J. Membrane Biol. 29, 345–372 (1976)

Ca Binding to the Human Red Cell Membrane: Characterization of Membrane Preparations and Binding Sites

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Received 5 December 1975; revised 26 February 1976

Summary. Inside out and right side out vesicles were used to study the sidedness of Ca binding to the human red cell membrane. It was shown that these vesicles exhibited only a limited permeability to Ca, enabling the independent characterization of Ca binding to the extracellular and cytoplasmic membrane surfaces. Ca binding was studied in 10 mM Tris HCl at pH 7.4, 22±2 °C and was shown to be complete in under 5 min. Scatchard plots were made from Ca binding data obtained at free Ca concentrations in the range of 10^{-6} to 10^{-3} M. Under these conditions inside out vesicles exhibit two independent binding sites for Ca with association constants of 1×10^5 and 6×10^3 M^{-1}, and right side out vesicles exhibit three independent binding sites with association constants of 2×10^5, 1.4×10^4 and 3×10^2 M^{-1}. Upon the addition of 0.1 M KCl a third, high affinity site was found on inside out vesicles with an association constant of 3×10^5 (in 0.1 M KCl). Ca binding to inside out vesicles increased nearly linearly with pH in the range of pH 4 to pH 11, while binding to right side out vesicles remained practically unchanged in the range of pH 7 to pH 9. Progressive increase of the ionic strength of the medium by the addition of K, Mg or Tris decreased Ca binding to inside out vesicles as did the addition of ATP. Comparison of a series of cation competitors for Ca binding sites on inside out vesicles at 0.003 mM Ca showed that La was the most effective competitor of all while Cd was the most effective divalent cation competitor of those tested. Our findings suggest that the effects of low concentrations of Ca at the inner surface of the red cell membrane are mediated primarily through Ca binding to site 1 (and possibly site 2) of inside out vesicles of which there are approximately 1.6×10^5 per equivalent cell.

The Ca ion is known to affect a wide variety of membrane properties and functions including nerve impulse transmission, cellular adhesion, intracellular communication and membrane permeability. There is therefore great interest in the Ca-membrane interaction which appears to be a central factor in these and other aspects of membrane function. In human red cells, Ca can influence the mechanical deformability (Weed, LaCelle & Merrill, 1969) and cation permeability of the membrane

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(Gardos, 1959; Romero & Whittam, 1971; Blum & Hoffman, 1971, 1972; Dunn, 1974). Ca can also alter the morphology of the cell itself (Weed & Chailley, 1973; Weed *et al.*, 1969; Dunn, 1974) and appears to induce a contraction of ghost membranes (Palek, Curby & Lionetti, 1971). Evidence presented by the above investigators strongly suggests that Ca must have access to the interior of the cell to initiate these changes, large variations of extracellular Ca being without effect. The human red cell is not unique in this respect; other phenomena which may be related to changes in intracellular Ca concentration include cell fusion (Ahkong, Tampion & Lucy, 1975), secretion (Cochrane & Douglas, 1974) and junctional membrane permeability (Loewenstein, 1973).

Since the effects of Ca on the red cell and other membranes seem to require its introduction to the interior of the cell, we decided to determine whether, and to what extent, Ca could interact with the cytoplasmic aspect of the membrane. Previous studies of Ca binding to the human red cell membrane have shown that, in both intact cells and ghosts, Ca binding is apparently due to a metabolically independent electrostatic adsorption to anionic membrane sites (Gent, Trounce & Walser, 1964; Seaman, Vassar & Kendall, 1969; Forstner & Manery, 1971; Long & Mouat, 1971).

Although there have been no detailed studies of Ca binding to the inner surface of the red cell membrane, Long and Mouat (1971) have shown that under certain conditions unsealed ghosts will bind approximately twice as much Ca as an equivalent number of intact red cells. This suggests that Ca binding sites are present at the internal surface of the membrane. In order to separate the Ca binding characteristics of the two sides of the membrane we have prepared red cell membrane vesicles whose orientation can be controlled such that either the cytoplasmic or extracellular membrane surface is exposed to the suspending medium (Steck, 1974*a*). Using these vesicles we have characterized the Ca binding sites at each membrane surface.

Materials and Methods

Water was deionized to a resistance greater than 5 $M\Omega/cm^3$. In general all chemicals were reagent grade. Sources of chemicals were: acrylamide, N – N′ methylenebiscacrylamide (BIS), Eastman Chemical Co.; sodium dodecyl sulfate (SDS), Fisher Scientific Co. Dextran T-110 was obtained from Pharmacia Fine Chemicals, neuraminidase (Type VI *clostridium perfringens*), bovine erythrocyte cholinesterase, sialic acid (95% pure) and Coomassie Brilliant Blue from Sigma Chemical Co. ^{45}Ca as $CaCl_2$ was obtained from New England Nuclear, as was ^{125}I-bovine serum albumin. Ultra pure KCl and HCl were obtained from Alfa Inorganics.

The stock Ca solutions for standardization of atomic absorption measurements and for binding experiments, were made by dissolving a weighed amount of Ca carbonate into a slight excess of ultra pure HCl and diluting with deionized water to a concentration of 100 mM. The concentration of these standards as determined by atomic absorption spectroscopy was always within a few percent of commercially available "Certified Atomic Absorption Standards (Fisher Scientific Co.).

Preparation of Inside Out and Right Side Out Vesicles

Inside out and right side out vesicles (*IOVs* and *ROVs*)[1] were prepared by a modification of the technique described by Steck (1974*a*) and Steck and Kant (1974). Since we have found that slight variations in Steck's protocol may alter the polypeptide composition of *IOVs*, our method of preparation is described in detail.

Blood was drawn into heparin from healthy donors by venipuncture on the same day it was to be used, and was washed 3 times in 150 mM NaCl, 5 mM Na phosphate, pH 8.0. All procedures were done at 0 °–4 °C unless otherwise specified. The washed cells were hemolyzed by pipetting 10 ml of packed cells into 230 ml of 5 mM Na phosphate, pH 8.0 (5P8). The hemolyzed cell suspension was spun at 27,000 × *g* for 25 min, the supernatant aspirated, and the ghost pellet resuspended in 5P8 and washed twice more in the same way. After each spin the small "button" of contaminating white cells which pellets beneath the ghosts was carefully removed (Fairbanks, Steck & Wallach, 1971).

After washing, the ghosts were either slightly pink (two washes) or creamy white (three washes). Each ghost pellet derived from 10 ml of packed cells was resuspended in 230 ml of 0.5 mM Na phosphate, pH 8.0 (0.5P8) and incubated on ice for 15 min. At the end of this time 0.23 ml of 0.1 M $MgSO_4$ (or enough to bring the suspension to 0.1 mM in Mg) was added to the aliquot of the suspension to be made into *ROVs*. Separate aliquots to be made into *IOVs* received no Mg. The suspensions were then centrifuged at 27,000 × *g* for one hr. After centrifugation the supernatants were carefully aspirated so as not to disturb the pellet, which was then incubated overnight at 4 °C, a procedure which evidently promotes vesicle sealing (Steck, 1974*a*).

After the overnight incubation, 2 ml of 0.5P8 (with 0.1 mM Mg for the *ROVs*) was added to the pellets and the thick suspensions were homogenized by passage six times through an 0.5″, 27 gauge hypodermic needle on a 2.5 ml plastic syringe. The suspensions were then diluted in 0.5P8 and carefully layered on a two to three fold excess volume of 1.03 g/ml Dextran 110 made in 0.5P8. Typically, an unfractionated suspension derived from 10 ml of packed cells was diluted to 12 ml, layered on a 26 ml Dextran shelf, and spun in a Beckman SW 27 rotor at 25,000 rpm for 4–6 hr. At the end of the spin, *IOVs* and *ROVs* (in their separate tubes), which do not penetrate the Dextran shelf, were removed by pipet and washed twice in a 100-fold excess volume of 10 mM Tris HCl, pH 7.4.

Assays and Enzymatic Treatments

Vesicle sidedness was established by determining the accessibility of sialic acid and acetylcholinesterase (AChE), markers for the extracellular membrane surface, and glyceraldehyde

1 *Abbreviations used: IOV*, inside out vesicle; *ROV*, right side out vesicle; AChE, acetylcholinesterase; G3PD, glyceraldehyde 3-phosphate dehydrogenase; 5P8, 5 mM Na phosphate, pH 8.0; 0.5P8, 0.5 mM Na phosphate, pH 8.0; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)-N—N′-tetraacetic acid; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin, EDTA, ethylenediamine tetraacetic acid.

3-phosphate dehydrogenase (G3PD), a marker for the intracellular membrane surface, as described by Steck (1974*a*).

