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Binding of Calcium and Magnesium Ions to Human Erythrocyte Membranes[1]

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Binding of calcium and magnesium ions to human erythrocyte membrane was investigated by determination of these cations by means of atomic absorption spectrophotometry. Roles of the membrane components in the binding were also studied.

Mean Ca and Mg contents of the erythrocyte stroma are 22 and 40 *n* moles, respectively, per stroma equivalent to 1 ml of the packed erythrocytes. About 60 and 40% of the membrane Ca are associated respectively with the outer and inner surface of the membrane, whereas about 15% of the membrane Mg are burried in the intra-membrane region and the remaining 85% are loosely adsorbed onto the membrane surfaces which are removable by simple saline washes.

The erythrocyte stroma can bind large amounts of Ca and Mg additionally from the surrounding medium. These "exogenous" divalent cations are, however, easily removed by simple saline washes and the binding seems to be non-specific with respect to the ion species. At least three different kinds of sites each are concerned in the binding of Ca or Mg to the membrane.

Membrane lipids, in addition to membrane proteins, are thought to play important roles in the association of the firmly-bound Ca (Ca originally present) in the membrane, but not so much in the additional binding of Ca from the medium.

Many previous workers reported the content of calcium in human erythrocyte using various techniques but their results are not necessarily in good agreement, mainly due to the insensitive or non-specific methods employed. Recently, however, Harrison and Long[3] have established, using atomic absorption spectrophotometry, the calcium content in human erythrocyte to be 15.8 μ moles per liter of the packed cells, which are almost exclusively located in the membrane. Long and Mouat[4] demonstrated that human erythrocyte can bind a maximum of 400 μ moles of calcium per liter of the packed cells in iso-osmotic sucrose medium and the cell ghost about 1000 μ moles per liter of the packed cell equivalent. They further showed that such binding of calcium is greatly influenced by the ionic strength of the surrounding medium. Forstner and Manery[5] reported that almost 80% of the additionally-bound calcium are associated with the protein and 20% with the lipid component of the erythrocyte membrane as demonstrated by radio-isotope tracer technique.

In the preceding paper,[6] the present authors reported the calcium and magnesium contents of whole erythrocyte and the stroma of five representative mammalian species including human. It was thus disclosed that, of the total calcium, 40—86% are located in the membrane, whereas only 2—6% of the total magnesium are present in the membrane. In every species examined, calcium content of the erythrocyte membrane fell in a range of $1.5—2.6 \times 10^{-18}$ moles per cell, but the magnesium content varied considerably ($1.6—12 \times 10^{-18}$ moles per cell).

1) Part of the present report was presented at the 91st Annual Meeting of the Japanese Pharmaceutical Society, Fukuoka, April 1971, and also at the 45th Annual Meeting of the Japanese Biochemical Society, Tokyo, November 1972.

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3) D.G. Harrison and C. Long, *J. Physiol.* (London), **199**, 367 (1968).

4) C. Long and B. Mouat, *Biochem. J.*, **123**, 829 (1971).

5) J. Forstner and J.F. Manery, *Biochem. J.*, **124**, 563 (1971).

6) T. Fujii, T. Sato and T. Hanzawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 171 (1973).

In the present paper, binding of calcium and magnesium ions to human erythrocyte membrane is more thoroughly investigated, as an approach to the elucidation of the physiological roles of these divalent cations in the erythrocyte membranes.

Experimental

Standard Solutions——Calcium- and magnesium-free water was prepared by distillation in an all-grass apparatus and then by passage through an ion-exchanger, and was used throughout the experiments.

A stock solution of calcium or magnesium standard was prepared by dissolving $CaCO_3$ or Mg metal in a slight excess of HCl and diluting with water to give final concentration of 1 mg Ca or 0.1 mg Mg per ml. The working standard solution was prepared by diluting this stock solution with water containing 0.03M $SrCl_2$ and 7% of $HClO_4$ to give final concentration of 0.4 μg Ca or 2.0 μg Mg per ml.

Determination of Calcium and Magnesium in Erythrocyte and the Stroma[6]——Erythrocyte suspension, equivalent to about 1 ml of the packed cells, or stroma suspension prepared from 2.5 ml of packed cells, was dried under air stream and digested with perchloric acid. The digestate was diluted with water containing 0.03M $SrCl_2$ to make the total volume of 5—10 ml. Calcium and magnesium were determined by atomic absorption spectrophotometry at the wave length of 422.7 and 285.2 nm, respectively, by means of Hitachi model 208 atomic absorption spectrophotometer.

