Calcium Binding by Human Erythrocyte Membranes

SIGNIFICANCE OF CARBOXYL, AMINO AND THIOL GROUPS

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1. The role of the ionized carboxyl groups of proteins of the erythrocyte membrane as Ca^{2+} receptor sites was investigated. A water-soluble carbodi-imide [1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate], referred to as carbodi-imide reagent, and glycine methyl ester were used to modify the free carboxyl groups of the membrane. The degree of modification was estimated from amino acid analyses, which showed the amount of glycine incorporated. As the concentration of carbodi-imide reagent was raised (0.1–0.4 M) incorporation of glycine increased and Ca^{2+} binding decreased by about 77%. At 0.4 M-carbodi-imide reagent all of the binding of Ca^{2+} to protein was abolished and it was estimated that about 37% of the side-chain carboxyl groups of aspartic acid plus glutamic acid had been blocked by glycine. 2. Acetylation of all of the free amino groups was achieved by incubating the erythrocyte 'ghosts' at pH 10.3 with acetic anhydride (10–15 mg/10 mg of 'ghost' protein). Acetylation increased by 1.5-fold the capacity of the 'ghost' to bind Ca^{2+} , indicating that the remaining carboxyl groups of aspartic acid and glutamic acid were made available for Ca^{2+} binding by this procedure. These findings support the concept that in normal 'ghosts', at pH 7.4, Ca^{2+} binding to free carboxyl groups is partially hindered by the presence of charged amino groups. 3. Treatment of 'ghosts' with *N*-acetylneuraminidase, which removed 94% of sialic acid residues, and treatment with 1 mM-*p*-chloromercuribenzoate did not alter Ca^{2+} binding. The major effect of 5.8 mM-*p*-chloromercuribenzoate upon 'ghosts' was to cause a solubilization of a calcium-membrane complex, which included about one-third of the 'ghost' protein. The molar ratio of Ca^{2+} : protein in the solubilized material was the same as that in the intact (untreated) 'ghosts'.

Forstner & Manery (1971) showed that as much as 283 μmol of Ca^{2+} /g of protein bind firmly to haemoglobin-free erythrocyte membranes under saturation conditions. In the technique used the membranes were separated by centrifugation from a ^{45}Ca -containing solution, and the pellets were washed three times in a Ca^{2+} -free medium before the determination of their ^{45}Ca content. In this technique therefore the strongly bound Ca^{2+} was measured, but probably not Ca^{2+} that might be associated with weak binding sites. Of this firmly bound Ca^{2+} 79% was associated with membrane protein and 16% with the lipid component; the latter was almost entirely confined to a lipid fraction containing phosphatidylserine, phosphatidylethanolamine and phosphoinositides, in a molar ratio of Ca^{2+} : phosphorus of 1:2. Ca^{2+} binding to erythrocyte 'ghosts' increased with increasing pH, suggesting that dissociable membrane anions were important Ca^{2+} -binding groups. The most likely

Ca^{2+} -binding sites associated with the 'ghost' protein are the carboxyl groups of sialic acid, aspartic acid and glutamic acid.

Erythrocyte 'ghosts' contain only 81 μmol of sialic acid/g of 'ghost' protein (calculated from the data of Eylar, Madoff, Brody & Oncley, 1962) and therefore this anion might be expected to account for only about 40 μmol of bound Ca^{2+} ($\text{CO}_2\text{H}:\text{Ca}^{2+}$ molar ratio assumed to be 2:1). Nevertheless, experiments were conducted to compare Ca^{2+} binding by 'ghosts' before and after treatment with neuraminidase to assess the importance of sialic acid as a receptor group for strongly bound Ca^{2+} .

The role in Ca^{2+} binding of other carboxyl groups of the membrane protein, such as those of the side chains of aspartic acid and glutamic acid, was investigated by the use of a water-soluble carbodi-imide reagent, which has been shown to modify free carboxyl groups (Hoare & Koshland, 1967). The

data presented show that the side-chain carboxyl groups of aspartic acid plus glutamic acid provided the major binding sites, since modification of about 37% of them almost completely abolished Ca^{2+} binding. This research has been published in abstract (Forstner & Manery, 1970). The rest of the protein carboxyl groups did not appear to be involved in binding Ca^{2+} at pH 7.4, which suggested that some membrane factor or component actually hindered Ca^{2+} binding at this pH.

If amino residues hinder Ca^{2+} binding to protein carboxyl groups by electrostatic stabilization of the carboxylate negative charge (which might be very strong if both groups are present in a hydrophobic region; Elmore, 1968), then modification of the amino groups by acetylation would be expected to remove the positive charges, which would result in greater Ca^{2+} binding to negative carboxyl groups at pH 7.4. Data presented in this paper show that acetylation of amino groups enhanced Ca^{2+} binding 1.5-fold, thus providing support for the concept of amino-carboxyl interaction.

Thiol residues are also possible Ca^{2+} -binding sites. Tolberg, Farmer & Macey (1970) reported that thiol groups might account for 30–40% of Ca^{2+} bound to 'ghosts'. Since acetylation probably blocks thiol groups (Riordan & Vallee, 1967) their involvement in Ca^{2+} binding was tested separately by treating the 'ghosts' with *p*-chloromercuribenzoate. The results indicated that thiol groups do not act as major Ca^{2+} receptor sites.

EXPERIMENTAL

Preparation of erythrocyte 'ghosts'. Haemoglobin-free 'ghosts' were prepared by the method of Dodge, Mitchell & Hanahan (1963). In the acetylation experiments and experiments with *p*-chloromercuribenzoate to be described, the 'ghosts' had been thawed and refrozen three times before use. A comparison of Ca^{2+} binding by intact and frozen-thawed 'ghosts' showed no significant differences.

