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Hereditary High Concentration of Glutathione in Canine Erythrocytes Associated With High Accumulation of Glutamate, Glutamine, and Aspartate

By Yoshimitsu Maede, Noriyuki Kasai, and Naoyuki Taniguchi

We have found a dog family in which there were five cases of increased red cell glutathione corresponding to four to five times the normal concentration without any clinical signs. In the present study, we mainly examined the concentrations of free amino acids in the erythrocytes, plasma, and urine of two of the dogs, and we demonstrated that the concentrations of glutamate, aspartate, and glutamine in their erythrocytes increases to 92, 63, and 13 times the mean value in the normal blood, respectively. There were no changes observed in the other amino acids

as compared to normal, although the glycine and histidine in the erythrocytes showed slight increases. The concentrations of amino acids in the plasma and urine of the dogs were almost equal to normal ones. The activities of some of the enzymes involved in the glutathione metabolism in the erythrocytes from the two dogs were all within the normal range. The increased level of glutathione could be explained by the fact that the feedback inhibition of γ -glutamyl cysteine synthetase by glutathione was released by the high levels of glutamate in the erythrocytes.

ERYTHROCYTE GLUTATHIONE is believed to participate in intracellular redox reactions and to protect the hemoglobin and some of the thiol-dependent enzymes or membrane proteins from oxidative stress. However, the current knowledge on glutathione metabolism in erythrocytes is not yet complete.

Previously, we reported on two canine cases of hemolytic anemia associated with increased concentrations of erythrocyte glutathione.¹ The two dogs showed severe anemia with hemoglobinuria after eating a small quantity of boiled onions. Glucose-6-phosphate dehydrogenase (G6PD) activity and adenosine triphosphate (ATP) content of the erythrocytes, hemoglobin electrophoresis, blood urea nitrogen (BUN), and the direct antiglobulin test of erythrocytes in both dogs tested at the time when they showed severe anemia were either normal or negative, except that the concentration of erythrocyte glutathione was 4–5 times the mean value found in normal blood. A dam and sibling of one of the dogs were also found to possess erythrocytes containing the high concentration of glutathione without any clinical signs or hematologic disorders except slight reticulocytosis. The hemolysis observed in the two dogs may have been produced by the feeding of onion, because onions fed to dogs produce severe hemolytic anemia.² The inheritance of this abnormality was of a recessive autosomal mode (Fig. 1). The propositi and dog I-3 of the family were mongrel dogs, which indicates that the gene for high concentration of erythrocyte glutathione may not be rare in dogs.

The present study was carried out to clarify the cause of the increased concentration of glutathione in the dog erythrocytes and demonstrated the marked accumulation of glutamate, which is a precursor of glutathione, glutamine, and aspartate in these cells.

MATERIALS AND METHODS

The pedigrees for the two mongrel dogs that possessed an increased concentration of erythrocyte glutathione, that is, I-1 and II-8, are shown in Fig. 1. Individuals I-4, II-7, and IV-1 also had erythrocytes containing a high concentration of glutathione. All of the other dogs examined in the family showed a normal concentration of erythrocyte glutathione.

In the present study, free amino acid concentrations in the erythrocytes, plasma, and urine of the two dogs (II-8, IV-1) with increased concentrations of erythrocyte glutathione and the other one (II-6) with normal level of erythrocyte glutathione were mainly examined. Glutathione concentration in both the erythrocytes and the plasma and some of the enzymes involved in the glutathione metabolism were also assayed. Six normal dogs were used as control. All of the dogs examined had been kept under the same conditions for 1–3 yr.

