THE STRUCTURE OF ERYTHROCYTE MEMBRANES STUDIED BY FREEZE-ETCHING

II. Localization of Receptors for Phytohemagglutinin and Influenza Virus to the Intramembranous Particles

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The surface membranes of bacterial, animal, and plant cells and the membranes of most intracellular organelles contain globular particles approximately 75 A in diameter located within the internal matrix of the membranes. These particles are exposed during splitting of the membranes by the freeze-cleaving procedure (1, 2).

In another paper[1] we have reported on a study of the structure of red cell membranes by freeze-etching after treatment with reagents that remove or alter specific components of the membrane. We concluded that the intramembranous particles are at least partially composed of protein located within the lipid regions of the membrane, and the evidence also suggested that glycoprotein molecules may be associated with the particles. In order to obtain more direct evidence that membrane glycoproteins are associated with the intramembranous particles we have attempted to map the distribution of specific receptor sites on the surfaces of human red blood cells using the freeze-etching technique outlined in another paper.[1] Phytohemagglutinin (PHA[2] from *Phaseolus vulgaris*) and influenza viruses were chosen as the two labeling reagents, since both can be prepared in pure form and their receptors are known to reside on different parts of the membrane glycoproteins.

The chemical structure of a PHA receptor has recently been described (3); it is a complex oligosaccharide that is covalently linked to asparagine residues of the polypeptide portion of the membrane glycoprotein. Receptors for influenza and other myxoviruses are composed of sialic acid residues, organized in various macromolecular forms. Sialic acid residues of the human red cell are found in a variety of different oligosaccharides (4), in contrast to the relatively specific PHA receptor sites. Es-

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sentially all the sialic acid of the human red cell can be removed from intact red cells by pronase treatment (5), indicating that the oligosaccharides containing these sialic acids are attached to polypeptide chains that are exposed to the external medium.

Both PHA and influenza viruses are large enough to be seen directly in freeze-etched preparations. However, in order to be certain of recognizing individual PHA molecules, we also conjugated purified preparations of PHA with ferritin.

The strategy of these experiments was (a) to determine the distribution of both receptors over the surfaces of normal red blood cells, and (b) to follow the change in distribution of receptors as a result of artificially altering the membrane organization, using methods described elsewhere.[1]

The results of these experiments indicate that both the PHA and the influenza receptor sites are distributed uniformly over the surface of red cells, and both appear to be linked in some as yet undefined way to the intramembranous particles.

Materials and Methods

Preparation of Red Blood Cells and Ghosts.—Red blood cells were obtained from human and animal whole blood collected in acid-citrate-dextrose and washed three times in phosphate-buffered 0.85% saline (PBS). Red blood cell ghosts were prepared by lysis in dilute phosphate buffer according to the procedure of Dodge et al. (6).

Trypsin Treatment of Red Cell Ghosts.—Freshly lysed normal human red cell ghosts were treated with trypsin at a ratio of 1 mg of packed ghosts in 1.5 ml of 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.5, and the incubation was carried on for 30 min at 37°C. Lima bean-trypsin inhibitor (Worthington Biochemical Corp., Freehold, N. J.) was added at the end of the incubation, and the ghosts were washed two times in 20 mM Tris-HCl buffer.

Neuraminidase Treatment of Red Blood Cells and Ghosts.—0.2 ml of packed, washed, intact red blood cells or of packed red cell ghosts were suspended in 9.8 ml of 0.02 M Tris-maleate-buffered 0.85% NaCl, pH 5.5. 50 μl of 1 mg/ml neuraminidase (Sigma Type VI from *Clostridium perfringens*, Sigma Chemical Co., St. Louis, Mo.) were added, and incubation was carried out at 37°C for 1 hr. The intact cells were centrifuged at 2500 rpm for 10 min, and the sialic acid released in the supernatant was measured by the Warren procedure (7). Very slight hemolysis was noted after 1 hr of incubation. The intact red cells were washed two times in PBS. The red cell ghosts that were treated with neuraminidase were centrifuged at 50,000 g for 20 min and were washed two times in either 10 mM phosphate buffer, pH 7.5, or in 10 mM Tris-HCl buffer, pH 7.5.

Treatment of Intact Red Cells, Red Cell Ghosts, and Trypsinized Ghosts with Phytohemagglutinin.—Purified erythroagglutinating phytohemagglutinin (PHA) was prepared from Bacto-phytohemagglutinin-P (Difco Laboratories, Inc., Detroit, Mich.) by the procedure described by Weber et al. (8), followed by chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). 0.3 ml of a 10% suspension of intact, washed red blood cells were added in small aliquots (approximately 0.05 ml) to 2.0 ml of 7 mg/ml purified PHA in phosphate-buffered saline. There was very slight clumping of the red cells at this concentration of PHA, but more dilute solutions of PHA resulted in more agglutination of the cells. The PHA-treated cells were lysed by the Dodge procedure (6), and the ghosts were washed once with 10 mM phosphate buffer, pH 7.5. The PHA-treated ghosts had a tendency to break up into smaller vesicles during subsequent distilled water washes, but this could be prevented by adding 1 mM MgCl$_2$ to the distilled water. These PHA-treated ghosts were then freeze-etched.

0.5-ml samples of freshly lysed red cell ghosts and ghosts that had been treated with trypsin (as described previously) were added in small aliquots to 2.0 ml of 7 mg/ml purified PHA in 20 mM phosphate buffer, pH 7.5. These ghosts were washed once with 10 mM phosphate buffer containing 1 mM $MgCl_2$, and then two times in distilled water containing 1 mM $MgCl_2$, and were freeze-etched.

Treatment of Red Cell Ghosts and Trypsinized Ghosts with Ferritin-Conjugated Phytohemagglutinin.—The ferritin conjugation method was based on that of Nicolson and Singer (9). 2.0 ml (containing 200 mg) of 6× crystalized ferritin (Pentex Biochemical, Kankakee, Ill.) was mixed with 40 mg of purified erythroagglutinating phytohemagglutinin (purification by the Weber procedure [8] and gel filtration on Sephadex G-200) in 8 ml of 50 mM phosphate buffer, pH 7.0; this was allowed to stir at room temperature while 0.8 ml of neutral 0.5% glutaraldehyde (Polysciences, Inc., Warrington, Pa.; N_2-sealed) was slowly added. The reaction was carried on, with constant stirring, for 1 hr at room temperature. The mixture was dialyzed against 0.1 M NH_4Cl in 50 mM phosphate buffer, pH 7.0, for 5 hr at 4°C, and then overnight against the phosphate buffer alone. The mixture was centrifuged at 10,000 g for 15 min, and the pellet was discarded. The supernatant was applied to a 2.5 × 100 cm Agarose A-1.5 (Bio-Rad 200-400 mesh Bio-Rad Labs, Richmond, Calif.) column and eluted with 50 mM phosphate buffer, pH 7.0. The excluded peak contained the PHA-ferritin conjugate, as determined by assay of agglutinating activity against red blood cells and OD at 440 mμ. The included volume contained unconjugated ferritin and PHA. The conjugate was concentrated threefold with a Diaflo (Amicon Corp., Lexington, Mass.) protein-concentrating apparatus.

Normal red cell ghosts and red cell ghosts that had been treated with trypsin for 30 min were added to the ferritin-PHA conjugate and were subsequently washed with 10 mM phosphate buffer and distilled water containing 1 mM $MgCl_2$, as described in the preceding section for PHA alone. The ferritin-PHA conjugate agglutinated the ghosts unless fairly concentrated conjugate preparations were used. Red cell ghosts were also treated with ferritin alone to be certain that ferritin was not binding nonspecifically to the membrane. Samples of normal and trypsinized conjugate-treated ghosts and ferritin-treated ghosts were taken for freeze-etching.