Ca^{2+} -binding procedure. Samples of 'ghosts' containing 4 mg of protein were incubated with 10 mM- CaCl_2 containing $1 \mu\text{Ci}$ of $^{45}\text{CaCl}_2$ in 0.01 M-tris-HCl buffer, pH 7.4, for 1 h at 22°C in a final volume of 10 ml. After incubation the 'ghosts' were centrifuged and washed three times, then solubilized in Nuclear-Chicago Solubilizer and their radioactivities counted. The detailed procedure has been described by Forstner & Manery (1971).

Incubation of 'ghosts' with neuraminidase. 'Ghosts' containing about 5 mg of protein were washed three times with 10 vol. of 0.5 M-acetic acid-KOH buffer, pH 5.0; they were then incubated at 37°C for 3 h on a Dubnoff metabolic incubator in a solution of the same buffer containing *N*-acetylneuraminidase (EC 3.2.1.18) enzyme (0.286 mg, type VI, Sigma Chemical Co., St Louis, Mo., U.S.A.) in a final volume of 4 ml. Control 'ghosts' were treated identically except that no enzyme was added. Samples were removed at various time-intervals, centri-

fuged at 39 100g for 10 min and washed twice with 10 vol. of acetate buffer. Both the 'ghost' pellet (1 ml) and combined supernatant and washing solutions were analysed for sialic acid content by the method of Warren (1959). For 'ghost' pellets, sialic acid analyses were performed after mild acid hydrolysis in 0.05 M- H_2SO_4 at 80°C for 1 h. *N*-Acetylneuraminic acid solutions (0–0.06 μmol /assay volume) were chosen as reference standards since this is the chief sialic acid found in erythrocytes (Eylar *et al.* 1962). Protein analyses (Lowry, Rosebrough, Farr & Randall, 1951) on both pellets and combined supernatant and washing solutions were performed to rule out the possible loss of protein from the 'ghosts' during the incubation or washing steps or both. No protein loss was observed.

The 'ghosts' were subsequently washed three times with 10 vol. of 0.01 M-tris-HCl, pH 7.5, and tested for their Ca^{2+} -binding capacity as described under ' Ca^{2+} -binding procedure'.

Treatment of 'ghosts' with carbodi-imide reagent. The method of Hoare & Koshland (1967) for modifying the carboxyl groups of purified proteins was applied to erythrocyte membranes; the efficiency of blockage was assessed by measuring incorporation of glycine into the protein. 'Ghosts' were washed twice with 10 vol. of 0.01 M-acetic acid, adjusted to pH 4.6 with NaOH, and divided into samples each containing 10 mg of protein in a volume of 2 ml. To modify protein carboxyl groups, carbodi-imide reagent [1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.)] was added to each 'ghost' sample, to give a final concentration of 0–0.4 M, along with glycine methyl ester to a final concentration of 1.33 M. The solutions were adjusted to pH 3.0 by dropwise addition of M-HCl; the final incubation volume was 10 ml. In samples used as controls the carbodi-imide reagent was replaced by acetate buffer. During incubation at 22°C for 3 h, the pH was readjusted as necessary to 3.0. Within about 30–60 min no further additions of HCl were required, indicating that the reaction was complete. After overnight storage at 4°C, the contents of the reaction vessels were adjusted to pH 2.0 and left for 20 min, to hydrolyse potential phosphoamide bonds that might have formed between membrane phospholipids and the glycine methyl ester. The 'ghosts' were then separated by centrifugation at 38 000g for 15 min, and washed twice in 10 vol. of 0.01 M-tris-HCl, pH 7.4, to remove excess of reagents and bring the pH to 7.4 before testing the Ca^{2+} -binding capacity. Since amides of carbon (i.e. R-CONH₂) are stable at pH 7.4, side-chain carboxyl groups which had been converted into glycine methyl ester amides would remain blocked during subsequent incubations with CaCl_2 .

Amino acid analysis of 'ghost' protein. To assess the efficiency of carboxyl-group blockage by carbodi-imide reagent-glycine methyl ester, some membrane samples were analysed for amino acid content before and after treatment with these reagents, and before and after exposure to Ca^{2+} . The extent of glycine incorporation by 'ghosts' so treated was used as an index of carboxyl modification.

For amino acid analysis 'ghost' samples were extracted three times with chloroform-methanol (1:1, v/v) (method of Burger, Fujii & Hanahan, 1968) and hydrolysed under

vacuum with 1 ml of constant-boiling 6M-HCl for 22 h at 110°C. The HCl was removed under vacuum and the residue dissolved in the sodium citrate buffer, pH 2.2, of Moore, Spackman & Stein (1958). The amino acids were analysed on a Beckman-Spinco automatic analyser, model 120 C, with 0.125 μ mol of norleucine as an internal standard (recovery 99–100%). With the exception of glycine, the amino acid profile thus obtained was essentially the same as those of Mazia & Ruby (1968) and Rosenberg & Guidotti (1968). The concentration of glutamic acid plus aspartic acid was 1.195 μ mol/mg of 'ghost' protein, and was obtained by subtraction of ammonia released during hydrolysis from the total apparent aspartic acid plus glutamic acid concentration after hydrolysis. This value was used as a reference standard and all amino acid values except glycine were 'normalized' to it, thus making various 'ghost' preparations strictly comparable. Such a procedure was adopted where exactly 0.25 mg of 'ghost' protein was not always available for analysis.

