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Increase of Na-K-ATPase Activity, Glutamate, and Aspartate Uptake in Dog Erythrocytes Associated With Hereditary High Accumulation of GSH, Glutamate, Glutamine, and Aspartate

By Yoshimitsu Maede, Mutsumi Inaba, and Naoyuki Taniguchi

We have found convincing evidence for the presence of Na-K-ATPase and high potassium (K) and low sodium (Na) concentrations in the erythrocytes of some dogs associated with hereditary high concentrations of erythrocyte glutathione and some amino acids, glutamate, glutamine, and aspartate. The Na-K-ATPase activity of the erythrocyte membranes of the dogs was about 3 times that of human erythrocyte membranes, whereas the enzyme activity was not detected in control dogs with a normal level of blood glutathione. The Michaelis constant of the enzyme for ATP (K_m , ATP) was $6.6 \times 10^{-3} M$ in the dogs' erythrocytes and $5.0 \times 10^{-4} M$ in the human erythrocytes.

MOST MAMMALIAN erythrocytes are able to maintain the low sodium (Na) and high potassium (K) concentrations in their cells as compared to those in plasma by the function of a membrane-bound enzyme, Na-K-activated adenosine triphosphatase (Na-K-ATPase).^{1,2} In contrast with those cells, dog and cat erythrocytes are known to contain low K and high Na concentrations and to lack the membrane Na-K-ATPase.¹ In the present study, however, we present convincing evidence for the presence of Na-K-ATPase and high K, low Na concentrations in the erythrocytes of some clinically normal dogs.

We have recently reported two canine cases of inherited high concentrations of reduced glutathione (GSH), glutamate, glutamine, and aspartate in their erythrocytes without any clinical signs.³ The inheritance of this abnormality was of a recessive autosomal mode. The increased level of GSH, which was 5–7 times the normal concentrations, could be explained by the fact that feedback inhibition of γ -glutamyl-cysteine synthetase by GSH was released by the high level of glutamate (about 92 times the normal) in their cells. In the course of trying to clarify the cause of the high accumulation of such amino acids in these cells, we found that these erythrocytes showed high activity of their membrane Na-K-ATPase and contained high K and low Na concentrations, which is not compatible with the current knowledge concerning Na-K-ATPase activity and Na, K concentrations in dog erythrocytes. Furthermore, we also found that these erythrocytes showed high uptake of glutamate and aspartate, which seemed to result in the high accumulation of the amino acids in their cells.

MATERIALS AND METHODS

The activity of membrane adenosine triphosphatase (ATPase), the concentration of Na, K, and amino acid transport in the

The concentration of K in the erythrocytes in the dogs examined was about 11 times that of the controls, whereas the erythrocyte Na concentration in the dogs was about one-third that of the controls. The concentrations of K and Na in the plasma of the dogs were equal to those of the controls. Furthermore, L-³H-glutamate and L-³H-aspartate uptake by those cells with high activity of Na-K-ATPase greatly increased, while L-³H-glutamine uptake was unchanged. It appeared that Na⁺ and K⁺ gradients created by Na-K-ATPase across the cell membrane might stimulate glutamate and aspartate uptake by the cells, thus causing the high accumulation of such amino acids in the cells.

erythrocytes of 9 dogs of one family (Fig. 1), including 5 dogs with a high level of erythrocyte-reduced glutathione (GSH) were examined. Three normal dogs other than those of the family were also examined as controls. Hematologic data were obtained by the usual standard procedures. The total number of red cells was determined with a hemocytometer. The PCV was determined by the microhematocrit method and Hb by a cyanmethemoglobin method. Reticulocyte counts were done on blood films stained supravitaly with new methylene blue. The osmotic fragility of erythrocytes was measured by the method of Beutler et al.⁴ with a minor modification using a series of dilutions of a buffer containing 180 g NaCl, 27.3 g Na₂HPO₄, and 4.86 g NaH₂PO₄ in 2000 ml. Erythrocyte-reduced glutathione (GSH) concentration of each dog was measured by the 5',5-dithiobis (2-nitrobenzoic acid) derivative.⁵ The concentrations of glutamate, glutamine, and aspartate in the erythrocytes were measured by two automatic amino acid analyzers as reported previously.³