Preparation of Washed Erythrocyte Suspension and the Stroma——Freshly-drawn ACD-blood 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 isotonic saline and resuspended in the saline.

These washed erythrocytes are hemolyzed in 20 volumes of hypotonic veronal buffer (30 imOsM, pH 7.4) and the stroma spun down at $15600 \times g$ for 30 minutes were further washed three times with the same hypotonic buffer to obtain hemoglobin-free stroma, according to the method of Dodge, Mitchell and Hanahan.[7]

Binding of Externally-added Calcium or Magnesium to Erythrocyte or the Stroma——Erythrocytes were treated at 4° for 15 minutes with an isotonic NaCl solution buffered with Tris, pH 7.4, containing 10 mM $CaCl_2$ or $MgCl_2$, and then washed twice with the buffered isotonic saline without the divalent cations. Stroma were treated at 4° for 15 minutes with hypotonic veronal buffer (30 imOsM, pH 7.4) containing 10 mM $CaCl_2$ or $MgCl_2$ and then washed twice with the hypotonic buffer without the divalent cations.

Removal of Calcium or Magnesium Ions from Erythrocyte or the Stroma——Erythrocytes were treated in a similar way as above with buffered isotonic saline containing 5 mM EDTA and then washed to remove EDTA. The stroma were treated with buffered hypotonic saline containing 5 mM EDTA and then washed.

Extraction of Lipids from the Stroma——Stroma prepared from 2.5 ml of packed erythrocytes were extracted twice with chloroform–methanol (1: 1, by volume) according to the method of Burger, Fujii and Hanahan[8] and the insoluble residue was collected by centrifugation. The combined extracts were evaporated to dryness and the residue was re-extracted with chloroform–methanol (2: 1, by volume). The extract was washed with Ca–Mg-free water and the organic phase was taken as the lipid fraction and the aqueous phase plus the residual portion of the first extraction as the non-lipid fraction.

Result and Discussion

The Amounts of Calcium and Magnesium originally Present in Erythrocyte Membrane

The amounts of calcium and magnesium detected in the stroma from intact human erythrocytes are shown in Fig. 1. The mean values±S.D. of the membrane-bound Ca and Mg, derived from 35 specimen of normal human erythrocytes, are 21.6±6.65 (range; 10.5—34.2) and 40.4±11.23 (range; 22.2—58.4) *n* moles per stroma equivalent to 1 ml of the packed erythrocytes, respectively.

In order to remove the divalent cations exposed on the outer surface of the erythrocyte membrane, the intact cells were washed with iso-osmotic NaCl containing 5 mM EDTA (pH 7.4) and stroma were prepared from these treated cells. Determination of Ca and Mg in the "stroma from EDTA-treated cells" revealed that about 60% of calcium and 20% of magnesium were removed by such treatment.

Stroma prepared from intact cells were treated with EDTA-containing medium. It is expected in this case that all the divalent cations exposed on either side of the membrane

7) J.T. Dodge, C. Mitchell and D.J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
8) S.P. Burger, T. Fujii and D.J. Hanahan, *Biochemistry*, **7**, 3682 (1968).

