# THE ORGANIZATION OF PROTEINS IN THE HUMAN RED BLOOD CELL MEMBRANE

## A Review

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## I. INTRODUCTION

The elucidation of the molecular architecture of cell membranes is a central goal for cell biology, as structure lies at the heart of function. The erythrocyte plasma membrane has long provided a favored testing ground for this inquiry. Human red blood cells are readily available, relatively homogeneous, and relevant to medicine. Their plasma membranes can be easily isolated intact and essentially free of contamination from other cells, organelles, and cytoplasmic contents. This membrane is complex enough to be interesting and, to some degree, representative, yet it is simple enough to be analyzed as a whole. These circumstances make it likely that the human red cell plasma membrane will be the first whose molecular anatomy is known in any degree of satisfying detail.

The literature concerning the proteins of erythrocyte membranes and membranes in general has been the subject of repeated review (1–9). This article will focus on the localization and modes of association of individual major polypeptides within the human red cell membrane.

## II. THE PROTEIN FRACTION

Red cell membranes can be purified after osmotic hemolysis in dilute, mildly alkaline buffer by repeated washing until the "ghosts" (or "stroma") are pearly white (cf. 10–12). The hemoglobin-free ghost has a mass of $11–12 \times 10^{-13}$ g (10). Taking the membrane density as 1.15 g/cm$^3$ (13) and its surface area as 140 $\mu$m$^2$ (14), a thickness of 75 Å may be calculated, in good agreement with electron microscope studies (15).

The proteins can be separated from the lipids by extracting membranes with polar organic solvents (10, 16–18). A few percent of the protein is lost to the organic phase, suggesting the presence of hydrophobic proteins or proteolipid complexes (16, 18). Approximately 52% of the membrane mass is protein, 40% is lipid, and 8% is carbohydrate (10, 18, 19). Only about 7% of this sugar is carried by glycosphingolipids (20); the rest takes the form of small oligosaccharides linked via *O*- and *N*-glycosidic bonds to serine (threonine) and asparagine residues in the membrane glycoproteins (cf. 19, 21).

The amino acid composition of the erythrocyte membrane protein fraction is not distinctively different from that determined for other membranes or, indeed, for mixtures of soluble proteins (4, 22). However, individual proteins, and, more particularly, topographically specified domains of certain polypeptides, have characteristic compositions which relate to their disposition in the

membrane (4, 9, 19, 23–25). There is a distinct excess of acidic (21%) over basic (12%) residues in the protein fraction (18), consistent with an apparent net isoelectric point of pH 4–5 (16, 17). While cysteine represents only about 1% of amino acid residues, the particular functional significance and chemical reactivity of its sulfhydryl group *in situ* have been the subject of several investigations (cf. 26, 27).

## III. THE POLYPEPTIDES

When red cell membranes are dissolved in an excess of sodium dodecyl sulfate (SDS), the polypeptides are denatured and separated from the lipids and from one another, as demonstrated by polyacrylamide gel electrophoresis (11, 28, 29). Despite the simplicity, sensitivity, and high resolution of this technique, it is still subject to certain limitations and anomalies (cf. 4). In addition, architectural and functional information is lost upon SDS treatment, unless such features are identified before membrane dissolution, as illustrated later.

Fig. 1 A is a densitometric scan of the Coomassie blue-stained ghost polypeptides on a polyacrylamide gel after electrophoresis in SDS. Table I lists estimates of the molecular weight and mass fraction of each major membrane component derived from such profiles. While these values are, at best, approximations, they are generally supported by independent evidence. For example, band *6*, the glyceraldehyde 3-P dehydrogenase (G3PD) protomer (30), is known by other means to have a molecular weight of ~35,000 (31) and to constitute 4–7% of the membrane protein (11, 30, 32). Bands *1*, *2*, and *5* have been selectively eluted from the membrane and shown to comprise 25–30% of its protein (11, 33–35). Gel filtration chromatography of ghost polypeptides dissolved in 6 M guanidine-HCl has provided independent support of the molecular weights calculated from SDS-gel mobilities (29, 36). Gel electrophoresis in other denaturing detergents and at other pH values (37) and the use of other protein stains (11) give similar profiles. There is good evidence that (with one exception, see below) the components listed in Table I are not aggregates or complexes, since they cannot be reduced in size by a variety of dissociating agents; however, they are quite vulnerable to proteolytic degradation (11, 38). Ghosts prepared by several different methods all yield similar electrophoretograms (39). The polypeptide profiles from a variety of mammalian erythrocyte sources closely resemble the human; the glycoprotein patterns are more diverse (33, 40–45). Some ghost-associated proteins may be lost during erythrocyte maturation, at least in the rabbit (46).

The Coomassie blue-stained polypeptides listed in Table I account for about three-quarters of the stained profile. There are also many minor components (such as bands *2.1–2.3*, and the broad, complex zone designated *4.5*), some of which are obscured by the major gel components. For example, there are only a few hundred $Na^+,K^+$-stimulated ATPase sites per cell (47), whose $^{32}PO_4$-acylphosphate intermediate comigrates with the vast excess of band *3* polypeptides (48) and is therefore not visualized in stained gels.

Still other components, the heavily glycosylated glycoproteins, are not readily detected by Coomassie blue and other conventional protein stains (4, 11), but are made visible by the periodic acid-Schiff (PAS) staining technique (Fig. 1 B). Their electrophoretic mobilities may be anomalous with respect to nonglycosylated reference proteins, making their molecular weight assignments ambiguous (cf. 49. 50). The PAS profile bears a close resemblance to the distribution of sialic acid in the gel, as determined by direct chemical analysis (11, 51, 52) and, more particularly, by the radioactivity profile of membrane proteins gently reacted with periodic acid (so as to oxidize only sialate residues) and then reduced by tritiated borohydride (53, 54).

The major sialoglycoprotein contains 64% carbohydrate, including 28% *N*-acetylneuraminic acid (19); it has been identified with the binding locus for influenza virus, certain blood group isoantibodies, and certain plant hemagglutinins (cf. 9, 19, 55, 56). The estimate of 500,000 sialoglycoprotein molecules per cell (Table I) is in excellent agreement with the prevalence of these lectin binding sites on human erythrocytes (55, 57). It now appears that bands PAS-1 and PAS-2 actually represent interconvertible forms of the same molecule (58). This unusual case should remind us that the SDS-gel electrophoresis system is not an ideal one (cf. reference 4).