Treatment of Intact Red Cells and Red Cell Ghosts with Influenza Virus.—Influenza virus was obtained as a gift from Eli Lilly & Co., Indianapolis, Ind. The virus was the PR8 strain, and had been treated with formalin, which abolishes infectivity but leaves the agglutination properties of the virus intact. This virus preparation had 65,000 hemagglutination units/ml.

Intact, washed red blood cells were added in small aliquots (approximately 0.05 ml) to 2.0 ml of various dilutions of the influenza virus in PBS. Dilutions of the virus in the range of 1:10 to 1:100 caused marked agglutination of the red cells, whereas adding the red cells to 1:3 or 1:2 dilutions of the virus resulted in almost no agglutination; since the red cells were maximally coated with virus, little bridging of the virus between red cells was possible. The virus-treated, intact red cells were lysed in the usual manner by the Dodge procedure (6); the ghosts were washed two times with 10 mM Tris-HCl, pH 7.5, and freeze-etched.

For preparations of virus-coated red cell ghosts, the ghosts were added to a 1:3 dilution of influenza virus, which resulted in slight clumping of the ghosts The ghosts were washed two times in 10 mM Tris-HCl and were freeze-etched.

Red cell ghosts that had been treated with trypsin or with neuraminidase, as described in the preceding sections, were added to a 1:3 dilution of influenza virus, after which the ghosts were washed two times in 10 mM Tris-HCl and were freeze-etched.

Preparation of Glycoproteins from Red Cells of Different Animals.—Human, sheep, porcine, bovine, and horse whole blood were collected in acid-citrate-dextrose; after centrifugation and removal of serum and buffy coat, the red cells were washed three times in 0.85% phosphate-buffered saline, and the cells were lysed by the Dodge procedure (6). The ghosts were washed twice with dilute phosphate buffer and then were dialyzed against distilled water overnight, and the membranes were lyophilized. The glycoproteins of the red cell membranes were extracted with lithium diiodosalicylate (LIS) according to the procedure described earlier

(10). The membranes were suspended in a solution of 0.3 M lithium diiodosalicylate in distilled water, at a concentration of 20 mg of membrane protein per milliliter of LIS solution. The mixture was stirred for 10 min at room temperature, and then 2 vol of distilled water were added to dilute the LIS concentration to 0.1 M. After 10 min, the mixture was centrifuged at 50,000 g for 90 min at 4°C, and the supernatant was mixed with an equal volume of cold 50% phenol in water for 10 min. This was then centrifuged at 4000 g for 1 hr at 4°C, and the aqueous phase was collected and dialyzed against distilled water for 36 hr, and then lyophilized. This material was washed once with cold 90% ethanol and twice with absolute ethanol, and the precipitate was dissolved in distilled water and lyophilized. The lyophilized material was weighed, and the sialic acid content was determined by the Warren procedure (7). Samples of the lyophilized material were taken up in 5% sodium dodecyl sulfate (SDS), dialyzed into 0.1% SDS in 0.1 M phosphate buffer, and run on 0.1% SDS polyacrylamide disc gels according to the procedure of Shapiro et al. (11). The gels were stained to show protein bands with Coomassie blue and with periodic acid–Schiff (PAS) to show bands containing carbohydrate.

Freeze-Etching Procedure.—Small droplets of red cell ghosts that had been treated with influenza virus, phytohemagglutinin, or phytohemagglutinin-ferritin conjugate were placed on 3-mm copper planchets and frozen in liquid Freon 22 (E. I. du Pont de Nemours & Co., Wilmington, Del.) cooled by liquid nitrogen. The specimens were freeze-cleaved at −150°C, and were deep-etched at −100°C for times varying from 1 min to 10 min, and shadowed with platinum-carbon in a Balzers freeze-etching apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) according to the method of Moor and Mühlethaler (12). The platinum-carbon replicas were floated off the specimens on distilled water, cleaned with Clorox, picked up on electron microscope grids, and examined in a Philips 200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

RESULTS

Phytohemagglutinin Labeling of Human Red Cell Ghosts.—Electron micrographs of red cell ghosts prepared from intact erythrocytes that had been treated with a concentrated solution of purified phytohemagglutinin and freeze-cleaved and deep-etched for 1 min are shown in Fig. 1. The external surface of the ghost membrane is covered by granular material, which is distributed on the membrane in slightly aggregated arrangements similar to the distribution of the 75-A particles on the cleaved face of the ghost. At high magnification, the granular material on the external membrane surface appears to consist of discrete units that are slightly smaller than the 75-A particles on the cleaved membrane faces.

Labeling of Normal and Trypsinized Human Red Cell Ghosts with Ferritin-Conjugated Phytohemagglutinin.—In order to identify the phytohemagglutinin molecules more easily in freeze-etched preparations of normal and trypsinized red cell membranes, the purified erythroagglutinating PHA was conjugated to ferritin; this procedure did not alter the erythroagglutinating activity of the PHA. Brief trypsin treatment of red cell ghosts causes marked aggregation of the 75-A intramembranous particles, as described in another paper[1] and shown in Fig. 2.

