)

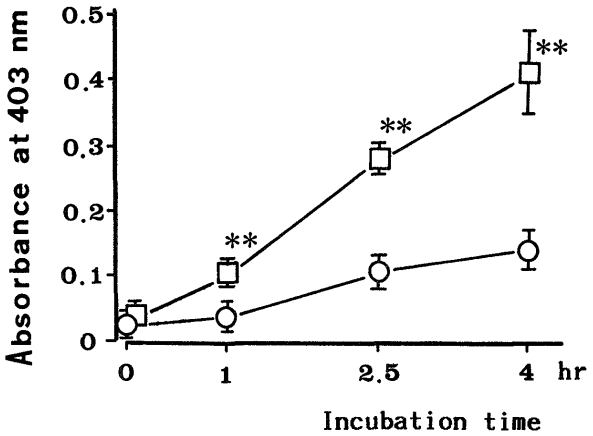


Fig. 3. Denatured hemoglobin in the membrane of HK cells (○) and LK cells (□) incubated with APH in the absence of glucose. Data are the mean ± SD of 3 animals. Significant difference between groups is indicated as **P<0.01 (Student's *t* test).

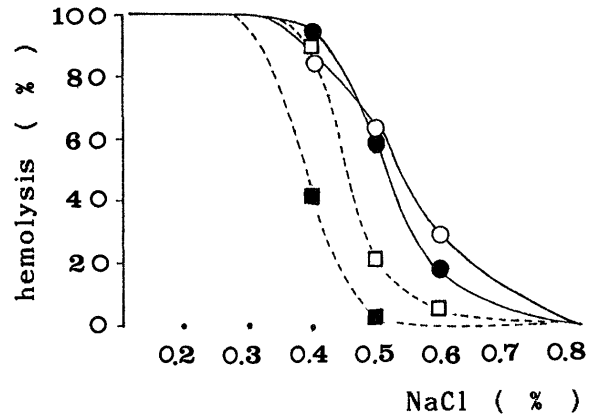


Fig. 4. Osmotic fragility curves before and after incubation with APH in the absence of glucose. ●, HK cells and ■, LK cells before incubation; ○, HK cells and □, LK cells after incubation. Each point represents the mean of 3 separate experiments.

with APH, there was a marked increase in denatured hemoglobin bound to the membrane, as indicated by elevated peak absorbance at 403 nm (Fig. 3). Denatured hemoglobin in LK cells increased more rapidly than in HK cells, and the difference increased further with time. Figure 4 shows osmotic fragility curves of HK and LK cells before and after 6 hr-incubation with APH in the absence of glucose. Before incubation with APH, HK cells underwent hemolysis to a greater extent in hypoosmotic NaCl solution than LK cells. At 6 hr, however, the osmotic fragility curve of HK cells had hardly deviated from its initial locus while that of LK cells markedly shifted from initial locus to a

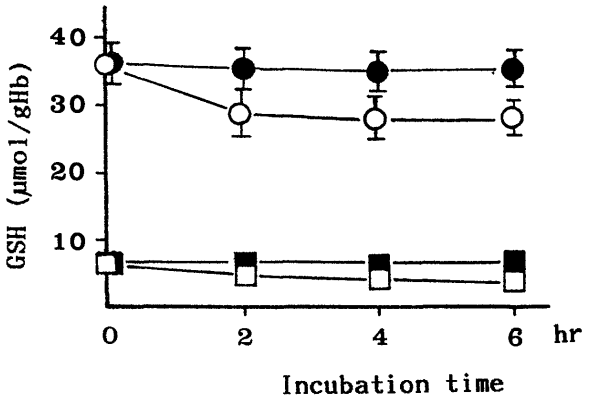


Fig. 5. Change of GSH concentration in HK cells (○) and LK cells (□) incubated with APH in the presence of glucose. Filled symbols indicate control experiments without APH in the medium. Data are the mean ± SD of 3 animals.