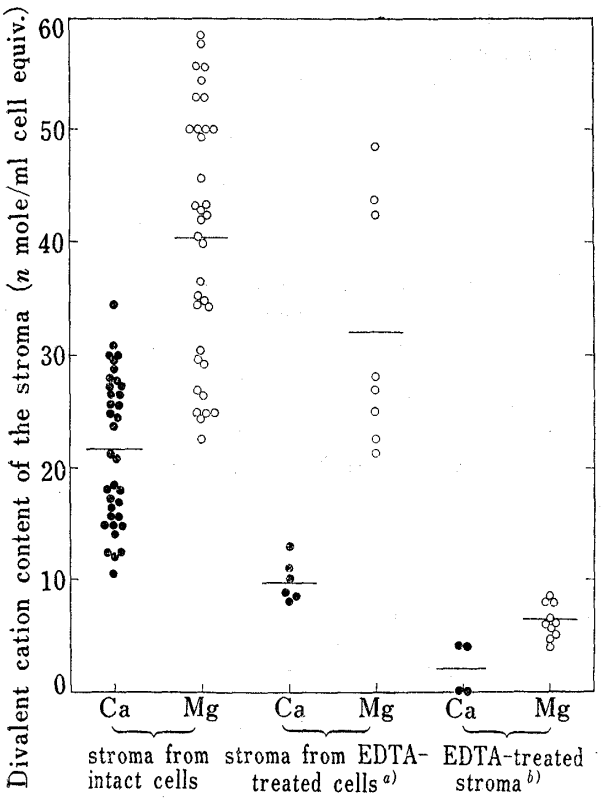

Fig. 1. Calcium and Magnesium Contents of Human Erythrocyte Stroma

The bar indicates the mean value.
a) Erythrocytes were washed with 3-fold volumes of isotonic NaCl, pH 7.4, containing 5mM EDTA. From these erythrocytes, stroma were prepared as usual.
b) Stroma were prepared from intact erythrocytes and were then washed with 20-fold volumes of hypotonic veronal buffer (30 imOsM), pH 7.4, containing 5 mM EDTA.

surfaces (outer and inner surfaces) are removed by this chelating agent which is permeable through the stroma but not through the intact erythrocyte membrane. Indeed, as is clear from Fig. 1, the "EDTA-treated stroma" have lost almost all of the membrane-bound Ca and about 85% of the membrane-bound Mg by such a treatment. Because a definite amount (15% of the total) of Mg still remains in such stroma preparation, this amount seems to correspond to those present in some intra-membrane region, not exposed on the membrane surfaces.

From the above data, it may be concluded that about 60% and 30—40% of the membrane Ca are associated respectively with the outer and inner surface of human erythrocyte membrane, whereas only about 20% of the magnesium are bound to the outer surface, another 15% burried in the membrane interior and the remaining 65% bound to the inner surface. To derive such a conclusion, we need to assume that EDTA molecule does not penetrate into intracellular space of the erythrocyte through the membrane pores. However, even if a small amount of EDTA molecules do penetrate through the intact membrane, they must immediately form a chelate compound with the magnesium ions present abundantly in the intracellular space[6] and therefore will hardly be able to act on the divalent cations bound to the inner surface of the membrane.

As will become evident later from Table II, when the stroma from intact cells are repeatedly washed with buffered isotonic saline, none of the membrane Ca is removed but approximately 80% of the membrane Mg are removed which almost correspond to the amount removable by EDTA washes. Therefore, we consider that the magnesium ions present on both surfaces of the membrane are only loosely adsorbed there, in contrast with the calcium ions on the surfaces which are relatively firmly bound and are only removed by an action of a chelating agent.

The Amounts of Calcium and Magnesium additionally Bound to Erythrocyte Membrane

When intact erythrocytes are incubated with buffered isotonic saline containing 10 mM $CaCl_2$, calcium content of the cells increases from 36.7 to 61.4 *n* moles per ml of the packed cells, as indicated in Table I. The difference means the amount of Ca additionally bound to the membrane from the surrounding medium. This amount of Ca is, however, completely removed by the subsequent EDTA wash. Thus, we can see this kind of Ca ions binds to the membrane outer surface.

As to the magnesium ions, the amount additionally bound to the membrane by a similar treatment could not be detected by the present method of determining the divalent cations in the digestate of the whole erythrocytes, owing to the fact that the whole erythrocytes contain predominantly large amount of intracellular magnesium ions in comparison with

Table I. Additional Binding of Calcium Ions to Intact Human Erythrocyte

Treatments of intact cells			Calcium content of the cells (n mole/ml cells)
1st Cells are treated with:	2nd	3rd	
No treatment (intact cells)			36.7
10 mM $CaCl_2$ in saline[a)]	saline	saline	61.4
10 mM $CaCl_2$ in saline	5 mM EDTA in saline	saline	31.7

a) Isotonic NaCl solution buffered with Tris (pH 7.4).

the amount bound to the membrane, as already disclosed by the preceding paper.[6)]

When the stroma are treated in a similar way with external Ca or Mg, they can bind far greater amount of the respective ion than the whole erythrocyte, because in the case of the stroma even the inner surface of the membrane is accessible to the divalent cations added to the external medium. An alternative explanation is that certain conformational change might occur in the course of hypotonic hemolysis to produce stroma from the intact membrane of the erythrocyte. Anyway, as is clear from Fig. 2, the stroma can bind large and variable amounts of the divalent cations from the surrounding medium, the amounts being dependent upon the ionic strength of the surrounding medium.[4)]