AChE activity was determined by a modification of the method of Ellman, Courtney, Andres and Featherstone (1961) as described by Steck (1974*a*). For the determination of vesicle sidedness, 10–50 μl of a vesicle suspension was briefly pre-incubated with an equal volume of either 5P8 or 0.2% Triton X – 100 in 5P8. The Triton treatment lyses the vesicles and does not inhibit AChE since the AChE activity of ghosts and *ROVs* remained the same after treatment with 0.1% Triton. The assay was also performed on intact red cells washed free of plasma. All red cells used in this study fell within the range of 8.88–12.0 units AChE/ml packed cells (one unit (u) of AChE activity is defined as that amount of enzyme which will hydrolyse 1 μM of ACh per min). This compares well with values given by Ellman *et al.* (1961) and Atlas, Shapiro and Green (1973) of 10 u/ml and 6–10 u/ml, respectively. The assay was further calibrated using bovine serum cholinesterase; calculated values of enzyme activity agreed with the manufacturer's value within 10%.

G3PD activity was determined by the procedure of Steck (1974*a*) which is a modification of the method of Cori, Slein and Cori (1948). In sidedness determinations G3PD activity of a vesicle suspension was determined either with or without a brief pre-incubation in 0.1% Triton X – 100 as described for the AChE assay. Units of G3PD activity were calculated as described by Steck (1974*a*). Although the activity of G3PD has been reported to be sensitive to Triton (Shin & Carraway, 1973) no change in the G3PD activity of unsealed (frozen-thawed) ghosts was observed at this low Triton concentration.

For the determination of sialic and vesicles or ghosts were suspended in 0.1 N H_2SO_4 at 80 °C for 1 hr. At the end of this time the vesicles were pelleted and sialic acid in the supernatant was determined by the method of Warren (1959). Calibration was performed with crystalline sialic acid as a standard.

Neuraminidase was used at a concentration of 0.06 μg/μg membrane protein. Membranes were incubated with the enzyme for 1 hr at 37 °C in 0.1 M Tris acetate at pH 5.7, followed by two washes in 20 volumes of ice cold 10 mM Tris HCl at pH 7.4. Membranes used as controls in sidedness determinations were carried through the same procedure in the absence of neuraminidase.

Lipids were extracted by a modification of the method of Burger, Fujii and Hanahan (1968) (Cohen, 1974). If the extract was to be used only for quantitative analysis of phosphate, it was gently boiled to dryness and the residue analyzed for phosphate by the method of Gomori (1942). Phosphate standards were made by dissolving reagent grade Na phosphate in deionized water. Each lipid extraction was performed on three independent samples which always agreed to within 5%. Lipid to protein ratios of vesicles obtained using these methods agreed well with published values as will be discussed in connection with Table 2.

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using standards made from crystalline bovine serum albumin.

SDS Polyacrylamide Gel Electrophoresis

Gel electrophoresis of membrane proteins followed the procedure of Fairbanks *et al.* (1971) with minor modifications. Gels of 5% acrylamide (recrystalized from chloroform) 0.19% BIS were run in constant bore glass tubing of 0.25″ internal diameter. Electrophoresis was carried out for 2–3 hr at a current of 5–7 mA/tube and a voltage of 6–9 V/cm. Tubes were removed individually from the lower bath as the tracking dye (Pyronin-Y) reached a mark 8 cm from the top of the gel. Gels were stained for 16 hr in 25% isopropyl alcohol, 10% acetic acid and 0.025% Coomassie Blue. The gels were then soaked in 10% acetic acid, 10% isopropyl alcohol for 8 hr, followed by 10% acetic acid until the background was clear. Gels were scanned on a microdensitometer (Joyce-Loebel Co., Gateshead on Tyne, England) at 515 mM.

Determination of Bound Ca

Binding experiments were done at 22±2 °C. Vesicles containing 0.1–0.5 mg protein/ml in 10 mM Tris HCl were diluted to a final volume of 2 ml with this same buffer containing the appropriate concentrations of ^{45}Ca and ^{40}Ca and other additions as described in the text. In all experiments three independent suspensions were made at each Ca concentration. After 0.5–1 hr the vesicles were pelleted at 40,000 × g in an SM 24 head of a Sorvall RC 2B refrigerated centrifuge (Ivan Sorvall, Inc., Newtown, Conn.) at 22 °±2 °C for 15 min. Control experiments demonstrated that vesicle recovery (as monitored by total AChE activity in the pellet) was 95–100% after each of three successive spins of a typical vesicle suspension. The AChE accessibility of *IOVs* before the addition of Triton increased by 2–3% after each successive pelleting and resuspension. After centrifugation the supernatant was either decanted or removed by Pasteur pipet and saved for analysis. The sides of the polycarbonate centrifuge tubes were carefully swabbed with Q-tips to remove any drops of supernatant remaining, taking great care to avoid disturbing the translucent pellet of vesicles. The pellet was then resuspended in 2 ml of 10 mM Tris and made 0.5% in $LaCl_3$, to minimize the adsorption of small amounts of Ca to the sides of the tube. A 25 µl sample of the resuspended pellet was pipetted into 10 ml of PCS solubilizer (Amersham Searle Inc., Arlington Heights, Ill.) and counted for ^{45}Ca in a Nuclear Chicago Liquid Scintillation Counter (Nuclear Chicago Inc., Des Plaines, Ill.) after 4–15 hr in the dark. Control experiments showed that at a given free Ca concentration ^{45}Ca counts increased linearly with the number of vesicles in the pellet. The supernatant corresponding to a given pellet was counted either by pipetting 10 µl into the scintillation vial containing the pellet counts and counting again or by counting in a separate vial. Both techniques gave identical results to within 5%. Counts in pellet and supernatant samples were never less than 10,000 above background.

In all cases careful controls were performed to determine whether differences in Ca concentration or in the composition of sample solutions had any effect on the efficiency of ^{45}Ca counting. Counting efficiency in PCS was found to be independent of: Ca concentrations in the range used in our experiments, water content (up to about 10%) and salts (to about 100 µl of 0.15 M KCl).

Specific activity of ^{45}Ca in the supernatant was determined by analyzing the solution for total Ca by atomic absorption spectroscopy. Before analysis the solutions were made 0.5% in $LaCl_3$ to minimize adsorption of Ca to the walls of the test tubes. Standards were made in 10 mM Tris HCl at pH 7.4 containing 0.5% $LaCl_3$; a correction for extraneous Ca (generally 0.002–0.003 mM) from the Tris and $LaCl_3$ was incorporated into the calibration procedure.

Ca bound to vesicles was computed by dividing the ^{45}Ca activity in the resuspended vesicle pellet by the specific activity of ^{45}Ca in the supernatant, after correction for trapped volume (*see* below). In some cases bound Ca was determined from the difference in ^{45}Ca activity of the stock Ca solution and the supernatant from the vesicle pellet. The two procedures agreed to within 10%. For convenience Ca binding was normalized to total membrane AChE activity. As discussed below, Table 2 gives factors which permit the renormalization of our data to either membrane protein or phospholipid.

Determination of Trapped Volume

Below about 0.1 mM total Ca, Ca bound to vesicles was determined by measuring disappearance of ^{45}Ca from the supernatant of the vesicle pellet. However, this technique could not be used at Ca concentrations much above 0.1 mM because the fractional decrease in supernatant counts became too small to be measured reliably. Consequently to determine bound Ca accurately at high Ca concentrations it was necessary to account for unbound Ca in the vesicle pellet.

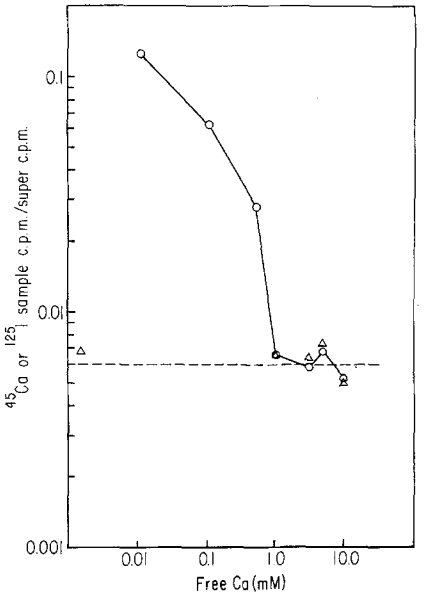

Fig. 1. Effect of increasing Ca concentration on the ratio of ^{45}Ca and ^{125}I in the vesicle pellet to that in the supernatant. *IOVs* were incubated as described in *Materials and Methods* in 0.01 to 10 mM Ca and ^{125}I-BSA with 1% w/v BSA carrier, all in 10 mM Tris-HCl at pH 9.0. Each point is the mean of three independent measurements on separate samples. The triangle to the far left is the ^{125}I ratio at no added Ca. The dotted line shows the mean value of the ^{125}I ratio. o—o, ^{45}Ca; Δ—Δ, ^{125}I

Vesicles were incubated as described above at various Ca concentrations in the presence of ^{125}I-bovine serum albumin (BSA) and 0.5% (w/v) unlabeled BSA. The vesicles were pelleted and samples of the supernatant and resuspended pellet were counted for ^{125}I activity. The trapped volume was calculated on the assumption that all of the BSA was external to the vesicles. All samples of ^{125}I-BSA were brought to the same final volume for counting in a Nuclear Chicago Model 422 Gamma counter.