Figs. 3 and 4 show a comparison of the external surfaces of nontrypsinized and trypsinized red cell ghosts treated with the ferritin-PHA conjugate before freeze-etching. The ferritin molecules are distributed uniformly over the ex-
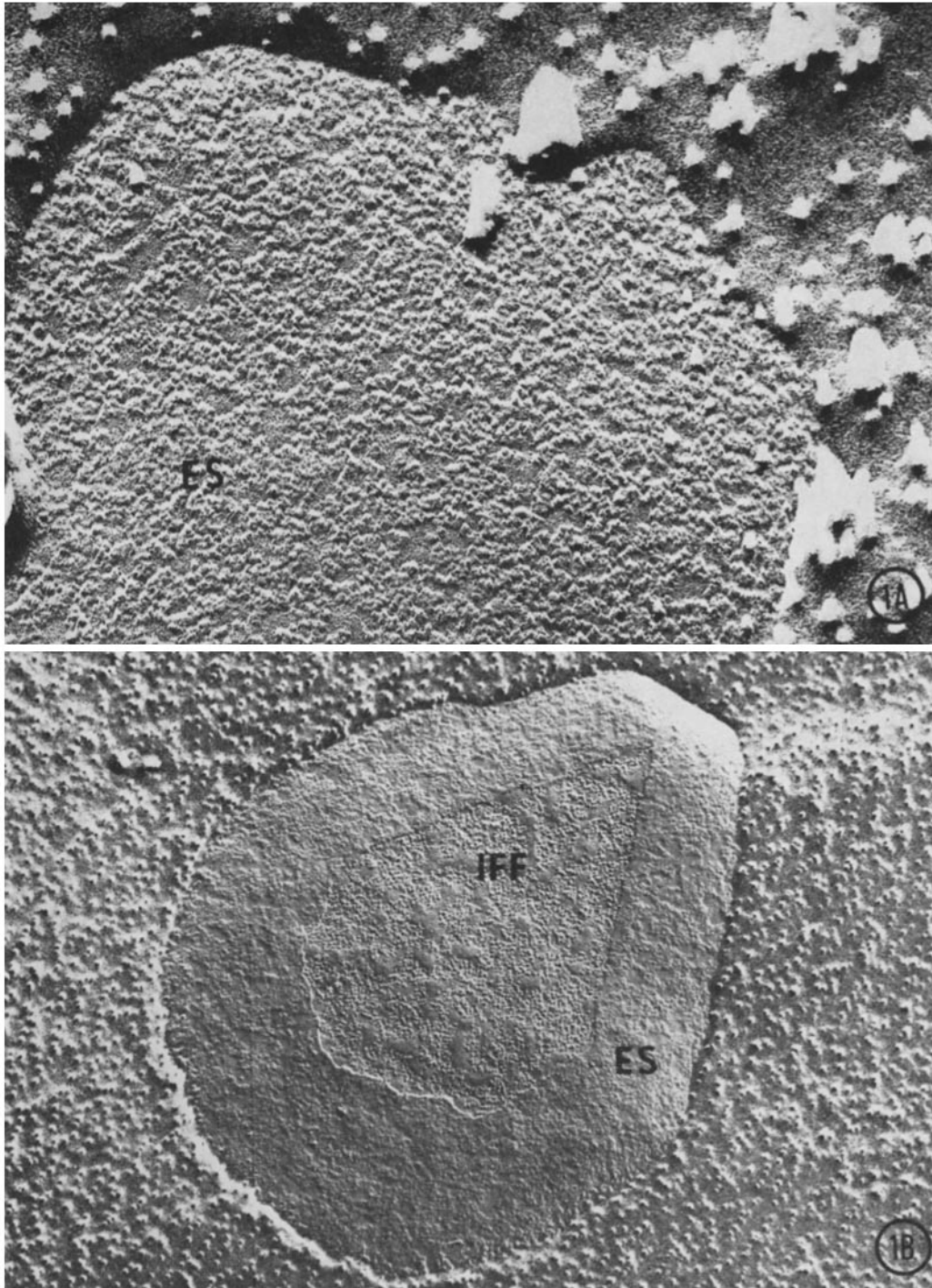
FIG. 1. Platinum-carbon replicas of red cell ghosts treated with purified PHA. Fig. 1 A demonstrates that the external surface (ES) of the ghost exposed by deep-etching is covered by granular material. Fig. 1 B shows that the granular material on the external surface (ES) of the membrane has a similar distribution as the 75-A particles on the inner fracture face (IFF). A, × 115,000; B, × 45,000.
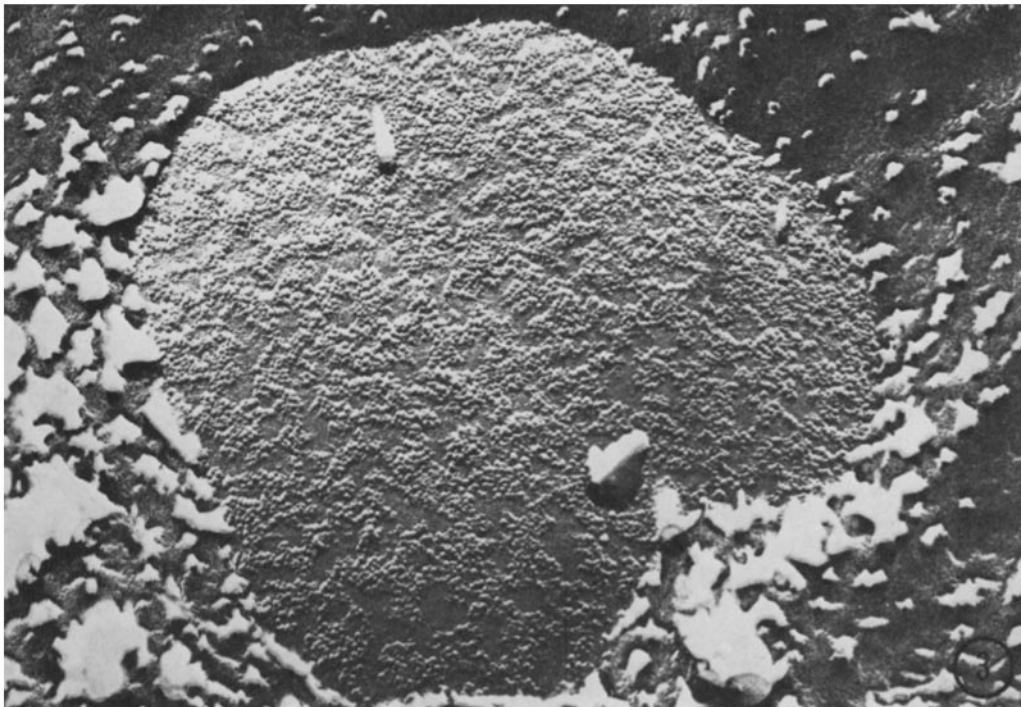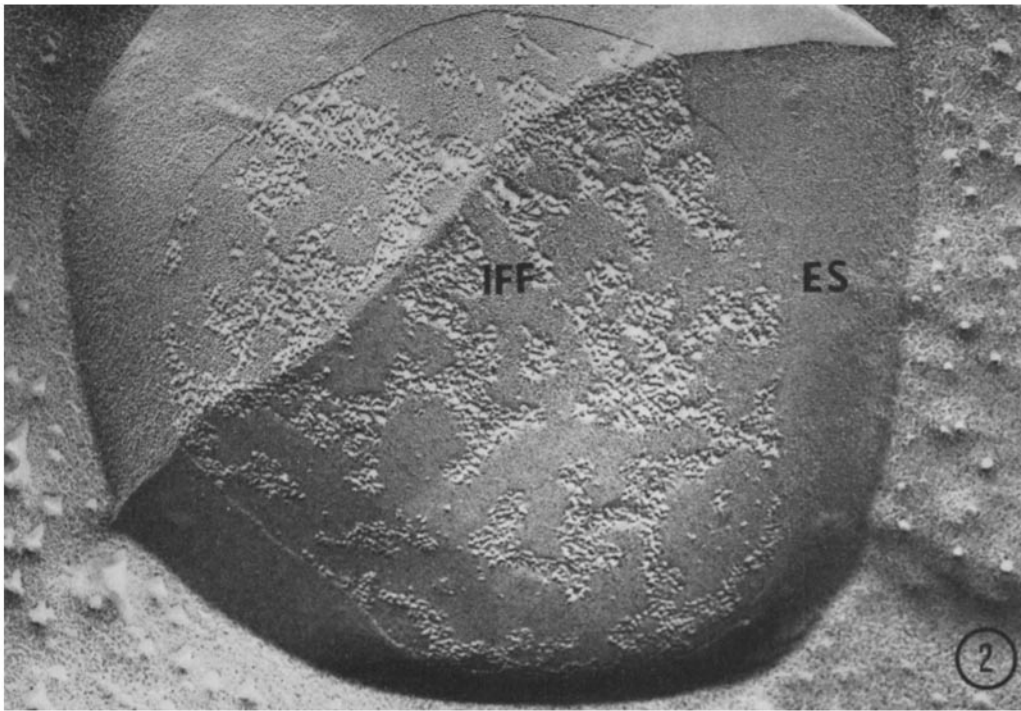
Fig. 2. Replica of a trypsinized red cell ghost. The 75-A particles on the inner fracture face (IFF) are markedly aggregated in a reticular pattern. × 100,000.