Ca²⁺ binding was measured and amino acid analyses were performed on 'ghost' samples that had been treated with carbodi-imide reagent-glycine methyl ester; therefore the consequences of modification of carboxyl groups upon Ca²⁺ binding in the same sample could be assessed.

Acetylation of 'ghosts'. The method used for acetylation of 'ghosts' was essentially that described by Riordan & Vallee (1967). Acetylation was performed at pH 10.3 and at 0°C in the presence of half-saturated sodium acetate by the addition of 10.9 M-acetic anhydride to give a final concentration of 0–50 mg/10 ml of 'ghost' protein. [¹⁴C]Acetic anhydride (Amersham Searle Corp., Arlington Heights, Ill., U.S.A.) with a specific radioactivity of 3081 d.p.m./ μ mol was employed to measure incorporation of acetyl groups into 'ghosts'. Solubilization and counting of ¹⁴C-labelled 'ghosts' was performed as described by Dryden & Manery (1970). After acetylation, 'ghosts' were centrifuged at 34800g for 10 min and washed (usually seven times) until no trace of radioactive acetate remained in the supernatant. The wash solution consisted of 10 vol. of Ca²⁺-free water for the first three washes, and 0.01 M-tris-HCl buffer, pH 7.4, for the last four.

It was observed that acetylated 'ghosts' became gel-like in consistency during the washing steps, and some loss of 'ghost' material into the supernatant was almost unavoidable. This was partially solved by using longer centrifugation times during the washing procedure to obtain tighter packing. It should be noted that all data are based on final protein determinations. After washing, the 'ghosts' were resuspended in tris-HCl buffer in a total volume of 4 ml. Samples were removed for estimation of ¹⁴C (3 ml) and protein (0.1 ml) concentrations, and the remaining membranes were later used for Ca²⁺-binding studies.

Because of the difficulty of counting both ¹⁴C and ⁴⁵Ca in the same 'ghost' sample, experiments were usually arranged in duplicate but identical tubes, such as to count only one isotope per tube. The results of acetyl incorporation and Ca²⁺ binding were thus strictly comparable in both sets of tubes.

Lipid extraction of 'ghosts'. Some experiments were done to observe the effect of acetylation on the distribution of ⁴⁵Ca in the membrane lipid and protein. Accordingly acetylated 'ghosts' containing bound ⁴⁵Ca

were extracted three times with chloroform-methanol (1:1, v/v), as described by Forstner & Manery (1971), and the distribution of Ca²⁺ was compared with its distribution in similarly extracted 'ghosts' that had not been acetylated.

Determination of free amino groups in 'ghosts'. Free amino groups were determined in acetylated 'ghosts' by the trinitrobenzenesulphonic acid method described by Shinoda & Satake (1961), Okuyama & Satake (1960) and Satake, Okuyama, Ohashi & Shinoda (1960) so that the proportion of the total amino groups which were altered during acetylation could be calculated. A standard curve was constructed with suitable dilutions of 1 mM-DL-leucine and used to determine free amino groups of test samples.

When the samples contained 'ghosts', the assay solution was cloudy and satisfactory spectrophotometer readings were impossible to obtain. To overcome this problem the 'ghosts' were solubilized in sodium dodecyl sulphate (1 mg/mg of protein) after the 'ghosts' had been acetylated and washed free of reagents. Once solubilized, the 'ghosts' could be used in the trinitrobenzenesulphonic acid assay without further treatment. Addition of sodium dodecyl sulphate (3 mg and 6 mg) to leucine standards as well as to reagent blanks produced no significant differences in extinction readings. The molar extinction coefficient of leucine calculated from the standard curve was 1.2×10^4 at 340 nm, 22°C, which is similar to the value of 1.4×10^4 reported by Okuyama & Satake (1960).

Treatment of 'ghosts' with p-chloromercuribenzoate. To investigate the role of thiol groups in Ca²⁺ binding by erythrocyte membranes, treatment with p-chloromercuribenzoate was carried out in two ways.

(a) Experiments were designed to block the thiol groups by pretreatment with p-chloromercuribenzoate before measurement of Ca²⁺ binding. A stock solution of 0.02 M-p-chloromercuribenzoate (Eastman Chemical Co., Rochester, N.Y., U.S.A.) was prepared in 0.1 M-sodium acetate buffer, pH 4.8, by the method of Riordan & Vallee (1967); enough of this stock was added to 'ghosts' containing 5 mg of protein to give final concentrations of p-chloromercuribenzoate from 0 to 1 mM. The total final volume was 2 ml and the mixtures were incubated at 22°C for 1 h. After centrifugation at 39100g for 10 min the 'ghost' pellet was washed four times with 10 vol. of 0.01 M-tris-HCl buffer, pH 7.4, until the supernatant was free of p-chloromercuribenzoate (checked by measurements of extinction at 233 nm; Riordan & Vallee, 1967). The 'ghosts' were then tested for their Ca²⁺-binding capacity.

(b) Experiments were done to measure the displacement of bound Ca²⁺ by p-chloromercuribenzoate. 'Ghosts' were first incubated in CaCl₂ containing ⁴⁵CaCl₂ and then centrifuged and washed three times as described under 'Ca²⁺-binding procedure'. Samples of 'ghosts' were removed for analysis of protein and radioactivity. The remaining membranes were incubated with 5.8 mM-p-chloromercuribenzoate as described under (a) above. After washing free of the reagent, the 'ghosts' and the supernatant solutions were analysed for protein and ⁴⁵Ca content. The total Ca²⁺ and protein lost from the ⁴⁵Ca-labelled 'ghost' samples could then be calculated.