Chemicals

Glutathione reductase, reduced and oxidized glutathione, reduced nicotinamide adenine dinucleotide (NADPH), creatine phosphokinase, pyruvate kinase, and L- α -glutamyl *p*-nitroanilide were obtained from C.F. Boehringer Mannheim, Germany. DL- α -amino-[1-¹⁴C] butyrate and [¹⁴C]-glycine were obtained from New England Nuclear. The Uridine-5'-monophosphate (UMP) was obtained from Yamasa-Shoyu Co., Japan. L- γ -Glutamyl-L- α -aminobutyrate was prepared as described previously.³ All other reagents used were provided by the courtesy of Wako Pure Chemical Industries, Ltd.,

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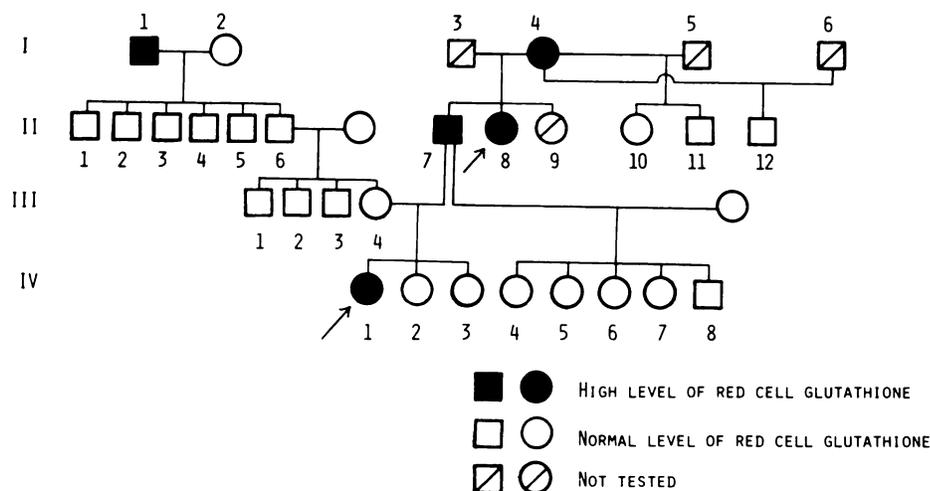


Fig. 1. Pedigree of dogs with high levels of red cell glutathione. Present cases are II-8 and IV-1 (arrows). Previously reported cases are I-1 and II-8.

Japan. All spectrophotometric measurements were performed with Gilford model 250.

Preparation of Erythrocytes, Plasma, and Urine

Venous blood was collected into heparinized tubes from the jugular vein of each dog. The blood was centrifuged at 2000 rpm for 10 min, and the plasma and a buffy coat were removed by aspiration. The red cells were washed 3 times with cold 0.9% sodium chloride solution. The washed cells were then filtered through microcrystalline cellulose- α -cellulose to remove the remaining leukocytes and platelets.⁴ The red cells were then resuspended in a cold 0.9% sodium chloride solution to yield hematocrit values of 40%–50% and used for analysis of free amino acids and assay of the enzymes. Urine samples from dog II-8, IV-1, and two of the controls were collected by catheter at the same time, and the creatinine content in each of the urine samples was measured with a Wako Creatinine Kit (Wako Pure Chemical Industries, Ltd., Japan), which is essentially based on the method of Bonsnes and Taussky.⁵

Analysis of Free Amino Acids

One milliliter of the red cell suspension was frozen in liquid nitrogen and thawed by water 3 times. One milliliter of ice-cold 5% sulfosalicylate was then added to the hemolysate and mixed thoroughly with a glass stick. The mixtures were centrifuged at 3000 rpm for 10 min. The plasma and urine samples were deproteinized by the addition of an equal volume of 5% sulfosalicylate, followed by centrifugation at 3000 rpm for 10 min. Deproteinized extracts of erythrocytes and the plasma and urine samples were diluted and subjected to amino acid analysis on an automatic amino acid analyzer (JLC-6AH, Nihon-Denshi Co., Ltd., Japan) using a single column (0.6 \times 45 cm) of resin (ICR-2, Nihon-Denshi) and a sodium citrate buffer elution system. The method employed was essentially based on that of Spackman et al.⁶ Elution was started with pH 3.2 buffer at 52.5°C. At approximately 85 min, the buffer was changed to pH 7.3 and the temperature to 65°C. This system, however, did not give a complete separation of glutamine, threonine, and asparagine. To measure these amino acids, both the erythrocytes and plasma extracts from dogs II-8, IV-1, and two controls were also analyzed by another amino acid analyzer (KLB-3B, Hitachi Co., Ltd., Japan) using a single column (0.9 \times 50 cm) of resin (Hitachi, 2612) with pH 2.8 lithium citrate buffer at 34.5°C.