There are other membrane glycoproteins which are not well represented by the PAS technique, presumably because they have a low sialic acid content. Band *3* (Fig. 1 A) is one example: it contains approximately 7% carbohydrate (1, 59) and contributes roughly 10% of the total membrane sugar, yet it is visualized in the PAS profile, at best, only as a small shoulder on the trailing

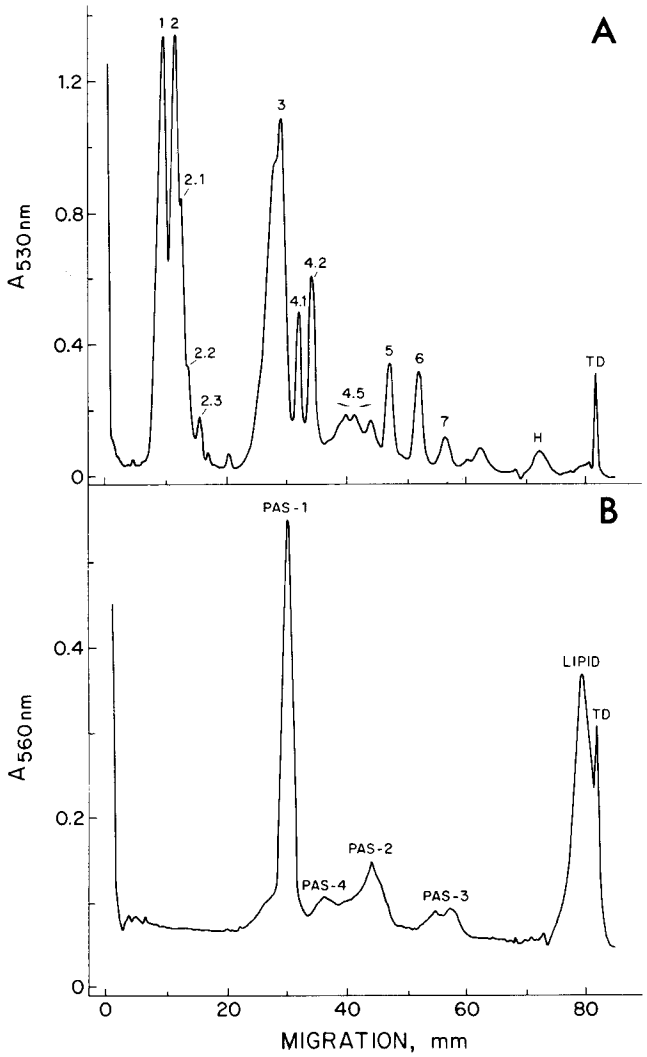


FIGURE 1 Erythrocyte membrane polypeptides and glycoproteins. (A) a densitometric scan of a gel stained for protein with Coomassie blue after electrophoresis of 10 $\mu$l of packed ghosts (40 $\mu$g protein). (B) a similar gel stained for carbohydrate with PAS reagent. Procedures and nomenclature are described in references 11 and 118. *H*, polypeptide chains from hemoglobin; *TD*, inked needle stab recording the position of the tracking dye. See Table I for data.

edge of the PAS-1 peak (Fig. 1 B). Similarly, treating membranes with galactose oxidase plus tritiated borohydride strongly labels several otherwise indistinct components in the poorly stained region of PAS-4 and PAS-2 (or zone *4.5*) (54, 60, 61). Such data make it clear that a precise enumeration of the red cell membrane polypeptides is an important, unfinished task.

## IV. ASYMMETRICAL DISTRIBUTION

Some models of membrane molecular organization have postulated that proteins can serve a structural (i.e. stabilizing or space filling) role in the membrane, contributing to its fabric rather than its function (cf. 62, 63). Such formulations do not require that the proteins be specifically arranged with respect to the membrane's two surfaces. A converse viewpoint recognizes that every biological membrane is interposed between two distinctly different aqueous spaces and acts differentially upon them. An asymmetry in the locus of at least some membrane proteins can be anticipated on this basis, reflecting the functional specialization of the two membrane surfaces. A

TABLE I

*The Major Erythrocyte Membrane Polypeptides and Glycoproteins*

| Component | mol wt* | Percent of stained protein‡ | Polypeptides/ Ghost§ | References for purification, characterization, and other designations |
|---|---|---|---|---|
| A. Polypeptides | | | | |
| *1* | 240,000 | 15.1 | 216,000 | Spectrin (110–112) |
| | | | | Tektin A (34) |
| *2* | 215,000 | 14.7‖ | 235,000‖ | Myosin-like polypeptides (35) |
| *3* | 88,000¶ | 24.1 | 940,000 | Component a (51) |
| | | | | Protein E (59) |
| | | | | Minor glycoprotein (1) |
| *4.1* | 78,000 | 4.2 | 180,000 | |
| *4.2* | 72,000 | 5.0 | 238,000 | |
| *5* | 43,000 | 4.5 | 359,000 | Actin-like polypeptide (35) |
| *6* | 35,000 | 5.5 | 540,000 | G3PD (30) |
| *7* | 29,000 | 3.4 | 403,000 | |
| B. Glycoproteins | | | | |
| PAS-1 | 55,000** | 6.7‡‡ (2.8) | 500,000 | Glycophorin (23) |
| PAS-2 | | | | Sialoglycoprotein (19) |
| | | | | ABH and MN isoantigen (23, 56) |

* Values from reference 123; comparable values found in references 11, 29, 35.

‡ Average values from a laboratory exercise where 21 medical students determined the distribution of Coomassie blue stain on gels of their own red cell membranes according to the procedure of Fairbanks et al. (11); comparable values are found in references 11, 35.

§ Calculated assuming $5.7 \times 10^{-10}$ mg of protein per hemoglobin-free ghost (11).

‖ Perhaps slightly overestimated because of overlap with band *2.1* (see Fig. 1 A).

¶ Value for peak of the stained band; tail extends to ~105,000 daltons.

** Provisional value based on references 23, 49, 56. PAS-1 and PAS-2 are taken as the same species (58).

‡‡ Calculated assuming this glycoprotein carries 70% of the membrane sialic acid (11, 50, 54) and that 1.6% of the membrane mass is sialic acid (11, 18, 19, 76, 86). Parenthetic entry is for protein portion alone (19).

central question is, therefore, to what extent and in what manner are individual proteins (and other constituents) asymmetrically oriented in the membrane.