Preparation of Membranes

Approximately 5 ml of venous blood was collected into heparinized tubes from the saphena of each dog. The blood was filtered through a column of microcrystalline cellulose and α -cellulose to remove leukocytes and platelets. The blood was then washed 3 times with 0.9% NaCl solution at 4°C, after which the erythrocytes were resuspended in a cold 0.9% NaCl solution to yield hematocrit values of 40%–50%. Hemoglobin-free membranes for measurement of ATPase activity were prepared by a modification of Dodge et al.⁶ Washed and resuspended erythrocytes were hemolyzed in 20 volumes of a hypotonic solution (0.01 M sodium barbital–0.05N HCl,

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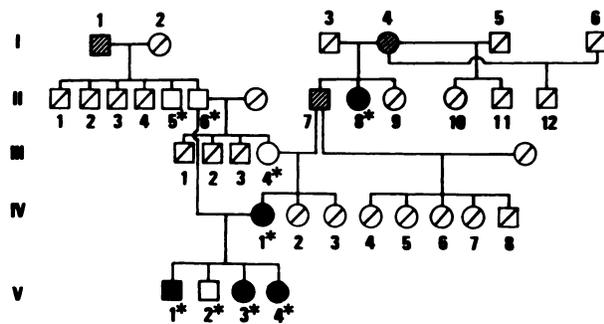


Fig. 1. Pedigree of a dog family including high GSH dogs. (■●) Dogs with high concentrations of red cell GSH, amino acids, potassium, and high activity of Na-K-ATPase. (◐◑) Previously reported dogs with high concentration of red cell GSH. The Na, K, and amino acid concentrations and ATPase activity were not examined. (◒◓) Dogs with normal concentrations of red cell GSH, amino acids, and potassium without Na-K-ATPase activity. (◔◕) Dogs with normal concentrations of red cell GSH. The Na, K concentrations and ATPase activity were not examined. (*) Examined in the present study.

pH 7.4, 30 mosmole) and centrifuged at 15,000 rpm and 4°C for 40 min. The supernatant was decanted and the membranes were washed 3 times with ice-cold hemolyzing solution. The resulting fluffy membranes were stored in an equal volume of 50 mM tris-HCl (pH. 7.5) at 4°C until used. All ATPase assays were performed on the same day that the membranes were prepared. For comparison with human erythrocyte ATPase, red cells were obtained from six healthy men and prepared by the same method.

ATPase Assay

The activity of total ATPase was assayed essentially by the method of Nakao et al.⁷ One-hundred microliters of the membrane suspension containing about 0.15 mg of protein was incubated for 1 hr at 37°C in 1.0 ml of a reaction mixture consisting of 3 mM ATP, 5 mM MgCl₂, 140 mM NaCl, 14 mM KCl, 1 mM EDTA, and 50 mM Tris-HCl (pH 7.5). The activity of Na-K-ATPase was measured by adding a concentration of 10⁻⁴ M ouabain to the reaction mixture. The reaction was stopped by the addition of 1.0 ml of 15% trichloroacetic acid. The mixture was shaken vigorously for 30 sec by a vortex mixer and then centrifuged at 3000 rpm for 15 min at 4°C. The released Pi in the supernatant was measured by the method of Fiske and SubbaRow.⁸ The activity was expressed as micromoles of Pi released per hour per milligram protein. The Na-K-ATPase activity was determined by numerical subtraction of ouabain insensitive ATPase activity from the total ATPase activity.

Estimation of Na, K Concentrations

For determinations of Na, K concentrations in the dog erythrocytes and plasma, blood was collected into a heparinized plastic tube from the saphena of each dog and the packed cell volume was determined by the microhematocrit method. Twenty-five microliters of whole blood was hemolyzed in 5 ml of a lithium standard solution containing 100 ppm lithium (Daiichi Chemical Co., Ltd., Japan). The remaining blood was centrifuged at 3000 rpm for 10 min at 4°C, and the plasma was separated for measurement of the electrolyte concentrations. The concentrations of Na and K in the whole blood and plasma were determined by a flame photometer (Hiranuma FPF-3A, Japan). Erythrocyte Na and K concentrations were calculated from the PCV and the difference between the analytical results from plasma and hemolyzed whole blood.⁹ The results were expressed as milliequivalent per liter of plasma or red blood cells.

All measurements were performed within 30 min after the blood collection.

