INTRAMEMBRANE PARTICLE AGGREGATION IN ERYTHROCYTE GHOSTS

I. The Effects of Protein Removal

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ABSTRACT

We have used freeze-etching and SDS-polyacrylamide gel electrophoresis to study the conditions under which the intramembrane particles of the human erythrocyte ghost may be aggregated. The fibrous membrane protein, spectrin, can be almost entirely removed from erythrocyte ghosts with little or no change in the distribution of the particles. However, after spectrin depletion, particle aggregation in the plane of the membrane may be induced by conditions which cause little aggregation in freshly prepared ghosts. This suggests that the spectrin molecules form a molecular meshwork which limits the translational mobility of the erythrocyte membrane particles.

Surface proteins are mobile in many cell membranes (Frye and Edidin, 1970; Brown, 1972; Cone, 1972; Pinto da Silva, 1972; Singer and Nicolson, 1972), and can readily be aggregated or capped by antibodies or lectins (Taylor et al., 1971; Loor et al., 1972; Edidin and Weiss, 1972). However, in many different types of cells, specific components are localized in limited areas of the plasma membrane (for recent descriptions, see Stirling 1972; Friend and Gilula 1972; Osterhelt and Stoeckenius, 1973; Staehelin et al., 1972), and in such cases there must be mechanisms which restrict the distribution of these membrane macromolecules. We have used the membrane of the human erythrocyte as a simple but highly specialized system in which to study one example of such restrictions.

The freeze-etch technique, when used in the preparation of electron microscope specimens, reveals a large number of approximately 85 Å particles on the fracture faces of the human erythrocyte (Engstrom, 1970; Pinto da Silva and Branton, 1970). These particular structures are commonly referred to as freeze-etch particles or intramembrane particles. There are now strong indications that such intramembrane particles represent proteins or protein aggregates intercalated into the membrane lipid bilayer (Branton and Deamer, 1971; Hong and Hubbell, 1972). Human erythrocyte membranes contain two major glycoproteins, the carbohydrate moieties of both being exposed on the cell surface (Steck, 1974). The one richest in sialic acid and other sugar residues has been named glycophorin (Marchesi et al., 1972).
and is also referred to as PAS-I (Fairbanks et al., 1971). The other contains less carbohydrate (Tanner and Boxer, 1972) and is commonly referred to as band 3 (Fairbanks et al., 1971). These two major glycoproteins carry important antigenic determinants including ABO and MN blood group polysaccharides, and influenza virus and lectin binding sites (Marchesi et al., 1972). Chemical labeling experiments (Breccher, 1971 and 1973; Segrest et al., 1973) and proteolytic dissection (Steck et al., 1971; Kant and Steck, 1972; Steck, 1972 b) suggest that these glycoproteins extend completely through the membrane to the cytoplasmic surface where they may interact with other membrane proteins. Experiments in our laboratory (Pinto da Silva et al., 1971) and elsewhere (Tillack et al., 1972) show that they are associated with and possibly form the principal components of the intramembrane particles.

Pinto da Silva (1972) reported that the intramembrane particles of erythrocyte ghosts can be aggregated by incubation at pH 5.5. The experiments we report here were designed to investigate this aggregation phenomenon in greater detail. They show that the fibrous membrane protein, spectrin (Marchesi et al., 1970; Clarke, 1971 a and b), which is located on the cytoplasmic side of the erythrocyte membrane (Nicolson et al., 1971), restricts the mobility of these intramembrane particles.

MATERIALS AND METHODS

Preparation of Isolated Erythrocyte Membranes

Human erythrocyte ghosts were prepared according to the procedure of Dodge et al. (1973). In summary, freshly drawn human blood (type O) was mixed with citric acid anticoagulant (Handbook of Clinical Data, 1968, The Chemical Rubber Co., Cleveland, Ohio, p. 386) and stored at 0.4°C for no longer than 1 h. The erythrocytes were then washed once in 16 vol of PBS, once in 40 vol of PBS, once in 40 vol of 310 PB, pH 7.6, and then lysed in 40 vbl of 20 PB, pH 7.6. After centrifugation, the ghosts were washed twice in 40 vol of 20 PB, pH 7.6, and once in 4 vol of 20 PB, pH 7.6. At this stage the ghosts were gray-white with only the slightest touch of pink. After each wash of the erythrocytes, the leukocytes forming the "buffy coat" were removed as thoroughly as possible by aspiration. Similarly, the cream-colored sticky pellet which formed beneath the loose ghost pellet was removed after each wash of the ghosts. Temperature was carefully maintained at 0.4°C. In experiments calling for fresh ghosts, the isolated erythrocyte membranes were stored on ice for no more than 1 h before use. Experiments in which protein release during different ghost pretreatments was assayed required the strictest standardization. Because these experiments required an entire day, the fresh ghosts were prepared on the previous afternoon and stored overnight (15 h) in a sealed tube on ice before use.