If BSA is excluded from the interior volume of vesicles, BSA in the pellet would be due only to that in the trapped volume. The ratio of ^{125}I-BSA in the pellet to that in the supernatant would therefore be dependent only on the trapped volume. Similarly, if Ca binding is saturable then at Ca concentrations well above saturation, the ratio of ^{45}Ca in the pellet to that in the supernatant will also be proportional to the trapped volume, since as total Ca is increased,^{45}Ca in the trapped volume will eventually greatly exceed bound ^{45}Ca. These assumptions are verified in Fig. 1 which shows the pellet-to-supernatant ratio of ^{45}Ca and ^{125}I as a function of free Ca for a typical suspension of *IOVs*. Increasing the free Ca above 0.5 mM changes the pellet-to-supernatant ^{45}Ca ratio of 0.0064 by less than 20% in contrast to the large decreases below 0.5 mM Ca. We may therefore conclude that essentially all of the ^{45}Ca counts in the pellet above 0.5 mM are due to trapped volume in this experiment. Fig. 1 also shows points corresponding to the ratio of ^{125}I-BSA in the pellet to the supernatant. In the range in which all of the Ca is in the trapped volume the pellet-to-supernatant distribution ratio for BSA (0.0062 ± 0.0008 SD) is the same as that for Ca (0.0064 ± 0.0006 SD). Hence the trapped volume accessible to Ca is identical to the trapped volume accessible to BSA.

In the experiment shown in Fig. 1 the trapped volume of 0.6% v/v resulted in a reduction in bound Ca by an average of 20% at 0.5 mM Ca. Trapped volumes of vesicle pellets ranged from 0.2 to 0.7% v/v in different experiments. At the highest Ca concentration used (5 mM) this could account for at most a 50% reduction in vesicle Ca. Generally, six independent trapped volume measurements were made in each experiment. These agreed to within 20%, the average being used for trapped volume corrections for all samples in that experiment. No consistent trend in trapped volume as a function of Ca concentration was observed.

Normalization of Ca Binding to AChE

Since it is not known which membrane components are responsible for Ca binding it was decided to normalize all binding to unit AChE activity (that is, total AChE activity after treatment with Triton X–100 as described in *Methods*). We reasoned that since variable amounts of some proteins may be lost from the vesicles during storage (*see* below) total protein content would be a poor normalization if some other membrane component or non-elutable protein were contributing to Ca binding. Consequently, the enzyme AChE was chosen because it is tightly bound to the membrane and not easily lost or inactivated. Mitchell, Mitchell and Hanahan (1965) have shown that AChE is not lost from ghosts under a variety of conditions and Bramley, Coleman and Finean (1971) reported some loss of ghost AChE activity only after incubation of ghosts in low ionic strength bicarbonate buffer. It is likely that some of this loss is the result of AChE internalization accompanying ghost vesiculation at low ionic strength. Further, we have found that *IOVs* and *ROVs* evidence no loss of total AChE activity for up to 10 days if kept at 4 °C.

Results

Characterization of Vesicles

Table 1 gives values obtained in sidedness assays typical of 35 vesicle preparations. These preparations varied considerably in each of the parameters listed. For instance, AChE accessibility of *IOVs* varied from about 4% to 32% among different preparations with an average of about 14%. During the course of the experiments, minor variations were introduced into the vesicle preparation technique to improve the yield or purity. Although all of the vesicles used in these studies were prepared as described in *Materials and Methods*, we have recently found that replacement of the 0.5P8 vesiculation medium with 1 mM Na phosphate pH 8.0 results in a significant increase in *IOV* yield (20–40%) with only a 10–15% decrease in vesicle purity (C. M. Cohen, *unpublished observations*).

Despite variations, the trends in Table 1 were always maintained; that is, vesicles prepared under conditions designed to foster the formation of *IOVs* never showed more than 32% accessibility of AChE, and in fact the accessibility was greater than 25% in only 8 of 35 preparations.

Table 1. Characterization of vesicle sidedness[a]

Preparation	Marker								
	AChE			G3PD			Sialic acid		
	−Triton (u/ml)	+Triton (u/ml)	Accessible (%)	−Triton (u/ml)	+Triton (u/ml)	Accessible (%)	−Neur. (nM/ml)	+Neur. (nM/ml)	Accessible (%)
IOVs	0.05	0.59	8.5	0.045	0.045	100	59.2	42.6	30
ROVs	0.76	0.76	100	0.004	0.38	1	97.1	9.75	90
Unsealed membranes[b]	0.76	0.76	100	0.06	0.06	100	54	0	100

[a] Enzyme activity and sialic acid were assayed as described in *Materials and Methods.* Accessibility of AChE and G3PD was calculated according to the formula:

% Accessibility = 100 × (enzyme activity of untreated preparation)/(enzyme activity of 0.1% Triton X-100 treated preparation).

Accessibility of sialic acid was calculated according to the formula:

% Accessibility = 100 × (sialic acid of untreated preparation-sialic acid of neuraminidase (neur.) treated preparation)/(sialic acid of untreated preparation).

[b] Unsealed membranes were derived from the pellet of the Dextran shelf on which *IOVs* were separated.

ROVs invariably showed nearly 0% accessibility of G3PD and 100% AChE accessibility. The unsealed membranes derived from the pellet of the Dextran shelf were generally 100% unsealed to both AChE and G3PD. No change in the sidedness of either *IOVs* or *ROVs* could be detected up to 10 days after preparation.

Table 1 shows that while the G3PD of *IOVs* is 100% accessible, its absolute magnitude (when normalized to AChE for instance) is much less than that of *ROVs*. This is so in spite of the fact that *IOVs* have nearly as much membrane bound G3PD as do *ROVs* as may be seen by comparing band 6, which is the monomer of G3PD (Kant & Steck, 1973), of *IOVs* and *ROVs* in Fig. 2. In subsequent experiments we have found that a brief preincubation of *IOVs* with EDTA increases their G3PD activity to values comparable to those of *ROVs*, while maintaining 100% accessibility of this enzyme. (C. M. Cohen, *unpublished observations*). Finally, it will be noted from Table 1 that sialic acid accessibility in *IOVs* is significantly greater than AChE accessibility. This difference is most likely due to vesicle breakdown resulting from the harsh conditions of the neuraminidase treatment required for the measurement of sialic acid accessibility, rather than being due to a disparity in the distribution of these components across the plane of the membrane.

Table 2. Composition of various membrane preparations[a]

	AChE		Sialic acid	Phospholipid
	(u/mg protein)	(u/μmole phospholipid)	(nmoles/mg protein)	(nmoles/mg protein)
Ghosts (other studies)	2.11[b]	2.3–4.6[c]	78[d]	674[d]
IOVs (other studies)	3.18[b]	–	162.3[b]	–
IOVs (this study)	3.04	3.81	135	796
ROVs (this study)	2.42	3.4	148	698

[a] AChE and sialic acid were determined as described in *Materials and Methods*. Phospholipid was determined after extraction of lipids as described in *Materials and Methods*.
[b] Steck and Kant, 1974.
[c] Juliano, 1973.
[d] Steck, Fairbanks and Wallach, 1971.

Composition of Vesicles

Table 2 shows that vesicles have higher AChE activity, sialic acid and phospholipid concentration when normalized to protein than do ghosts, presumably because of protein loss from the membrane during preparation (*see* below). The table also shows that our data on *IOVs* agree well with those of other investigators. These data, along with the sidedness studies in Table 1, provide evidence that *IOVs* and *ROVs* constitute a highly reproducible preparation for the study of the red cell membrane.

Protein loss from vesicles is particularly important since, as will be mentioned in the *Discussion*, a fraction of Ca bound to *IOVs* may be associated with easily eluted peripheral membrane proteins. Fig. 2 shows a densitometer scan of a Coomassie Blue stained SDS polyacrylamide gel separation of proteins associated with the membranes of freshly prepared *IOVs*, *ROVs* and ghosts. The major bands are labeled in accordance with the numbering procedure of Fairbanks *et al.* (1971) and Steck and Yu (1973). Many investigators have demonstrated the elution of certain membrane bound proteins in media of low ionic strength (Marchesi, Steers, Marchesi & Tillack, 1970; Hoogeveen, Juliano, Coleman & Rothstein, 1970; Reynolds & Trayer, 1971). In accord with those findings, Steck (1974*a*) has shown that *IOVs*, which are prepared in a low ionic strength medium, are greatly depleted of spectrin (bands 1 and 2) and band 5.