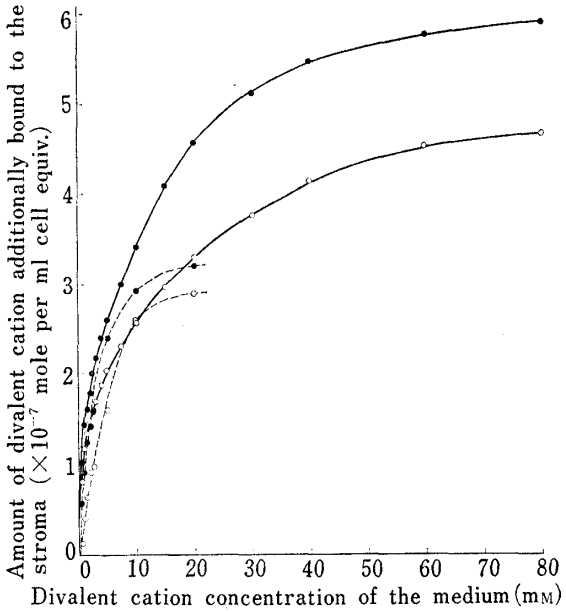

Fig. 2. Effect of the Medium Calcium or Magnesium Concentrations on Their Binding to Human Erythrocyte Stroma

● : calcium, ○ : magnesium, } isotonic medium, } hypotonic medium

The figure indicates that in the isotonic medium, the maximal binding of Ca or Mg takes place at the medium Ca or Mg concentration of about 20 mM, while in hypotonic medium it takes place at about 60—80 mM. These maximal amounts of additionally-bound Ca and Mg are 322 and 290 in isotonic medium and 592 and 466 n moles per stroma equivalent to 1 ml of the cells in hypotonic medium, respectively. These amounts of Ca correspond to approximately 15 times (in isotonic medium) and 27 times (in hypotonic medium) the amount originally present in the erythrocyte membrane. If incubated in salt-free medium such as in sucrose solution, the stroma should bind far more amount of Ca from the medium, as reported by Long and Mouat[4)] (about 60 times the amount originally present), but no experiment in such a medium was conducted in the present study.

Characteristics of the Association of Exogenous Calcium or Magnesium with the Stroma

The nature of the association of external medium Ca or Mg with the erythrocyte stroma were investigated in more detail as follows. Table II shows that the binding of both exogenous Ca and Mg is weak and almost non-specific. When the stroma are treated with buffered isotonic saline containing 10 mM $CaCl_2$ or 10 mM $MgCl_2$, the Ca and Mg content of the stroma increased from 17.0 to 290 and 42.8 to 286 n moles per stroma equivalent to ml cells. Upon re-treatment of such stroma preparation with simple saline, the divalent cation content was

reduced almost to the original level (22.7 and 34.1 for Ca and Mg, respectively), indicating that the divalent cations once bound were then replaced by high concentration of the monovalent cation in the medium. Similar replacement was also demonstrated by the re-treatment of the stroma having increased Ca or Mg content with the other kind of the divalent cations (Ca-stroma with Mg^{2+} and Mg-stroma with Ca^{2+}), as shown in Table II.

Table II. Removal of Additionally-bound Calcium or Magnesium from Human Erythrocyte Stroma by Washing with Salt Solution

Treatments of stroma[a]		Divalent cation content of the stroma (n mole/stroma equiv. to ml cells)	
1st	2nd		
Stroma are treated with:		Ca	Mg
No treatment (intact stroma)		17.0	42.8
Saline[b]	—	18.5	8.2
5 mM EDTA in saline	saline	1.0	6.2
10 mM $CaCl_2$ in saline	—	290	7.4
10 mM $MgCl_2$ in saline	—	17.5	286
10 mM $CaCl_2$ in saline	saline	22.7	
10 mM $MgCl_2$ in saline	saline		34.1
10 mM $CaCl_2$ in saline	10 mM $MgCl_2$ in saline	17.2	275
10 mM $MgCl_2$ in saline	10 mM $CaCl_2$ in saline	283	10.7

a) After each treatment, stroma were washed twice with divalent cation-free hypotonic veronal buffer.
b) Isotonic NaCl solution buffered with Tris (pH 7.4).