Glutathione Assay

Total glutathione concentration was determined essentially by the method of Tietze.⁷ For determination of glutathione, 1 volume of 12% trichloroacetic acid (TCA) was added to 2 volumes of the erythrocyte suspension. After centrifugation at 1000 g for 10 min, the supernatant was washed with ether to remove TCA, the ether was evaporated under nitrogen stream, and the supernatant was divided into two portions. One portion was used for the determination of total glutathione concentration. The other portion was incubated with 1 mM N-ethylmaleimide (NEM) at 0°C for 10 min, followed by extraction with ether to remove excess NEM. Ether was again removed under nitrogen stream and the remaining solution was used for assay of oxidized glutathione (GSSG) concentration. The reaction mixtures contained 100 mM phosphate buffer with 5 mM EDTA (pH 7.5), 4 mM NADPH, glutathione reductase (6 U/ml), 10 mM 5,5'-dithiobis(2-nitrobenzoate), and TCA extract.

Enzyme Assays

Hemolysate was prepared by repeated freezing by liquid nitrogen and thawing and used for the following enzymic assays unless otherwise specified. Glutathione reductase was assayed by the method of Beutler.⁸ The reaction mixtures contained 50 mM Tris-HCl buffer (pH 8.0), 0.25 mM EDTA, 3.3 mM GSSG, 2 mM NADPH, hemolysate, and in the absence and in the presence of 10 μ M Flavin adenine dinucleotide (FAD). The activity was measured by following the oxidation of NADPH at 340 nm and expressed as units per gram hemoglobin (Hb). γ -Glutamyl cysteine synthetase was assayed by the methods described by Griffith et al.⁹ Enzymatic activity was determined by measuring the formation of L- γ -glutamyl-(¹⁴C)-L- α -amino butyrate of Pi. The reaction mixtures contained 150 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 10 mM ATP, 10 mM phosphoenolpyruvate, 25 mM MgCl₂, 10 mM glutamate, 10 mM L- α -[¹⁴C] aminobutyrate, 0.25 mM EDTA, 4 U of pyruvate kinase, and hemolysate. The enzymic activity was expressed as nmoles of Pi or L- γ -glutamyl-L- α -aminobutyrate formed per hour per gram Hb. Glutathione synthetase was assayed by Oppenheimer et al.¹⁰ The reaction mixtures contained 100 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 5 mM L- γ -glutamyl-L- α -aminobutyrate, 10 mM ATP, 5 mM [¹⁴C]-glycine, 50 mM MgCl₂, 0.5 mM EDTA, 5 mM phosphocreatinine, 0.1 U of creatine phosphokinase, and hemolysate. The activity was expressed as nmoles of [¹⁴C]-tripeptide formed per hour per gram Hb. Pyrimidine

5'-nucleotidase was assayed by the method of Paglia et al.¹¹ Reaction mixtures contained 5 mM Tris-HCl buffer (pH 8.0), 100 mM MgCl₂, 20 mM UMP, and dialyzed hemolysate.¹¹ Activity was expressed as micromoles of Pi released per hour per gram Hb. γ -Glutamyl transpeptidase activity in the plasma was determined by the method of Tate and Meister,¹² in the presence of 3 mM L- γ -glutamyl-*p*-nitroanilide, 20 mM glycylglycine, and 100 mM Tris-HCl buffer (pH 8.0). The activity was expressed as nmoles of *p*-nitroaniline released per minute per milliliter plasma. The activities of both glutathione peroxidase¹³ and γ -glutamyl cyclotransferase³ were determined as described previously.