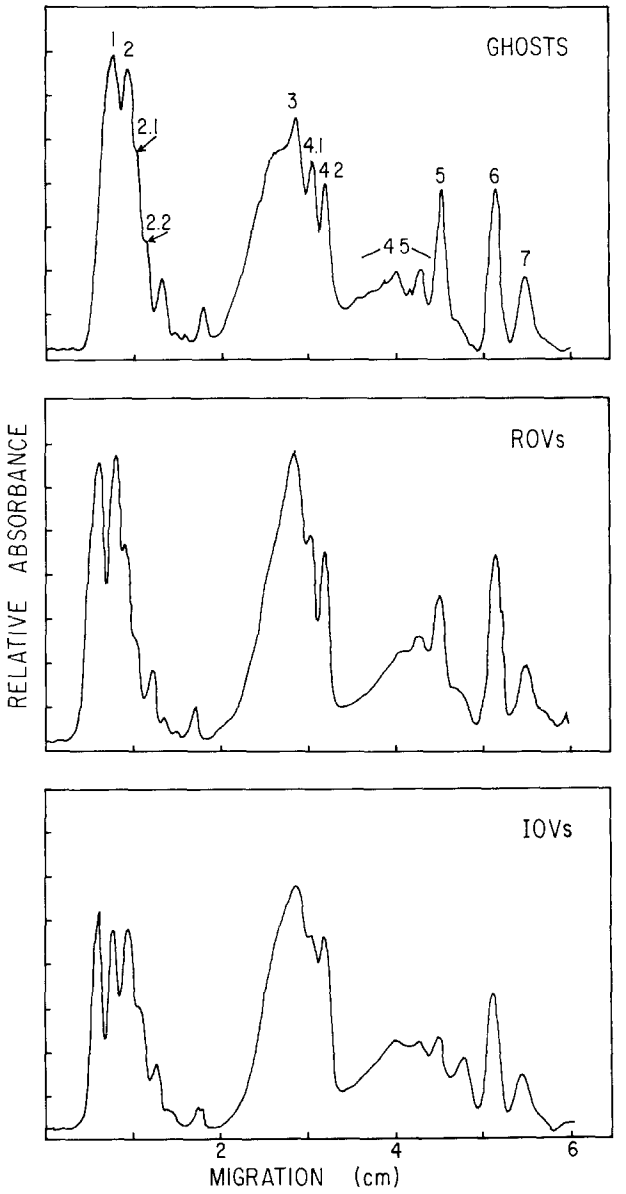

Fig. 2. Densitometer scan of a Coomasie blue stained SDS polyacrylamide gel separation of ghost, *ROV* and *IOV* membrane proteins

We have found that *IOVs*, although depleted of spectrin and band 5 with respect to their parent ghost suspension, possess quite variable amounts of these proteins. For example, vesicles prepared after long (1 hr or more) incubation of ghosts in 0.5P8 possess less spectrin than those prepared after a short (5 min or less) incubation. In addition, recent

experiments indicate that *IOVs* may be prepared by incubating ghosts in 1 or 2 mM Na phosphate pH 8.0; these vesicles have a significantly higher spectrin content than those formed from 0.5P8 (C. M. Cohen, *unpublished data*). In agreement with Steck (1974*a*) we find that *ROVs* have more nearly the normal complement of spectrin plus band 5 than do *IOVs*. *IOVs* stored in 10 mM Tris HCl, pH 7.4 and 4 °C for one week or more also evidenced some loss of bands 1, 2, 4.2 and 5. Consequently, Ca binding experiments were confined to vesicles less than 6 days old.

Ca Permeability of Vesicles

The Ca content of a vesicle pellet consists of Ca bound to the external surface of the vesicles, which we wish to measure, and Ca which may have leaked inside the vesicles. If internal Ca is appreciable compared to bound Ca, corrections are required. Ca binding to *IOVs* and *ROVs* was examined to determine whether there was a time dependent uptake of Ca which could be attributed to passage of Ca into the vesicle interior. Fig. 3 shows that Ca binding to *IOVs* and *ROVs* is essentially time independent, and complete in under 10 min. Ca bound to *IOVs* and *ROVs* increased 13% and 8%, respectively, after a two hr incubation. This increase is likely to be due to a very slow uptake of Ca since about 10% of the Ca of vesicles incubated in 0.05 mM Ca for 2 hr, as in Fig. 3, could not be washed from

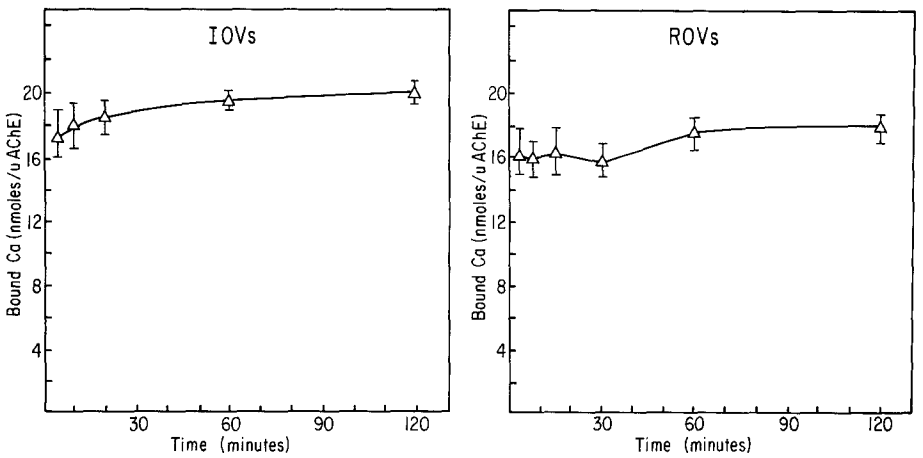

Fig. 3. Time dependence of Ca binding to *IOVs* and *ROVs*. Vesicles were incubated in 10 mM Tris-HCl at pH 7.4 with 0.05 mM Ca for times ranging from 5 to 120 min. Samples of the suspension taken at various times were pelleted and analyzed for bound Ca

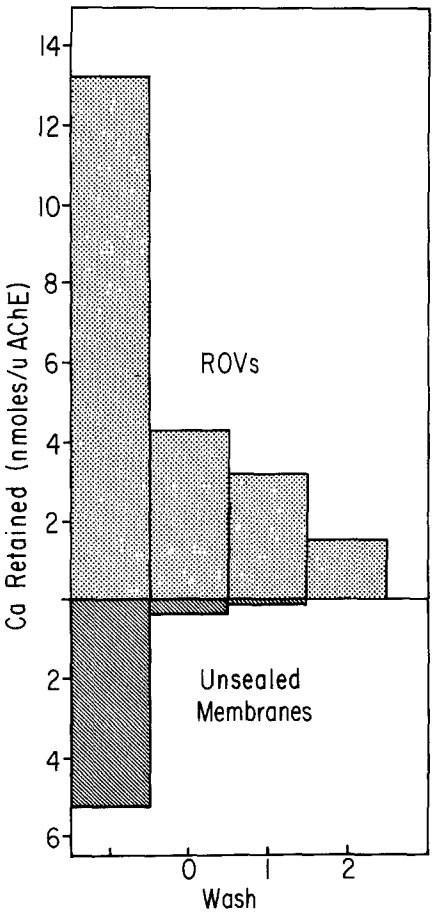

Fig. 4. Effect of washing on Ca retention by *ROVs*. *ROVs* were formed in the presence of ^{45}Ca and $CaCl_2$ at a final concentration of 0.05 mM Ca, added just before the vesiculated ghost pellet was homogenized. Vesicles were then separated on a Dextran shelf. The column to the left of the "0 wash" column represents the Ca content of vesicles which had been pelleted and resuspended in Ca free Tris after removal from the Dextran shelf. Each subsequent wash consisted of resuspending the vesicle pellet in a 50-fold excess of 10 mM Tris HCl at pH 7.4, and a subsequent centrifugation. The bottom half of the figure gives the corresponding results obtained with unsealed membranes derived from the pellet at the bottom of the Dextran shelf from which the vesicles were derived. After washing, these unsealed membranes showed 100% and 85% accessibility of AChE and G3PD, respectively. Each column is the mean of three independent determinations on separate samples

the vesicles by EGTA, although inclusion of EGTA during the 2 hr incubation prevented both binding and uptake resulting in no Ca being associated with the vesicles.

As a further test of Ca permeability, *ROVs* were formed in the presence of ^{45}Ca, added just after the membranes were pelleted from the 0.5P8,

0.1 mM Mg incubation medium. After removal from the Dextran shelf the *ROVs* were washed in Ca free 10 mM Tris, and aliquots were taken for ^{45}Ca determination. The upper part of Fig. 4 shows ^{45}Ca in *ROVs* as a function of the number of times they were washed in a 50-fold excess volume of 10 mM Tris. The lower part of the figure shows ^{45}Ca in the unsealed membrane pellet from which the *ROVs* were separated by the Dextran shelf.

