

**Calcium and Magnesium Contents of Mammalian Erythrocyte Membranes<sup>1)</sup>**

TATSUZO FUJII, TAKASHI SATO, and TAKASHI HANZAWA

*Faculty of Pharmacy, Meijo University<sup>2)</sup>*

(Received July 3, 1972)

Calcium and magnesium contents of whole erythrocytes and of erythrocyte membranes (stroma) were determined by atomic absorption technique on five mammalian species, man, rabbit, pig, cow and dog.

In every species, the magnesium content of the whole erythrocyte is predominantly higher than the calcium content. Of the total magnesium, about 2—6% are localized in the membrane, whereas 40—86% of the whole cell calcium are found in the membrane. Except that the magnesium content of bovine erythrocyte is remarkably lower (about 1/7) than those of the other species, there seems to be no big species difference in these cation contents in the whole erythrocytes.

The amounts of calcium localized in the membrane were found to be in a range of  $1.5\text{--}2.6 \times 10^{-18}$  mole per cell. The membrane magnesium contents vary considerably among the species examined and fall in a range of  $1.6$  (cow) —  $12$  (dog)  $\times 10^{-18}$  mole per cell, with the intermediate values on the other three species of  $4.0$ ,  $4.1$  and  $5.6 \times 10^{-18}$  mole per cell. The membrane Mg/Ca molar ratios range between 0.65—6.5.

Intracellular concentrations of calcium and magnesium were calculated from the differences between the amounts in the whole cell and those in the membrane, assuming that no release of these cations occurs in the course of preparation of stroma.

It is a well-known fact that divalent cations, such as calcium and magnesium ions, play important roles in the cellular membranes, but informations concerning the amounts of these cations present in association with membranes are rather scanty. Recently, several reports were published on the detailed investigations on the amounts of these ions bound to human erythrocyte membranes.<sup>3)</sup> However, with reference to the divalent cation contents of the erythrocyte membranes of the other mammalian species, practically no report is available, at least as far as known to the present authors. Considering a fact that the chemical composition and certain properties of the erythrocyte membranes vary considerably depending on the animal species examined,<sup>4)</sup> it seems to be of particular interest to clarify the possible species differences, if any, in the amounts of these physiologically important cations present in the membranes as well as in the intracellular fluids of mammalian erythrocytes in general. The present investigation was undertaken along this line.

**Experimental**

**Preparation of Washed Erythrocyte Suspension and Erythrocyte Stroma**—Freshly-drawn blood, with heparin added as anticoagulant, was centrifuged at  $900 \times g$  for 15 minutes. After removing the plasma and buffy coat, the precipitated red blood cells were washed three times with physiological saline, and resuspended in the saline. The hematocrit value was determined by a conventional method.

- 1) Part of the present report was presented at the 91st Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, April 1971, and also at the General Meeting of Tokai Branch of the same society, Nagoya, November 1971.
- 2) Location: *Yagoto Urayama 15, Tenpakucho, Showaku, Nagoya.*
- 3) a) D.G. Harrison and C. Long, *J. Physiol.* (London), **199**, 367 (1968); b) C. Long and B. Mouat, *Biochem. J.*, **123**, 829 (1971); c) J. Forstner and J.F. Manery, *Biochem. J.*, **124**, 563 (1971); d) J. Forstner and J.F. Manery, *Biochem. J.*, **125**, 343 (1971).
- 4) L.L.M. van Deenen and J. de Gier, in "The Red Blood Cell," ed. C. Bishop and D.M. Surgenor, Academic Press, New York, 1964, p. 243.

The washed erythrocytes were hemolyzed in about 20 volumes of hypotonic veronal buffer (30 imOsM, pH 7.4). The resulting stroma were spun down at  $15\ 600\times g$  for 30 minutes and washed with the same hypotonic solution. This procedure was repeated three times to give hemoglobin-free stroma.<sup>5)</sup>

**Determination of Calcium and Magnesium in the Whole Erythrocytes and in the Stroma**—Erythrocyte suspension, equivalent to about 1 ml of the packed cells, or the stroma suspension containing the stroma from 2.5 ml of packed cells or about 6.3 mg of the stromal protein, were digested with perchloric acid for 1.5—2 hours after evaporated to dryness. The digestate was diluted with water to make the total volume of 5—10 ml, with an addition of  $\text{SrCl}_2$  in a final concentration of 0.03 mole/liter. Calcium and magnesium were determined by atomic absorption spectrophotometry at the wave length of 4227 and 2852 Å, respectively, by means of Hitachi model 208 atomic absorption spectrophotometer.