Freeze-Etch Electron Microscopy

A ghost suspension (1.0 μl) was transferred to copper disks and rapidly quenched in liquid Freon 22 (chlorodifluoromethane) cooled to its freezing point by liquid N2. Freeze-fracturing or freeze-etching was done by standard techniques (Fisher and Branton, 1974) in a Balzers apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). Replicas were cast by using Pt-C and C anodes heated by electron bombardment from tungsten cathodes (Moor, 1971). Replicas were cleaned in household bleach, mounted on bare 400-mesh grids, and examined in a Siemens IA electron microscope.

SDS-Polyacrylamide Gel Electrophoresis

The procedures used were essentially those of Fairbanks et al., 1971, except that the gels contained 10% glycerol and were simultaneously fixed and stained for protein in freshly made 0.05% Coomassie brilliant blue in 35% TCA for 15-20 h. Diffusion destaining in 7% HAc required about 1 h. When desired, further destaining was accomplished in a 10% isopropanol-10% HAc solution. Alternatively, gels were stained for carbohydrate with PAS (Fairbanks et al., 1971).

Pretreatment Procedures

10.0 ml of liquid was added to 1.0 ml of packed fresh ghosts. The osmolarity, pH, and temperature of the added liquid were such as to achieve the desired pretreatment conditions in the resultant 11.0 ml of suspended ghosts, which were then incubated at the desired temperature. Even when not specifically mentioned, 0.5 mM NaN3 was included to inhibit bacterial growth in all pretreatments at 37°C lasting more than 30 min.
Aggregation Conditions

(a) Standard pH 5.5 incubation. After the desired pretreatment incubation, 1 ml of the 11-ml ghost suspension was cooled to 0-1°C and added to 39 ml of 20 PB, pH 5.5, on ice. This suspension was spun down at 20,000 g for 10 min at 0-4°C and the pellet resuspended in 40 ml of 20 PB, pH 5.5, on ice. After incubation on ice for 30 min the suspension was again centrifuged at 20,000 g for 10 min at 0-4°C. Samples of the pellet were then frozen for freeze-fracturing. (b) Where other aggregation conditions were employed, they are described in the text.

Assay of Protein Release

1.0 ml of the pretreated ghost suspension was assayed for total protein (Lowry et al., 1951).

To measure protein which had been released from the membranes by the pretreatment, the following procedure was adopted. The remaining 9 ml of ghost suspension was sonicated for 1 s with the microtip on a commercial sonifier (model 185W, Branson Instruments Co., Stanford, Conn.) at 30 W. The sample was then shaken gently for 20 s to facilitate release into free solution of any dissolved protein molecules which had remained entrapped within the ghosts. 8.5 ml of this sonicated suspension were centrifuged at 100,000 g for 30 min at 0-4°C, and three 1.0-ml samples of the supernate were then taken for Lowry total protein assay. The percentage of protein released (R) as a result of the pretreatment was then calculated with the formula:

$$R = \frac{C_1}{C_2} \times 100\%,$$

where $C_1$ = milligrams protein per milliliter of the supernate after sonication and centrifugation, and $C_2$ = milligrams protein per milliliter of ghost suspension before sonication and centrifugation (total protein). Three times-crystallized human serum albumin (Sigma Chemical Corp., St. Louis, Mo.) was used as a standard for the Lowry protein assay. 4 ml of the same supernate was frozen, concentrated by lyophilization, and dissolved for analysis on SDS-polyacrylamide gels.

Glutaraldehyde Cross Linking

At ice temperature, 500-μl samples of packed fresh or pretreated ghosts were added to 4.5 ml of 20 PB, pH 7.6, containing concentrations of glutaraldehyde from 0 mM to 150 mM. The pretreated ghosts had been incubated in 20 PB, pH 8.5, at 37°C for 22 h and then washed once in 80 vol of 20 PB, pH 7.6, before addition to the glutaraldehyde-containing solution. After incubation on ice in the glutaraldehyde solution for 90 min, each 5-ml suspension of ghosts was diluted to 40 ml with 20 PB, pH 7.6, and centrifuged at 20,000 g for 10 min at 0-4°C. The pellet was resuspended in 40 ml of 20 PB, pH 7.6, and sufficient cold CHsNH2-HCl pH 7.0 solution was added to bring its concentration up to 2.5 mM. The suspension was then left on ice for 30 min to allow reaction between any free aldehyde groups and the CHsNH2-HCl. The ghosts were harvested by centrifugation and washed once by resuspension and centrifugation in 40 ml of ice-cold 20 PB, pH 7.6.