It is now clear that the nature of additional binding of the external divalent cation to erythrocyte stroma is quite different from the binding of the "endogenous" calcium originally present in the erythrocyte membrane. As indicated in the upper part of the Table II, the latter type of calcium ions are not removed by simple saline washes and can be removed only

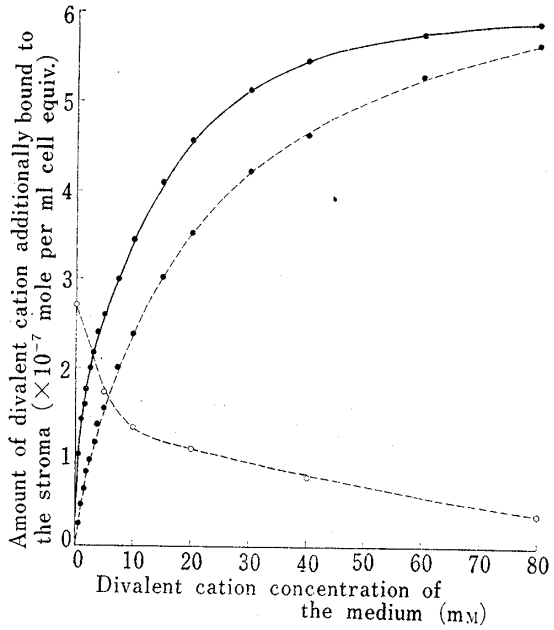

Fig. 3. Inhibitory Effect of Magnesium on Additional Binding of Calcium to Erythrocyte Stroma

—●—: calcium binding in the absence of $MgCl_2$ in medium
···●···: calcium binding in the presence of 10 mM $MgCl_2$
···○···: magnesium binding in the presence of 10 mM $MgCl_2$

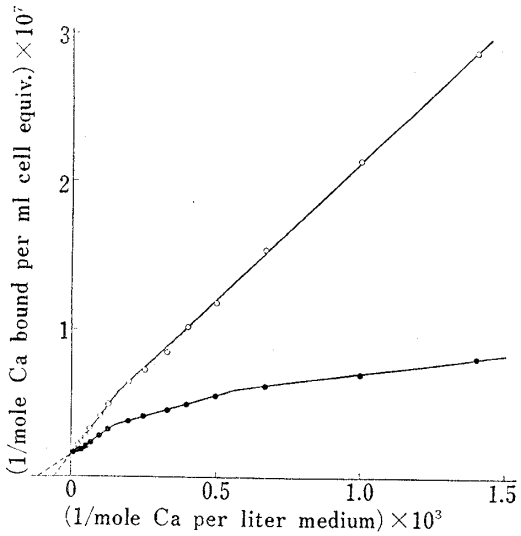

Fig. 4. Double Reciplocal Plot of Additionally-bound Calcium of the Stroma *versus* Calcium Concentration of the Medium (Data of Fig. 3 are re-plotted.)

—○—: in the presence of 10 mM $MgCl_2$
—●—: in the absence of $MgCl_2$ in medium

by chelating action of EDTA. As already referred to, the magnesium ions detected in the stroma are similar to those bound additionally from exogenous source in their affinity with the membrane binding sites (easily removable by simple saline washes). Rather, these Mg ions are said to represent those temporarily adsorbed onto the membrane from the intracellular fluid and remained there even in the course of hemolysis and the subsequent hypotonic washes.

Fig. 3 shows the inhibitory effect of Mg ions on Ca binding and of Ca ions on Mg binding of the stroma. The stroma were incubated with hypotonic veronal buffer containing $CaCl_2$ in varied concentrations between 0 and 80 mM in the absence or presence of 10 mM $MgCl_2$. In every concentration of the medium calcium, inhibitory effect of Mg ions on the Ca binding is notable. Similarly, binding of Mg in the medium was markedly affected as the result of increasing calcium concentration of the medium. Thus, under co-existence of the same concentration (10 mM) of $MgCl_2$ and $CaCl_2$, the Ca and Mg binding was decreased by about 30 and 51%, respec tively.

The data of the upper two curves of Fig. 3, namely those for Ca binding in the absence or presence of medium Mg, are re-plotted in the double-reciprocal plot as presented in Fig. 4. The graph clearly indicates that Mg in the medium inhibited competitively the Ca binding of the membrane.

Using the data of the binding in hypotonic medium in Fig. 2, Scatchard plots[9] were drawn as shown in Fig. 5. There seems to be at least three different types (A, B and C in the figure) of the binding sites in erythrocyte stroma for calcium ions and the same is true for magnesium ions. The site A represents the one with the highest affinity and the site C with the lowest affinity. The numbers of the binding sites and the association constants for these cations were derived from these plots and are shown in Table III. It appears that with each site, the numbers of the sites and the association constants for Ca and Mg are considerably similar.