Amino Acids Uptake Studies

Erythrocyte preparation. Venous blood was collected into heparinized tubes from the jugular vein of each dog. After centrifugation at 2000 rpm for 10 min, the buffy coat was discarded. Erythrocytes were washed 3 times with an ice-cold incubation medium containing 150 mM NaCl, 5 mM KCl, 15 mM MOPS (pH. 7.5), 2 mM MgCl₂, 10 mM glucose, and 0.1% bovine serum albumin. The erythrocytes were then resuspended in the same incubation medium to yield hematocrit values of 15%–20%. The cells were used within 2 hr of sampling.

Materials. L-³H-glutamate (34 Ci/mmole), L-³H-aspartate (5.1 Ci/mmole), and L-³H-glutamine (27 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, England. All other reagents were from Wako Pure Chemical Industries, Japan.

Uptake assays. Uptake for glutamate, glutamine, and aspartate by erythrocytes from high and normal GSH dogs were measured by the method of Young et al.¹⁰ with a minor modification. One-hundred-fifty microliters of the erythrocyte suspension were incubated at 37°C with 150 μl of the incubation medium containing the radioactive amino acid (0.5 μCi/ml) and the appropriate concentration of each of the amino acids (0.5–500 μM). Incubations were stopped at predetermined time intervals (10 sec–60 min) by the addition of 1 ml of the ice-cold medium. The cells were rapidly washed 3 times with 1 ml of this medium by centrifugation at 15,000 rpm for 10 sec using a microcentrifuge, and then lysed with 0.5 ml of 0.5% Triton X-100 in water. The samples were deproteinized by the addition of 0.5 ml of 5% of trichloroacetic acid, followed by centrifugation at 15,000 rpm for 2 min. Radioactivity in the protein-free supernatants was measured by a liquid scintillation spectrometry using scintisol EX-H (Wako Pure Chemical Industries, Ltd., Japan) as the scintillant. Each measurement was performed in duplicate. The rate of uptake was expressed as micromoles amino acid per minute per liter of red cells.

RESULTS

Hematologic Data

The concentrations of GSH, glutamate, glutamine, and aspartate in the erythrocytes of high GSH dogs and normal dogs, including 5 dogs of the family, are shown in Table 1, along with some hematologic observation. Five dogs of the family (II-8, IV-1, V-1, V-3, and V-4) showed a high concentration of erythrocyte GSH, glutamate, glutamine, and aspartate. Dog V-2 and the other dogs of the family examined showed normal levels of erythrocyte GSH and amino acids. The red cell count, hemoglobin concentration, and mean corpuscular hemoglobin concentration (MCHC) in the high GSH dogs were significantly lower ($p < 0.001$) and the mean cell volume (MCV) was higher ($p < 0.001$) than those in the normal GSH dogs, although the results were all within normal range.¹¹ Osmotic fragility of erythrocytes of the high GSH dogs showed a marked increase as compared to that of normal dogs (Fig. 2). This is not consistent with the previous observation in dog II-8 showing normal osmotic fragility of the cells.¹² This contradiction,

Table 1. Hematologic Data About the Dogs Used in These Experiments

	High GSH Dogs (n = 5)	Normal GSH Dogs (n = 7)
GSH (mg/dl RBC)	409.7 ± 39.2	78.4 ± 10.5*
Amino acid (nmole/ml RBC)		
Glutamate	13,577 ± 916	292 ± 21*
Aspartate	8,168 ± 1,978	191 ± 68*
Glutamine	11,794 ± 1,116	1,288 ± 607*
Ht (%)	48.8 ± 6.0	49.6 ± 2.7
RBC (10 ⁴ /μl)	619.7 ± 81.7	823.1 ± 70.4*
Reticulocyte (%)	2.2 ± 1.2	1.4 ± 0.4
Hb (g/dl)	13.8 ± 1.9	16.8 ± 0.9*
MCV (cu μ)	79.0 ± 3.5	60.5 ± 3.3*
MCH (pg)	22.3 ± 1.4	20.6 ± 1.7
MCHC (%)	28.3 ± 1.3	34.0 ± 2.4*

Data are expressed as mean ± SD.

**p* < 0.001 according to the Student's *t* test when compared to high GSH dogs.

however, may be explained by the fact that the dog had shown high reticulocytosis in the peripheral blood when it was examined, resulting from acute hemolytic anemia.