Effects of GSH, Glutamate, and Aspartate on γ -Glutamyl Cysteine Synthetase

Inhibition of γ -glutamyl cysteine synthetase by GSH was examined by the method of Richman and Meister.¹⁴ The hemolysate was applied on a column of Sephadex G-25 (PD-10, Pharmacia, Sweden), which had been equilibrated with 100 mM Tris-HCl buffer (pH 8.2) and the desalted hemolysate was used for the following experiments. The reaction mixtures contained 100 mM Tris-HCl buffer (pH 8.2), 10 mM L-glutamate, 10 mM α -L-aminobutyrate, 1 mM ATP, 4 mM MgCl₂, 0.4 mM EDTA, 0.1% bovine serum albumin, desalted hemolysate, and reduced glutathione (GSH) (0–10 mM). The effect of glutamate, or aspartate, on the enzyme activity was examined in the reaction mixtures containing 100 mM Tris-HCl buffer (pH 8.2), 10 mM L- α -aminobutyrate, 1 mM ATP, 4 mM MgCl₂, 0.4 mM EDTA, 0.1% bovine serum albumin, 2 mM GSH, desalted hemolysate and L-glutamate (0.5–10 mM), or L-aspartate (0.5–10 mM). All reaction mixtures were incubated at 37°C for 30 min, and the Pi released was determined by the method of Fiske and SubbaRow.¹⁵

RESULTS

Free Amino Acid Concentrations

The concentrations of free amino acids in the erythrocytes, plasma, and urine from the two dogs (II-8, IV-1) with increased concentration of erythrocyte glutathione and the control dogs are shown in Table 1. A marked change observed in the erythrocytes of the two dogs was the greatly increased concentration of glutamate, aspartate, and glutamine, which were approximately 92, 63 and 13 times the mean value in the control erythrocytes, respectively. The concentration of glycine and histidine in the erythrocytes also increased slightly, while the other amino acids did not change in concentrations as compared to the controls. Dog II-6 of the family showed normal concentration of erythrocyte free amino acids (data not shown). Glutathione was present in large concentrations in the erythrocyte extracts from all of the dogs examined, while it was not determined in the plasma extracts, and it covered the peak of aspartate in the sodium citrate buffer system. In the lithium citrate buffer system, however, glutathione and aspartate were separated and formed clearly demonstrable peaks. Thus, the concentrations of total glutathione in the erythrocyte from the two dogs and two of the controls were calculated as GSH. The concentration of total glutathione in the

Table 1. Amino Acids Concentrations in Erythrocytes, Plasma, and Urine

	Erythrocytes (nmoles/ml RBC)			Plasma (nmoles/ml Plasma)			Urine (nmoles/mg of Creatinine)		
	Dog II-8	Dog IV-1	Control Dogs (n = 6) Mean \pm SD	Dog II-8	Dog IV-1	Control dogs (n = 6) Mean \pm SD	Dog II-8	Dog IV-1	Control Dogs*
Aspartate	5,043	5,007	80*	0	0	0			
Threonine	198	157	160*	200	196	332*	502	295	474
Glutamine	9,624	8,926	690*	1,092	835	985*			
Serine	326	202	164 \pm 40	108	162	210 \pm 155	444	356	407
Asparagine	Trace†	Trace	Trace	72	74	85*			
Glutamate	14,243	12,565	146 \pm 26	122	103	112 \pm 28	128	139	175
Proline	273	230	161 \pm 39	204	117	142 \pm 68	57	74	37
Glycine	329	262	157 \pm 36	164	159	277 \pm 132	151	141	244
Alanine	342	374	351 \pm 58	566	354	385 \pm 103	286	634	498
Half-cysteine	Trace	Trace	Trace	19	32	22 \pm 10	204	100	135
Valine	207	171	155 \pm 23	224	153	145 \pm 44	227	140	299
Methionine	55	60	36 \pm 52	50	44	47 \pm 15	184	149	205
Isoleucine	62	60	57 \pm 6	83	62	48 \pm 14	21	60	20
Leucine	108	87	82 \pm 18	172	123	94 \pm 28	15	16	15
Tyrosine	143	129	112 \pm 25	65	34	35 \pm 9	18	15	28
Phenylalanine	85	84	74 \pm 22	68	46	51 \pm 16	Trace	12	6
Histidine	669	404	132 \pm 16	142	72	87 \pm 21			
Lysine	486	531	381 \pm 62	202	201	168 \pm 85			
Arginine	407	340	310 \pm 61	182	98	145 \pm 63	12	27	31
Glutathione (total, as GSH)	9,958	8,368	1,438*	0	0	0			

*Values are expressed as mean of two control dogs.