RESULTS

Ca²⁺ binding by sialic acid. The sialic acid content of five separate samples of intact erythrocyte 'ghosts' ranged from 20 to 25 $\mu\text{g}/\text{mg}$ of 'ghost' protein; this value is equivalent to 81 $\mu\text{mol}/\text{g}$ of protein (mol.wt. of *N*-acetylneuraminic acid, 309), and agrees with the data of Eylar *et al.* (1962).

When 'ghosts' were treated with neuraminidase, almost all of the sialic acid was removed after 15 min of incubation. After 150 min 91% of the sialic acid had been lost from the 'ghosts' and 84% was recovered in the respective supernatant solutions. However, there was no significant difference in Ca²⁺ bound by control and neuraminidase-treated 'ghosts'. Since only a small amount of Ca²⁺ could be bound to sialic acid in control 'ghosts' (about 40 $\mu\text{mol}/\text{g}$ of protein; see the introduction), the normal variation in binding data may have masked the expected loss. Alternatively, Ca²⁺ may normally be weakly bound to sialic acid carboxyl groups, and the washing steps employed during the Ca²⁺-binding procedure (see the Experimental section) may have removed most of sialic acid-bound Ca²⁺ ions.

Effect of carbodi-imide reagent-glycine methyl ester on glycine incorporation and Ca²⁺ binding. Treatment of 'ghosts' with carbodi-imide reagent-glycine methyl ester did not alter the amino acid profile except for glycine content, which rose markedly (7.0–17.5 mol/100 mol) with increasing concentrations of carbodi-imide reagent (0–0.4 M). In preliminary experiments, Ca²⁺ binding was depressed by all concentrations of carbodi-imide reagent employed (0.04–0.4 M) but maximal re-

duction (77–79%) was observed with 0.4 M-carbodi-imide reagent at pH 3.0 in the presence of 1.33 M-glycine methyl ester. In subsequent experiments therefore these conditions were always employed.

To find out whether the observed reduction in Ca²⁺ binding was due to an effect of carbodi-imide reagent on the protein or lipid components of the membrane, 'ghosts' which had been treated with 0 and 0.4 M-carbodi-imide reagent and glycine methyl ester were labelled with ⁴⁵Ca by the standard Ca²⁺-binding procedure and then extracted with chloroform-methanol (1:1, v/v). Control 'ghosts' (no carbodi-imide reagent) contained 666 d.p.m. of ⁴⁵Ca/mg of protein, whereas those exposed to 0.4 M-carbodi-imide reagent and glycine methyl ester had only 148 d.p.m. All of the latter, i.e. 100% of the Ca²⁺ bound to treated 'ghosts', was found in the lipid extract, whereas the lipid phase of the control membranes contained about 25% of the total counts, which is the proportion usually associated with this phase in untreated 'ghosts' (Forstner & Manery, 1971). Thus the protein binding of Ca²⁺ had been completely abolished by carbodi-imide reagent, but the amount of lipid-bound Ca²⁺ was unaltered. This is not surprising when it is recalled that phosphoamide bonds are unstable and that the membranes were exposed to pH 2.0 to hydrolyse any such bonds that might have been formed as a result of carbodi-imide reagent-glycine methyl ester treatment (see the Experimental section).

In Table 1 the relationship between concentration of carbodi-imide reagent, total Ca²⁺ binding and incorporation of glycine is presented. As the concentration of carbodi-imide reagent increased

Table 1. *Effect of carbodi-imide reagent-glycine methyl ester on Ca²⁺ binding and incorporation of glycine*

'Ghosts' were incubated with 1.33 M-glycine methyl ester and carbodi-imide reagent (0–0.4 M) as described in the Experimental section. After washing, they were incubated in 10 mM-CaCl₂ + 1 μCi of ⁴⁵CaCl₂ in 0.01 M-tris-HCl buffer, pH 7.4, in a total volume of 10 ml. Each Ca²⁺ value is the mean from two separate experiments for each concentration of carbodi-imide reagent. Each value of glycine incorporation is the mean of two separate amino acid analyses for each concentration of carbodi-imide reagent; the differences between the two were not greater than 5 $\mu\text{mol}/\text{g}$ of 'ghost' protein. In untreated (no carbodi-imide reagent) samples the glycine content was 0.504, and the aspartic acid plus glutamic acid content was 1.195 $\mu\text{mol}/\text{mg}$ of 'ghost' protein (used for obtaining data in column 8).

Carbodi- imide (M)	Ca ²⁺ bound to whole membranes (μmol/g of protein)	Ca ²⁺ bound to membrane protein (μmol/g)	Decrease in Ca ²⁺ bound				Incorporation of glycine	
			To whole membrane		To protein alone		-CO ₂ H groups blocked (% of total Asp + Glu) (8)	Glycine incorporated (μmol/g of protein) (9)
			(μmol/g of protein)		(μmol/g of protein)			
			(%) (4)	(5)	(%) (6)	(7)		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
0	191	153	0	0	0	0	0	0
0.1	158	120	17.3	33	21.6	33	20.1	240
0.2	72	34	62.2	119	78.0	119	36.6	432
0.4	43	5	77.4	148	96.5	148	70.1	836

from 0 to 0.4M, the total Ca²⁺ bound decreased from 191 to 43 μ mol/g of protein (column 2), which represents a 77.4% decrease in binding (column 4). The incorporation of glycine increased by 836 μ mol/g of protein (column 9), an amount which is, in theory, capable of having modified about 70% of the total free carboxyl groups of aspartic acid and glutamic acid (column 8). (This is based on the observed value for aspartic acid plus glutamic acid being 1195 μ mol/g of 'ghost' protein; see the Experimental section.)