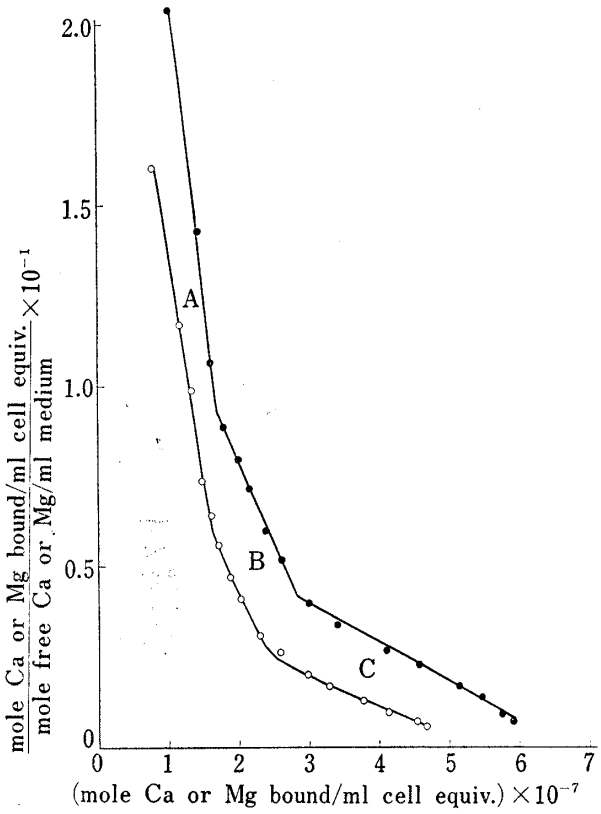

Fig. 5. Scatchard Plots for the Binding of Calcium and Magnesium by Erythrocyte Stroma in Hypotonic Medium
(Data of the binding in hypotonic medium in Fig. 2 are re-plotted.)
—●—: calcium A, B and C indicate three binding sites.
—○—: magnesium

From these facts and also from the characteristics of the binding as already revealed, it seems to be probable that both of these divalent cations share the identical binding sites on the membranes, which are supplied by at least three different kinds of the membrane components.

The Membrane Components Responsible for the Calcium Binding

Amounts of calcium associated with the lipid and non-lipid components of the erythrocyte membrane are shown in Table IV. In intact stroma, the percentage of calcium bound to the stromal lipid fraction was 34% of the total. On the other hand, the situation is different

9) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).

Table III. Characteristics of Three Kinds of Binding Sites of Erythrocyte Stroma for Calcium and Magnesium

	Binding sites (mole Ca or Mg bound per ml cell equiv.) $\times 10^{-7}$		Association constants (mole free Ca or Mg per ml medium)$^{-1} \times 10^{6}$	
	Ca	Mg	Ca	Mg
A	2.23	2.12	1.77	1.18
B	3.73	3.02	0.46	0.42
C	6.65	5.42	0.11	0.08

The data are derived from the Scatchard plots as shown in Fig. 5.

Table IV. Distribution of Endogenous and Exogenous Calcium between Lipid and Non-lipid Components of Human Erythrocyte Stroma

Stroma preparation	Stroma components[a)]	Ca content	
		n mole per ml stroma equiv. to ml cells	% of total
Intact stroma (with only endogenous Ca)	lipid	8.5	34
	non-lipid	16.2	66
Stroma treated with 10 mM $CaCl_2$ (with endogenous and exogenous Ca)	lipid	24.7	7
	non-lipid	312	93
The difference (exogenous Ca)	lipid	16.2	5
	non-lipid	296	95

a) After extraction of lipids from the stroma preparation with chloroform–methanol, the extract was washed with cation-free water and the organic phase was taken as the lipid fraction, and the aqueous phase plus the residual portion of extraction as non-lipid fraction.

in the case of the stroma saturated with calcium ions *in vitro*. In such stroma, only 7% of the calcium are lipid-bound, and if we consider only the amount of the "exogenous" Ca adsorbed, about 5% are bound to stromal lipids.

According to the tracer experiment by Forstner and Manery,[5)] about 20% of the radioactive Ca incorporated from the medium are bound to the stromal lipid fraction. The discrepancy between their result and ours may be due to the fact that a part of the calcium originally present in the membrane is exchangeable with the radioactive calcium ions externally added, and thus the value obtained by the tracer technique falls between the above-mentioned values (34 and 5%) obtained by the direct chemical determination of Ca in the lipid fraction of the intact and Ca-saturated stroma.