ATPase Activity

Table 2 shows the effect of three cations, Mg, Na, and K, on the ATPase activity in the dog erythrocyte membranes. In the presence of the three cations, the

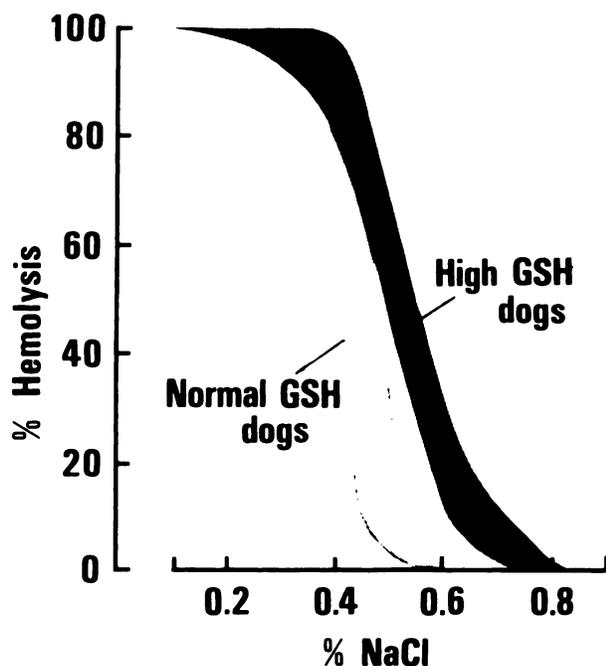


Fig. 2. Osmotic fragility curves of erythrocytes in high and normal GSH dogs. Each curve consists of data from 5 dogs with high GSH blood and from 7 dogs with normal GSH blood. Fresh blood from each dog was used to obtain the osmotic fragility curve.

Table 2. Effects of Magnesium, Sodium, Potassium, and Ouabain on ATPase Activity in Dog Red Cell Membranes

Ionic Composition	ATPase Activity (μmole Pi/hr/mg Protein)	
	High GSH Dogs (n = 5)	Normal GSH Dogs (n = 7)†
1. Mg, Na, K	1.10 ± 0.15	0.64 ± 0.11
2. Mg, Na, K, ouabain	0.65 ± 0.09	0.64 ± 0.11
3. Mg, Na	0.75 ± 0.08	0.63 ± 0.11
4. Na, K	0.06*	0.03*

The reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 1 mM ATP, 1 mM EDTA, and the indicated cations or ouabain.

The concentrations of the indicated ions were as follows: magnesium 5 mM, sodium 140 mM, and potassium 14 mM. Ouabain was used at a concentration of 0.1 mM.

Data are expressed as mean ± SD.

*Data from one dog.

†Four dogs of the family are included.

activity of ATPase in the membranes of the high GSH dogs was about 1.7 times that of the normal dogs. The addition of 0.1 mM ouabain to the reaction mixtures containing the three ions resulted in a marked decrease of ATPase activity in the high GSH dogs' erythrocyte membranes, whereas the ouabain did not affect the enzyme activity in the normal GSH dogs. Moreover, the enzyme activity in the erythrocytes of the high GSH dogs was almost equal to that of the normal dogs when the reaction mixtures contained ouabain at a concentration of 0.1 mM. In the absence of Mg, ATP was not hydrolyzed by the enzyme in either of the erythrocyte membranes. These results demonstrated clearly that two types of ATPase were present in the erythrocyte membranes of the high GSH dogs, one of which was Mg-dependent and not inhibited by ouabain (Mg-ATPase), and the other that was greatly activated by the presence of the three cations, Mg, Na, and K, and completely inhibited by ouabain (Na-K-ATPase). The latter, Na-K-ATPase, was not detected in the erythrocyte membranes of the normal dogs.

Figure 3 shows the effect of pH on the ATPase activity in the erythrocyte membranes of a high GSH dog. Both the ouabain-sensitive and insensitive ATPase activities in the erythrocyte membrane incubated at various pH values rose sharply to a peak at pH 7.5, which indicates a pH optimum in that vicinity.

Figure 4 shows the effect of various concentrations of ouabain on the ATPase activity of erythrocyte membranes from a high GSH dog and a control dog. Ouabain inhibited the enzyme activity in the high GSH dog, but had no effect on the enzyme activity in the control dog. This inhibition was apparently observed at the concentration of 10⁻⁵M ouabain and was maximal at 10⁻³M.