†Trace amount less than 10 nmoles of amino acids.

Table 2. Glutathione Concentration and Enzyme Activities in Erythrocytes and Plasma

Glutathione and Enzymes	Dog II-8	Dog IV-1	Control Subjects (n = 6) Mean \pm SD
Erythrocytes			
Reduced glutathione (μ mole/ml RBC)	7.27	8.15	1.02 \pm 0.37
Oxidized glutathione (nmole/ml RBC)	3.53	4.43	6.86 \pm 1.74
Glutathione reductase with FAD (U/g Hb)	18.1	19.1	11.5 \pm 1.4
Glutathione reductase without FAD (U/g Hb)	9.6	12.9	8.9 \pm 1.7
Glutathione peroxidase (U/g Hb)	403	347	484 \pm 141
γ -Glutamyl cysteine synthetase (nmole/hr/g Hb)	8.8	ND*	10.0 \dagger
Glutathione synthetase (nmole/hr/g Hb)	21.3	ND	18.3 \dagger
γ -Glutamyl cyclotransferase (nmole/hr/g Hb)	21.9	ND	19.7 \dagger
Pyrimidine 5'-nucleotidase (μ mole/hr/g Hb)	10.2	ND	6.2 \dagger
Plasma			
Reduced glutathione (μ M)	0.74	1.02	0.83 \pm 0.38
γ -Glutamyl transpeptidase (nmole/min/ml)	1.37	2.00	1.21 \pm 0.08

*ND, not done.

 \dagger Values are expressed as mean of two control dogs.

erythrocytes of the dogs was about 7 times that of the control dogs. The concentrations of some amino acids in the urine, such as aspartate, which is a minor constituent of the canine urinary amino acids, glutamine, histidine, and lysine, were not determined because many ninhydrin-positive substances obscured their presence. The results for the control subjects were in agreement with other reports on free amino acid concentrations in the erythrocytes, plasma, and urine of dogs.^{16,17}

Glutathione Concentration in Erythrocytes and Plasma

The reduced glutathione concentrations measured by the enzymatic method in the erythrocytes of the two dogs were about 7 times mean values in the control erythrocytes, while the oxidized glutathione concentrations in the cells of the two dogs were almost equal to or lower than the controls. There were no changes in the concentration of plasma glutathione in the two dogs as compared to the controls (Table 2). Dog II-6 showed normal level of glutathione in the erythrocytes and plasma.

Enzyme Activities

The results of assays of the enzymes are summarized in Table 2. The activities of all the enzymes examined in the two dogs were within normal ranges, except the activity of glutathione reductase, which showed an increase when FAD was added to the reaction system.

Effects of GSH and Glutamate on γ -Glutamyl Cysteine Synthetase

The activities of γ -glutamyl cysteine synthetase in erythrocytes from II-8, IV-I, and one control dog were inhibited by 18%, 54%, and 26% in the presence of 2

mM GSH (physiologic concentration of GSH in canine erythrocytes was about 1 mM as shown in Table 2) and by 45%, 53%, and 62% in the presence of 10 mM GSH in the reaction mixtures, respectively (Fig. 2). On the contrary, γ -glutamyl cysteine synthetase was markedly activated by increasing the concentration of L-glutamate, in spite of the presence of 2 mM GSH in the reaction mixtures (Fig. 3). L-Aspartate did not affect the enzyme activity under these conditions (data not shown).