A simple approach to the anisotropy of the erythrocyte membrane has been to expose the intact red cell to probe molecules presumed not to pass through the membrane. The membrane constituents which react are judged to be confined to its outer surface (as in references 64–66). For example, acetylcholinesterase can be inactivated by exposing intact erythrocytes to proteases (cf. 52, 67, 68), and sialic acid can be quantitatively released from red cells by treating them with sialidase (69). Proof that these components are confined to the outer surface of the membrane, however, calls for demonstration that the same response does not occur when the cytoplasmic surface is subjected to an identical treatment. Such corollary data will rule out the possibilities that the component can move back and forth between the membrane surfaces (as has been suggested at times for membrane phospholipids [70] and proteins [71, 72]), that the probe has access to both surfaces, that some copies of the component are actually present at each surface, etc. The *inaccessibility* of a membrane component at the *cytoplasmic* surface provides strong evidence for its exclusive localization at the external surface.

An indirect approach to the analysis of the cytoplasmic surface of the red cell membrane has been to compare the reactivity of the membrane in the intact cell with that of the isolated, unsealed ghost; the difference between the two preparations is ascribed to the accessibility of the cytoplasmic surface in the latter (e.g. 50, 73, 74). However, there are more direct and selective approaches to the cytoplasmic surface. Advantage may be taken of the fact that red cell membranes can be at least partially resealed, after they are osmotically lysed, by appropriate manipulation of the ionic milieu (cf. 12, 75). Probes the size of proteins and even small solutes can thus be equilibrated into the interior space of ghosts during or after hemolysis

and then trapped inside by resealing. Washing the resealed ghosts removes the probe from the external space, so that the cytoplasmic surface can be subsequently examined by activating the probe.

Another strategem for the selective exploration of the cytoplasmic surface is to generate and purify highly impermeable inside-out (or inverted) vesicles from unsealed ghosts (75–79). Impermeable right-side-out vesicles can be prepared by closely related procedures (75, 77–79).[1] Separation of inside-out from right-side-out species has been effected on the basis of physicochemical differences between the two membrane surfaces by free-flow electrophoresis (79) and two-phase aqueous partition (75).

Finally, cytochemical and electron microscope techniques permit visual detection of specific labels at the two surfaces of unsealed ghosts (e.g. 80–85).

While each strategy has its limitations, the data from all are in good mutual agreement and in strong support of a simple, far reaching hypothesis: namely, that there is an absolute asymmetry in the distribution of every protein constituent between the two surfaces of the red cell membrane. That is, no component is symmetrically represented at both membrane faces.

This premise is illustrated in Table II, which summarizes the sidedness of a variety of erythrocyte membrane markers. All of the species listed as outer surface components are reactive to nonpenetrating probes in intact erythrocytes, resealed ghosts, and/or right-side-out vesicles. More critically, none is accessible in inside-out vesicle preparations, beyond a small fraction (~10%) attributable to contamination, until the permeability barrier is destroyed with detergents (75). Conversely, the inner-surface components are fully accessible in inside-out vesicles but are not detected in intact cells, sealed ghosts, or right-side-out vesicles, except for a low level compatible with contamination (75). All markers are fully accessible in unsealed ghosts, but electron microscope analysis of the distribution of electron-dense stains has corroborated their asymmetric disposition in this case.

The components listed in Table II make up perhaps only a small fraction of the total membrane protein mass, so that a separate inquiry into the sidedness of the major membrane polypeptides was undertaken in several laboratories. Intact human red cells were exposed to enzymes such as proteases (52, 60, 65, 86–89), lactoperoxidase plus $^{125}I^-$ and $H_2O_2$ (64, 74, 90, 91), and galactose oxidase plus $B^3H_4$ (54, 60, 61), or to small, presumably nonpenetrating covalent ligands such as [$^{35}$S]diazonium benzene sulfonate (52, 66, 87), [$^{35}$S]formylmethionyl sulfone methylphosphate (51), trinitrobenzene sulfonate (60, 92, 93), and disulfonic stilbene derivatives (94, 95). The membranes were then isolated and the affected polypeptides analyzed by gel electrophoresis. These several approaches demonstrated that two major membrane glycoproteins, bands *3* and PAS-1, and several poorly resolved glycoproteins were reactive in the intact cells. When unsealed ghosts were exposed to the same agents, all of the major membrane polypeptides seemed to be reactive. No component in these ghosts appeared inert or hidden, although the various polypeptides did not react equally with all probes.

TABLE II

*Markers of Erythrocyte Membrane Sidedness*

| |
|---|
| Outer surface |
| Acetylcholinesterase (52, 60, 67, 68, 78) |
| Sialic acid (60, 69, 76, 78, 79) |
| Nicotinamide adenine dinucleotidase (158, 159) |
| Ouabain binding site (160, 161) |
| Carbohydrates (54, 60, 83) |
| Inner surface |
| NADH diaphorase (32, 60, 78, 82) |
| G3PD and its binding site (32, 60, 139) |
| Cyclic AMP binding site (106, 139) |
| Protein kinase (106) |
| ATPase (80, 162) |

A simple interpretation has been drawn from these data: components *3*, PAS-1, and a number of minor glycoproteins are present at the outer surface of the membrane, while components *1*, *2*, *2.1*, *2.2*, *2.3*, *4.1*, *4.2*, *5*, *6*, *7*, and several trace polypeptides are confined to the cytoplasmic surface.

Certain objections to the labeling studies must be considered. First, how can we be sure that a peak of radioactivity which coincides with a major stained gel band is carried by that component and not by a comigrating minor species? For example, band *3* had sometimes been equated incorrectly with PAS-1 and with a $Na^+$, $K^+$-ATPase polypeptide because of similarity in their mobilities. This problem is especially troublesome where the

[1] In one study (86; see also reference 5) confusion of inverted and normally oriented vesicles led to inappropriate conclusions, which subsequent analysis has clarified (60).

amount of label incorporated is only a small mole fraction of the putative target polypeptide present (e.g. references 51, 74). One answer to this problem, to isolate the individual labeled components in pure form, is sure, but difficult. As a simple alternative, certain constituents have been identified through the selective alteration of their relative electrophoretic mobility by cross-linking (95), proteolysis (73, 89), or modification of electrophoretic conditions (49, 50). A related problem with labeling techniques is that they may not clearly demonstrate whether all copies of a given component are accessible at a single surface or only a fraction (e.g. one-half). Controlled proteolysis can usually clearly demonstrate which gel components are being attacked, and to what extent; it has provided a valuable counterpart to the labeling studies. (In no case has controlled protease treatment been found to disrupt the membrane permeability barrier.)