Figure 5 shows the ATPase activity in the high GSH

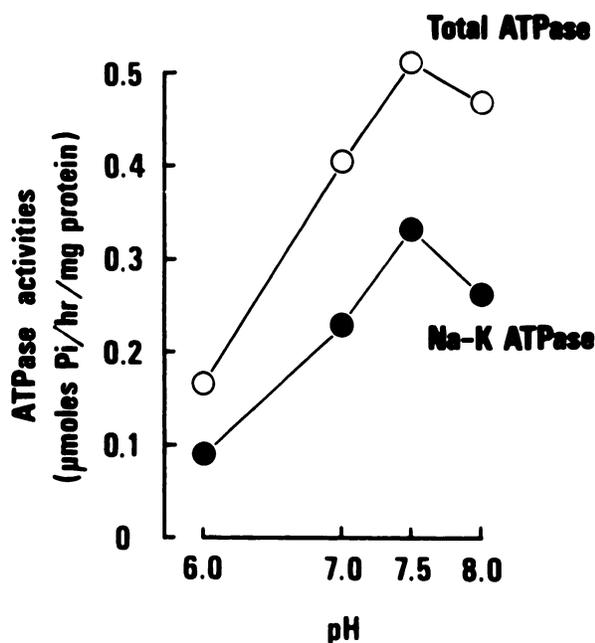


Fig. 3. Effect of pH on total and Na-K-ATPase activities in the red cell membranes of a high GSH dog.

dog and human erythrocyte membranes at various concentrations of ATP. The activity of ouabain-sensitive ATPase in the dog peaked at an ATP concentration of about 5 mM, whereas in the human erythrocytes it peaked at a concentration of 2 mM. The Michaelis constant of the ouabain-sensitive ATPase for ATP (K_m ATP) was estimated by the Lineweaver-Burk plot. The K_m ATP of the high GSH and human

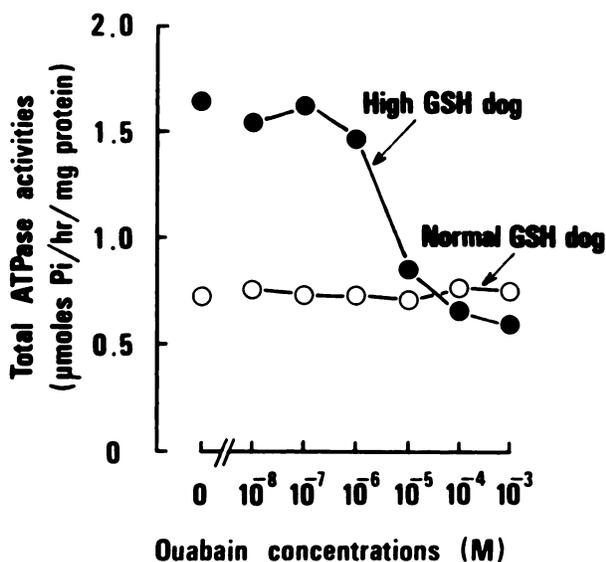


Fig. 4. Effects of ouabain at various concentrations on ATPase activity in red cell membranes in a high GSH dog and in a normal GSH dog.

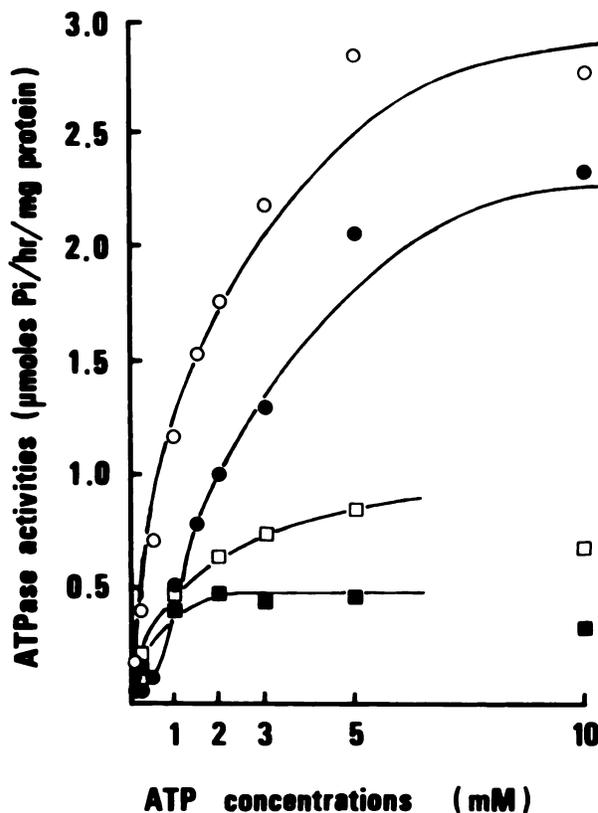


Fig. 5. Effects of ATP at various concentrations on ATPase activity in the erythrocyte membranes of a high GSH dog (●, ○) and a normal human (■, □). (○ □) Total ATPase activity. (● ■) Na-K-ATPase activity.