Trypsin Digestion of Intact RBCs

A solution of 10 mg/ml trypsin (Worthington Biochemical Corp., Freehold, N.J.) in 1 mM HCl was made up just before use. Blood was drawn and the erythrocytes washed three times, as for the preparation of ghosts. The erythrocytes were then washed in 40 vol of room temperature 12 mM Tris-HCl-130 mM NaCl-10 mM CaCl2; the pH of this Tris-salt solution had previously been adjusted at 37°C to pH 8.1. The washed erythrocytes were resuspended in the same buffer to make a 20% hemocrit, as determined by use of a microcapillary centrifuge run for 3 min at 11,300 rpm. Trypsin was added to a concentration of 35 μg/ml and the suspension incubated at 37°C for 45 min. The cells were then pelleted by centrifugation at 800 g for 10 min, the supernate was removed and saved for carbohydrate analysis, and the cells were resuspended in 40 vol of ice-cold PBS containing 2.5 μg/ml soybean trypsin inhibitor (Worthington Biochemical Corp.). After a 5-10-min incubation on ice, the cells were pelleted at 800 g for 10 min, then washed once in 40 vol PBS at 0-4°C and once in 310 PB, pH 7.6 at 0-4°C. The erythrocytes were then lysed and the ghosts isolated as previously described.

The effects of trypsin digestion were observed by comparing the Coomassie brilliant blue and PAS gel staining patterns of ghosts made from the trypsin-digested erythrocytes with those of fresh ghosts. A phenol-sulfuric acid assay (Dubois et al., 1956) was used to determine the amount of carbohydrate released into the supernate by the trypsin treatment. To take into account the small amount of lysis and concomitant sugar release during the incubation in the trypsin digestion medium (maximally, 5% of the total hemoglobin was released by lysis), a family of calibration curves for the phenol-sulfuric acid assays was made using glucose solutions to which known amounts of erythrocyte lysate were added. The actual amount of lysis in each assay sample was determined by measuring the hemoglobin absorption at 490 mm before addition of the phenol-sulfuric acid reagents. The appropriate calibration curve was then used.

Neuraminidase Digestion of Intact Erythrocytes

Blood was drawn and the erythrocytes were washed three times as described for preparation of ghosts. The erythrocytes were then washed once in 40 vol of ice-cold 20 mM Tris-maleic acid, 145 mM NaCl, pH 5.5. 4 ml of these washed, packed erythrocytes were then incubated for 60 min at 37°C after resuspension in 150 ml of the
same buffer containing two units of Sigma type VI
Neuraminidase from Clostridium perfringens. No lysis
was detected during this 60-min incubation, after which
the erythrocytes were washed once in 40 vol of 310 PB,
pH 7.6, at 0-4°C. The erythrocytes were then lysed as
described earlier. The ghosts made from these neurami-
dase-treated erythrocytes were pinker than those made
from non-neuraminidase-treated cells.

The effect of the neuraminidase treatment was assayed
by measuring the amount of NANA which remained
bound to these ghosts. 500 μl of packed ghosts were
added to 200 μl of 0.4 N H₂SO₄. After incubation at
80°C for 60 min, the suspension was centrifuged at
25,000 g for 20 min and 200 μl of clear supernate were
withdrawn for the NANA assay (Warren, 1959). Stan-
dards containing known amounts of NANA were sub-
jected to identical treatment, including the centrifugation
routine, to provide a calibration curve.

The total protein content of the packed ghosts was also
determined by the Lowry procedure. Hemoglobin ac-
counted for no more than 5% of the total protein content
in the pinkish neuraminidase-treated ghosts.

Quantitation of Intramembrane
Particle Aggregation

The degree of particle aggregation observed in each
freeze-fracture replica was quantified by visual compari-
son with the set of six aggregation states, 0-5, (Fig. 1)
used as our standards. These standard aggregation states
illustrate easily distinguishable points within the continua-
to the fully dispersed state typical of untreated
erthrocytes and untreated ghosts, to a state in which
the particles are very aggregated. In each case, the degree
of aggregation recorded was the average value determined
after examining at least 10 fracture faces in the replica.
Although we recorded observations only of the particle-
rich fracture face on the half-membrane closest to the
erythrocyte cytoplasm, the complementary fracture faces
showed a complementary pattern of particles and depres-
sions. The uncertainty limits shown on our graphs
represent the range of aggregation states of more than
two-thirds of the fracture faces observed, as well as the
uncertainty (no greater than 0.5 units) in assigning a
numerical value to a given fracture face. Overall, this
simple comparative approach to the quantitation of
particle aggregation proved to be both reproducible and
observer-independent.