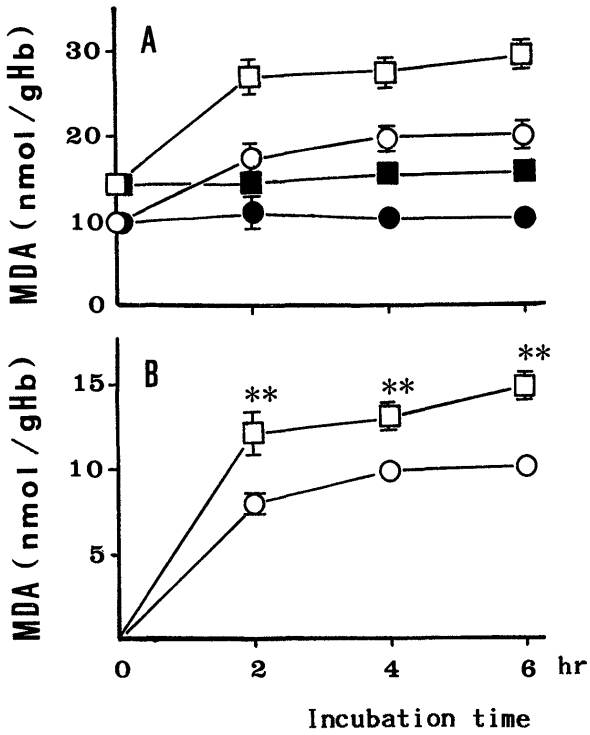


Fig. 6. (A) Change of MDA concentration in HK cells (○) and LK cells (□) incubated with APH in the presence of glucose. Filled symbols indicate control experiments without APH in the medium. Data are the mean ± SD of 3 animals. (B) Net increase in MDA from initial values in HK cells (○) and LK cells (□). Values were calculated from data A. Significant difference between groups is indicated as **P<0.01. (Student's *t* test)

higher NaCl concentration. The effect of APH is thus shown to be less in HK cells than in LK cells. When HK and LK cells were incubated with APH in the presence of glucose, GSH in both cells decreased 20% of initial values at 2 hr, and no further

change was observed thereafter (Fig. 5). MDA formations in HK and LK cells incubated with APH in the presence of glucose were less than those in the absence of glucose (Fig. 6A). Net increase in MDA from basal or initial value in HK cells was significantly less than that in LK cells throughout a 6 hr period (Fig. 6B).

To determine whether lipid structures differ in HK and LK cells, and if such differences are responsible for those in the extent of lipid peroxidation, total lipid, total phospholipid, total and free cholesterol, total sugar from glycolipid, and total fatty acid were determined (Table 1). Also, the relative amounts of phospholipid subclasses were determined, and fatty acid compositions analyzed. The results obtained for the two cell types were essentially the same, except HK cells contained more saturated C-16 fatty acid and less C-18 fatty acid. The total amount of fatty acid and unsaturated carbon chains per cell were essentially the same for both cells.

## DISCUSSION

The data presented above clearly indicate that the membrane of HK cells is more resistant against oxidative damage induced by APH. Lipid peroxidation in HK cells was significantly lower, and denatured hemoglobin and osmotic fragility increased more slowly in HK than in LK cells. These results are consistent with Maede's report showing that Heinz body count and turbidity of hemolysate are appreciably lower in HK than in LK cells when red cells of both types are incubated with 30 mM APH [13]. APH is a well-known reagent that generates superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) in erythrocytes [5], and accumulation of these activated oxygens can directly produce lipid peroxidation of the membrane but will also cause hemoglobin denaturation [3]. Since denatured hemoglobin is believed to become attached to the SH group of the membrane protein and thereby impairs cell deformability and osmotic resistance, hemoglobin denaturation is considered to be another important process of oxidative damage to the erythrocyte membrane leading to hemolysis. To protect the membrane from oxidative damages, $O_2^-$ is reduced to $H_2O_2$ by superoxide dismutase (SOD), and $H_2O_2$ to $H_2O$ by catalase and GSH [14]. Reduced glutathione is essential to the reduction of $H_2O_2$ and lipid peroxides in membranes through its oxidation to GSSG by glutathione peroxidase (GPx) functioning as a catalyst [7]. HK and LK cells were previously shown to have about the same activities in SOD, catalase and GPx, and GSH content in HK cells to be 5 times that in LK cells [18]. The hemoglobin of HK cells withstands oxidation induced by sodium nitrite. HK cells possess greater ability of reducing methemoglobin back to oxyhemoglobin owing to GSH presence in great excess and increase in glycolysis [10, 18]. Similar results were obtained for lipid peroxidation and hemoglobin denaturation induced by APH in this study. Oxidative damage induced by APH was more

Table 1. Lipid distribution in the membranes of HK and LK cells. Values are the mean±SD of 3 animals. Significant difference between groups is indicated as *P<0.05 (Student's *t* test)

| | HK cells | LK cells |
|---|---|---|
| Total lipid ($\times 10^{-13}$g/cell) | 4.96±0.22 | 5.02±0.23 |
| Total cholesterol | 1.10±0.04 | 1.10±0.05 |
| Free cholesterol | 0.82±0.02 | 0.84±0.03 |
| Total sugar from glycolipid | 0.22±0.02 | 0.21±0.01 |
| Total phospholipid | 2.48±0.06 | 2.51±0.07 |
| Phospholipid composition (%) | | |
| phosphatidyl ethanolamine | 26.8±0.5 | 27.6±1.5 |
| phosphatidyl serine | 16.4±0.7 | 14.9±1.2 |
| phosphatidyl choline | 44.5±1.0 | 45.4±1.7 |
| Sphingomyelin | 12.6±0.7 | 12.2±0.9 |
| Total fatty acid ($\times 10^{-13}$Eq/cell) | 1.00±0.12 | 1.02±0.03 |
| Fatty acid composition (%) | | |
| 16: 0 | 23.2±1.6* | 19.3±2.3 |
| 18: 0 | 23.4±2.0 | 24.7±1.9 |
| 18: 1 | 12.9±1.0 | 13.7±0.8 |
| 18: 2 | 11.6±1.5 | 12.7±1.6 |
| 20: 4 | 29.0±2.4 | 29.7±1.8 |