erythrocyte membranes were $6.6 \times 10^{-3} M$ and $5.0 \times 10^{-4} M$, respectively.

The ATPase activities observed in the dog and human erythrocyte membranes are summarized in Table 3. The activity of Mg-ATPase, ouabain-insensitive ATPase, in the high GSH dogs was almost equal to that of the normal GSH dogs, but it was 1.7 times that

Table 3. ATPase Activity of Dog Red Cells With High and Normal GSH and Human Red Cells

Subjects	ATPase Activity (μ mole Pi/hr/mg Protein)		
	Total ATPase	Mg-ATPase	Na-K-ATPase
High GSH dogs (n = 5)	1.87 \pm 0.19	0.78 \pm 0.08	1.09 \pm 0.14
Normal GSH dogs (n = 7)*	0.64 \pm 0.15	0.64 \pm 0.14	0 \pm 0.03
Human (n = 6)	0.85 \pm 0.17	0.47 \pm 0.10	0.38 \pm 0.10

The condition of ATPase assays were as follows: the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 3 mM ATP, 1 mM EDTA, 5 mM $MgCl_2$, 140 mM NaCl, 14 mM KCl, and 0.1 mM ouabain. The reaction was carried out at 37°C for 60 min.

Data are expressed as mean \pm SD.

*Four dogs of the family are included.

Table 4. Na, K Concentrations in Red Cells and Plasma of High GSH and Normal GSH Dogs

Subjects	Na, K Concentration (meq/liter)			
	Erythrocyte		Plasma	
	Na	K	Na	K
High GSH dogs (<i>n</i> = 5)	54.1 ± 21.6	123.6 ± 5.9	148.9 ± 3.3	4.4 ± 0.3
Normal GSH dogs (<i>n</i> = 7)*	153.0 ± 44.9	10.7 ± 3.1	148.2 ± 3.4	4.9 ± 0.9

Data are expressed as mean ± SD.

*Four dogs of the family are included.

of the human erythrocyte membranes. The activity of Na-K-ATPase, ouabain-sensitive ATPase, in the high GSH dogs was about 3 times that of the human erythrocyte membranes.

Na, K Concentrations in Erythrocytes and Plasma of Dogs

The results are shown in Table 4. There was an apparent inverse relationship between the high GSH and the normal dog erythrocytes in terms of the concentration of Na and K. That is, the high GSH dog erythrocytes contained high K and low Na, whereas the normal cells contained low K and high Na concentrations as compared to their concentrations in the plasma. The concentrations of Na and K in the plasma of the high GSH dogs was equal to those of the normal dogs. The results for Na, K concentrations in the erythrocytes and plasma of the normal dogs were in agreement with other reports on the concentrations of the electrolyte in dog erythrocytes.^{2,9}

Amino Acid Uptake by Erythrocytes

As shown in Fig. 6 and Table 5, L-³H-glutamate and L-³H-aspartate uptake by high GSH erythrocytes greatly increased as compared to normal GSH cells. That is, L-glutamate and L-aspartate uptake by high GSH cells at an extracellular concentration of 50 μM were about three-fold higher than by normal GSH

cells. In addition, L-glutamate and L-aspartate uptake by both the high and normal GSH cells were saturable and entirely consistent with simple Michaelis-Menten kinetics: apparent K_m values for L-glutamate and L-aspartate were 25.6 μM and 19.2 μM in the high GSH cells, and 28.6 μM and 17.0 μM in the normal GSH cells, respectively. In contrast to these characteristics observed in glutamate and aspartate uptake, glutamine uptake by both types of cells was linear, and there was almost no difference between the high and the normal cells (Fig. 6).