DISCUSSION

Glutamate and two of its γ -linked derivatives, glutamine and glutathione, play central roles in the

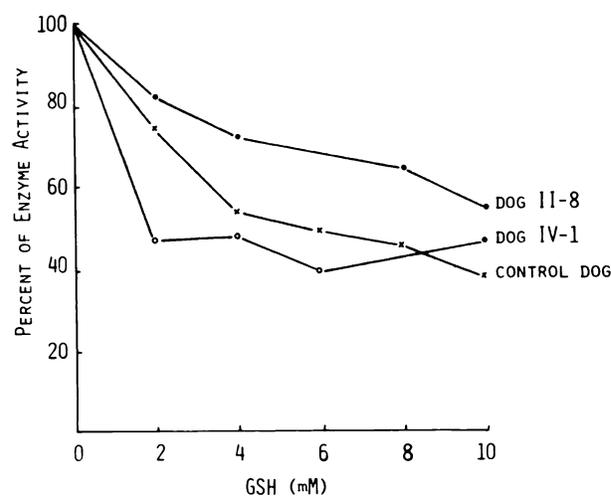


Fig. 2. Effect of GSH on γ -glutamyl cysteine synthetase activity. Reaction mixtures contained 100 mM Tris-HCl buffer (pH 8.2), 10 mM L- α -aminobutyrate, 10 mM L-glutamate, 1 mM ATP, 4 mM MgCl₂, 0.4 mM EDTA, 0.1% bovine serum albumin, desalted hemolysate, and GSH (0.5–10 mM). After incubation for 30 min, released Pi was determined as described in Materials and Methods.

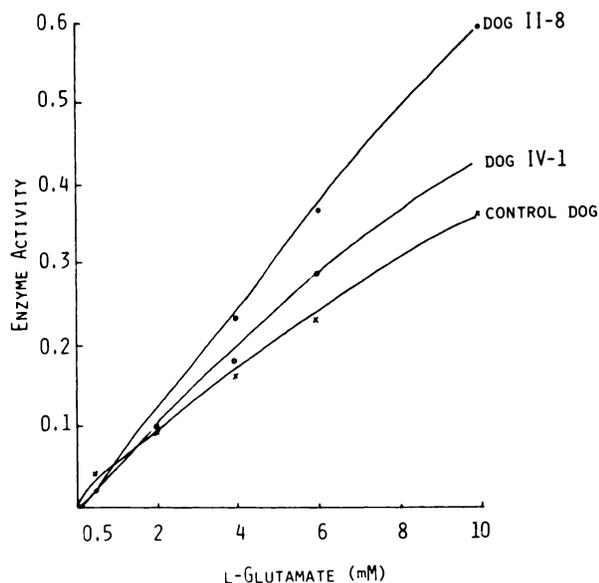


Fig. 3. Effect of L-glutamate on γ -glutamyl cysteine synthetase activity. Reaction mixtures contained L-glutamate (0.5–10 mM), 100 mM Tris-HCl buffer (pH 8.2), 1 mM ATP, 4 mM MgCl₂, 10 mM L- α -aminobutyrate, 0.4 mM EDTA, 0.1% bovine serum albumin, desalted hemolysate, and 2 mM GSH. After incubation for 30 min, released Pi was determined as described in Materials and Methods. Enzyme activity is expressed as optical density at 660 nm.

metabolism of amino acids and ammonia in many tissues.¹⁸ Both glutamate and aspartate are also highly concentrated in the mammalian central nervous system,^{19,20} and they are thought to play an important role as an excitatory neurotransmitter.^{21,22} However, the striking increase in the concentrations of such amino acids and glutathione in the erythrocytes observed in the present study has not been reported previously.

Glutathione is synthesized from glutamate together with cysteine and glycine by the catalytic action of two enzymes, γ -glutamyl cysteine synthetase and glutathione synthetase (Fig. 4), in erythrocytes as well as in many other cells of a variety of species.^{18,23} The concentration of glutathione in normal mammalian erythrocytes is maintained fairly constant²⁴ by the inhibitory action of glutathione itself to γ -glutamyl cysteine synthetase¹⁴ and by the transport of excessive glutathione to the outside of the cells,²⁵ although their mechanisms have not been clarified completely. On the other hand, human erythrocytes have the capacity to synthesize glutathione exceeding the rate of glutathione turnover,²³ which indicates that there is a considerable reserve capacity for glutathione synthesis in the cells. Thus, increased concentrations of erythrocyte glutathione can occur under a number of conditions,^{26,31} e.g., in patients with myeloproliferative syndrome²⁷ resulting from increased activity of glutathione synthetase in their erythrocytes and in rabbits