It is important to recognize that even though we have
found this method of quantitation very convenient in
conveying our results, our standard aggregation states
(Fig. 1) do not form a proportional scale. Thus particles
on fracture faces rated 4 are not necessarily twice as
aggregated as those on fracture faces rated 2. In other
words, the quantitative implications of our graphs should
not be overinterpreted. They are intended merely to
summarize the results of many observations, some of
which are illustrated in accompanying electron micro-
graphs.

RESULTS

Pretreatment

We found that various incubation conditions
employing high temperature, high pH, or low ionic
strength rendered the intramembrane particles of
erthrocyte ghosts more susceptible to subsequent
aggregation. These pretreatment conditions did
not in themselves cause particle aggregation, but
did allow particle aggregation to occur when the
pretreated membranes were then subjected to the
standard pH 5.5 aggregation incubation (see Ma-
terials and Methods). Fresh nonpretreated ghosts
subjected to the same standard pH 5.5 incubation
showed little or no particle aggregation. Therefore,
we could assess the effectiveness of various pre-
treatments by measuring the degree of particle
aggregation induced by the standard pH 5.5 incu-
bation.

We found the effectiveness of a pretreatment in
20 PB, pH 7.6, increased with temperature and
with length of incubation (Fig. 2). Although incu-
bation in 20 PB, pH 7.6, at 0°C was not an
effective pretreatment, incubation at 37°C was
effective. However, the absence of any pretreat-
tment temperature optimum, as well as the results
of other experiments (not shown) which used the
serine protease inhibitors phenylmethane sulpha-
yl fluoride and 2,2′-dibromopropamidine iseth-
ionate (Geratz, 1969), indicated that proteolysis
could not explain the pretreatment phenomenon.

Using SDS-polyacrylamide gel electrophoresis,
we found that large amounts of bands 1, 2, and 5
(for nomenclature see Steck, 1974) were lost from
the ghosts during effective pretreatment incuba-
tions (Figs. 3, 4). The gels further showed that this
solubilization of spectrin (bands 1 and 2) and band
5 was not accompanied by any extensive proteo-
lytic breakdown. PAS-stained gels (not shown)
indicated no significant loss of glycoproteins even
after incubation of the ghosts at 37°C for 22 h.

If the release of spectrin and band 5 were the
only molecular events underlying the pretreatment
effect, there should be a quantitative relationship
between the amount of these proteins released and
the effectiveness of a pretreatment incubation. We
therefore undertook a series of experiments in
which the amount of protein released from ghosts
during different pretreatment incubations (Fig. 5

ELGSAETER AND BRANTON Intramembrane Particle Aggregation in Erythrocyte Ghosts 1021
FIGURE 1 Particle aggregation scale. The photographs illustrate the range of aggregation starting with untreated, freshly prepared ghosts where the particles are dispersed (AGN 0), to trypsin-treated ghosts where the particles show maximal aggregation (AGN 5). × 60,000.
FIGURE 2. The effect of pretreatment temperature and time on subsequent particle aggregation in erythrocyte ghosts. (A) Pretreatment of fresh ghosts in 20 PB, pH 7.6, at the indicated temperatures. After pretreatment the ghosts were incubated in 20 PB pH 5.5 for 30 min on ice, and then examined by freeze-etching to assess the pretreatment effectiveness. Filled squares: particle aggregation of ghosts pretreated for 30 min; open circles, triangles, and squares, pretreated for 3, 6, and 24 h. (B, C) Examples of freeze-etch electron micrographs (× 60,000) used to determine degree of aggregation at B and C above.

A, C, and E) was correlated with the degree of particle aggregation after a subsequent standard pH 5.5 incubation (Fig. 5 B, D, and F). SDS-polyacrylamide gels of the supernates and pellets after these pretreatments show that spectrin and band 5 were the only major proteins released in appreciable amounts under all the conditions examined (Fig. 4). Furthermore, a quantitative relationship between the amount of protein (spectrin and band 5) released and the effectiveness of all the pretreatment incubations could be established (Fig. 6). The pretreatment effect was thus dependent on the amount of spectrin and band 5 released, and was independent of the conditions used to cause this release.