DISCUSSION

Our previous study³ demonstrated that two dogs with an inherited high concentration of erythrocyte GSH showed a high accumulation of glutamate, aspartate, and glutamine in their erythrocytes, which increased to 92, 63, and 13 times the normal value, respectively. In addition, the activity of γ-glutamyl cysteine synthetase in the erythrocytes, which plays a catalytic action in glutathione synthesis, was inhibited by the presence of GSH in a reaction system, but it was greatly activated by increasing the concentration of L-glutamate in the system. It thus appeared that the feedback inhibition of γ-glutamyl cysteine synthetase by GSH was released by the high concentration of glutamate in the erythrocytes, which might result in an increased concentration of GSH in the cells. However,

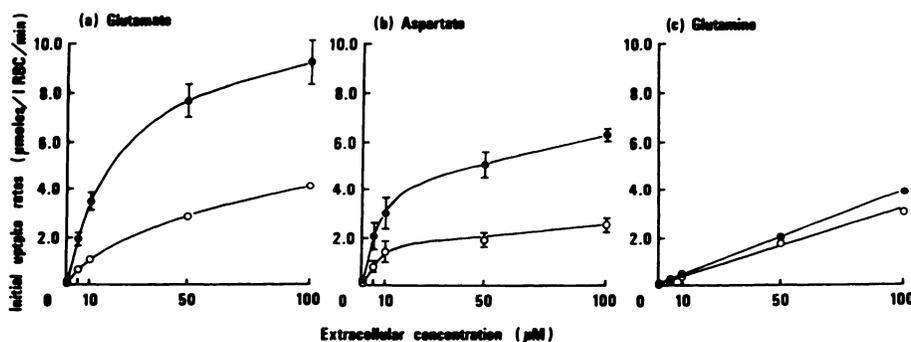


Fig. 6. Concentration dependence of L-glutamate, L-aspartate, and L-glutamine uptakes by high and normal GSH erythrocytes. (●) High GSH dogs (*n* = 5). (○) Normal GSH dogs (*n* = 6). Data are expressed as mean ± SD, except (C) glutamine uptake, where mean values of each of three dogs were plotted. Erythrocyte suspension (Ht:20%) was incubated for 60 sec at 37°C with a radioactive amino acid in the medium containing 150 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 10 mM glucose, 0.1% BSA, 15 mM MOPS (pH 7.5 at 37°C), and each amino acid at indicated concentrations. After incubation, radioactivity of the cells was measured as described in Materials and Methods.

Table 5. Amino Acid Uptake by Dog Erythrocytes With High and Normal Levels of GSH

Subjects	Initial Uptake Rates ($\mu\text{mole/liter RBC/min}$)		
	L-Glutamate	L-Aspartate	L-Glutamine
High GSH dogs (<i>n</i> = 5)	7.44 \pm 0.43	4.79 \pm 0.23	2.00*
Normal GSH dogs (<i>n</i> = 6)	2.55 \pm 0.73	1.63 \pm 0.49	1.72*

Amino acids were present at an extracellular concentration of 50 μM .

Data are expressed as mean \pm SD.

*Mean values of three dogs.

the question of why the levels of glutamate, aspartate, and glutamine became so extremely elevated in the high GSH erythrocytes has remained to be solved. In the present study, we obtained two very valuable results to answer this question.

First, the present study clearly demonstrated that the erythrocytes of the 5 dogs examined, which were characterized by high concentrations of GSH, glutamate, glutamine, and aspartate, had two types of ATPase activity. One type of enzyme was Mg-ATPase, which was stimulated by magnesium and not inhibited by ouabain, and the other type was greatly stimulated by the presence of three cations, magnesium, sodium, and potassium, and completely inhibited by ouabain at a concentration of 10^{-4}M . These characteristics of this enzyme are consistent with those of Na-K-stimulated adenosine triphosphatase, Na-K-ATPase, which is a membrane-bound enzyme believed to play an active role in transporting sodium out of and potassium into cells.^{13,14} Furthermore, the cells contained high K and low Na concentrations as compared to their concentrations in plasma, which indicates that Na and K ion gradients across the cell membranes are probably formed by the action of Na-K-ATPase ($[\text{Na}^+]_{\text{out}} > [\text{Na}^+]_{\text{in}}$, $[\text{K}^+]_{\text{out}} < [\text{K}^+]_{\text{in}}$). These results apparently conflict with the widely accepted view that dog erythrocytes contain high Na and low K concentrations ($[\text{Na}]_{\text{out}} \doteq [\text{Na}]_{\text{in}}$, $[\text{K}]_{\text{out}} \doteq [\text{K}]_{\text{in}}$) and lack the membrane Na-K-ATPase.^{1,2,9}