The analysis repeated 10 times on the same erythrocyte suspension gave the value of coefficient of variation of less than 10% for both calcium and magnesium (for example: mean value  $\pm$  standard deviation for calcium in  $\mu\text{g}$  per ml packed cell was  $1.58 \pm 0.15$ ). Therefore, the method was considered to be applicable practically.

**Result and Discussion**

**Calcium and Magnesium Contents of the Whole Erythrocytes**

Graphs in Fig. 1 show calcium and magnesium contents per cell of man, rabbit, pig, cow and dog erythrocytes. The calcium contents are relatively similar each other among all these mammalian species examined, being in a range of  $0.8\text{--}1.6 \times 10^{-16}\text{g/cell}$ . The situation is, however, different with the magnesium content. The amount of magnesium in cow erythrocyte is exceptionally low ( $7.3 \times 10^{-16}\text{g/cell}$ ) in comparison with the other four species which contain about  $52\text{--}58 \times 10^{-16}\text{g}$  of magnesium per cell. Such result confirms the pre-

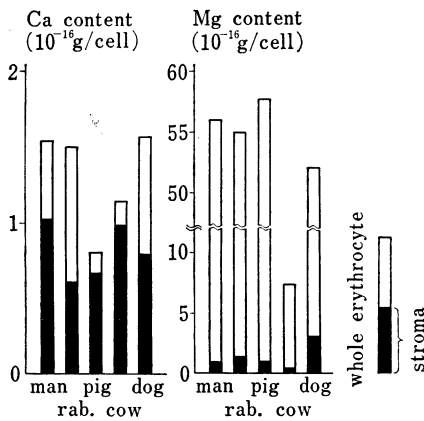


Fig. 1. Calcium and Magnesium Contents of Whole Erythrocyte and of the Membrane (stroma)  
rab.=rabbit

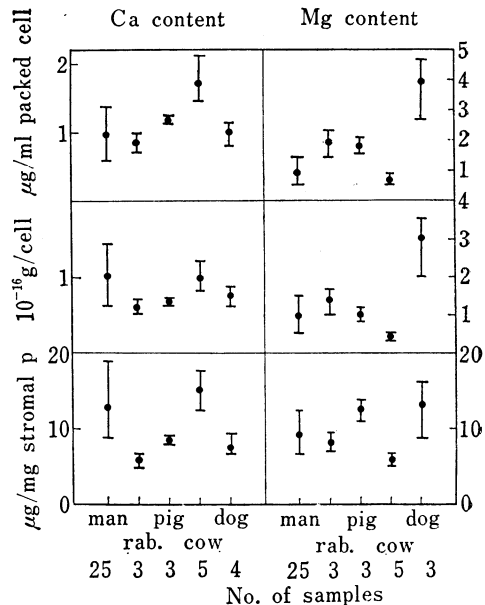


Fig. 2. Calcium and Magnesium Contents per Different Basis of Erythrocyte Membrane (stroma)  
range: |—|; rab.=rabbit  
mean value: ●

5) J.T. Dodge, C. Mitchell, and D.J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).

vious finding reported by Burger, Fujii and Hanahan<sup>6)</sup> that bovine erythrocyte contains remarkably low amount of magnesium in comparison with human erythrocyte.

The values in Fig. 1 are the means of the data on 3 different individuals, except those for human erythrocyte on which analysis was performed on 6 different samples. The data on human erythrocyte thus fall in the following ranges:

Ca  $1.17-1.79 \times 10^{-16}$ g/cell, or  $1.12-1.71$   $\mu$ g/ml packed cell.

Mg  $45.9-69.8 \times 10^{-16}$ g/cell, or  $43.9-66.7$   $\mu$ g/ml packed cell.

According to Harrison and Long,<sup>3-a)</sup> the corresponding values are:

Ca  $0.198-1.072$   $\mu$ g/ml cell (on 15 individual specimen), mean value 0.634

Mg  $58.1-118.2$   $\mu$ g/ml cell (on 10 individual specimen), mean value 79.7

It is understood that the values on human erythrocytes obtained by the present authors are appreciably higher on calcium, and lower on magnesium than those reported by Harrison and Long. The cause of such disagreement is unknown.