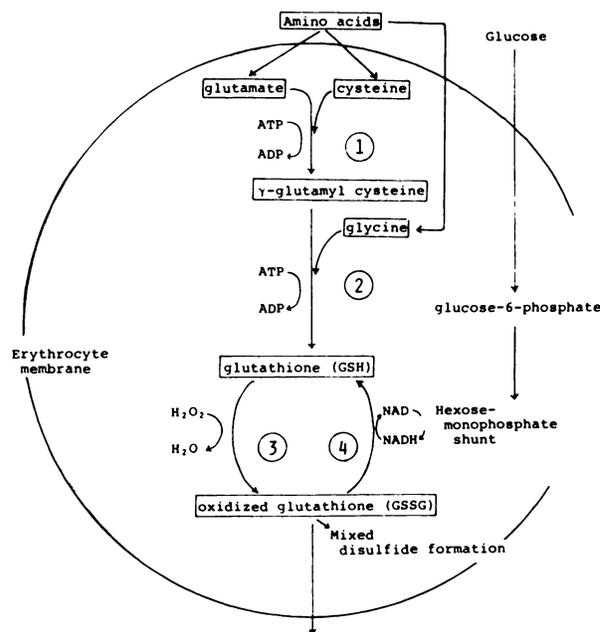


Fig. 4. An outline of glutathione metabolism in erythrocyte. (1) γ -Glutamyl cysteine synthetase; (2) glutathione synthetase; (3) glutathione peroxidase; (4) Glutathione reductase. (Adapted from a diagram by Prins and Loos.²⁶)

given diaminodiphenylsulfone or methylene blue.²⁶ Valentine et al.²⁸ observed a marked increase of glutathione concentration in human erythrocytes with a deficiency of pyrimidine 5'-nucleotidase. Kondo et al.³² suggested that the high concentration of erythrocyte glutathione in patients with pyrimidine 5'-nucleotidase deficiency was due to the inhibitory effect of the accumulated nucleotides on glutathione transport. In the present study, however, the activities of both glutathione synthetase and pyrimidine 5'-nucleotidase in the erythrocytes of a dog possessing a high concentration of erythrocyte glutathione were found to be within normal ranges. Paniker and Beutler²⁶ suggested that the increase of erythrocyte glutathione in rabbits injected with methylene blue was due to a kinetic change in the K_m of glutathione synthetase for γ -glutamyl cysteine. Smith et al.³¹ observed that the concentration of glutathione, glutamate, and pyrrolid-2-one-5-carboxylate increased in the erythrocytes when methylene blue was administered to rabbits. From this observation they suggested that changes in substrate concentration (particularly of glutamate) might be more important than enzymic changes in controlling glutathione and that the methylene-blue-induced increase in erythrocyte glutathione was due to the drug's effect on glutamate concentration. Nevertheless, they could not explain how methylene blue increased the concentration of erythrocyte glutamate. Under normal conditions, glutathione is believed to

exert a feedback inhibition on γ -glutamyl cysteine synthetase that catalyzes the initial step of glutathione biosynthesis from glutamate in the γ -glutamyl cycle.¹⁸ If glutathione fails to inhibit the enzyme, there is a marked accumulation of intracellular glutathione. In the present study, the activity of γ -glutamyl cysteine synthetase in the erythrocytes of the two dogs as well as that of the normal dog was inhibited apparently by glutathione. On the other hand, the inhibition of the enzyme produced by the glutathione was overcome by increasing the concentration of L-glutamate in the reaction system, which was in good agreement with the results of Richman and Meister.¹⁴ Although several factors may account for this abnormal concentration of erythrocyte glutathione in the present cases, the most

conceivable one is that increased concentrations of glutamate stimulated the increase of glutathione concentration in the cells. Although we cannot explain at present why the levels of glutamate, glutamine, and aspartate became so extremely elevated in the erythrocytes, further studies should lead to a greater understanding of amino acid metabolism within erythrocytes and the role of glutathione in amino acid transport across the erythrocyte membrane.^{33,34}

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