A more serious question concerns the impermeability of the membrane to the small covalent ligand molecules. Several workers have shown that reagents believed to readily cross the membrane barrier exhibit the same selective labeling of components *3* and PAS-1 as do the purported nonpenetrating probes (92, 96, 97). The inference is that the selectivity of protein labeling in intact membranes may not directly reflect membrane asymmetry, and that the enhanced labeling of all proteins in isolated, unsealed ghosts could result from alterations in their conformation or local environment, rather than increased accessibility. (There have been other reports which suggested that a membrane's molecular organization may be altered by its isolation [98–101]) Bretscher (102) has responded to these objections by suggesting that, under the conditions employed, the pH at the cytoplasmic surface of the membrane in intact cells would be lower than in unsealed ghosts, thus reducing the labeling of inner surface proteins by the permeable reagents. However, granting Bretscher's argument still does not permit us to conclude that the small probes discussed above cannot cross the membrane. Furthermore, even external lactoperoxidase may catalyze the iodination of hemoglobin within intact red cells under untoward conditions, presumably by the release of some form of membrane-permeating active iodine (103).

Nevertheless, selective examination of the external and cytoplasmic membrane surfaces and the use of specific macromolecular probes have supported the thesis stated above. Resealed ghosts closely resemble the intact erythrocyte in their responsiveness (25, 60, 88), so that neither the removal of the cytoplasmic contents nor the stress of osmotic lysis causes a general enhancement in the reactivity of the polypeptides. (The same is not true of the red cell lipids [cf. reference 98].) It is possible that resealing could heal not just the holes in the ghost but whatever other hypothetical architectural derangements accompany hemolysis. However, electron microscope studies on unsealed ghosts show no loss of the asymmetric localization of membrane carbohydrates (83), ATPase (80), or spectrin (84). Furthermore, there is no significant difference in reactivity between the intact cell membrane and sealed right-side-out vesicles which are derived from *unsealed* ghosts (60).

When selective radioiodination of the cytoplasmic surface was performed by trapping lactoperoxidase inside resealed ghosts, several polypeptide species were labeled which were unreactive in intact cells (90, 104). Finally, membrane polypeptides not reactive in intact cells are cleaved by proteases in sealed inside-out vesicles (60). Since outer surface constituents such as those listed in Table II are not accessible in these vesicles, it is clear that the reaction transpires at the cytoplasmic surface.

An important conclusion of several of these studies is that at least two glycoproteins, components *3* and PAS-1, span the membrane thickness asymmetrically. Since it is clear that some portion of these proteins is accessible at the outer surface, the argument underlying this thesis is that another domain of each is demonstrable only at the cytoplasmic surface. Bretscher (50, 73) showed that more peptide fragments from each of these two proteins were labeled by [$^{35}$S]formylmethionyl sulfone methylphosphate in unsealed ghosts than in intact cell membranes. He attributed this difference to unique portions of each polypeptide being confined to the cytoplasmic side of the membrane. (However, it has been pointed out that even minor changes in the environment or conformation of these proteins during ghost preparation could account for the increased labeling, since there is no direct evidence that the tag is actually affixed at the inner surface.) Segrest et al. (25) have shown that while the N-terminal portion of the sialoprotein (which bears the carbohydrate) is accessible to lactoperoxidase in intact cells and resealed ghosts, the C-terminal region is iodinated by lactoperoxidase only in unsealed ghosts. Their

sequence data also support a model which places the hydrophilic N- and C-terminal domains of glycophorin, respectively, at the outer and inner membrane surfaces, connected by a roughly 30-residue hydrophobic segment which would span the apolar core. Reichstein and Blostein (90) found that lactoperoxidase sequestered within freshly resealed ghosts labeled band *3* but not band PAS-1, while both were labeled, of course, at the outer surface. More recently, Morrison et al. (104) have reported that lactoperoxidase does indeed tag PAS-1, PAS-2, and PAS-3 when sealed into well-washed and resealed ghosts. The failure of other workers to label the sialoglycoproteins in similar experiments (90) is ascribed to the persistence of trapped catalase and glutathione peroxidase in their resealed ghosts, since these cytoplasmic enzymes would consume the $H_2O_2$ required in the iodination reaction (104).

That both components *3* and PAS-1 extend to the cytoplasmic surface has been demonstrated using inside-out vesicles. The PAS-1 sialoglycoprotein is digested by proteases in both normally oriented and inverted membranes, but the susceptibility patterns are distinctly different for each (60). Radioiodination of the amino-terminal region of PAS-1 proceeds preferentially in sealed right-side-out vesicles, while in sealed inside-out vesicles labeling of the carboxy-terminal portion is enhanced (I. Kahane, V. T. Marchesi, and T. L. Steck. 1974. Unpublished observations). Component *3* is digested in intact cells by chymotrypsin and Pronase, but not trypsin; however, trypsin attacks this polypeptide in inside-out vesicles (60). Furthermore, component 3 can be cross-linked to a dimer by disulfide bond formation when the oxidizing agent is presented to sealed inside-out but not right-side-out vesicles (105). Finally, band *3* provides sites for G3PD binding (32, 105) and for phosphorylation (section VI and reference 106) only at the cytoplasmic membrane surface. That the outer surface portion of these two glycoproteins is not available in the inside-out vesicles has been explicitly demonstrated by their resistance to sialidase (76, 77), galactose oxidase (54, 60), and certain proteases (60).

In summary, each major polypeptide can be assigned to one surface or the other, with the penetrating glycoproteins spanning the membrane asymmetrically, sugar-side out. The data provide no support for (structural) proteins which are indifferent to vectorial topography or have more than one disposition. There is also no indication of polypeptide movement through the membrane thickness or rotation about an axis lying parallel to the plane of the membrane.

Purified ghosts and inside-out and right-side-out vesicles derived therefrom conserve the detailed asymmetric disposition of constituents inferred from probing the intact cell. This point is crucial, since it supports the assumption that isolated membrane derivatives are suitable for topographic analysis of the proteins (but perhaps not the lipids [reference 98]). Finally, the evidence for asymmetrical penetration of some glycoproteins through the membrane suggests: (*a*) that they might be bound hydrophobically in the lipid core, and (*b*) that they might be involved in transmembrane (e.g., transport) functions. There conjectures are considered further below.

## V. ASSOCIATIONS AND DISSOCIATIONS

Every protein in the erythrocyte membrane appears to be bound in a specific and characteristic fashion. Many solubilizing agents dislodge certain components quite selectively, and, in doing so, reveal something about their mode of attachment to the membrane.

### *A. High Ionic Strength Elution*

It has been widely observed that normally soluble glycolytic enzymes are recovered on isolated human and bovine erythrocyte membranes (cf. 30, 32, 107, 108). Because these enzymes can be readily released by raising the ionic strength to physiologic levels, it could be argued that they, like hemoglobin (109), can be adsorbed by ghosts in vitro through nonspecific electrostatic forces in dilute salt solutions. However, recent evidence points to specificity in the association of at least G3PD (band *6*) with the human red cell membrane. This enzyme binds to the membrane (probably via component *3* [105]) only at the cytoplasmic surface, from which it can be completely released by millimolar levels of certain glycolytic intermediates (32, 108).