prominent in LK than in HK cells. Lipid peroxidation in both HK and LK cells incubated with APH in the absence of glucose was more marked than that in the presence of glucose. Glutathione was rapidly consumed and depleted in the absence of glucose, while decreased only 20% in the presence of glucose. Glucose is essential to the regeneration of GSH since glucose metabolism provides NADPH required for reducing GSSG to GSH [9, 15]. HK cells quite likely have greater protection from oxidative damage induced by APH owing to excess GSH in HK cells.

Physiological properties of red cells from HK and LK dogs differ in several respects, and these are related to differences in ion transport across the membrane. HK cells possess high Na, K-ATPase activity, and the presence of the enzyme leads to abnormalities of these cells, such as enhanced amino acid uptake and high GSH concentration [8, 11]. Moreover, HK cells have a tendency to hemolyze and cell volume of HK cells is greater than that of LK cells [12, 17]. LK cells have the Na-Ca exchange and Na-H exchange transporters instead of Na, K pump, both of which are involved in the regulation of cell volume [19, 20]. In HK cells, Cl-dependent K transport may act as a volume regulator when the cells are swollen [6]. These differences in the membrane functions may possibly arise from differences in the membrane structures. To determine whether lipid structure differ in HK and LK cells, an examination was made of the lipid patterns of HK and LK cells. They were found essentially the same except for only a slight difference in fatty acid compositions. Fatty acids in the membrane of erythrocytes mainly constitute phospholipids [16]. Lipid peroxidation occurs in unsaturated fatty acids, and susceptibility to oxidation is related to the degree of unsaturation. Of five fatty acids found in the membrane of canine erythrocytes in this study, unsaturated fatty acids were oleic acid (18:1), linoleic acid (18:2), and arachidonic acid (20:4). Composition of these unsaturated acids were similar in HK and LK cells. Peroxidizability index (PI) calculated from the equation PI = monoenoic % $\times$ 0.025 + dienoic % $\times$ 1 + trienoic % $\times$ 2 + tetraenoic % $\times$ 4 [28] were 127.9 $\pm$ 11.1 in HK cells and 131.8 $\pm$ 8.8 in LK cells, and they were not significantly different. Thus, lipid patterns of HK and LK cells would hardly account for differences in the extent of lipid peroxidation with APH in the two cell types.

It should be pointed out that they have almost the same amounts of total lipid, total phospholipid and total and free cholesterol even with appreciable differences in cell volume (MCV, 91.5 and 71.5 fl in HK and LK cells, respectively). Hematological studies of various mammals show erythrocyte size to vary considerably according to species, and total lipid, total phospholipid and cholesterol contents to increase with cell volume [16]. HK cells should thus have smaller amount of membrane lipids in consideration of their size. Small amount of lipids may thus be reasonably expected to form loosely arranged lipid bilayers and this should be at least one reason why HK cells hemolyze more easily than LK cells.

REFERENCES

1. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234: 466–468.
2. Beutler, E., Duron, O., and Kelly, B. M. 1963. Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* 61: 882–888.
3. Carrell, R. W., Winterbourn, C. C., and Rach-Milewitz, E. A. 1975. Activated oxygen and hemolysis. *Br. J. Haematol.* 30: 259–264.
4. Folch, J., Lees, M., and Sloane-Stanley, G. H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497–509.
5. French, J. K., Winterbourn, C., and Carrell, R. W. 1978. Mechanism of oxyhemoglobin breakdown on reaction with acetylphenylhydrazine. *Biochem. J.* 173: 19–26.
6. Fujise, H., Yamada, I., Masuda, M., Miyazawa, Y., Ogawa, E., and Takahashi, R. 1991. Several cation transport and volume regulation in high K dog red blood cells. *Am. J. Physiol.* 260: C 589–597.
7. Hast, C. W. M., Kamp, D., and Deuticke, B. 1979. Formation of disulfide bonds between glutathione and membrane SH groups in human erythrocytes. *Biochim. Biophys. Acta* 557: 363–371.
8. Inaba, M. and Maede, Y. 1984. Increases of Na gradient dependent L-glutamate and L-aspartate transport in high K dog erythrocytes associated with high activity of Na, K-ATPase. *J. Biol. Chem.* 259: 312–317.
9. Kosower, N. S., Vanderhoff, G. A., and London, I. M. 1967. The regulation of reduced glutathione in normal and glucose-6-phosphate dehydrogenase deficient human red blood cells. *Blood* 29: 313–319.
10. Maede, Y. and Inaba, M. 1987. Energy metabolism in canine erythrocytes associated with inherited high Na and K-stimulated adenosine triphosphatase activity. *Am. J. Vet. Res.* 48: 114–118.
11. Maede, Y., Inaba, M., and Taniguchi, N. 1983. Increase of Na, K-ATPase activity, glutamate, and aspartate uptake in dog erythrocytes associated with hereditary high accumulation of glutathione, glutamate, glutamine, and aspartate. *Blood* 61: 493–499.
12. Maede, Y., Kasai, N., and Taniguchi, N. 1982. Hereditary high concentration of glutathione in canine erythrocytes associated with high accumulation of glutamate, glutamine,