### Calcium and Magnesium Contents of Erythrocyte Membranes (Stroma)

In Fig. 1, the black bar represents the amount of the divalent cation under examination present in erythrocyte membrane (stroma). Thus it is clear that about 40—86% of the whole cell calcium are localized in the membrane, whereas only 2—6% of the total magnesium are found in the membrane. More detailed informations on the amounts of these cations present in association with the membranes are presented in Fig. 2 which shows the calcium and magnesium contents per different basis, namely per ml packed cell,<sup>7)</sup> per cell and per mg stromal phosphorus (indicative of the membrane phospholipids), and also as the mean values and the ranges

Though the interpretation of the results is somewhat different with the different basis employed, still we have not too much difficulty to reveal the general tendency in the distribution of these divalent cations in erythrocyte membranes. Thus, it is evident that the erythrocyte membrane calcium contents are relatively uniform in every mammalian species examined, with the mean values between  $0.6-1.0 \times 10^{-16}$ g/cell,  $0.8-1.7$   $\mu$ g/ml packed cell or  $6-15$   $\mu$ g/mg stromal phosphorus. On the contrary, the membrane magnesium contents vary considerably, from the lowest extreme (cow erythrocyte) through the highest extreme (dog erythrocyte), giving the range of values of  $0.4-3.0 \times 10^{-16}$ g/cell,  $0.7-3.9$   $\mu$ g/ml packed cell or  $6-13$   $\mu$ g/mg stromal phosphorus.

TABLE I. The Amounts of Calcium and Magnesium in the Membrane (Stroma) and Intracellular Fluid of Mammalian Erythrocytes

Species	Content in the membrane (stroma)			Intracellular concentration <sup>a)</sup>		
	Ca mole/cell	Mg mole/cell	Mg/Ca molar ratio	Ca mole/liter	Mg mole/liter	Mg/Ca molar ratio
Man	$2.57 \times 10^{-18}$	$3.99 \times 10^{-18}$	1.6	$12.2 \times 10^{-6}$	$21.6 \times 10^{-4}$	177
Rabbit	1.50	5.64	3.8	30.9	30.5	99
Pig	1.65	4.07	2.5	6.70	42.0	627
Cow	2.45	1.60	0.65	7.22	4.98	69
Dog	1.93	12.5	6.5	25.9	26.1	101

a) The intracellular concentration was calculated from the differences between the amount in the whole cell and that in the membrane (stroma).

From the above-mentioned values, the average calcium and magnesium contents in mole/cell basis are calculated on each species and are presented in Table I. In addition, Mg/Ca

6) S.P. Burger, T. Fujii, and D.J. Hanahan, *Biochemistry*, **7**, 3682 (1968).

7) The numbers of erythrocytes contained per ml packed cell for man, rabbit, pig, cow and dog are 0.956, 1.39, 1.81, 1.75 and  $1.30 \times 10^{10}$ , respectively.

molar ratios are shown there. In this way, the erythrocyte membrane calcium is found to be present in an order of  $1.5\text{--}2.6 \times 10^{-18}$  mole per cell, while the membrane magnesium is in a wider range of  $1.6$  (cow)— $12$  (dog)  $\times 10^{-18}$  mole per cell, with intermediate values on the other three species being  $4.0$  (man),  $4.1$  (pig), and  $5.6$  (rabbit). In consequences, the Mg/Ca molar ratios in the mammalian erythrocyte membranes range between  $0.65\text{--}6.5$ .

The big differences in the erythrocyte membrane magnesium contents among the five mammalian species under investigation can not be ascribed to the differences in the intracellular magnesium concentrations which will be described later (Table I), because the dog erythrocyte, of which membrane has markedly higher Mg content, contains only an intermediate level of the intracellular magnesium ions, although the bovine erythrocyte with very low membrane magnesium also has very low intracellular magnesium concentration. It might be possible that the membrane magnesium contents are related to the phosphatidylcholine contents of the membranes, considering the findings reported previously that the dog erythrocyte contains relatively larger percentage (47%) of this phospholipid per total phospholipid basis and the cow erythrocyte contains very little or no phosphatidylcholine,<sup>8,9</sup> while the erythrocyte membranes of the other three species contain the intermediate percentages of this phospholipid (23—34%). Of course, it is equally possible that such a parallelism is only a matter of accidental happening.