The data in Fig. 4 is consistent with a slow loss of ^{45}Ca from the *ROVs* with each wash, in marked contrast to the rapid loss of ^{45}Ca from the unsealed membranes shown in the lower part of the figure. We may conclude that the reason *ROVs* retain so much of their internal Ca is that they possess only a limited permeability to this ion. The wash out profile of *ROVs* and unsealed membranes formed at other Ca concentrations was similar to Fig. 4. The Ca retained by *ROVs* after each wash was proportional to the initial concentration of added Ca for the three concentrations used (0.007 mM, 0.05 mM, and 0.1 mM Ca).

No reliable data could be obtained on Ca efflux from *IOVs* due to the restriction of having to form these vesicles at very low Ca concentrations. It was feared that forming vesicles at moderate Ca concentrations such as those used for *ROVs* would induce the formation of leaky vesicles (Steck, 1974*a*).

An additional confirmation of the relative impermeability of both types of vesicles to Ca comes from the data of Table 3. If the vesicles are impermeable to Ca then the sum of Ca bound to *IOVs* and *ROVs* at a given free Ca concentration should equal the Ca bound by an equivalent suspension of unsealed membranes. It may be seen from Table 3 that the sum of Ca bound to the two types of vesicles is comparable to that bound by unsealed membranes, particularly at low Ca concentrations. This

Table 3. Total membrane Ca binding capacity[a]

Free Ca (mM)	Ca bound to *IOVs* plus *ROVs* (nmoles/u AChE)	Ca bound to unsealed membranes (nmoles/u AChE)
0.02	30±5 (SD)	28±5
0.05	40±5	50±10
0.2	80±10	110±10
0.5	110±10	150±10

[a] The unsealed membranes were taken from the pellet of a Dextran shelf on which *IOVs* were separated; AChE and G3PD were both 100% accessible. *IOVs* showed 15% AChE accessibility and *ROVs* 2% G3PD accessibility.

confirms that Ca influx into either type of vesicle is too slow to account for a significant fraction of the Ca in a vesicle pellet.

It is interesting to compare the data in Fig. 4 with that of Kant and Steck (1972) on the Na permeability of *IOVs* and *ROVs*. These authors present a Na wash out profile for *IOVs* and *ROVs* formed in the presence of 0.5 mM Na which is quantitatively comparable to our results with Ca. Their *ROVs* retained about 25 nmoles Na/u AChE after two washes in Na free buffer. Similarly, *ROVs* formed in 0.2 mM Ca retained 7.3 nM Ca/u AChE. Since the proportion of ion retained to the initial external concentration is about the same in both cases this suggests that the *ROV* membrane is about as impermeable to Ca as it is to Na.

Ca Binding to Vesicles

Fig. 5 shows the effect of increasing $CaCl_2$ concentration on Ca binding to *IOVs* and *ROVs*. It is clear that, up to about 2.0 mM Ca, *IOVs* bind twice as much Ca as do *ROVs*. Scatchard plots were made from Ca binding data to determine the apparent binding constants and saturation

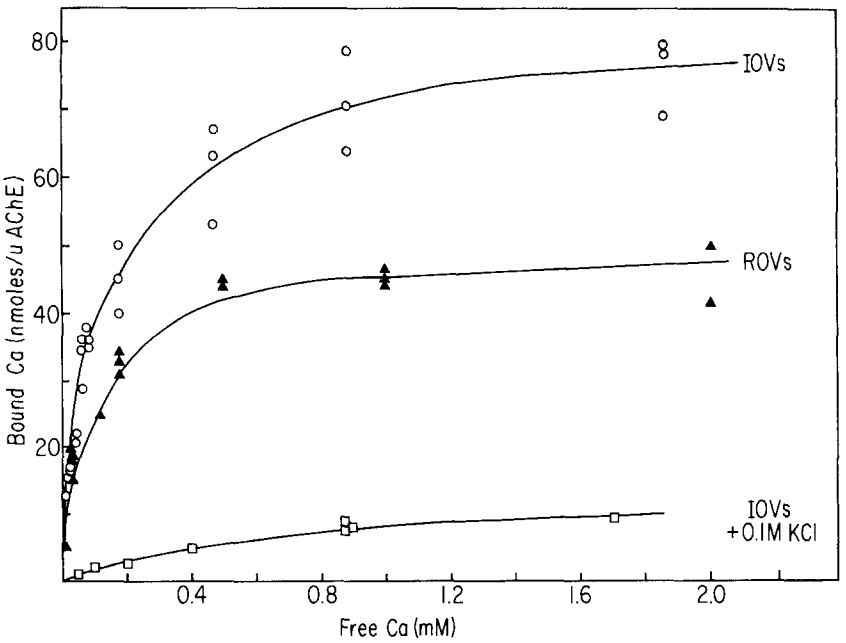

Fig. 5. Effect of increasing Ca concentration and KCl on Ca binding by *IOVs* and *ROVs*. Binding was measured, as described in *Materials and Methods*, in 10 mM Tris HCl at pH 7.4 with or without 0.1 M KCl

binding capacities of both types of vesicles. Fig. 6 shows one plot typical of Ca binding to *IOVs* at pH 7.4 in 10 mM Tris HCl. As mentioned previously, Ca bound to vesicles was normalized to total membrane AChE activity. Since this normalization was retained in our Scatchard plots, the intercepts of these plots on the abscissa are equal to the number of Ca binding sites per unit AChE. The convention of normalizing binding to number of sites per molecule obviously could not be used here due to the multi-component nature of these vesicles. A Scatchard plot of binding to a single class of independent sites is a straight line whose slope is equal to the apparent binding (association) constant and whose intercept on the abscissa is the total concentration of binding sites (Scatchard, 1949). Since the points in Fig. 6 do not form a straight line it is evident that there

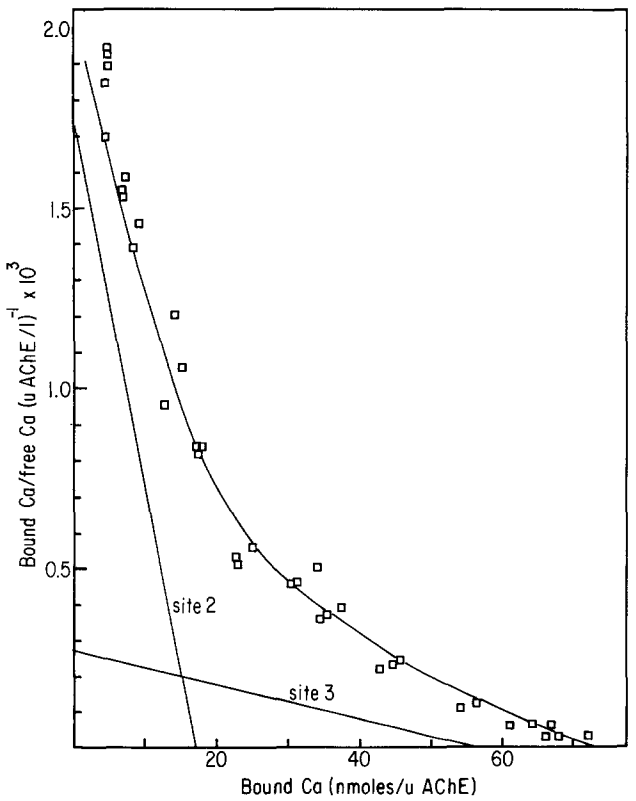

Fig. 6. Scatchard plot of Ca binding to *IOVs* in 10 mM Tris HCl at pH 7.4. Binding was measured at Ca concentrations of 0.002 to 2.5 mM. The two straight lines represent the individual Scatchard plots for the two sites. When Ca binding to these two sites is added in bound *vs.* free coordinates their sum gives the best least squares fit to the data. This sum is shown, transformed back to Scatchard coordinates, as the curved line through the data points. Pooled data from three vesicle preparations

is more than one class of binding site present or that Ca binding exhibits a negative cooperativity. We have interpreted the data on the basis of a number of independent classes of sites in accord with evidence given below. If we assume, as a first approximation, that there are only two independent classes of sites and that each Ca ion occupies a single site, the data may be fit to Eq. (1):

$$Ca_b = \frac{N_2 K_2 [Ca]}{1 + K_2 [Ca]} + \frac{N_3 K_3 [Ca]}{1 + K_3 [Ca]} \quad (1)$$

Ca_b and [Ca] represent the concentrations of bound and free Ca respectively and N_i and K_i the concentration and apparent binding constant of each class of sites. The sites in Fig. 6 are labeled 2 and 3 because, as will be shown later, there is a site of higher affinity than 2 which will be called site 1. Our data were fit to Eq. (1) by a standard nonlinear regression protocol (Snedecor & Cochran, 1967) adapted for iteration by computer. The computer program required an initial estimate for each of the four binding parameters (N_2, N_3 and K_2, K_3) along with the data. As a starting point, the first estimates were obtained from the slopes and intercepts on the abscissa of two straight lines drawn through the upper left and lower right extremities of a line connecting the data points. The line connecting the data points in Fig. 6 is the best least squares fit to the data generated from the computed binding parameters which are listed in Table 4. It should be noted that the two straight lines which are the

Table 4. Summary of Ca binding parameters

	Apparent binding constant (M^{-1})	Ca binding capacity (nmoles/u AChE)	Number of sites per equivalent red cell [a]
ROVs			
site 1	2 ± 0.3 [b] $\times 10^5$	3 ± 1	1.6×10^6
site 2	$1.4 \pm 0.05 \times 10^4$	32 ± 1	1.7×10^7
site 3	$3 \pm 0.3 \times 10^2$	55 ± 4	3.0×10^7
IOVs			
site 1	$3 \pm 1.6 \times 10^5$ [c]	0.3 ± 0.1	1.6×10^5
site 2	$1 \pm 0.05 \times 10^5$	18 ± 1	9.8×10^6
site 3	$6 \pm 0.2 \times 10^3$	53 ± 1	2.9×10^7

[a] Calculated using a value of 10 u AChE/ml packed red cells, and assuming each Ca ion occupies a single site.