### *B. Low Ionic Strength Elution*

Three prominent polypeptides, components *1*, *2*, and *5*, are selectively liberated when ghosts are placed in a low ionic strength, mildly alkaline medium free of divalent cations (11, 34, 110–113). These polypeptides (originally called spectrin [110]) have been identified with the fibrillar material observed at the inner surface of ghosts, since

(*a*) those fibrils disappear during the elution process, and (*b*) fibrils form when the ionic strength of the spectrin eluates is increased (110, 114). The eluted proteins behave hydrodynamically as small, highly asymmetric oligomers (34, 112), so that an electrostatic disaggregation or depolymerization of the fibrils presumably accompanies and may actually bring about their release. It could be that bands *1*, *2*, and *5* actually bind very weakly to the membrane but remain in place because of their extensive self-association within the ghost (see below).

### C. Elution by Protein Perturbants

A variety of reagents, known to denature or covalently modify proteins, selectively solubilize a certain group of membrane polypeptides, leaving the remainder still associated with all of the lipid and carbohydrate in a membranous residue. Among these agents are 6 M guanidine hydrochloride (115), hydrochloric (116), or acetic acid (117), 0.1 N NaOH, 40 mM lithium diiodosalicylate (LIS), succinic anhydride, dimethyl maleic anhydride, 1–5 mM *p*-chloromercuribenzoate (all, reference 118), and *p*-chloromercuribenzene sulfonate (27, 118). The polypeptides released, accounting for one-half of the membrane protein, are, in each case: bands *1*, *2*, *2.1*, *2.2*, *2.3*, *4.1*, *4.2*, *5*, and *6*, plus several minor, unnamed species (Fig. 2). (Not surprising is the observation that the polypeptides most readily eluted by the various perturbing agents are components *1*, *2*, *5*, and *6;* i.e., those species released by mere manipulation of the ionic milieu.) Retained in the residual membranes are components *3* and *7*, the poorly resolved zone termed *4.5* (Fig. 2), and the entire PAS profile.

The inference drawn from these effects (118) is that the nonglycosylated half of the protein mass is associated with the membrane by site-specific bonding which is undone by perturbation of protein structure. The eluted species could be called "peripheral" or "extrinsic" (2, 3) in the sense that they are dissociated from the lipid stratum without its dissolution; however, their selective release does not necessarily imply loose binding or an exogenous origin. It is noteworthy that all of the perturbant-eluted polypeptides are uniquely associated with the cytoplasmic surface of the membrane.

In contrast, the membrane glycoproteins seem to be tightly bound to the membrane, so that modification or denaturation does not extract them. Hydrophobic anchoring, as opposed to stereospecific binding, seems likely, particularly in view of their increased content of apolar residues (118) and evidence that at least two members of this group span the membrane. It is noteworthy that all of the polypeptide species accessible at the outer surface of the membrane are in this group. Only one perturbant-resistant component is found uniquely at the cytoplasmic surface: band *7*.

### D. Elution by Nonionic Detergents

Certain nonionic detergents can bring membrane lipids into micellar solution and displace the lipid from hydrophobic proteins without denatura-

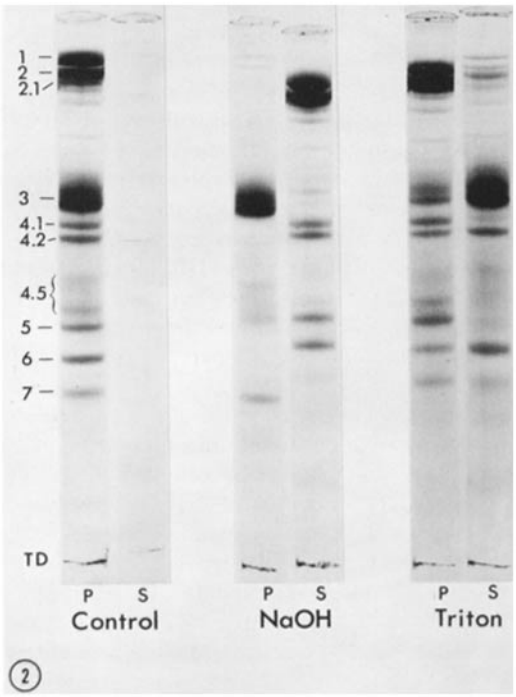


FIGURE 2 Selective solubilization of polypeptides from ghosts by protein perturbants and nonionic detergents. Isolated ghosts were incubated in 5 mM sodium phosphate buffer, pH 8 (left); water adjusted to pH 12.0 with NaOH (middle); and 0.5% Triton X-100 in 56 mM sodium borate, pH 8.0 (right). After centrifugation, aliquots of pellet (*P*) and supernatant (*S*) fractions derived from 10 μl of ghosts were electrophoresed and the gels stained with Coomassie blue. *TD*, tracking dye. (Reprinted by copyright permission from *J. Supramol. Struct.* 1973, **1**:220 and 233.)

tion (cf. 119–121). We would anticipate, therefore, that polypeptides anchored in the membrane through apolar association with lipids could be solubilized as detergent complexes, leaving protein tertiary structure and quaternary associations intact.

The selective action of Triton X-100 on human ghosts (119) is nearly reciprocal to that seen with the perturbants (Fig. 2). Released in a truly soluble, lipid-free form are bands *3*, *4.2*, *6*, and (not shown here) the entire PAS-stained profile. Somewhat more than half of the membrane phospholipid (principally the glycerolipids) is also solubilized. The remaining polypeptides (primarily bands *1*, *2*, *4.1*, *5*, and *7*) are retained in ghost-shaped residues which appear in electron micrographs to be comprised of a dense reticulum of finely filamentous material studded with segments of lipid (primarily sphingolipids) (Fig. 3).

This filamentous matrix is probably attributable to the "spectrin" polypeptides (bands *1*, *2*, and *5*), since (*a*) they comprise at least half of the protein in the residues; (*b*) no Triton residues are observed if these components are previously selectively eluted by low ionic strength treatment; and (*c*) spectrin has previously been identified with inner surface fibrillar material (110, 111, 114). The spectrin filaments may thus form a submembrane continuum held together by self-association, rather than binding to the lipid part of the membrane (119).

Components *3*, *4.2*, and *6* also seem to retain specific quaternary associations in Triton X-100 solution (105, 122). Band *3* behaves like a dimer, just as in the membrane (123), and band *6* remains tetrameric, as judged by its unaltered sedimentation properties and G3PD activity. All of band *6* is specifically bound, apparently cooperatively, to component *3* in the Triton X-100 extract, two G3PD tetramers per band *3* dimer, as is also the case in ghosts (105).