#### Concentrations of Calcium and Magnesium in the Intracellular Fluid of Erythrocytes

Intracellular concentrations of these two divalent cations were calculated by the differences between the amounts in the whole cell and those in the membrane (stroma), assuming that no release of these cations occurs in the course of hypotonic hemolysis procedures. As shown in Table I, the calcium concentrations vary considerably among the mammalian species examined, ranging from  $6.7\text{--}31 \times 10^{-6}$  mole/liter. As to the value on human erythrocyte, Schatzman and Vincenzi<sup>10</sup> already reported a value of  $4.1 \times 10^{-5}$  mole/liter, which is approximately three times higher than our data ( $1.2 \times 10^{-5}$  mole/liter). Such discrepancy might be due to difference in the metabolic conditions of the erythrocyte preparations employed, because intracellular calcium concentration tends to be increased remarkably in energy-depleted state of the erythrocytes, as demonstrated, for example, by Weed, LaCelle and Merrill.<sup>11</sup>

In contrast to the intracellular calcium, the erythrocyte intracellular magnesium concentrations are distributed in relatively narrow range, namely  $22\text{--}42 \times 10^{-4}$  mole/liter, except a value of  $5 \times 10^{-4}$  mole/liter for bovine red blood cell.

The Mg/Ca molar ratio in the intracellular fluid of each mammalian species is also presented in Table I. It is interesting to note that these values are not too much variable among the species (69—177) with an exception of swine erythrocyte (627). It may be supposed that the relative concentration of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the intracellular fluid of erythrocyte is maintained at rather an uniform level in all four mammalian species investigated, except in the case of swine erythrocyte which contains the lowest concentration of calcium and the highest concentration of magnesium, giving the exceptionally high intracellular Mg/Ca molar ratio.

It is to be worth of note that the above-mentioned species differences can not be referred to the known species differences in the mono-valent cation concentrations. As is well known, the cow and dog erythrocytes are  $\text{Na}^+$ -rich cells which almost lack  $\text{Na}^+\text{--K}^+$ -sensitive ATPase activity and have high  $\text{Na}^+$  and low  $\text{K}^+$  concentrations equal to those in the surrounding plasma, whereas the other three species have  $\text{K}^+$ -rich erythrocytes which have a marked concentration gradient on these mono-valent cations between the intra- and extracellular fluid,<sup>4</sup> as are the cases for the tissue cells in general. The reasons why bovine erythrocyte has ex-

8) G.J. Nelson, *Biochim. Biophys. Acta*, **144**, 221 (1967).

9) T. Sato and T. Fujii, *Chem. Pharm. Bull.* (Tokyo), **19**, 377 (1971).

10) H.J. Schatzman and F.E. Vincenzi, *J. Physiol.* (London), **201**, 369 (1969).

11) R.I. Weed, P.L. LaCelle, and E.W. Merrill, *J. Clin. Invest.*, **48**, 795 (1969).

exceptionally low magnesium ion concentration and also why swine red blood cell has exceptionally high intracellular Mg/Ca ratio, are complete obscure at the present time. These problems will be reinvestigated by the direct determination of these cations in the erythrocyte intracellular fluid to be performed in near future in our laboratory.

#### **Considerations on the Possible Correlationship between the Calcium and Magnesium Contents and the Membrane Stability**

It was reported previously that acetylcholinesterase of bovine erythrocyte is easily released from the erythrocyte membrane by repeated washing with hypotonic or hypertonic buffer and is recovered in a form of lipoprotein particles, whereas no such a release occurs in the case of human erythrocytes, and also that the divalent cations such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in very low concentrations is effective in preventing the release.<sup>6)</sup> It was then suspected that such an instability of bovine erythrocyte membrane is connected in some way to the remarkably low magnesium content of the whole erythrocyte actually determined, in comparison with the human erythrocyte.<sup>6)</sup> It was later demonstrated, however, that swine erythrocyte behaves just like the bovine cell in this respect,<sup>12)</sup> while the dog erythrocyte just like human cell.<sup>13)</sup> Considering these results and the results obtained in the present study, the species differences in the above-mentioned erythrocyte membrane stability can not be traced back to the differences in the divalent cation contents of the erythrocyte membrane or of the intracellular fluid. Namely, in any sense, no common pattern in calcium or magnesium content or concentration, characteristic to both cow and pig erythrocytes on one hand and to man, rabbit and dog erythrocytes on the other hand, is recognizable. It might be possible that certain type of the membrane-bound calcium or magnesium (for example, the cation bound to particular proteins or phospholipids, the bound cation exposed to the outer membrane surface, *etc.*) is responsible for keeping the stability of the membranes. Such possibility is now under examination in our laboratory.

12) T. Fujii, Y. Komatsu, and M. Murofushi, *Chem. Pharm. Bull. (Tokyo)*, **19**, 2325 (1971).

13) D.J. Hanahan, in "The Red Blood Cell," ed. C. Bishop and D.M. Surgenor, Academic Press, New York, 1964, p. 89.