Fig. 3. Replica of a normal red cell ghost treated with ferritin-PHA conjugate and deep-etched. The external surface of the cell is exposed, and it appears uniformly covered by ferritin molecules. × 65,000.

ternal surface of the normal ghost; however, the ferritin molecules on the surface of the trypsinized ghost are distributed in a reticular arrangement very similar to the distribution of the intramembranous particles within trypsinized red cell membranes (see Fig. 2). Control experiments in which normal or trypsinized ghosts were treated with ferritin that had not been conjugated to PHA
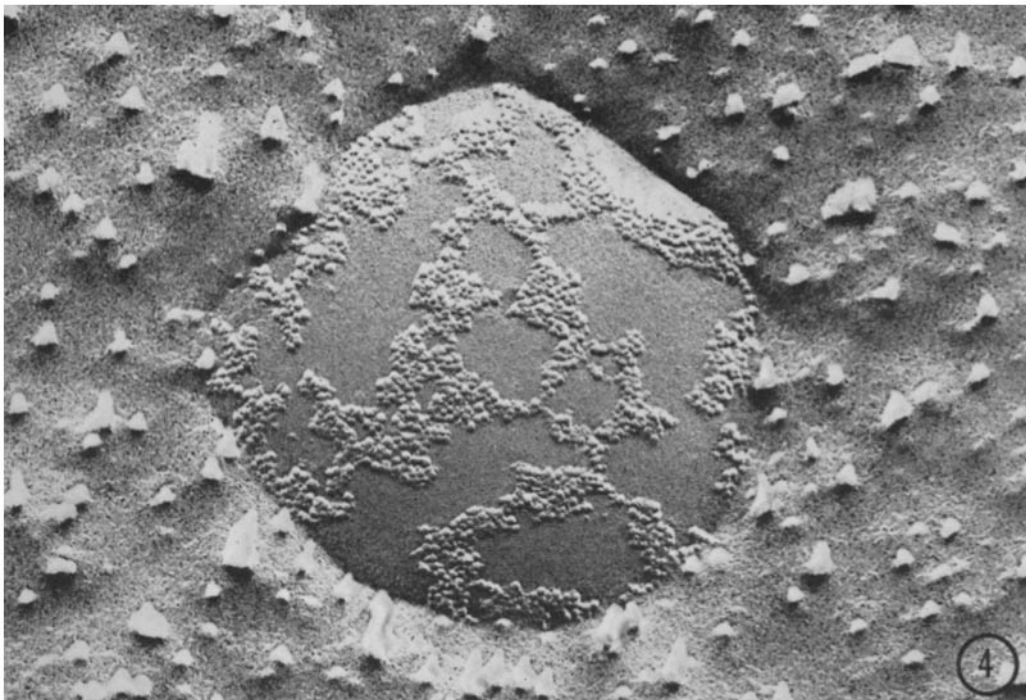

Fig. 4. Replica of a trypsinized red cell ghost treated with ferritin-PHA conjugate and deep-etched. The external surface of the membrane shows aggregates of ferritin molecules and large intervening bare areas. This distribution of ferritin-PHA is similar to the distribution of 75-A intramembranous particles on the fracture faces of trypsinized ghosts (see Fig. 2). X 80,000.

never showed ferritin molecules on membrane surfaces, eliminating the possibility of nonspecific adsorption of ferritin to the membrane.

Red cell ghosts that have been trypsinized, treated with ferritin-PHA conjugate, and then freeze-cleaved and deep-etched are shown in Fig. 5. This preparation permits a comparison of the localization of the ferritin-PHA conjugates on the external surface of the trypsinized ghosts with the appearance of the intramembranous particles on the cleaved interior face of the ghost membrane. The ferritin-PHA conjugates are aggregated on the external membrane surface,
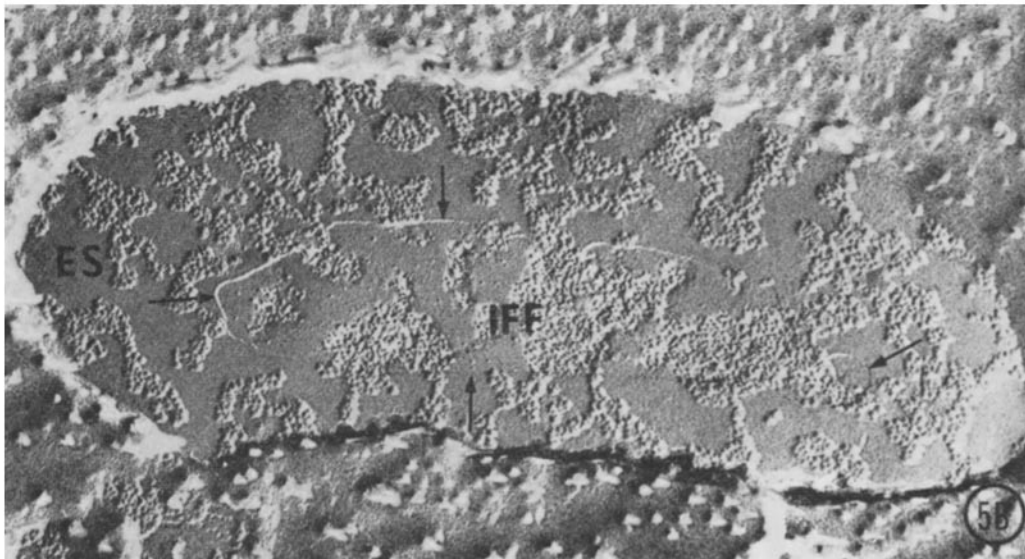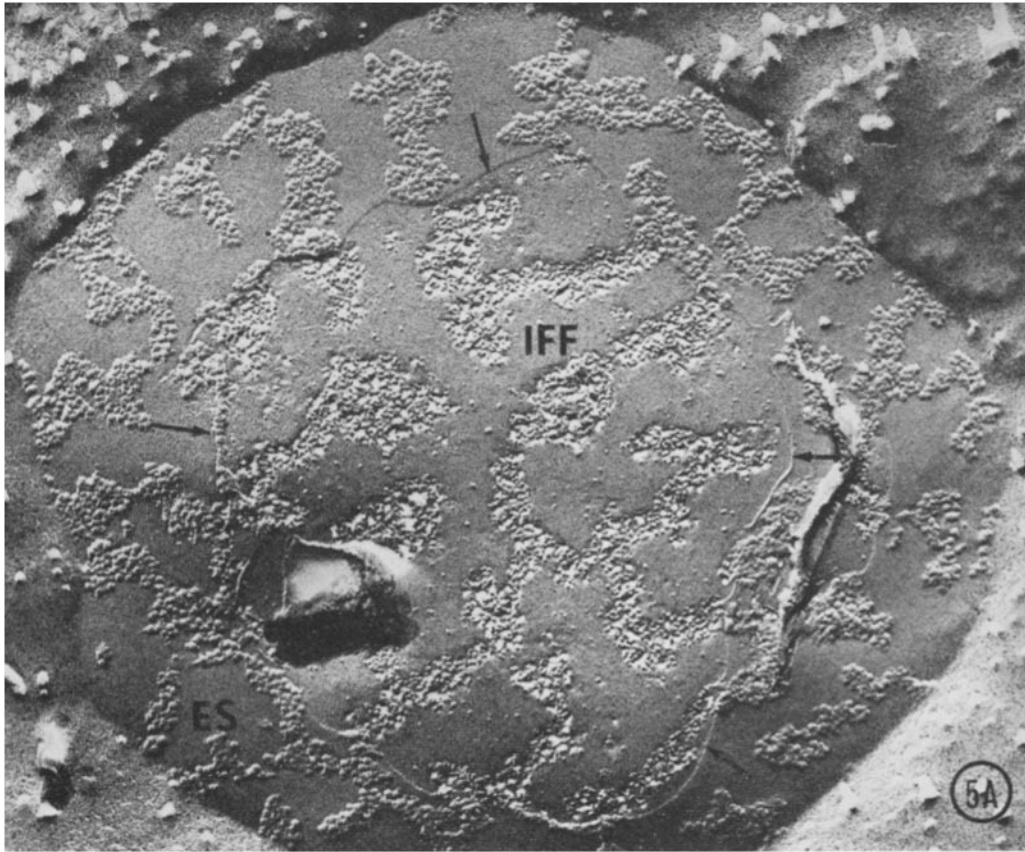
FIG. 5 A and B. Replicas of freeze-cleaved and deep-etched trypsinized red cell ghosts treated with ferritin-PHA conjugate. There is a striking correlation between the distribution of ferritin-PHA on the external surface (ES) of the membrane and the distribution of the

and these aggregates appear to be continuous with aggregates of the 75-A intramembranous particles on the cleaved membrane faces. Furthermore, the aggregates of intramembranous particles are depressed into the cleaved face of the membrane, as a result of indentation of the membrane by the overlying ferritin molecules. These results suggest that the ferritin-PHA conjugates attach to receptor sites on the membrane that are directly over the intramembranous particles and that when the particles are mobilized within the membrane by trypsinization, the PHA receptor sites travel with the intramembranous particles. A diagrammatic summary of the relationship of the PHA receptor sites on membrane glycoproteins to the underlying 75-A intramembranous particles is presented in Fig. 6.