Since the lipid had been found previously to be responsible for about one-fifth of the total membrane-bound Ca²⁺ (Forstner & Manery, 1971), and since carbodi-imide reagent did not affect lipid binding of Ca²⁺, the amount of Ca²⁺ bound to protein alone was estimated (column 3) by subtracting a maximum of 20% of 191 (i.e. 38 μ mol) from each figure in column 2. When the values of column 3 were translated into percentage reduction of Ca²⁺ bound to protein alone (column 6), it was evident that Ca²⁺ binding to protein was almost abolished (96.5% reduction) by 0.4M-carbodi-imide reagent.

Glycine incorporation by sialic acid groups. Carbodi-imide reagent might be expected to modify residues in the membrane protein other than aspartic acid and glutamic acid carboxyl groups. These include sialic acid carboxyl groups, phenolic hydroxyl groups of tyrosine (Carraway & Koshland, 1968) and the thiol groups of cysteine (Carraway & Triplett, 1970).

To measure the extent of glycine incorporation into sialic acid after modification by carbodi-imide reagent, sialic acid was removed with neuraminidase

and the 'ghosts' were then treated with glycine methyl ester and carbodi-imide reagent (0 and 0.4M). With the exception of glycine, the amino acid compositions of carbodi-imide-treated, neuraminidase-treated and untreated control 'ghosts' were identical. However, the glycine incorporation (Table 2) in those treated with carbodi-imide reagent-glycine methyl ester and neuraminidase was much less (0.44, range 0.32–0.56 μ mol/mg) than in the 'ghosts' treated with the reagent only (0.84 μ mol/mg). Thus $0.84 - 0.44 = 0.40$ (range 0.52–0.27) μ mol of glycine appears to have been incorporated into sialic acid carboxyl groups; the apparent percentage of aspartic acid and glutamic acid modified by carbodi-imide reagent is thereby decreased from 70 to 37% (range 27–47%).

Treatment with carbodi-imide reagent-glycine methyl ester and Ca²⁺ binding by denatured protein. Not all of the free carboxyl groups of aspartic acid and glutamic acid were modified by 0.4M-carbodi-imide reagent, and yet Ca²⁺ binding to the protein component of the 'ghosts' appeared to have been virtually abolished by this treatment. The possibility thus exists that some carboxyl groups were inaccessible to Ca²⁺ and to carbodi-imide. Accordingly, an attempt was made to 'expose' more membrane carboxyl groups by denaturing the protein by lipid extraction. The insoluble, denatured protein thus obtained was treated with carbodi-imide reagent-glycine methyl ester and the Ca²⁺-binding capacity and incorporation of glycine were measured. Both were found to be unaltered by this type of denaturation. The data in Table 3 show that glycine incorporation into the denatured protein was sufficient to block

Table 2. *Effect of carbodi-imide reagent-glycine methyl ester on incorporation of glycine into neuraminidase-treated erythrocyte 'ghosts'*

'Ghosts' containing 10 mg of protein were treated for 2h with sufficient neuraminidase to remove total membrane sialic acid (see the Experimental section). After incubation, 'ghosts' were washed and reincubated at pH 3.0 with glycine methyl ester (1.33M) and carbodi-imide reagent (0, 0.4M). After washing to remove excess of reagents, the lipid of the 'ghosts' was extracted with chloroform-methanol (1:1, v/v) and their amino acid composition was determined. Each value in the table represents the average of the number of amino acid analyses shown under *n*. The % of Asp+Glu modified is based on a total of 1.195 μ mol of Asp+Glu/mg of protein.

<i>n</i>	Carbodi-imide (m) (1)	Glycine (μmol/mg of protein) (2)	Incorporation of glycine	
			(μmol/mg of protein) (3)	(% of Asp+Glu modified) (4)
No treatment with neuraminidase				
(1)	0	0.50	0	0
(2)	0.4	1.34	0.84	70
Treated with neuraminidase				
(2)	0	0.50	0	0
(4)	0.4	0.95	0.44	37

Table 3. *Effect of treatment with carbodi-imide reagent-glycine methyl ester on Ca²⁺ binding and incorporation of glycine into denatured 'ghost' protein*

'Ghost' samples containing 10 mg of protein were extracted with chloroform-methanol (1:1, v/v) (Forstner & Manery, 1971). The resulting denatured protein residue was incubated with glycine methyl ester (1.33 M) and carbodi-imide reagent (0 or 0.4 M) at pH 3.0 for 3 h at 22°C. After being washed twice, the 'ghosts' were reincubated with 10 mM-CaCl₂ + 1 μ Ci of ⁴⁵CaCl₂ and Ca²⁺ binding to each sample was determined. Amino acid analyses were performed on the remaining ⁴⁵Ca-labelled 'ghost' protein to determine glycine incorporation. Each value represents a separate incubation of 'ghost' protein, with Ca²⁺ and protein analyses performed in duplicate. To calculate the data of column (6) the total Asp + Glu content was assumed to be 1195 μ mol/g of protein.