[b] All errors are ±SEM.

[c] This binding constant was determined in the presence of 0.1 M KCl plus 10 mM Tris at pH 7.4 and is therefore not directly comparable to the other binding constants listed which were measured in the absence of potassium. *See* text for discussion.

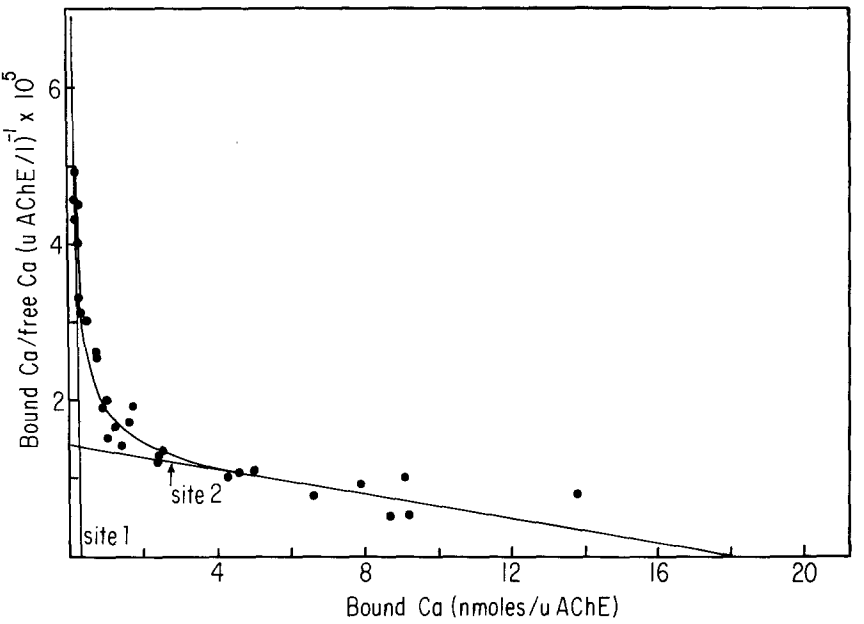

Fig. 7. Scatchard plot of Ca binding to *IOVs* in 0.1 M KCl, 10 mM Tris HCl at pH 7.4. Binding was measured at Ca concentrations of 0.003 to 1.8 mM

individual Scatchard plots for the two classes of sites are significantly different from the first estimates for these sites described above.

The fact that the first four points lie above the curve in Fig. 6 at low bound Ca concentration suggested that there may be a third, higher affinity, binding site present on *IOVs*. Therefore the binding contributed by the lower affinity component was subtracted from the total binding and the results plotted as a separate Scatchard plot. Although this procedure is more amenable to solution than attempting to analyze the data as a whole in terms of three binding sites, it still did not allow the demonstration of a third binding site.

If there were a site of higher affinity than site 2 in *IOVs* it might be detected more easily in the presence of a large excess of a competitor for Ca binding sites. Hence Ca binding to *IOVs* was measured in the presence of 0.1 M potassium. This data is shown in bound versus free coordinates in Fig. 5, and as a Scatchard plot in Fig. 7. Fig. 5 shows that the Ca binding has been reduced considerably by the addition of potassium. The Scatchard plot in Fig. 7 may be divided into two components; the lower affinity component having an intercept of 18 ± 0.5[2] nmoles/u AChE and a

2 Unless otherwise indicated, all errors in Ca binding parameters are ±SEM calculated from the nonlinear regression fit protocol by a standard method (Snedecor & Cochran, 1967).

slope of $8 \pm 0.3 \times 10^2$ M^{-1} and the high affinity component having an intercept of 0.3 ± 0.1 nmoles/u AChE and a slope of $3 \pm 1.6 \times 10^5$ M^{-1}. It is easily shown that while the equation for a Scatchard plot of binding to a single class of sites is given by:

$$Ca_b/[Ca] = K[N - Ca_b]$$

in the presence of a competitor the equation becomes:

$$Ca_b/[Ca] = \frac{K}{1 + K'[X]}[N - Ca_b]$$

where K' is the binding constant of the competitor and $[X]$ is the concentration of unbound competitor. If potassium is a competitive inhibitor of Ca binding its addition should reduce the apparent Ca binding constant while leaving the total number of sites of each class (the intercept of the Scatchard plot with the abscissa) unchanged. Since the intercept of site 2 in the presence of potassium (18 ± 0.3 nmoles/u AChE) agrees well with that of the high affinity site (site 2) of Fig. 6 (18 ± 1 nmoles/u AChE) we may reasonably conclude that these components represent the same site and that potassium is competing for Ca binding sites. Thus, the high affinity binding site seen in the presence of potassium represents an additional class of sites which could not be resolved in the absence of 0.1 M potassium. This class will be called site 1 of *IOVs* and has an N of 0.3 nmoles/u AChE. Since its binding constant was measured in 0.1 M potassium plus 10 mM Tris it is likely that the value in the absence of potassium, the condition under which all other binding constants were measured, would be greatly increased. We may estimate what this value might be by the following calculation. Since the reduction of the Ca binding constant of site 2 by 0.1 M potassium depends only upon the concentration of potassium and its affinity for site 2 we may calculate from the reduction of K_2 from 1×10^5 to 8×10^2 M^{-1} that potassium binds to site 2 of *IOVs* with an apparent binding constant of $1.2 \pm 0.06 \times 10^3$ M^{-1}. We have used the approximation that $\text{potassium}_{\text{total}} = \text{potassium}_{\text{free}}$ and have assumed that the stoichiometry of potassium and Ca binding to a single site is the same. Assuming further that the apparent binding constant of potassium to site 1 of *IOVs* is the same as that for site 2, we can calculate that the adjusted binding constant of Ca for site 1 (in the absence of 0.1 M potassium) would be $K_1 = 4 \pm 2 \times 10^7$ M^{-1}, a binding constant much greater than that of any other site on *IOVs* or *ROVs* (*see* below). A summary of the Ca binding parameters is presented in Table 4 which also shows the approximate number of Ca ions bound to each site per equivalent red cell.

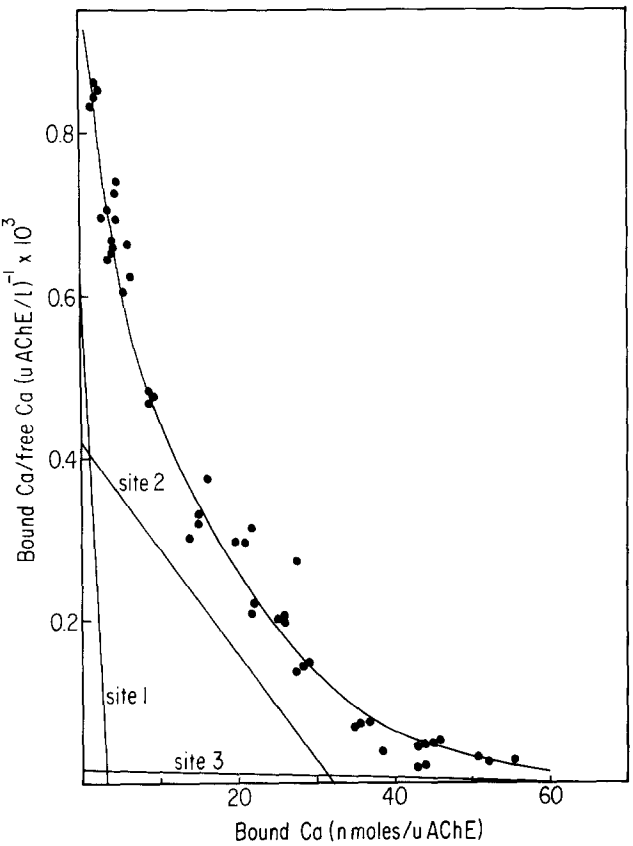

Fig. 8. Scatchard plot of Ca binding to *ROVs* at pH 7.4. Binding was measured at Ca concentrations of 0.003 to 5.0 mM. Pooled data from three vesicle preparations

Fig. 8 shows a Scatchard plot of Ca binding to *ROVs* at pH 7.4 in 10 mM Tris HCl. The low affinity region of the Scatchard plot was first fit with two binding sites. The computed Ca binding capacity of site 3 at each Ca concentration was then subtracted from the data points in bound *vs.* free coordinates at the corresponding concentrations. The resulting data were then refit by equation 1 to two binding components. From this second fit K and N for sites 1 and 2 were obtained. Finally, all three components were added together to yield the fit shown in Fig. 8. This figure shows that the data may be fit with three binding sites whereas no fit could be obtained using only two binding sites. The parameters for the three sites of *ROVs* are given in Table 4. It should be emphasized that although *IOVs* bind more Ca than *ROVs* at 2 mM Ca the total binding capacity of both vesicles (given by the intercepts on the abscissa of their respective Scatchard plots) is about the same.