TRYPSIN TREATMENT
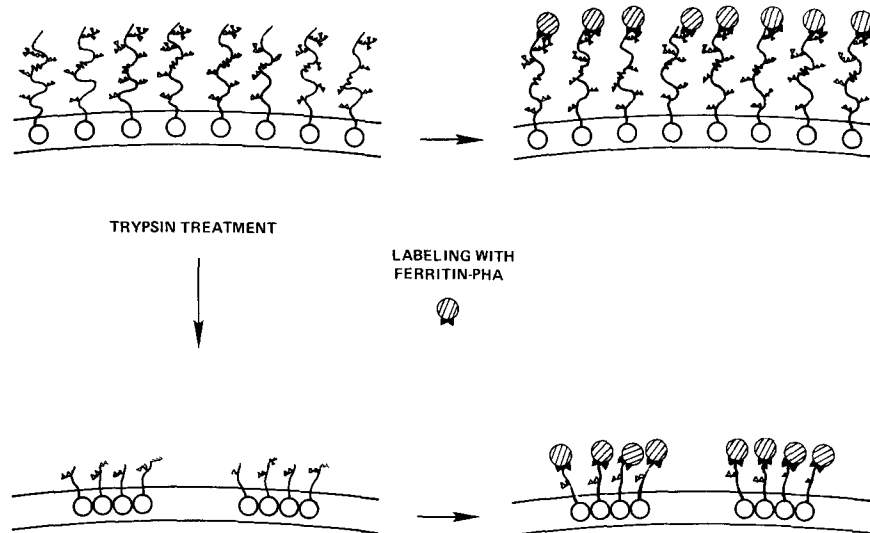
LABELING WITH
FERRITIN-PHA

Fig. 6. Diagram illustrating the labeling of membrane glycoproteins with ferritin-PHA before and after trypsinization of the red cell membranes.

Influenza Virus on Normal Red Cell Ghosts.—An electron micrograph of the PR8 strain of influenza virus negatively stained with 2% potassium phosphotungstate is shown in Fig. 7. The virus particles measure 1300 A in length and 700 A in width, and most appear to be bifid in shape. Spikes approximately 100 A in length cover the surface of the virus, and these have been identified as the hemagglutinin and neuraminidase components of the virion (13).

75-A intramembranous particles on the inner fracture face (IFF). *Arrows* mark the junction between the IFF exposed by cleavage and the true external surface exposed by etching. The aggregates of intramembranous particles appear continuous across the cleavage line with the aggregates of ferritin-PHA. The aggregates of intramembranous particles are depressed into the inner fracture face by the overlying ferritin-PHA. A, $\times$ 73,000; B, $\times$ 65,000.
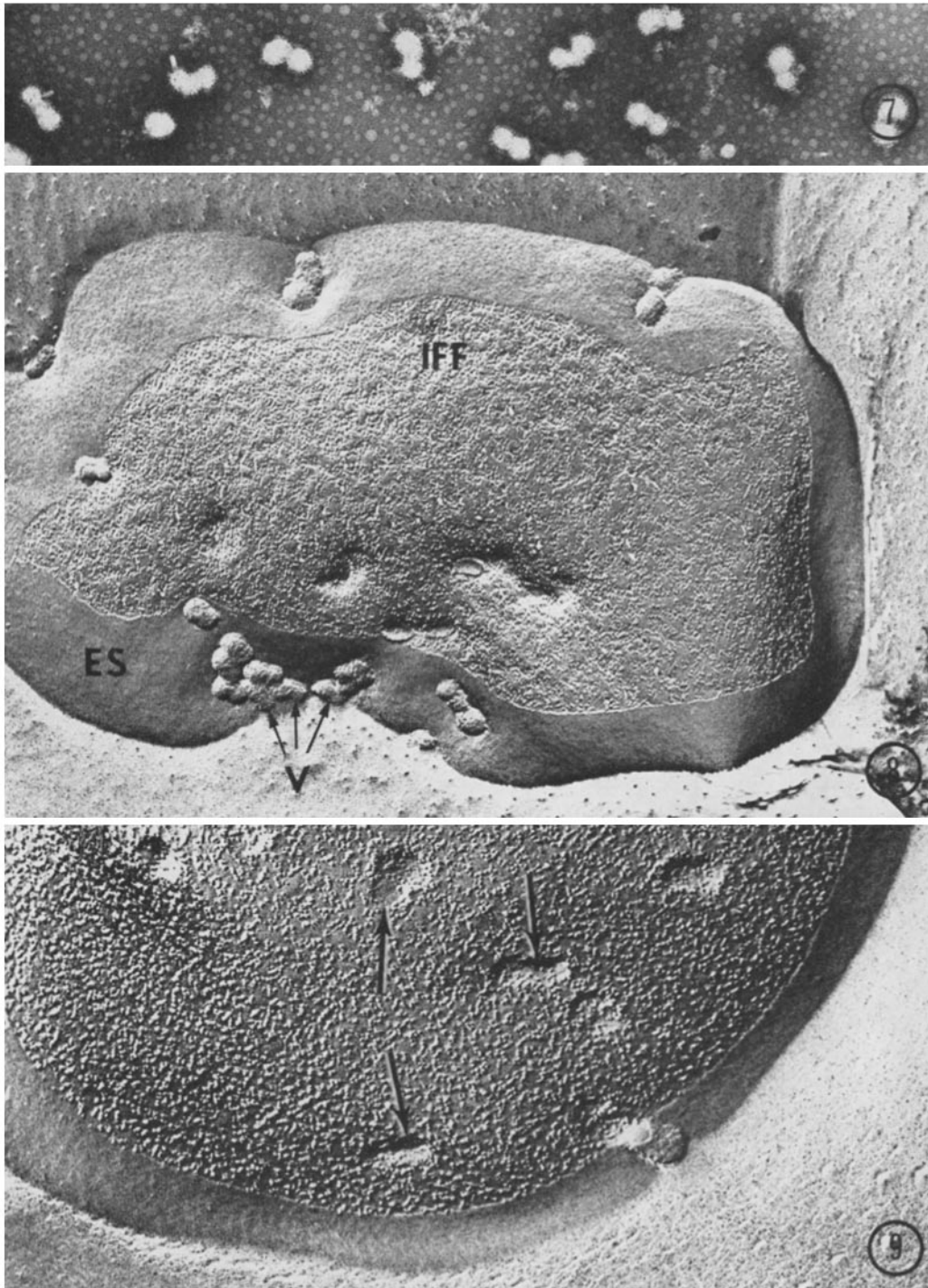
Fig. 7. Influenza virus negatively stained with 2% phosphotungstate. × 27,000.