Finally, band *4.2* may be in the form of a tetramer, since formaldehyde rather specifically cross-links it to a 270,000-dalton form, both while membrane bound and in Triton solution (123). This polypeptide also appears tightly bound to band *3*, since it copurifies with the latter in a variety of systems, unless perturbants are first employed to break the complex (105, 122). These data make clear why bands *4.2* and *6* are selectively eluted with the glycoproteins by Triton X-100 (Fig. 2).

### *E. Detection of Associations by Electron Microscopy*

As estimated by freeze-fracture electron microscopy, each human red blood cell membrane has about 500,000 intramembrane particles with an average diameter of approximately 80 Å, which are believed to be composed of protein (14). The particles in erythrocytes and freshly isolated membranes are laterally dispersed with no apparent ordering. It is not now known if they are free to move or are fixed in place under these conditions. Treatment of ghosts, but not erythrocytes, with digestive enzymes and certain alterations in the pH or ionic strength of the medium causes the particles to cluster (cf. 14, 124). This shift in distribution could represent either an initiation of particle translation or an aggregation or particles already in motion.

It has proved difficult to identify the dispersed intramembrane particles with a specific protein. However, after the induction of clustering, the distribution of particle aggregates parallels the pattern of binding sites for these ligands: anti-A blood group antibody (125), kidney bean phytohemmaglutinin (126), influenza virus (126), and colloidal iron hydroxide (127). These activities are all associated (at least in part) with the major sialoglycoprotein (9, 19, 23, 55), which, furthermore, has a transmembrane disposition and an abundance (Table I) appropriate to being the intramembrane particle. On the other hand, the sialoglycoprotein (and, more particularly, its hydrophobic transmembrane segment) appears too small to account for the size of the intramembrane particle (24; however, see reference 128). It has been suggested, therefore, that the particles represent groups of laterally associated membrane-penetrating proteins, perhaps involving all of the glycoprotein species (7, 9). What is not known, however, is whether the various membrane glycoprotein species are normally laterally associated, since this grouping could result from the membrane perturbations required in these experiments. Two cross-linking studies on human (123) and bovine (129) red cell membranes failed to detect any complexes formed by the sialoglycoproteins; however, Ji (130) has recently reported that new PAS-stained gel bands appear after dimethyl adipimidate treatment of ghosts. Finally, it should be noted that band *3*, of itself, could account for all the intramembrane particles. As spherical 180,000

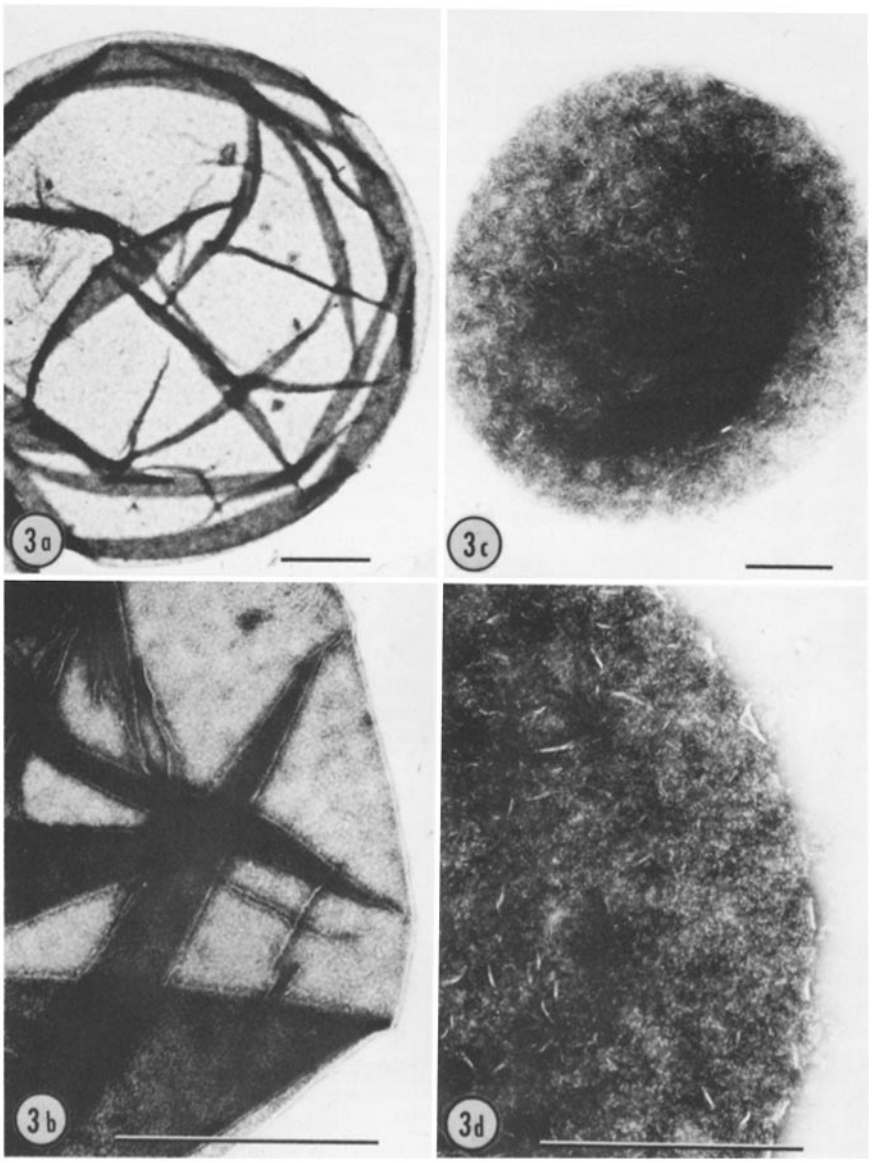


FIGURE 3 Triton X-100-extracted ghosts. Ghosts were incubated in 5 mM sodium phosphate (pH 8.0) with or without 0.5% Triton X-100, then negatively stained with aqueous 1% uranyl acetate. (*a* and *b*) control ghosts; (*c* and *d*) extracted ghosts. Calibration bar is 1 $\mu$m throughout. (Reprinted by copyright permission from *J. Supramol. Struct.* 1973, **1**:233.)

dalton dimers, this component would contribute $\sim 5 \times 10^5$ particles of 75-Å diameter to the plane of fracture.