Carbodi-imide (M)	Ca ²⁺ bound (μ mol/g of protein)	Decrease in Ca ²⁺ bound (%)	Glycine content (μ mol/g of protein)	Incorporation of glycine (μ mol/g of protein)	Modification of free -CO ₂ H of Asp + Glu (% of total)
0	99	0	504	0	0
	136		504	0	0
0.4	19	84	1315	811	67.9
	19		1199	695	58.2

Table 4. *Effect of p-chloromercuribenzoate on the displacement of Ca²⁺ from ⁴⁵Ca-labelled membranes*

Erythrocyte membranes (4 mg of protein) were carried through the Ca²⁺-binding procedure (see the Experimental section). After being washed three times, a portion of the 'ghosts' (1–2 mg of protein) was reincubated at 22°C (pH 6.5) for 60 min in a total final volume of 1.05 ml with water (control) or *p*-chloromercuribenzoate (PCMB; 5.8 mM).

	Before PCMB treatment		After PCMB treatment			Ghost 'protein' solubilized by PCMB (% of initial protein)
	'Ghost' protein (mg) (1)	Ca ²⁺ bound (μ mol/mg of protein) (2)	Supernatant Ca ²⁺ (μ mol/0.5 ml of supernatant) (3)	Supernatant protein (mg/0.5 ml of supernatant) (4)	Supernatant Ca ²⁺ /protein (μ mol/mg) (5)	
Control	2.40	0.109	0.018	0	—	—
(no PCMB)	1.92	0.141	0.019	0	—	—
Treated	2.07	0.129	0.040	0.310	0.129	22.4
(5.8 mM-PCMB)	1.06	0.156	0.048	0.308	0.156	43.5

58–68% of aspartic acid plus glutamic acid free carboxyl groups, which is not significantly different from the 70.1% modification (Table 1) in intact 'ghosts'. As well, carbodi-imide reagent almost abolished Ca²⁺ binding to the denatured protein, the decrease being 84%. Therefore this form of denaturation did not significantly expose carboxyl groups to make them more reactive with either carbodi-imide reagent-glycine methyl ester or Ca²⁺ ions. Other denaturants (urea, guanidine hydrochloride) were not employed because apparently they cause membrane solubilization.

Role of thiol groups. The participation of free thiol groups was investigated by determining the effect of *p*-chloromercuribenzoate on Ca²⁺ binding. Pretreatment of 'ghosts' with 0–1 mM-*p*-chloromercuribenzoate caused no significant alteration in subsequent Ca²⁺ binding. However, when 'ghosts' were preincubated with Ca²⁺ and then treated with 5.8 mM-*p*-chloromercuribenzoate,

(Table 4), some displacement of membrane Ca²⁺ was observed (column 3). In addition there was a concomitant loss of membrane protein into the supernatant (column 6), which entirely accounted for the observed Ca²⁺ displacement (compare columns 5 and 2). It appeared therefore that *p*-chloromercuribenzoate did not interfere with specific Ca²⁺-binding sites on the membrane. Instead, *p*-chloromercuribenzoate effected a partial solubilization of a membrane-Ca²⁺ complex.

It was not known how effectively *p*-chloromercuribenzoate blocked thiol groups, and there was therefore a possibility that the lack of effect of the compound on 'ghost' Ca²⁺ binding resulted from insufficient blocking of thiol groups by the reagent. However, Van Steveninck, Weed & Rothstein (1965) have shown that 1 mM-*p*-chloromercuribenzoate is able to modify at least 25% of 'ghost' thiol groups (equivalent to 103 μ mol/g of protein) and Tolberg *et al.* (1970) have reported

that 1 mM-*p*-chloromercuribenzoate displaced 30–40% of bound Ca²⁺. Therefore a decrease in Ca²⁺ bound/mg of protein should have been observed in our data if thiol groups were quantitatively of importance.

Free amino groups. Since only a fraction of the total carboxyl groups (about 37%) appeared to participate in Ca²⁺ binding to 'ghost' protein, and since denaturation of the protein by chloroform-methanol did not increase the availability of carboxyl groups, it was considered possible that free amino groups (NH₃⁺) at pH 7.4 might hinder Ca²⁺ binding by stabilizing carboxylate (–CO^{2–}) through electrostatic interaction. Free ε-NH₃⁺ groups of lysine (pK 8–10; Greenstein & Winitz, 1961) have a concentration of 0.427 μmol/mg of protein as determined from amino acid analyses. Histidine imidazole groups (pK 6.5–7.0) and phospholipid NH₃⁺ groups contribute fewer amino groups (less than 0.150 μmol/mg of protein; Van Deenen & DeGier, 1964). Thus experiments were designed to block the major NH₃⁺-containing species through the use of acetic anhydride (see the Experimental section), to see if an increase in Ca²⁺ binding to 'ghosts' would result.

Fig. 1 shows that, after acetylation, Ca²⁺ binding was enhanced by about 1.5-fold in incubations containing equal quantities (by wt.) of acetic

anhydride and 'ghost' protein. The total decrease in free amino groups was 0.561 μmol/g of 'ghost' protein, which is estimated to represent about 97% of the total available. Maximum incorporation of acetyl groups was 1.069 μmol/g of 'ghost' protein; thus residues other than free amino groups must have also been acetylated. The excess of acetyl incorporation (i.e. 1.069–0.561 = 0.508) was probably into tyrosine, serine and threonine, or thiol groups or all these, present in the membranes (Vallee & Riordan, 1969).