and aspartate. *Blood* 59: 883–889.
13. Maede, Y., Kuwabara, M., Sasaki, A., Inaba, M., and Hiraoka, W. 1989. Elevated glutathione accelerates oxidative damage to erythrocytes produced by aromatic disulfide. *Blood* 73: 312–317.
14. McMahon, S. and Stern, A. 1979. The interrelationship of superoxide dismutase and peroxidatic enzymes in the red cell. *Biochim. Biophys. Acta* 566: 253–258.
15. Metz, E. N., Balcerzak, S. P., and Sagne, A. L. 1974. Regeneration of reduced glutathione in erythrocytes: Stoichiometric and temporal relationship to hexose monophosphate shunt activity. *Blood* 44: 691–697.
16. Nelson, G. T. 1967. Lipid composition of erythrocytes in various mammalian species. *Biochim. Biophys. Acta* 144: 221–232.
17. Ogawa, E., Fujise, H., and Kobayashi, K. 1988. A dog possessing high glutathione (GSH) and K concentrations with an increased Na, K-ATPase activity in its erythrocytes. *Exp. Anim. (Tokyo)* 37: 187–190.
18. Ogawa, E., Nagaoka, A., Fujise H., and Takahashi, R. 1989. Methemoglobin formation and reduction in canine erythrocytes with inherited high Na, K-ATPase activity. *Jpn. J. Vet. Sci.* 51: 1185–1192.
19. Parker, J. C.1983. Passive calcium movements in dog red blood cells: anion effects. *Am. J. Physiol.* 244: C 318–323.
20. Parker, J. C. and Castranova, V. 1984. Volume-expansion sodium and proton movements in dog red blood cells. *J. Gen. Physiol.* 84: 379–401.
21. Parpart, A. K., Lorenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, A. M. 1947. The osmotic resistance of human red cells. *J. Clin. Invest.* 26: 636–641.
22. Radin, N. S., Lavin, F. B., and Brown, J. R. 1956. The preparative isolation of cerebrosides. *J. Biol. Chem.* 219: 977–983.
23. Skipski, V. P., Peterson, R. F., and Barklay, M. J. 1964. Quantitative analysis of phospholipids by thin layer chromatography. *Biochem. J.* 90: 374–378.
24. Stoffel, W., Chu, F., and Ahres, E. H. 1959. Analysis of long-chain fatty acid by gas-liquid chromatography. *Anal. Chem.* 31: 307–308.
25. Thiele, O. W. und Denden, A. 1967. Über die Vertailung der Lipide in der Cornea des Rindes. *Z. Physiol. Chem.* 348: 1097–1101.
26. Tomoda, A., Tsuji, A., and Yoneyama, Y. 1981. Involvement of superoxide anion in the reaction mechanism of hemoglobin oxidation by nitrite. *Biochem. J.* 193: 169–179.
27. Watkins, J. A., Kawanishi, S., and Caughey, W. 1985. Autoxidation and reduction of hemoglobin A free from other red cell components: a minimal mechanism. *Biochem. Biophys. Res. Comm.* 132: 742–748.
28. Wittig, L. A. and Horwitt, M. K. 1964. Effect of degree of fatty acid unsaturation in tocopherol deficiency induced creatinuria. *J. Nutr.* 82: 19–25.
29. Yagi, K. 1976. A simple fluorometric assay for lipid peroxide in blood plasma. *Biochem. Med.* 15: 212–216.
30. Yoshimitsu, T. 1983. Lipid peroxidation of human erythrocyte hemolysate. *Hiroshima Daigaku Igaku Zasshi* 31: 947–961 (in Japanese).
31. Zak, B. 1954. Rapid estimation of free and total cholesterol. *J. Lab. Clin. Med.* 24: 1307–1315.