We noted that removal of spectrin and band 5 from the ghosts caused them to lose their disklike shape and become swollen. During pretreatment incubations at low ionic strength and high pH, when large amounts of spectrin and band 5 were...
FIGURE 3  Ghost protein composition before and after pretreatment. Fresh ghosts were pretreated by incubation at 37°C in 20 PB, pH 8.5, for 22 h. The pretreated ghosts were washed in 40 vol of 20 PB, pH 7.6, at 0-4°C before being dissolved for SDS-polyacrylamide gel electrophoresis. The gels of the samples prepared from fresh and pretreated ghosts were run simultaneously and stained identically with Coomassie brilliant blue. Top gel, control, fresh ghosts (protein, 50 µg). Bottom gel, pretreated ghosts (protein, 30-40 µg).

FIGURE 4  SDS-polyacrylamide gels showing protein release during pretreatments. S, supernate; P, pellet after pretreatment in: (A) 2 PB, pH 7.5, on ice for 4 h. (B) 2 PB, pH 9.4, on ice for 23 h. (C) 20 PB, pH 7.5, at 37°C for 4 h. (D) 20 PB, pH 8.5, at 37°C for 4 h. (E) 20 PB, pH 9.5, on ice for 4 h.

released, some or all of the ghosts tended to break down into small 0.5-1-µm diameter vesicles. However, we could find no clear relationship between vesicle size and particle aggregation within a given sample. Morphologically intact but swollen ghosts generally showed the same degree of particle aggregation as the small vesicles present in the same preparation.

If spectrin and band 5 release were crucial to the pretreatment phenomenon, we reasoned it should be possible to reduce the effectiveness of a pretreatment by cross linking these proteins to prevent their release from the ghost during the pretreatment incubation. To test this argument we performed a series of experiments using the following protocols: (a) glutaraldehyde cross linking → pretreatment → particle aggregating conditions → freeze-etching; (b) pretreatment → glutaraldehyde cross linking → particle aggregating conditions → freeze-etching. For both protocols, we used a series
of glutaraldehyde concentrations ranging from 0 to 150 mM. At all glutaraldehyde concentrations above 10 mM, protein release during subsequent pretreatment incubation (protocol a) was less than 15–20% of the amount released without prior glutaraldehyde treatment (protocol b).

The ghosts treated with 10 mM or greater glutaraldehyde according to protocol (b) showed significantly greater particle aggregation than ghosts treated according to protocol (a) (Fig. 7). The lack of particle aggregation after protocol (a) (Fig. 7 C) cannot be explained by assuming that glutaraldehyde treatment destroys a mechanism responsible for pH-dependent particle aggregation, because the glutaraldehyde-treated ghosts in protocol (b) (Fig. 7 F) showed strong pH-dependent particle aggregation. Thus, the ghosts in Fig. 7 C must show less particle aggregation, because the release of spectrin and band 5 from these ghosts had been inhibited by glutaraldehyde cross links.

**Particle Aggregation Properties of Fresh and Pretreated Ghosts**

To elucidate the pH and salt dependence of particle aggregation and to determine the extent to which aggregation depends upon pretreatment, we subjected fresh and pretreated ghosts to a range of pH and salt conditions. The results of these experiments (Figs. 8–10) emphasize the extent to which particle aggregation is dependent upon the events which occur during pretreatment. Regardless of which aggregating conditions were used, the particles in fresh ghosts showed little or no aggregation. On the other hand, low pH (Fig. 8) increased salt concentrations (Fig. 9), or a combination of high salt and low pH (Fig. 10) induced marked aggregation in the pretreated ghosts depleted of spectrin and band 5.

Other experiments (Elgsaeter, 1974) have shown that aggregation can be reversed. When ghosts are returned from the aggregation conditions to ice-cold 20 PB, pH 7.6, the particles return to a fully dispersed state (aggregation state 0–0.5).

**Digestion of Surface Proteins**

Both spectrin and band 5 reside on the cytoplasmic side of the erythrocyte membrane (Steck, 1974). We therefore reasoned that if their removal were the event underlying the pretreatment phenomenon, removal of the NANA moieties from, or trypsin digestion of the outer surface of intact erythrocytes should not eliminate the pretreatment dependence of the pH-induced particle aggregation. Incubation of intact cells in either neuraminidase or trypsin (Fig. 11) confirmed this reasoning and indicated that neither of these incubations could substitute for a pretreatment which removed spectrin and band 5. However, removal of the sialic acid from intact erythrocytes did enhance particle aggregation in pretreated ghosts made from these cells (compare squares in Figs. 8 and 11). In fact, the particle aggregation curve of pretreated ghosts made from neuraminidase-treated intact red blood cells in 20 PB was similar to that of non-neuraminidased, pretreated ghosts in 20 PB containing 290 mosM NaCl (compare squares in Figs. 10 and 11).