Distribution of bound Ca²⁺ after acetylation. By way of confirming that the protein component of the membranes rather than the lipid components had been acetylated by acetic anhydride, the acetylated membranes that had been incubated with ⁴⁵Ca were extracted with chloroform-methanol and the distribution of radioactivity in the resulting phases was determined (Table 5). The observed distribution of recovered Ca²⁺ was 12.7, 85.4 and 1.9% for lipid, protein and aqueous phases respectively. The expected values for the distribution of bound Ca²⁺ were calculated on the assumption that the increase in Ca²⁺ bound by acetylated membranes (265–190 = 75 μmol, column 1) was due to increased protein binding alone. Of the total 265 μmol of Ca²⁺/g of protein observed it was assumed that 152 (control value, column 3) + 75 = 227 μmol would be bound to protein, which is equivalent to 227/265 × 100 = 85.6% of the total bound Ca²⁺. It was also assumed that lipid-bound Ca²⁺ in acetylated 'ghosts' would be the same as in control 'ghosts', i.e. 28.5 μmol or 10.7%, and aqueous-phase Ca²⁺ would be 9.5 μmol or 3.6%. These expected values are very close to the observed Ca²⁺ distribution, suggesting that the main effect of acetylation was to increase the capacity of 'ghost' protein to bind Ca²⁺. The small amount of Ca²⁺ observed within the aqueous phase (1.9%) tends to rule out the possibility that acetylation might have acted non-specifically to cause an inward flux of Ca²⁺ ions into a soluble interior compartment of the 'ghost' vesicles.

Fig. 1. Effect of acetylation on Ca²⁺ binding. 'Ghosts' (10 mg of protein) were acetylated by incubation with [¹⁴C]acetic anhydride (0–50 mg) and half-saturated sodium acetate, pH 10.3, at 0°C for 2 h in a final volume of 4 ml (see the Experimental section). After washing free of excess of reagents, radioactivities of samples were counted to obtain data on incorporation of acetyl groups (O). Acetylated (non-radioactive) 'ghosts' (4–5 mg of protein) were carried through the Ca²⁺-binding procedure, washed three times and sampled for counting ⁴⁵Ca (●). Free NH₂ groups (Δ) were estimated by the trinitrobenzenesulphonic acid reaction after solubilization of the membranes in sodium dodecyl sulphate (1 mg/mg of protein). Each point represents the mean of two to four separate experiments, and the bars show the total range of values obtained.

DISCUSSION

Binding of Ca²⁺ by carboxyl groups. The aim of this research was to identify the protein anionic sites responsible for the firm binding of Ca²⁺ ions previously described by Forstner & Manery (1971). Treatment with 0.4 M-carbodi-imide reagent-glycine methyl ester abolished Ca²⁺ binding to the protein component. However, the amount of glycine incorporated (836 μmol/g of protein; Table 1, column 9), which provided a measure of the number of groups blocked, far exceeded the reduction in Ca²⁺ bound (148 μmol/g of protein; Table 1, column 5). Since carbodi-imide reagent

Table 5. *Effect of acetic anhydride on the distribution of Ca^{2+} in the lipid and protein components*

Two separate membrane suspensions (containing 10 mg of protein) were incubated for 2 h at 0°C with acetic anhydride (3 mg) and half-saturated sodium acetate, pH 10.3 (final volume, 4 ml). After washing free of excess of reagents, the 'ghosts' were exposed to ^{45}Ca , washed three times and extracted with chloroform-methanol (1:1, v/v); all details are given in the Experimental section. ^{45}Ca was determined in duplicate samples of each phase formed. The expected values of Ca^{2+} distribution were calculated on the assumption that the difference in Ca^{2+} bound between acetylated and non-acetylated 'ghosts' (i.e. 75 $\mu\text{mol/g}$ of protein) was due entirely to increased Ca^{2+} binding to the protein component.

	Total Ca^{2+} bound	Lipid-bound Ca^{2+}		Protein-bound Ca^{2+}		Aqueous-phase Ca^{2+}	
	($\mu\text{mol/g}$ of 'ghost' protein) (1)	($\mu\text{mol/g}$ of 'ghost' protein) (2)	(% of total bound Ca^{2+}) (3)	($\mu\text{mol/g}$ of 'ghost' protein) (4)	(% of total bound Ca^{2+}) (5)	($\mu\text{mol/g}$ of 'ghost' protein) (6)	(% of total bound Ca^{2+}) (7)
Control 'ghosts'	190	28.5	15	152	80	9.5	5
Acetylated 'ghosts' (observed values)	265	33.7	12.7	226.3	85.4	5.0	1.9
Acetylated 'ghosts' (expected values)		28.5	10.7	227	85.6	9.5	3.6

is primarily a blocking agent for carboxyl groups, one would expect the glycine to be incorporated at the sites of the free carboxyl groups of sialic acid, aspartic acid and glutamic acid. Results (Table 2) show that sialic acid was responsible for the incorporation of about 400 $\mu\text{mol/g}$ of protein. This leaves 436 μmol of glycine to be associated with glutamic acid and aspartic acid. We have shown that removal of sialic acid has little or no effect on the firmly bound Ca^{2+} that we were measuring. Hence it is reasonable to conclude that 436 μmol of glycine occupied the carboxyl sites on the glutamic acid and aspartic acid residues, which bound 148 μmol of Ca^{2+} in the controls. The molar stoichiometry of Ca^{2+} bound to carboxyl groups is therefore 148:436 or approximately 1:3. Attention is drawn to the fact that Gent, Trounce & Walser (1964) had previously suggested that erythrocyte membranes had a trivalent binding site for Ca^{2+} .

Erythrocyte membranes contain a total of 1195 μmol of glutamic acid and aspartic acid/g of membrane protein. Hence incorporation of 436 μmol of glycine represents a modification of only 37% of the total free carboxyl groups of these amino acids.