Second, amino acid uptake measurements revealed that L-glutamate and L-aspartate uptake by high GSH erythrocytes greatly increased, while there were almost no differences in glutamine uptake between the high and normal GSH erythrocytes. Furthermore, while glutamine uptake in the high and normal GSH cells was linear, glutamate and aspartate uptake in both types of cells were saturable and entirely consistent with simple Michaelis-Menten kinetics, which suggest that dog erythrocytes may possess an energy-dependent transport system for glutamate and aspartate (Fig. 6). This result is consistent with the recent

report by Ellory et al.¹⁵ that dog red cells possess a high-affinity, Na-dependent, stereospecific selective transport system for glutamate and aspartate, although erythrocytes of most species are essentially impermeable to such amino acids.¹⁶

A transport system for glutamate and aspartate has been found in other tissues.¹⁷⁻²⁰ Schneider et al.¹⁸ examined the characteristics of the L-glutamate transport system in renal brush border membrane vesicles. They found that a Na⁺ gradient-dependent transport system for L-glutamate, shared by L-aspartate, was stimulated by an extravesicular > intravesicular Na⁺ gradient and that it was increased additionally by the presence of an intravesicular > extravesicular K⁺ gradient. The same phenomenon was also found in brush border membrane vesicles from rat renal proximal tubules where glutamate and aspartate were reabsorbed via the same system,¹⁹ and in membrane vesicles isolated from a rat cerebral cortex,²⁰ where such amino acids were highly concentrated.²¹ Roskoski et al.²⁰ found that membrane vesicles from synaptosomal fractions were active in transporting radioactive L-glutamate and L-aspartate in a Na⁺-dependent manner and that the amino acid transports were inhibited by abolishing both the Na⁺ and K⁺ gradients. These findings strongly suggest that sodium and potassium ion gradients across cell membranes ($[\text{Na}^+]_{\text{out}} > [\text{Na}^+]_{\text{in}}$, $[\text{K}^+]_{\text{out}} < [\text{K}^+]_{\text{in}}$) provide the driving forces for glutamate and aspartate uptake by cells possessing such an amino acids transport system. In this respect, Na-K-ATPase is believed to play a key role in creating the Na and K ion gradients.^{13,14}

Thus, the most reasonable explanation for the abnormal accumulation of glutamate and aspartate observed in the high GSH erythrocytes is that the Na and K ion gradients created by the Na-K-ATPase across the erythrocyte membranes stimulated the active transport of glutamate and aspartate into the cells, thus causing the high intracellular accumulation of such amino acids. In contrast to this, glutamine uptake by both the high and normal GSH cells was linear, which indicates that an active transport system for glutamine was not present in the dog erythrocytes. The accumulation of glutamine observed in high GSH cells, therefore, might be due to a high concentration of glutamate in the cells, since glutamate is a precursor of glutamine as well as glutathione and it can be converted to glutamine by a catalytic action of glutamine synthetase,²² which is present in erythrocytes²³ as well as in other tissues.

Hematologic parameters such as hematocrit, hemoglobin concentration, red cell and reticulocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin

concentration (MCHC) measured in the high GSH dogs were all within the normal range, except the reticulocyte count, which increased slightly in dogs V-1 and V-4. However, the red cell count, hemoglobin concentration, and MCHC were significantly lower and the MCV significantly higher than in the normal GSH dogs, while the MCH was unchanged. This suggests that the cell water in high GSH erythrocytes is abnormally higher than in normal GSH cells, which seems to be closely related to the increase of osmotic fragility observed in high GSH erythrocytes. The significance of this phenomenon is not clear at this point, although it has been reported that potassium

influx in dog erythrocytes greatly increased and sodium influx decreased when the cell water content increased.^{24,25}

Although some unsolved questions remain in the present cases, it is evident that dogs such as those employed in the present study are undoubtedly useful genetic variants for the study of membrane transport of amino acids and glutathione, not only in erythrocytes but in other tissues as well. In addition, the genes for those abnormalities observed in the present cases may not be rare in dogs, because all dogs of the family were mongrel dogs.

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