**DISCUSSION**

All of our results show that spectrin and band 5 was released from erythrocyte ghosts before in-

![Figure 5](https://example.com/figure5.png)

**Figure 5** Membrane protein release and particle aggregation at pH 5.5 after the indicated pretreatments. A, C, and E show the percent of total membrane protein released; B, D, and F show particle aggregation after a standard pH 5.5 incubation. Circles, 0.5-h pretreatment incubation. Squares, 4-h pretreatment incubation. Triangles, 23-h pretreatment incubation. (A) and (B) Pretreatment at indicated pH values in 20 PB on ice. (C) and (D) Pretreatment at indicated pH values, in 2 PB on ice. (E) and (F) Pretreatment at indicated pH values in 20 PB at 37°C.
FIGURE 6 Particle aggregation as a function of membrane protein release. (A) The results of the experiments shown in Fig. 5 are replotted to show the relation between the protein released in different pretreatments and particle aggregation after the standard pH 5.5 incubation. Circles, squares, and triangles as in Fig. 5. (B-E) Examples of freeze-etch electron micrographs (× 60,000) used to determine the degree of aggregation at B–E above. The examples in B, C, and D are shown to emphasize the fact that no significant aggregation could be induced until more than ca. 20% of the membrane protein had been released.
cubation at pH 5.5 induced significant aggregation of the intramembrane particles. Although a number of glycosidase and protease activities have been identified in human red blood cell membrane preparations (Bernacki and Bosmann, 1972) and although Engstrom (1970), and Tillack et al. (1972) observed that proteolytic enzymes cause particle aggregation, we observed no pretreatment temperature optimum (Fig. 2) and found that protease inhibitors did not significantly alter pretreatment effectiveness. SDS-polyacrylamide gels (Fig. 4) showed that proteolytic breakdown was slight, particularly during effective pretreatment incubations at 0–1°C. Thus, our data indicate that spectrin and band 5 removal, rather than proteolytic breakdown, accounts for the effectiveness of a pretreatment. Furthermore, glutaraldehyde cross linking showed that the pretreatment conditions per se were ineffective when release of spectrin and band 5 were inhibited by cross linking (Fig. 7), and neuraminidase or trypsin treatment showed that removal of spectrin and band 5 was required for subsequent particle aggregation even after extensive modification of the red blood cell membrane outer surface (Fig. 11). Although we cannot rule out the possibility that both protein release and enhanced particle aggregation result from other, undetected events, the simplest interpretation of our results is that removal of spectrin and band 5 is required before a pH 5.5 incubation can induce particle aggregation.

The molecular interactions that explain how removal of spectrin and band 5 enhances particle aggregation during subsequent low pH incubations remain to be elucidated. Although band 5 is removed under all the conditions which we have found lead to removal of spectrin, the interaction of this polypeptide may not be important in restricting particle aggregation. SDS-polyacrylamide gel electrophoresis showed that in many cases most of band 5 was removed from the membrane during the early stages of pretreatment before significant amounts of spectrin were removed (Clarke, 1971 b; Shotton, Elgsaeter, and Branton, unpublished observations). Because band 5 accounts for less than 4% of the membrane protein (Fairbanks et al., 1971), and because more than 20% of the total membrane protein must be released for an effective pretreatment (Fig. 6), we infer that band 5 may not play a significant role in preventing the low pH-dependent particle aggregation.