Other possible binding sites. Before one can conclude that all of the Ca^{2+} bound to the protein of erythrocyte 'ghosts' is bound to 37% of the free carboxyl groups of glutamic acid and aspartic acid other possible binding sites must be considered. Treatment of the 'ghosts' with carbodi-imide reagent-glycine methyl ester was carried out under

conditions which minimized interactions with groups other than the carboxyl. However, under certain conditions of pH etc., tyrosine hydroxyl and thiol groups are susceptible to modification by carbodi-imides (Hoare & Koshland, 1967; Carraway & Koshland, 1968; Carraway & Triplett, 1970).

The extent to which tyrosine phenolic hydroxyl groups (200 $\mu\text{mol/g}$ of protein, from amino acid analyses) were modified at pH 3.0 in our experiments is not known, so that we have no estimate of the amount of glycine they might have incorporated. It is unlikely, however, that tyrosine provided Ca^{2+} -binding sites, for the following reasons: (a) with a pK of ≥ 10 tyrosine phenolic hydroxyl groups would not be dissociated at pH 7.4 and so could not bind Ca^{2+} ; (b) acetic anhydride probably modifies tyrosine residues, yet Ca^{2+} binding increased after treatment with this agent whereas it decreased after treatment with carbodi-imide reagent. Since these two types of modification produced opposite effects on Ca^{2+} binding, these effects were not likely to have been mediated by the common action of hydroxyl group blockade.

The same considerations apply to thiol groups, which are susceptible to both carbodi-imides (Carraway & Triplett, 1970) and acetic anhydride (Vallee & Riordan, 1969). In addition, *p*-chloromercuribenzoate did not appear to have interfered with specific Ca^{2+} -binding sites in the membrane. *p*-Chloromercuribenzoate caused a partial solubilization of the 'ghosts' in our experiments and Ca^{2+} was removed with the protein solubilized. Smith & Verpoorte (1970) observed up to 40% membrane

solubilization by 1mM-*p*-chloromercuribenzoate. These findings may explain the data of Tolberg *et al.* (1970), who measured a displacement of 30–40% of adsorbed Ca²⁺ in erythrocytes and ghosts after treatment with 1mM-*p*-chloromercuribenzoate but do not report a protein loss.

It is reasonable to conclude that tyrosine hydroxyl and thiol groups are not involved in Ca²⁺ binding. If they incorporate glycine in these experiments, the percentage of glutamic acid and aspartic acid carboxyl groups involved in Ca²⁺ binding would be decreased below 37%.

Influence of amino groups on Ca²⁺ binding. The lack of involvement in Ca²⁺ binding of about 63% of the free carboxyl groups of amino acids can be attributed to the neutralization of carboxylate anions by charged amino groups. After acetylation, which virtually abolished the total free amino groups of the membrane (Fig. 1), Ca²⁺ binding was increased from 191 to 608 μ mol/g of protein. Thus it seems likely that in normal erythrocyte membranes free amino groups at pH 7.4 are close enough to free carboxyl groups that their electrostatic interaction can prevent Ca²⁺ binding. If the carboxyl and amino groups are contained within hydrophobic areas, the electrostatic interaction between them might be very strong (Elmore, 1968). The fact that more Ca²⁺ ions are bound to normal membranes as the pH rises above 7.4 (Forstner & Manery, 1971) supports the concept that amino groups are involved as outlined above, because the dissociation of NH₃⁺ groups at high pH would 'free' carboxyl groups, making them accessible to Ca²⁺. Irons & Perkins (1962) observed that, in serum albumin, acetylation of amino groups resulted in a marked increase in Ca²⁺ binding to carboxyl groups. Thus the regulation of Ca²⁺ binding by amino-carboxyl interaction may be a mechanism that operates in other proteins as well as in those of erythrocyte membranes.

Sialic acid. We have suggested (see the Results section) that sialic acid does not bind Ca²⁺ ions firmly enough to withstand the three washing steps involved in the Ca²⁺-binding procedure. There is some support for the view that sialic acid anions have a lower affinity for Ca²⁺ than the amino acid carboxyl groups. Sialic acid undoubtedly binds cations because of its low pK of 2.6 (Eylar *et al.* 1962) and its terminal position on oligosaccharide side chains of glycoproteins (Gottschalk, 1966). There is strong evidence that sialic acid carboxyl groups provide most of the total surface anions of the erythrocyte and regulate its electrophoretic mobility. Weiss & Mayhew (1967) and Seaman, Vassar & Kendall (1969) demonstrated that Ca²⁺ decreased this electrophoretic mobility through neutralization of part of this surface negative charge. In both of these studies another set of

Ca²⁺-binding sites was detected after removal of sialic acid by neuraminidase. These sites bound Ca²⁺ more strongly than neuramate anions and were presumed to be the carboxyl groups of glutamic acid and aspartic acid.

Weed, LaCelle & Merrill (1969) have investigated factors that influence the deformability of erythrocytes. Their studies indirectly support the view that there is low affinity of sialic acid for Ca²⁺ ions, and also suggest a functional importance for the more strongly bound Ca²⁺. In aged erythrocytes the chief factor responsible for decreased deformability appeared to be a high Ca²⁺ content of the membrane. When the membranes were treated with sialidase they lost their sialic acid, but no change in deformability (a measure of membrane elasticity) occurred. By inference then, the membrane Ca²⁺ that confers resistance to deformation is not removed by sialidase. Firmly bound Ca²⁺, which is not associated with sialic acid, may therefore be of importance in controlling the deformability characteristics of circulating erythrocytes.

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