An electron micrograph of a red cell ghost that has been incubated with a
relatively dilute suspension of influenza virus (approximately 1:50 dilution
of stock) and then freeze-cleaved and deep-etched for 3 min is shown in Fig. 8.
Viral particles are scattered over the deep-etched outer surface of the ghost,
and they appear to indent the membrane where they are attached. The viral
particles have a coarse outer surface, corresponding to the neuraminidase and
hemagglutinin spikes. The freeze-cleaved faces of virus-treated red cell ghosts
show characteristic "footprints" where virions were attached to the membrane
(Figs. 8 and 9). In some instances the virus has pushed in the membrane,
leaving a depression in the fracture face where the overlying half of the mem-
brane containing the virus has been cleaved away. In other instances the plane
of cleavage through the red cell membrane has jumped up to the overlying
virus membrane and then back down to the red cell membrane, leaving part of
the virus capsule lying on top of the red cell membrane fracture face. As
illustrated diagrammatically in Fig. 10, these depressions and partial virus
capsules on the fracture face of the freeze-cleaved red cell membranes allow
one to determine where viral particles were located on the red cell ghosts even
though all or part of the virions have been cleaved away with the external
leaflet of the membrane. The viral footprints were much larger than the 75-A
intramembranous particles on the cleaved faces of the ghost membranes.
Thus, the only way to determine whether the virus receptor site was directly
over the intramembranous particles was to trypsinize the red cell ghosts to
aggregate the particles before treating them with the virus. These results are
presented in the following section.

Treatment of intact red cells or ghosts with neuraminidase, which removes
all of the sialic acid of the membrane, prevents the agglutination of the cells
or ghosts with influenza virus. Freeze-etching of red cell membranes that had
been treated with neuraminidase and subsequently incubated with influenza
virus showed an absence of any virions on the membrane surfaces. This ruled
out the possibility that the virus was nonspecifically adsorbed to the membrane
surfaces.

Influenza Virus on Trypsinized Red Cell Ghosts.—Brief treatment of red cell
ghosts with trypsin has been shown[1] (Fig. 2) to aggregate the 75-A globular
particles seen on freeze-cleaved faces of ghost membranes, leaving large bare
areas between the clumps of particles. Red cell ghosts that have been treated
with trypsin are still agglutinated by influenza virus since only about 50%

FIG. 8. Replica of a normal red cell ghost treated with a dilute suspension of influenza
virus. Virions (V) are attached to the external surface (ES) of the membrane. Depressions and
partial viral capsules (arrows) are seen on the inner fracture face (IFF) of the membrane.
X 37,000.

FIG. 9. Replica of a normal red cell ghost treated with influenza virus. Depressions (arrows)
are present on the inner fracture face (IFF) as a result of indentation of the membrane by the
virions attached to the external surface. X 75,000.

of the sialic acid on the membrane surface is released by trypsin. Electron micrographs of freeze-etched red cell ghosts that had been trypsinized for ½ hr and then mixed with a 1:3 dilution of stock influenza virus are shown in Fig. 11. Influenza virus is present on the external surfaces of the trypsinized ghosts. The cleaved faces of the membranes now show aggregation of the 75-A intra-

RED BLOOD CELL
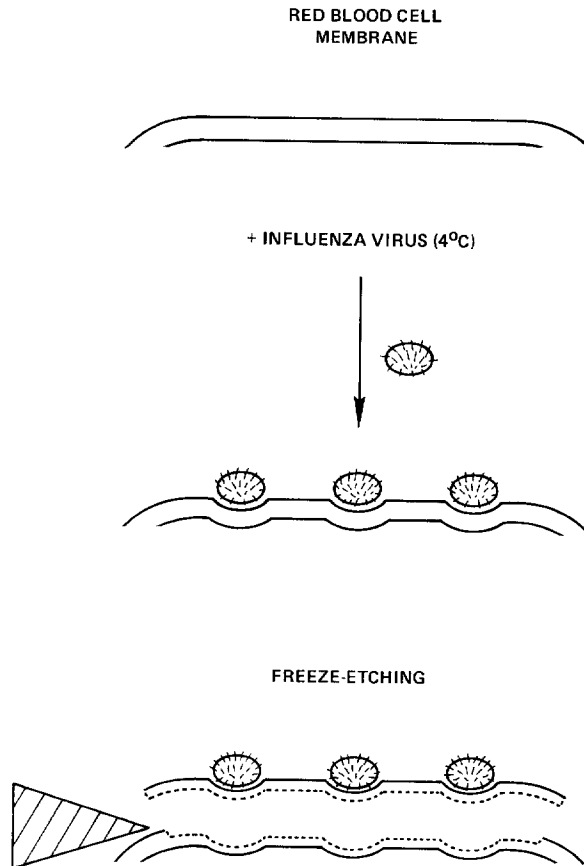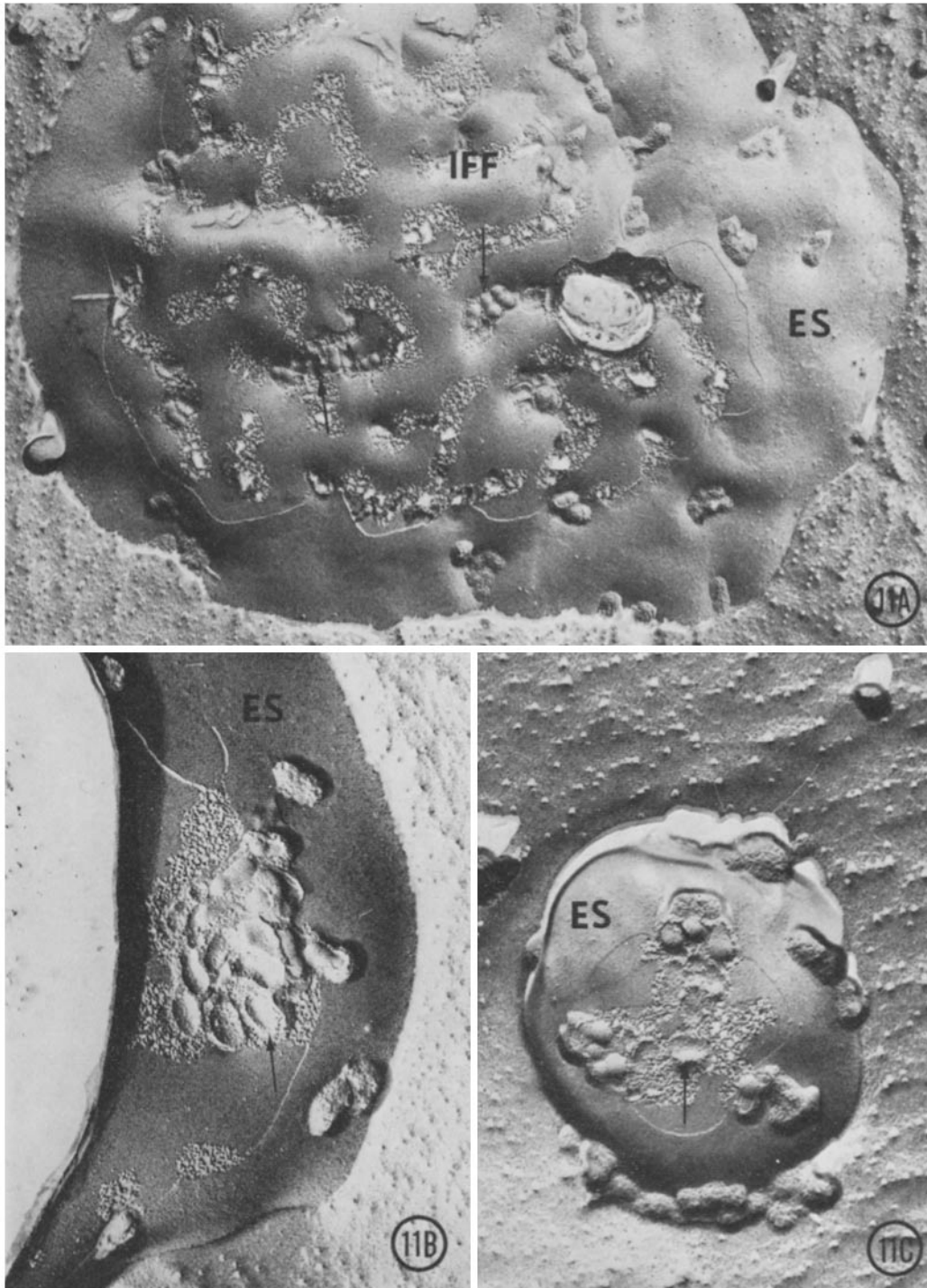MEMBRANE

+ INFLUENZA VIRUS (4°C)

FREEZE-ETCHING

Fig. 10. Diagram illustrating freeze-cleavage of a red cell membrane with attached virions, leaving characteristic "footprints" on the freeze-cleaved faces of the membrane.

membranous particles, and the viral depressions or partial viral capsules are located exclusively over these aggregates of particles. In numerous freeze-etched micrographs of trypsinized red cell ghosts that were exposed to influenza virus, the viral footprints were always located over the aggregates of 75-A particles and were never present on bare areas of the cleaved membrane faces. Thus it appears that the influenza virus attaches only to those regions of the external red cell membrane that are directly over the 75-A globular particles.