It is thought that both glycoporphin and band 3 are associated with intramembrane particles (Pinto da Silva et al., 1971; Tillack et al., 1972), and that both project to the cytoplasmic surface of the membrane (for a review of this evidence see Steck, 1974) where they could interact with spectrin. Considerations based on the number of particles per erythrocyte (ca. 6 x 10^9), the mean interparticle distance in the untreated erythrocyte ghost membrane (aggregation state 0, Fig. 1; mean interparticle distance ca. 200 Å), the length of the spectrin molecule (ca. 2,100 Å [Clarke, personal communication]), and the number of spectrin molecules per erythrocyte (170,000 molecules each of bands 1 and 2 per ghost [Fairbanks et al., 1971]) show that there are three or four particles per spectrin molecule, and that each spectrin molecule is long enough to span approximately 10 particles. A photograph through a scale model showing the relative abundance of the spectrin molecules and intramembrane particles (Fig. 12) indicates that there is sufficient spectrin on the cytoplasmic surface of the erythrocyte membrane to form a meshwork which alone could restrict particle mobility by either specifically binding to or physically entrapping the particles. Loosening or disrupting the meshwork by removal of spectrin molecules from the membrane would be expected to relieve this restriction, in accord with our observations of increased ease of particle aggregation after the pretreatments we have described. However, little particle aggregation was induced in ghosts from which less than 20% of the membrane protein had been removed. Since spectrin comprises approximately 25–30% of the erythrocyte membrane protein (Fairbanks et al., 1971), it appears that substantial disruption of the spectrin meshwork (greater than 20% membrane protein release) is required before particle aggregation occurs in our standard low pH incubation.

Spectrin could be exerting its influence in other less direct ways (for instance via membrane phospholipids), and other structural models for the erythrocyte membrane should not be excluded before there is clear biochemical evidence concerning the nature of the interaction between spectrin and the components of the particles. At this time, the scanty evidence for such interaction is conflicting. While some cross linking studies (Niehaus and Wold, 1970; Steck, 1972 a; Capaldi, 1973) have failed to show any association between spectrin...
FIGURE 8 Aggregation as a function of pH. (A) Particle aggregation after incubation of fresh ghosts (circles) or pretreated ghosts (squares) in 20 PB on ice for 30 min at the indicated pH values. For pretreatment, ghosts had been incubated in 20 PB, pH 8.5, at 37°C for 22 h. (B, C) Examples of freeze-etch electron micrographs (× 60,000) used to determine the degree of aggregation at B and C above.

FIGURE 7 The effect of glutaraldehyde on subsequent particle aggregation. The left column (A–C) illustrates freeze-etched ghosts subject to protocol (a) (glutaraldehyde cross linking before pretreatment); the right column (D–F) ghosts subject to protocol (b) (glutaraldehyde cross linking after pretreatment). (A, D) Controls incubated in 150 mM glutaraldehyde for 30 min and pretreated in 20 PB, pH 8.5, at 37°C for 22 h, but not subjected to aggregation conditions. (B, E) Controls incubated in 0 mM glutaraldehyde for 30 min and pretreated in 20 PB, pH 8.5, at 37°C for 22 h and then subjected to aggregation conditions. (C, F) Ghosts incubated in 150 mM glutaraldehyde, and pretreated in 20 PB, pH 8.5 at 37°C for 22 h and then subjected to aggregation conditions. Throughout this experiment the aggregation conditions used were 30 min incubation in ice-cold 20 PB-290 mosM NaCl, pH 5.0, but other similar experiments using the standard pH 5.5 incubation produced similar though less dramatic results. × 60,000.
Figure 9  Aggregation as a function of salt concentration. (A) Particle aggregation after a 30-min incubation of pretreated ghosts in ice-cold 20 PB, pH 7.6, to which the indicated amounts of NaCl had been added. For pretreatment, ghosts had been incubated in 20 PB, pH 8.5, at 37°C for 22 h. (B, C) Examples of freeze-etch electron micrographs (× 60,000) used to determine the degree of aggregation at B and C above.

and glycoproteins, other cross linking studies (Ji, 1974; F. Richards, personal communication; J. Singer, personal communication) and antibody studies (Nicolson and Painter, 1973) do suggest associations between glycophorin, spectrin, and band 3. Although we have shown a correlation between spectrin release and subsequent particle aggregation during low pH incubation, our results only suggest, and do not establish, a mechanistic connection between the two events.
Figure 10  Aggregation as a function of pH in high salt medium. (A) Particle aggregation after incubation of fresh ghosts (circles) or pretreated ghosts (squares) in 20 PB-290 mosM NaCl on ice for 30 min at the indicated pH values. For pretreatment, ghosts had been incubated in 20 PB, pH 8.5, at 37°C for 22 h. (B, C) Examples of electron micrographs (× 60,000) used to determine the degree of aggregation at B and C above.
The idea that some of the erythrocyte membrane proteins form a meshwork is not new (Katchalsky et al., 1960; Palek et al., 1971; Jacob et al., 1971; MacGregor and Tobias, 1972; Evans, 1973; Yu, et al., 1973), but it has been difficult either to prove or disprove. Our suggestion of a spectrin meshwork that binds to the intramembrane particles has some structural implications which we have verified: (a) in order to form a meshwork which limits the particle mobility, most of the spectrin molecules should be up against the membrane in the fresh ghost; (b) because the lipids in the membrane must occupy a definite surface area, fresh ghosts should become distorted on exposure to conditions which cause strong precipitation or aggregation of spectrin molecules. If the aggregating forces in the spectrin meshwork are sufficiently strong, tangential pressure in the membrane lipid bilayer should cause particle-free lipid vesicles to bleb off from the shrinking ghost; (c) because all the spectrin molecules are not removed during pretreatment, the residue of spectrin that remains dangling from the intramembrane particles could, under spectrin-aggregating conditions, also drag the particles into an aggregated state. The experiments verifying these points will be published in subsequent papers.