The membranes of *IOVs* and *ROVs* are obviously heterogeneous systems with a potential for complex interactions among diverse Ca binding components. This suggests the possibility that the shape of the Scatchard plots is the result of such interactions, leading, in particular, to negative cooperativity in Ca binding. We cannot exclude this possibility completely on the basis of the data given here. However, our interpretation of the nonlinear Scatchard plots on the basis of independent binding sites as opposed to negative cooperativity is strongly supported by the foregoing data for *IOVs*. That is, the addition of 0.1 M potassium to *IOVs* reduces the apparent binding constant of site 2 while leaving the total number of sites unchanged in a manner consistent with site 2 being independent of either 1 or 3. The conclusion that *ROVs* possess three independent of both 1 and 3. The conclusion that *ROVs* possess three and 3 by neuraminidase treatment while the binding constant and number of Ca ions bound to site 2 remain unchanged (C. M. Cohen & A. K. Solomon, *manuscript in preparation*).

pH Dependence of Ca Binding

Fig. 9 shows the pH dependence of Ca binding to *IOVs* and *ROVs*. The data were obtained in two experiments covering the range of pH 4 to pH 11. These experiments were performed at a Ca concentration of 0.05 mM which resulted in 20 ± 1 (SD) nmoles Ca/u AChE bound to *IOVs* and 18 ± 2 (SD) nmoles Ca/u AChE bound to *ROVs* at pH 7.4. A calculation based on the data of Table 4 shows that between pH 7 and pH 8, at this Ca concentration, the major contribution to Ca binding comes from sites 1 and 2 of both *IOVs* and *ROVs*. Consequently the pH dependence shown represents the combined contribution of at least these two sites. These experiments were performed in the presence of 0.1 mM Tris to provide some buffering between pH 7.4 and pH 9.0. This low concentration of Tris was used because, as will be shown below, Tris acts as a competitor for Ca binding. Hence, deprotonation of Tris at high pH could cause an apparent increase in Ca binding due to decreased Tris competition for Ca binding sites.

Fig. 9 shows that both *IOVs* and *ROVs* increase their binding capacity with pH. This result is in accord with the findings of Forstner and Mannery (1971) who studied the pH dependence of Ca binding to ghosts. Our data

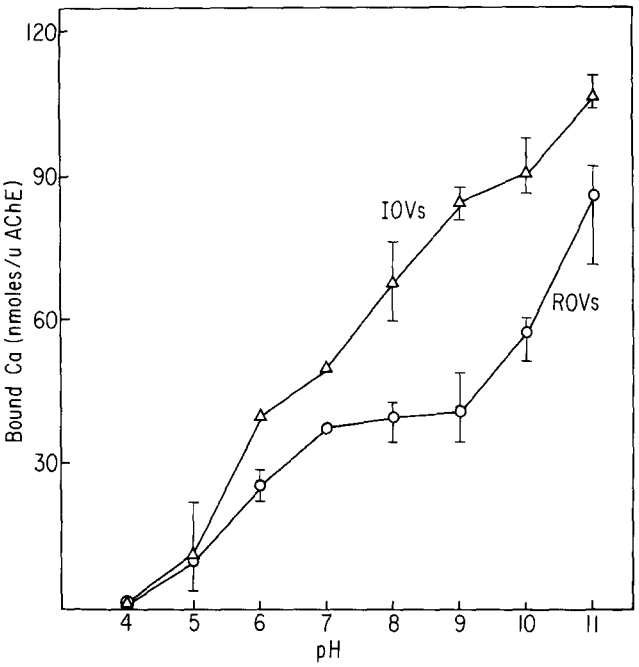

Fig. 9. pH dependence of Ca binding to *IOVs* and *ROVs*. Vesicles were incubated in 0.1 mM Tris HCl containing 0.05 mM Ca. Three independent suspensions were made at each pH which was adjusted to ± 0.05 pH units using a semi-micro electrode on a digital pH meter. The pH was checked and readjusted if necessary at the end of 10 min and at 45 min, just before pelleting. The points are the mean of three independent determinations, and the bars show the range of the data

provide the additional result that the pH dependence of binding to the internal membrane surface is distinctly different from that of the external surface. In particular, between pH 7.0 and 9.0, *ROVs* change their Ca binding very little whereas *IOVs* almost double their binding capacity. Ca binding to *IOVs* and *ROVs* could not be reliably compared below about pH 4.5 because *IOVs* formed a flaky precipitate which could not be resuspended easily for ^{45}Ca counting. *ROVs* remained in solution at the lowest pH tested. A Scatchard plot of Ca binding to *IOVs* at pH 9.0 showed that site 3 had increased its binding capacity significantly (120 ± 5 nmoles/u AChE at pH 9.0 compared to 53 ± 1 nmoles/u AChE at pH 7.4, both in 10 mM Tris). Due, however, to the large pH dependence of Tris competition for Ca binding sites reliable conclusions about the pH dependence of Ca binding to this or other individual sites cannot be drawn.

Competition for Ca Binding Sites and the Effect of ATP

Fig. 10 shows the effect of various competitors on Ca binding to *IOVs* at a Ca concentration of 0.05 mM. To induce a 60% reduction in Ca binding, Mg is roughly 20-fold more effective as a competitor than either potassium or Tris. It is interesting to note that Tris competes for Ca binding sites somewhat more effectively than potassium, an observation of relevance for Ca binding experiments done in Tris. The apparent binding constants derived from the Scatchard plots of binding to *IOVs* and *ROVs* may be adjusted for the presence of Tris just as the constant for site 1 of *IOVs* was corrected for the presence of 0.1 M potassium. Fig. 10 shows that Tris competition for Ca binding sites is similar to that of potassium at 0.05 mM Ca, so we may use the potassium binding constant as a first approximation for that of Tris. On this basis, the apparent binding constant of site 2 of *IOVs* measured in 10 mM Tris should be multiplied by a factor of 13 to adjust for the presence of Tris. Fig. 10 also shows the competition of Mg for Ca binding at a Ca concentration of 0.003 mM. At this lower Ca concentration Mg is a less potent inhibitor of binding. Further information on the specificity of binding at low Ca concentration is given in Table 5 which shows the inhibition of Ca binding by a series of di- and trivalent cations. It is interesting to note that Cd which produces the maximum inhibition of binding among all the divalent ions tested, is also the most effective inhibitor of Ca binding to the external surface of intact red cells (Long & Mouat, 1971). In order to compare the relative binding affinity of these cations with that of Ca, unlabeled Ca was added to *IOV* suspensions containing 0.003 mM ^{45}Ca to a final Ca concentration of 0.1 mM. The consequent reduction in bound ^{45}Ca is included in Table 5 for comparison.

Since it has been suggested that intracellular ATP plays an important role in the regulation of Ca binding by the inner surface of the red cell membrane (Chau-Wong & Seeman, 1971), we investigated the effect of ATP on Ca binding to *IOVs*. We found that ATP significantly decreased the amount of Ca bound to the vesicles, in agreement with the results of Chau-Wong and Seeman. At a Ca concentration of 0.025 mM in 10 mM Tris HCl, pH 7.4, Ca binding was reduced by 63% by 0.05 mM ATP (tri sodium salt) and 83% by 0.1 mM ATP. It appears, however, that not all of the reduction in binding can be accounted for by chelation of free Ca by ATP. Using a value of 8.5×10^3 M^{-1} for the association constant of Ca – ATP (Nanninga, 1961) it may be calculated that at 0.1 mM ATP the free Ca is reduced from 0.025 mM to about 0.012 mM. (This experiment