FIGS. 11 A, B, and C. Replicas of trypsinized red cell ghosts treated with influenza virus. The 75-A particles on the inner fracture face (IFF) have been aggregated by brief trypsin treatment of the membranes before incubation with influenza virus. Intact virions are present on the external surface (ES) of the membranes. Viral "footprints" (arrows) appear exclusively over the aggregates of particles on the IFF of the membranes. A, × 37,000; B, × 60,000; C, × 60,000.

Relation of Red Cell Membrane Sialic Acid Content to Number of Intramembranous Particles.—An attempt was made to correlate the number of glycoprotein molecules in red cell membranes of different animals with the number of 75-A particles within their membranes. Red cell membranes from different species had approximately equal numbers of intramembranous particles per unit surface area when examined by freeze-etching.[1] Although each species had only one major glycoprotein molecule in its red cell membrane (Fig. 12),
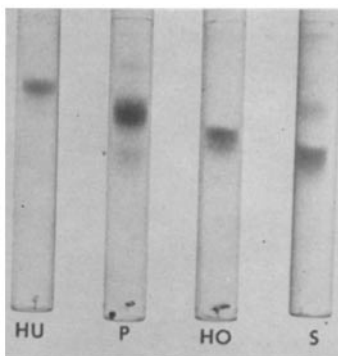

Fig. 12. 10% SDS polyacrylamide disc gels of human (HU), pig (P), horse (HO), and sheep (S) glycoproteins stained with PAS. The glycoproteins were isolated by extraction of the red cell membranes with LIS.

TABLE I

Relation of Membrane Sialic Acid (SA) to Intramembranous Particles

Erythrocyte source	Particles per μ^2	Molecules SA per μ^2 (ref. 14)	Calculated molecules SA per particle	SA content of glycoprotein	Ratio: $\dfrac{\text{SA of glycoprotein}}{\text{SA of particle}}$
				%	
Human	4500	150,000	33.3	20	0.60
Sheep	4900	120,000	24.5	16	0.65
Cow	4200	100,000	23.8	12.5	0.53
Pig	4700	53,000	11.3	6.5	0.58

red cells from different animals had previously been found to have widely different amounts of sialic acid content per unit surface area of the erythrocyte (14). If glycoprotein molecules from the different red cells all had the same amount of sialic acid per molecule, then there was a discrepancy between the number of glycoprotein molecules and the number of particles per unit area. This problem was resolved when the glycoprotein molecules from each type of red cell were extracted and the content of sialic acid was determined (see Table I).

The ratio of sialic acid content of the glycoprotein from each species of

erythrocyte was compared with the calculated number of molecules of sialic acid per intramembranous particle, and this gave a constant number, indicating that the differing amounts of sialic acid per unit surface area correlated with different amounts of sialic acid on each glycoprotein molecule. This supports the idea that erythrocytes of different species do have the same number of glycoprotein molecules per unit surface area as they have intramembranous particles.
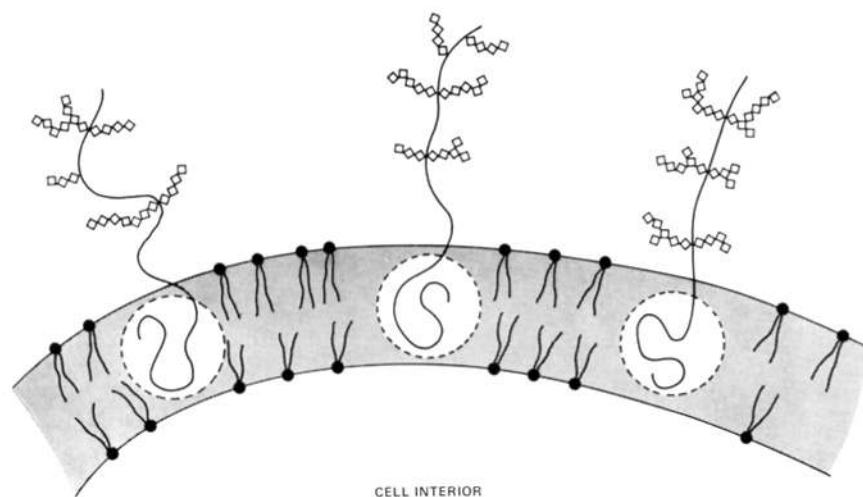

FIG. 13. Diagram showing the proposed orinetation of the major glycoprotein of the red cell membrane. Oligosaccharide chains attached to a polypeptide backbone are exposed at the cell surface and carry receptors for phytohemagglutinin and influenza virus. The hydrophobic end of the glycoprotein molecule is buried in the lipid layer of the membrane and forms a part of the intramembranous particle seen in freeze-etched membranes.

DISCUSSION

The results presented here indicate that the major glycoprotein of the human red cell membrane is oriented at the cell surface, as illustrated schematically in Fig. 13. The glycoprotein, which appears to be a single polypeptide chain with a molecular weight of 55,000 (15), extends out from the cell surface so that most of its oligosaccharide chains are exposed to the external medium. Trypsin digestion of intact cells or ghosts cleaves off several different glycopeptides; but since each glycoprotein molecule has multiple copes of both the PHA receptor and viral attachment sites (16), the residual glycoprotein fragment remaining in the membrane still has receptors for both ligands.

This model of the surface orientation of the glycoprotein is consistent with earlier results showing that essentially all the sialic acid of human red cells is located at sites that are accessible to exogenous neuraminidase (14). More

recent studies in which new ways to label proteins on cell surfaces with lacto-peroxidase (17) or with formylmethionyl methyl phosphate (18) are used also confirm that portions of the glycoproteins are exposed on the cell surface.

The correspondence between the glycoprotein receptor exposed at the cell surface and the underlying intramembranous particles strongly suggests that parts of the glycoproteins are physically attached to the particles. PHA and influenza viruses localize to sites on the membrane over the particles in both normal cells and those modified by trypsin.

In addition to these labeling experiments, the calculated number of receptors for PHA and for other glycoprotein-bound receptors compares remarkably well with the total number of intramembranous particles per cell as determined by direct counts of freeze-etched preparations (Table II). A recent study on the localization of blood group A activity indicates that this antigen also localizes to sites that correspond to particles (22).