In a more general sense, point (c) suggests that in pretreated ghosts the distribution of intramembrane particles will reflect the distribution and aggregation properties of the residual spectrin. Nicolson and Painter (1973) were led to a similar conclusion when they discovered that bivalent antispectrin acting at the inside surface of the membrane can aggregate the negative charge sites associated with the sialic acid groups on the outside surface of the ghost, since it is known that sialic acid residues are attached to the glycoprotein molecule associated with the particles (Marchesi et al., 1972). Nicolson and Painter noted that the antibody-induced aggregation of charged sites required incubation at elevated temperature. Our experiments suggest that their incubation conditions may have served the same role as our pretreatment in loosening the spectrin meshwork. It is noteworthy that incubation even in the 20 PB pH 7.6 solutions which we and many others routinely use to prepare ghosts can act as an effective pretreatment at 37°C or higher (Fig. 2).³

Although we intend to present evidence and discussion regarding the mechanism underlying pH-induced particle aggregation in a subsequent report, the experiments in this paper do show that particle aggregation in pretreated ghosts is promoted by increased ionic strength (Figs. 9 and 10), or by neuraminidase treatment (Fig. 11). Both of these treatments would reduce charge repulsion between the particles, either by Debye shielding or by removing the sialic acid residues that give the glycoproteins a net negative charge in the pH range we have studied. This reduction in charge repulsion could explain the increased particle aggregation observed in these cases.

Changes in the distribution of membrane macromolecules have been interpreted as evidence for membrane mobility or fluidity. Strictly speaking, observations of particle aggregation or patching which we and others have made (Taylor et al., 1971; Pinto da Silva, 1972; Edidin and Weiss, 1972) should not be confused with direct measurements of macromolecular mobility (Frye and

³It may be that Pinto da Silva (1972) observed pH-dependent particle aggregation at 0-4°C without prior pretreatment because he used ghosts stored for up to 3 days or ghosts inadvertently exposed to temperatures higher than 0-4°C.

![Figure 11](https://example.com/fig11.png)

**Figure 11** Particle aggregation in ghosts derived from neuraminidase- or trypsin-treated erythrocytes. Particle aggregation after incubation in 20 PB on ice for 30 min at the indicated pH values. (A) Dashed line, fresh ghosts from normal erythrocytes; circles, fresh ghosts from trypsin-treated erythrocytes; triangles, fresh ghosts from neuraminidase-treated erythrocytes, but ghosts pretreated in 20 PB, pH 8.5, at 37°C for 22 h. Neuraminidase digestion removed 100 mM NANA/mg membrane protein or about 92% of the total membrane sialic acid. Trypsin digestion released 50 μg carbohydrate/ml packed erythrocyte, removed the PAS-1 band from SDS-polyacrylamide gels and left the Coomassie brilliant blue staining pattern virtually unchanged. (Because of the probability of proteolytic cleavage during subsequent incubation at 37°C, pretreated ghosts derived from trypsin-digested cells were not studied). (B–E) Examples of freeze-etch electron micrographs (× 60,000) used to determine the degree of aggregation at B, C, D, and E above. For the aggregation observed in pretreated undigested ghosts under these conditions, see Fig. 8.
FIGURE 12 A scale model to illustrate the amount of the fibrous protein spectrin bound to the erythrocyte membrane. The black beads represent the intramembrane particles. The particle distribution is an actual projection from a freeze-fracture electron micrograph of a fresh ghost. Twisted strings are used to depict the spectrin fibers, which were assumed to be dimers of bands 1 and 2 (Clarke, 1971) and to lie in a plane parallel to the membrane lipid bilayer. The model is not intended to illustrate the detailed arrangement of the spectrin fibers, but only to illustrate that there are enough spectrin molecules present to form an extensive meshwork on the cytoplasmic surface of the erythrocyte membrane.

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