Two laboratories have recently presented evidence that the lateral motion of the sialoglycoproteins and the membrane-penetrating particles is constrained by the spectrin polypeptides (bands *1*, *2*, and *5*). Nicolson and Painter (131, 132) reported that treating ghosts with antisera prepared against spectrin promoted redistribution of the colloidal iron hydroxide binding pattern which identifies the sialoglycoproteins at the external membrane surface. Elgsaeter and Branton (124) have found that gently perturbing spectrin by incubating ghosts in low ionic strength, mildly alkaline solution is associated with an increased clustering of the

intramembrane particles during subsequent exposure of the membranes to media favoring particle aggregation (e.g., low pH or high ionic strength). The presumption is that spectrin filaments are interacting with membrane-penetrating proteins at the cytoplasmic surface. These authors favor an interpretation involving a direct association of penetrating proteins with spectrin. However, it is perhaps more likely that the penetrating proteins are entrapped within the interstices of the dense filamentous reticulum without being specifically bound, since the glycoproteins can be easily separated from the spectrin by mild nonionic detergent treatment which does not destroy other quaternary protein associations (119). These alternative mechanisms might be distinguished by a search for direct binding of solubilized bands *3* and PAS-1 to spectrin isolates.

## VI. PHOSPHORYLATIONS

When intact human erythrocytes are incubated in the presence of $^{32}PO_4$, the membrane protein and lipid fractions both become labeled (133). The same is true for isolated ghost membranes incubated with $\gamma$-[$^{32}$P]ATP (48, 134–138). The identity of the labeled polypeptides has been investigated using polyacrylamide gel electrophoresis in SDS. Avruch and Fairbanks (48) have detected a 105,000-dalton polypeptide in which the bound $^{32}PO_4$ turned over rapidly. They provided strong evidence that this component is the acylphosphate intermediate of the $Na^+$, $K^+$-ATPase. Williams (134) has also found a $^{32}$P-labeled gel band of approximately 98,000 daltons which showed certain features ascribable to a phosphorylated intermediate of the ATPase, yet differed from the aforementioned component (48) in certain important respects.

Stable phosphoserine and phosphothreonine linkages to proteins have also been demonstrated after incubation of ghosts in $\gamma$-[$^{32}$P]ATP (137, 138). Most heavily labeled is a gel peak which appears to correspond to band *2* (215,000 daltons); a 90,000-dalton component (presumably band *3*) is tagged less well and a 50,000-dalton peak is only slightly labeled. Micromolar levels of cyclic AMP greatly stimulate the labeling of the 50,000-dalton peak; increase in the 90,000-dalton peak is moderate, while the labeling of the 215,000-dalton component is not stimulated (135, 137, 138). A cyclic AMP-stimulated protein kinase activity can also be demonstrated in human erythrocyte membranes using exogenous histones as a substrate (136, 137). The enzyme appears to reside exclusively at the cytoplasmic side of the membrane (106), a further argument for the presence of the phosphorylated proteins at that surface. Whether the cyclic AMP binding site, which has also been ascribed to the inner surface of the erythrocyte membrane (106, 139), is the same as the regulatory site of the protein kinase is uncertain, since the cyclic AMP concentration required for half-maximal saturation of the binding site (3 nM) is 10–100 times lower than that required for half-maximal stimulation of phosphorylation (135, 136, 139). Photoaffinity labeling was recently used to demonstrate that the 50,000-dalton polypeptide whose phosphorylation is most stimulated by cyclic AMP probably corresponds to this cyclic AMP binding site (140).

Are the membrane proteins normally phosphorylated *in situ?* Several workers (16–18) have found residual phosphorus in the membrane protein fraction after exhaustive lipid extraction. While this could be ascribed to tightly bound phospholipid, it could also represent phosphoproteins, with approximately one to two phosphates per 50,000 daltons of protein. Since this level of phosphorylation greatly exceeds the extent of incorporation achieved in vitro, it may be that the latter is limited by preexisting specific phosphoprotein complexes.

## VII. CONCLUSIONS AND CONJECTURES

Fig. 4 summarizes some of the data discussed above. It is intended to depict the topography of the nine or ten principal polypeptides which comprise about 80% of the protein mass. It ignores their characteristic functions, molecular sizes, and abundance. Each polypeptide can be assigned to one surface of the membrane or the other. Two polypeptides which seem to extend across the membrane do so anisotropically, with their carbohydrate portions detectable only at the outer surface. All of the outer surface proteins seem very firmly anchored, presumably by penetration into or through the hydrophobic core of the membrane. The constituents confined to the inner surface are (with the exception of band *7*) bound by more readily reversed association, presumably with specific sites. Oligomers among like polypeptides are present, which in turn form complexes with certain other proteins at the cytoplasmic surface.

This scheme is necessarily provisional and inaccurate. The number and identity of the minor glycoprotein species is uncertain. Many or even all of them could span the membrane (cf. 104), but a

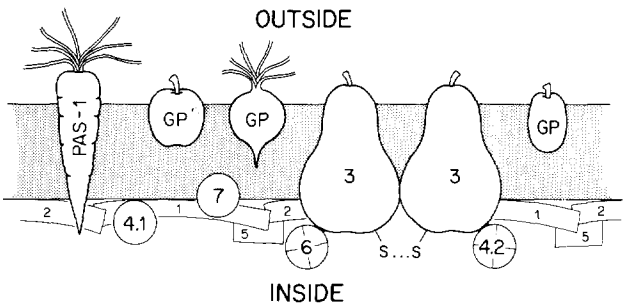


FIGURE 4 A possible arrangement for the major erythrocyte membrane polypeptides. The lipid permeability barrier is shaded. The glycoproteins penetrate into or through the hydrophobic zone, with all their carbohydrates exposed to the extracellular space. The other polypeptides are, in general, less firmly anchored at the cytoplasmic face, where they participate in specific interprotein associations. See text for details and reservations.

rigorous identification of which species extend to the cytoplasmic surface is presently wanting. Furthermore, it is not known whether band *4.2* and band *6* (G3PD) tetramers can bind independently to band *3* monomers or even dimers, particularly since G3PD binding to the dimers appears cooperative (105). Finally, the representation of bands *1*, *2*, and *5* is not intended to convey a model, but rather our present ignorance of their associations *in situ*. It is known that bands *1* and *2* can be coupled by covalent cross-linking reagents, apparently each to itself as well as to one another, both on ghosts (123, 141) and in solution (9, 34, 123); however, band *5* appears not to participate in these complexes. Indeed, the former two polypeptides can be separated from the latter after gentle elution (34, 124), although Guidotti has argued for their persistent association in solution (35). As discussed below, there is diverse evidence favoring the view that bands *1*, *2*, and *5* could be depicted as an actomyosin system.