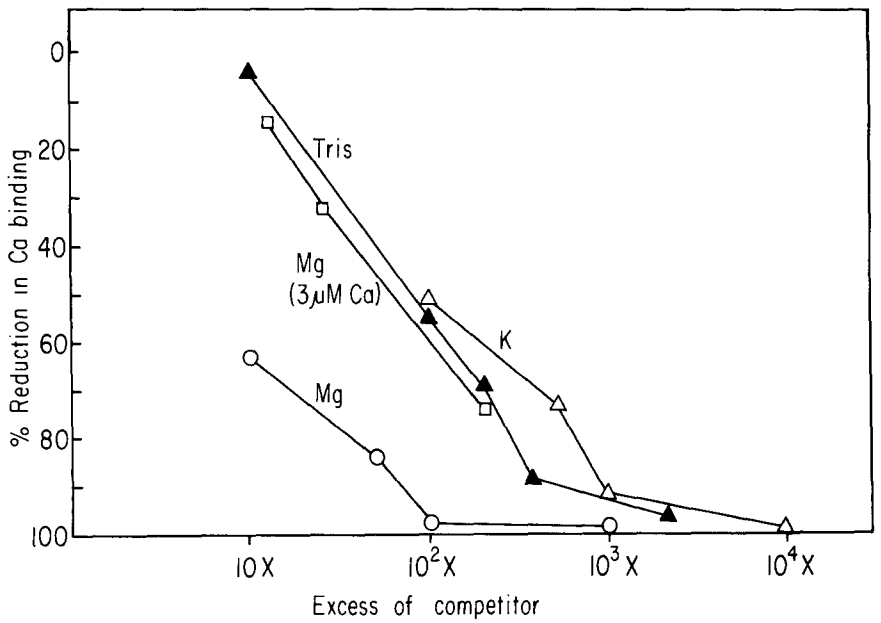

Fig. 10. The effect of various cations on Ca binding to *IOVs*. Unless indicated otherwise binding was measured at 0.05 mM Ca in 10 mM Tris HCl. Each point is the mean of three independent determinations on separate samples. The range of data about each point is less than 10% of the plotted values

Table 5. Inhibitory potency of di- and tri-valent cations on Ca binding to *IOVs*[a]

Cation	Reduction in Ca binding (%)
La	83
Cd	81
Mn	67
Zn	63
Ca	54
Sr	42
Ba	37
Mg	30
Hg	18

[a] *IOVs* were incubated for 10 min in 0.003 mM Ca, 10 mM Tris HCl at pH 7.4 with the cations indicated, at concentrations of 0.1 mM. The % reduction in Ca binding was determined by comparison with Ca bound in the absence of competing cations under identical conditions.

was done at a very dilute vesicle concentration so that in no case did Ca binding by vesicles change the free Ca concentration by more than 10%.) According to the Ca binding data for *IOVs* this decrease in free Ca would result at most in a 30–40% reduction in bound Ca, in contrast to the

reduction of 83% obtained in the experiments. A possible explanation for this discrepancy is that ATP, as a divalent cation chelator, may induce the elution of peripheral proteins associated with the surface of *IOVs* (Marchesi & Steers, 1968). In a control experiment we have found, however, that *IOVs* incubated in 0.5 mM ATP under conditions identical to those used above show no appreciable alteration in polypeptide composition on SDS polyacrylamide gels. The discrepancy, therefore, remains unexplained.

Discussion

We have used inside out and right side out vesicles to study Ca binding to the two surfaces of the human red cell membrane. The view that these vesicles are representative of the membrane in its native configuration is supported by a considerable body of experimental data (Steck, 1974*a*). Both *IOVs* and *ROVs* possess all of the major membrane associated proteins of ghosts, although in different proportions. Furthermore, proteolytic digestion and chemical and enzymatic labeling studies have shown that the trans-membrane disposition of the major membrane proteins of these vesicles is identical to that of ghosts and intact cells (Steck, 1972; Boxer, Jenkins & Tanner, 1974). In addition, these vesicles apparently maintain the phospholipid asymmetry of their parent ghost membranes (Kahlenberg, Walker & Rohrlick, 1974). We have presented information on the composition and sidedness of both *IOVs* and *ROVs* not only for their pertinence to our Ca binding studies but also in the hope that a detailed and independent confirmation of their properties will emphasize the usefulness and reproducibility of these preparations.

One observation is of particular relevance to the use of *IOVs* for Ca binding studies. Fig. 2 shows that *IOVs* evidence a marked depletion of the membrane proteins spectrin (bands 1 and 2) and band 5, both of which are loosely bound to the inner surface of ghosts and presumably intact cells (Steck, 1972; 1974*b*). It has been reported that a group of proteins consisting primarily of spectrin and some low molecular weight polypeptides extracted from ghosts is capable of binding large amounts of Ca (LaCelle, Kirkpatrick, Udkow & Arkin, 1973). If these Ca binding sites are in fact associated with spectrin and/or band 5 then it would seem that the depletion of these proteins from *IOVs* would result in a corresponding decrease in the Ca binding capacity of vesicles compared to ghosts or intact cells. Consequently, in estimating the number of Ca binding sites of red cells from *IOV* Ca binding data as was done in Table 4 it must be

kept in mind that if any of these sites correspond to spectrin and/or band 5 they may be increased two to threefold in number in cells or ghosts due to their higher spectrin plus band 5 content. In recent experiments, however, we have failed to detect any high affinity Ca binding to purified spectrin in solution (C. M. Cohen, *unpublished data*) suggesting that spectrin by itself is not likely to be the locus of either site 1 or 2 of *IOVs*.

Our data show that *IOVs* bind Ca to three classes of binding sites, a finding consistent with the view that the effects of Ca on the inside of the human red cell are mediated through specific interactions at the inner membrane surface, an hypothesis advanced by several authors (Weed *et al.*, 1969; Palek *et al.*, 1971; LaCelle *et al.*, 1973). It would seem likely that the effects of low concentrations of intracellular Ca on the red cell membrane are mediated primarily through Ca binding to site 1 (and possibly 2) of *IOVs*, of which there are approximately 1.6×10^5 per cell.

Although there have been no previous studies of Ca binding to the inner surface of the red cell membrane, Long and Mouat (1971) have shown that unsealed ghosts will bind approximately twice as much Ca as a corresponding number of intact red cells at 2.5 mM Ca. Using a value of 10 u AChE/ml packed red cells to convert Ca bound per liter packed red cells to Ca bound per u AChE, Long and Mouat's data may be shown to be equivalent to 102 nmoles Ca/u AChE bound to unsealed ghosts at 2.5 mM Ca. Our data gives a value of 125 ± 27 (S.D.) nmoles/u AChE when the binding capacity *IOVs* and *ROVs* (pH 7.4, 10 mM Tris HCl) are added, in satisfactory agreement with the results of Long and Mouat.

Long and Mouat studied Ca binding to intact human red cells and reported three classes of binding sites with apparent binding constants of 6×10^4, 3.6×10^3 and 6×10^2 M^{-1}. Comparison of these values with the apparent binding constants of *ROVs* shown in Table 4 reveals only minimal agreement. Long and Mouat do not, however, indicate how they have decomposed their Scatchard plot. It is clear from Fig. 8 for instance, that attempting to derive the slopes of the individual components of the Scatchard plot from the line through the data points will give unreliable values. Further, the intercept on the abscissa of our Scatchard plot for *ROVs* (90 ± 4 nmoles/u AChE or 0.90 ± 0.04 mmoles/liter packed cells) which is equal to the Ca binding capacity at saturation does not agree well with the intercept of Long and Mouat's plot (0.45 mmoles/liter packed cells), nor is the shape of our plot in the low bound Ca region similar to theirs. We feel that this discrepancy is a consequence of the different conditions of binding in these two studies (10 mM Tris at pH 7.4 in this study, and isosmotic sucrose in Long and Mouat's study) and does not reflect a major difference in the surface of an *ROV* and an intact red cell.

Several authors have demonstrated the presence of a "tightly bound" pool of Ca in the intact red cell. This Ca cannot be removed from intact cells by repeated washing in Ca free isotonic media. Harrison and Long (1968), in carefully controlled experiments, showed that intact cells retained 0.015 mmoles Ca/liter packed cells (or approximately 1.5 nmoles/u AChE) after repeated washing in isotonic NaCl. Fujii, Sato and Hanzawa (1973) and Lichtman and Weed (1973) in similar experiments obtained values of 0.025 mmoles/liter and 0.0154 mmoles/liter respectively. We have repeated Harrison and Long's original procedure (Cohen, 1974) and have obtained a value of 0.017 ± 0.005 (SD) mmoles/liter packed cells. Harrison and Long also showed that this tightly bound Ca could be removed from intact cells by chelating agents, suggesting that the Ca is bound to the external cell surface. It would seem likely that the high affinity site of *ROVs* (site 1, Fig. 8) could be the locus of this tightly bound Ca, since Table 4 shows that this site will bind 3 ± 1 nmoles Ca/u AChE in satisfactory agreement with the value of 1.5 nmoles/u AChE given for the "tightly bound" Ca above.

The authors would like to express their sincere thanks to Dr. John W. Peterson, III, for his invaluable assistance with the computer analysis of the Scatchard plots, and to Dr. T.L. Steck for his helpful advice on the preparation of vesicles during the initial stages of this work. This work was supported by the National Institutes of Health grants, TG-2T01 GM 00782-16 and 5 R01 HL 14820-03.

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