The chemical basis for the association between the glycoproteins and the

TABLE II

Comparison of Total Number of Particles on Human Erythrocytes to Estimated Number of Antigenic and Receptor Sites

Particles per human erythrocyte	500,000
A_1 blood group antigen sites (19)	800,000–1,000,000
A_2 blood group antigen sites (19)	300,000
B blood group antigen sites (19)	500,000–800,000
Phytohemagglutinin receptor sites (20, 21)	340,000–500,000

intramembranous particles remains undefined. However, on the basis of recent studies on the chemical properties of the isolated glycoprotein[3] it is possible to speculate that a portion of the glycoprotein may be associated via hydro-phobic interactions with other components (proteins or lipids) in the membrane to form the globular particles. Nonpolar amino acids are concentrated in the C-terminal third of the polypeptide chain of this glycoprotein, and peptides derived from this end of the molecule by tryptic digestion or cyanogen bromide cleavage are insoluble in aqueous media. This part of the molecule also has a small amount of tightly bound lipid. These properties are consistent with the idea that the C-terminal end of the glycoprotein is anchored in the lipid portion of the membrane. The part of the C-terminal segment of the glycoprotein that might be inserted in the membrane is estimated to have a molecular weight of 10,000–15,000, and this would be insufficient to account for the mass of the intramembranous particle. It is likely, therefore, that the internalized portion of the glycoprotein interacts with other polypeptides, and possibly lipids, to form the particle.

[3] Segrest, J. P., R. L. Jackson, and V. T. Marchesi. Manuscript in preparation.

The success of freeze-etching in mapping receptor sites for phytohemag-glutinin and influenza virus on red blood cell membranes indicates that this technique can be used for many other types of antigenic localization and mapping on the surface of different types of cells. Ferritin and viral conjugation techniques have permitted mapping of antigenic sites on human red blood cells (23) and normal and malignant mouse cells (24–26) by electron microscopic studies of thin sections, but this does not give a three-dimensional representation of the sites on cell surfaces. Serial sectioning of individual mouse thymocytes and lymphocytes has been used for topographical analysis of H-2 antigens on the cell surface (27), but this is a laborious technique that cannot be used to sample large numbers of cells. Freeze-etching offers the opportunity to visualize large regions of the cell surface at high resolution, and mapping of receptor or antigenic sites with appropriate labels should be easily accomplished.

SUMMARY

The distribution of specific glycoprotein receptors on the external surfaces of red cells was mapped, by the freeze-etching technique, to determine if the receptors coincided with the underlying 75-A intramembranous particles. Phytohemagglutinin, ferritin-conjugated phytohemagglutinin, and influenza virus were used as labeling agents since they can be seen by freeze-etching techniques and each reacts with a different site on the same glycoprotein molecule. The distribution of these labels was studied on intact human red cells, isolated ghost membranes, and trypsin-treated ghost membranes.

The results show that the receptors for these labels are distributed uniformly over the surfaces of normal red cell membranes in the same apparent distribution as that of the 75-A particles within the membrane. The association between the external receptors and the underlying particles is especially evident when trypsin-treated ghost membranes are labeled: the labeled receptor sites and the intramembranous particles both form sharply defined, reticulated networks, which overlap. These results provide further support for the idea that membrane-bound glycoproteins are oriented so that their carbohydrate-rich segments, which bear the antigenic sites and receptors, are exposed to the external medium, while hydrophobic segments of the same molecules interact with lipids, and possibly other proteins, to form the intramembranous particles.

REFERENCES

1. Tillack, T. W., and V. T. Marchesi. 1970. Demonstration of the outer surface of freeze-etched red blood cell membranes. *J. Cell Biol.* **45**:649.
2. Pinto da Silva, P., and D. Branton. 1970. Membrane splitting in freeze-etching: covalently bound ferritin as a membrane marker. *J. Cell Biol.* **45**:598.

3. Kornfeld, R., and S. Kornfeld. 1970. The structure of a phytohemagglutinin receptor site from human erythrocytes. *J. Biol. Chem.* **245:**2536.

4. Winzler, R. J. 1970. Carbohydrates in cell surfaces. *Int. Rev. Cytol.* **29:**77.

5. Cook, G. M. W., and E. H. Eylar. 1965. Separation of the M and N blood group antigens of the human erythrocyte. *Biochim. Biophys. Acta.* **101:**57.

6. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100:**119.

7. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234:** 1971.

8. Weber, T., C. T. Nordman, and R. Gräsbeck. 1967. Separation of lymphocyte-stimulating and agglutinating activities in phytohaemagglutinin (PHA) from *Phaseolus vulgaris. Scand. J. Haematol.* **4:**77.

9. Nicolson, G. L., and S. J. Singer. 1971. Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy: application to saccharides bound to cell membranes. *Proc. Nat. Acad. Sci. U.S.A.* **68:**942.

10. Marchesi, V. T., and E. P. Andrews. 1971. Glycoproteins: isolation from cell membranes with lithium diiodosalicylate. *Science (Washington).* **174:**1247.

11. Shapiro, A. L., E. Viñuela, and J. V. Maizel, Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **28:**815.

12. Moor, H., and K. Mühlethaler. 1963. Fine structure in frozen-etched yeast cells. *J. Cell Biol.* **17:**609.

13. Laver, W. G., and R. C. Valentine. 1969. Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. *Virology.* **38:**105.

14. Eylar, E. H., M. A. Madoff, O. V. Brody, and J. L. Oncley. 1962. The contribution of sialic acid to the surface charge of the erythrocyte. *J. Biol. Chem.* **237:**1992.

15. Segrest, J. P., R. L. Jackson, E. P. Andrews, and V. T. Marchesi. 1971. Human erythrocyte membrane glycoprotein: a re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* **44:**390.

16. Jackson, R. L., J. P. Segrest, and V. T. Marchesi. 1971. Further characterization of the principal glycoprotein of the human red cell membrane. *Fed. Proc.* **30:** 1280a. (Abstr.)

17. Phillips, D. R., and M. Morrison. 1971. Exposed protein on the intact human erythrocyte. *Biochemistry.* **10:**1766.

18. Bretscher, M. S. 1971. Human erythrocyte membranes: specific labelling of surface proteins. *J. Mol. Biol.* **58:**775.

19. Race, R. R., and R. Sanger. 1968. *In* Blood Groups in Man. F. A. Davis Company, Philadelphia, Pa. 49.

20. Steck, T. L., and D. F. H. Wallach. 1965. The binding of kidney-bean phytohemagglutinin by Ehrlich ascites carcinoma. *Biochim. Biophys. Acta.* **97:**510.

21. Kornfeld, S., and R. Kornfeld. 1969. Solubilization and partial characterization of a phytohemagglutinin receptor site from human erythrocytes. *Proc. Nat. Acad. Sci. U.S.A.* **64:**1439.

22. Pinto da Silva, P., S. D. Douglas, and D. Branton. 1971. Localization of A antigen sites on human erythrocyte ghosts. *Nature (London).* **232:**194.

23. Haberman, S., P. Blanton, and J. Martin. 1967. Some observations on the ABO antigen sites of the erythrocyte membranes of adults and newborn infants. *J. Immunol.* **98:**150.

24. Hämmerling, U., T. Aoki, E. de Harven, E. A. Boyse, and L. J. Old. 1968. Use of hybrid antibody with anti-γG and anti-ferritin specificities in locating cell surface antigens by electron microscopy. *J. Exp. Med.* **128:**1461.

25. Aoki, T., U. Hämmerling, E. de Harven, E. A. Boyse, and L. J. Old. 1969. Antigenic structure of cell surfaces. An immunoferritin study of the occurrence and topography of H-2, θ, and TL alloantigens on mouse cells. *J. Exp. Med.* **130:**979.

26. Aoki, T., E. A. Boyse, L. J. Old, E. de Harven, U. Hämmerling, and H. A. Wood. 1970. G(Gross) and H-2 cell-surface antigens: location on Gross leukemia cells by electron microscopy with visually labeled antibody. *Proc. Nat. Acad. Sci. U.S.A.* **65:**569.

27. Stackpole, C., T. Aoki, E. A. Boyse, L. J. Old, J. Lumley-Frank, and E. de Harven. 1971. Cell surface antigens: serial sectioning of single cells as an approach to topographical analysis. *Science (Washington).* **172:**472.