Thus far, all red cell membrane carbohydrates —both glycoprotein and glycolipid—appear localized to the external surface; furthermore, all the major outer surface proteins bear sugar (cf. 54). NADase and acetylcholinesterase should therefore be examined for the presence of bound carbohydrate to further test this hypothesis. Similarly, we might enquire whether a glycoprotein subunit of the red cell $Na^+,K^+$-ATPase (if analogous to other tissues; references 72 and 142) is exposed to the external surface (where it could bind ouabain), since the nonglycosylated polypeptide subunit is phosphorylated at the cytoplasmic side. There is now an ample and rapidly growing literature indicating that outer surface antigens, enzymes, receptors, and numerous proteins of presently unknown function are glycosylated in a variety of cells. Clearly the trend, if not the rule, is that proteins intrinsic to the outer surface of plasma membranes generally bear carbohydrate, while those confined to the cytoplasmic side do not (cf. also reference 85).

The strict asymmetry in the sidedness of the red cell membrane proteins must reflect their function. Some enzymes at the external surface presumably serve the needs of the organism rather than the cell. For example, the outer surface acetylcholinesterase can destroy the neurotransmitter circulating in the plasma without it being transported across the impermeable erythrocyte membrane. Externally oriented NADase, like the 5′-nucleotidase found on the surface of other cells (143), might be part of a mechanism generating permeant nucleosides from impermeant nucleotides for intracellular salvage. Conversely, inner surface enzymes (Table II) would appear to address intracellular homeostasis.

Polypeptides which span the membrane are logical candidates for transport sites. However, it is conceivable that oligomeric protein complexes could also play such a role without any single polypeptide constituent being accessible at both membrane surfaces. $Na^+,K^+$-ATPase (cf. 142) and cytochrome oxidase (144) might be examples. On the other hand, a deeper understanding of the structure of proteins within the lipid core might make it clear that the assembly of a transmembrane permeation mechanism out of more than a single polypeptide is unfavorable.

Cabantchik and Rothstein (89, 94, 95) have recently shown a close correlation between the inhibition of the facilitated diffusion of $Cl^-$ and $SO_4^=$ through the red cell membrane by a radioactive stilbene derivative and its selective covalent labeling of band *3*. Complete inhibition occurred when $3 \times 10^5$ molecules of inhibitor were bound per cell, at the outer surface.

There is also indirect evidence consistent with the premise that band *3* provides $\sim 3 \times 10^5$ sites to facilitate the diffusion of D-glucose across the membrane (145–147). To speculate briefly, there are enough band *3* polypeptides—and even dimers—to account for these various capacities. That band *3* is comprised of more than one molecular form is suggested by its complex staining profile (Figs. 1 and 2). This hypothesis is also favored by a recent report that several dansylated amino acids, presumed to represent amino-terminal residues, can be recovered from the band *3* region of polyacrylamide gels (148). However, Tanner and Boxer (59) found no free N-terminus in purified band *3* so that the dansylated residues (148) could have derived from other polypeptides of similar mobility (e.g., PAS-1). The band *3* polypeptide behaves homogeneously with regard to cross-linking (123), proteolytic cleavage (52, 60, 73, 88), and other features (59). It has been suggested that the dispersion in its stained electrophoregram could be caused by a variation in its carbohydrate complement (54). At present, therefore, we cannot dismiss any of these possibilities: (*a*) the primary polypeptide structure of band *3* is homogeneous, yet it facilitates the transport of more than one solute (but cf. 149); (*b*) band *3* is a family of closely related polypeptides, specialized to facilitate different solutes across the membrane; and (*c*) band *3* is not the source of specificity in the facilitated diffusion of these solutes. If band *3* does facilitate the diffusion of solutes, its dimerization, its phosphorylation, and binding of band *6* (G3PD) and band *4.2* take on added interest.

We have considered data which indicate that bands *1*, *2*, and *5* constitute a densely self-associated reticulum of microfilaments adherent to the cytoplasmic surface of the red cell membrane. This matrix could offer the tough and inextensible skeletal support which the delicate and deformable fluid lipid bilayer (with its integrated proteins) might require to withstand the rigors of prolonged shear stress in the circulation (cf. also 35). The "two-ply" structure created by the apposition of the fibrous meshwork to the supple lipid stratum might help to account for the complex mechanical properties of the membranes observed in a variety of biophysical studies (cf. 14, 150–152). This filamentous system could define and control cell shape, and perhaps respond to cytoplasmic or even extracellular signals via energy-dependent structural modification.

In support of these concepts are these observations: (*a*) Selective elution of bands *1*, *2*, and *5* is accompanied by the breakdown of ghosts via endocytic vesiculation, implying a specific destabilization of the membrane (76, 110). (*b*) Guidotti, having reviewed literature pointing to the presence of actin- and myosin-like proteins at other cell surfaces (1), suggested (35) that bands *1*, *2*, and *5* may be "erythrocyte actomyosin," by virtue of their molecular weight, amino acid composition, morphology, and associated $Ca^{++}$-dependent ATPase activity (114). While the precision of this analogy remains to be defined, recent studies have indeed indicated that purified component *5* can be polymerized at high ionic strength into fibrils which resemble actin in their capacity to stimulate myosin ATPase activity and in their pattern of arrowhead decoration by heavy meromyosin (M. Sheetz, R. G. Painter, and S. J. Singer, personal communication). (*c*) Mg-ATP stimulates the phosphorylation of band *2* (135, 137, 138), the breakdown of bovine ghosts by endocytic vesiculation (153), and the emergence in ghost preparations of an infrared spectrum identified with the anti-parallel beta conformation (154), all of which could conceivably relate to changes in the state of the spectrin system. (*d*) Striking increases in the rigidity of erythrocytes occur after ATP depletion and $Ca^{++}$ uptake, which are reversed by $Ca^{++}$ removal and increased levels of Mg-ATP; isolated ghosts share some of these properties (8, 155). (*e*) Finally, Jacob et al. (156) have implicated submembrane microfilamentous proteins in the abnormalities of shape and turgor seen in erythrocytes from patients suffering from hereditary spherocytosis and erythrocytes treated with colchicine, vincristine, and strychnine. Seeman et al. (157), however, have provided evidence that these alkaloids could be affecting the lipid portion of the membrane instead.

In conclusion, some organizational features of the conspicuous proteins in the human erythrocyte

membrane can presently be perceived at sufficient resolution to stimulate precise hypotheses concerning their activities in vivo. Such inquiry should guide future developments in this area, as function lies at the heart of membrane structure.

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*Note Added in Proof:* Recently, additional evidence was obtained supporting the transmembrane disposition of band *3* (163).

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