The Relevance of Research on Red Cell Membranes to the Understanding of Complex Human Disease: A Personal Perspective

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Annu. Rev. Pathol. Mech. Dis. 2008. 3:1–9

The *Annual Review of Pathology: Mechanisms of Disease* is online at pathmechdis.annualreviews.org

This article's doi:
10.1146/annurev.pathmechdis.3.121806.154321

1553-4006/08/0228-0001$20.00

Key Words

erythrocyte, spectrin, glycophorin, cytoskeleton

Abstract

Molecular analysis in the service of research on human disease has finally come of age, as the chapters within this volume testify. Many technical advances, among them the development of recombinant DNA and its many applications, opened the way to study cells and processes that were unapproachable in the 1960s, when I first began my research career. The state of molecular biological studies at that time limited studies of human cell membrane proteins to experimental material most available and accessible, making the human erythrocyte membrane the favored target. I describe here how studies of red blood cell membrane proteins evolved and how results from those studies still inform present-day research.

INTRODUCTION

The current explosion in biomedical research is the consequence of many technical advances, among them the development of recombinant DNA and its many applications, which opened the way to studying cells and processes that were unapproachable in the early days of post–World War II biochemistry and its even more primitive cousin, cell biology. A major problem in the early days was the availability of experimental material in quantities large enough to isolate specific biomolecules and study them with the then available techniques. It was not an accident that liver biochemistry was a major focus in the study of intermediary metabolism or that hemoglobin and myoglobin were among the first molecules to be studied at the atomic level. The same applied to the study of cell membranes. Equally important for mammalian cell biology, and for a foundation to study complex human diseases, was the human erythrocyte membrane, the most accessible and abundant source of membrane material. Lacking the power of recombinant DNA technology, the earliest attempts to characterize membrane proteins relied on old-fashioned protein-isolation methods. Because these required large amounts of high-quality experimental material, almost all the early attempts to isolate membrane proteins from mammalian cells used red blood cell (RBC) membrane ghosts as starting material.

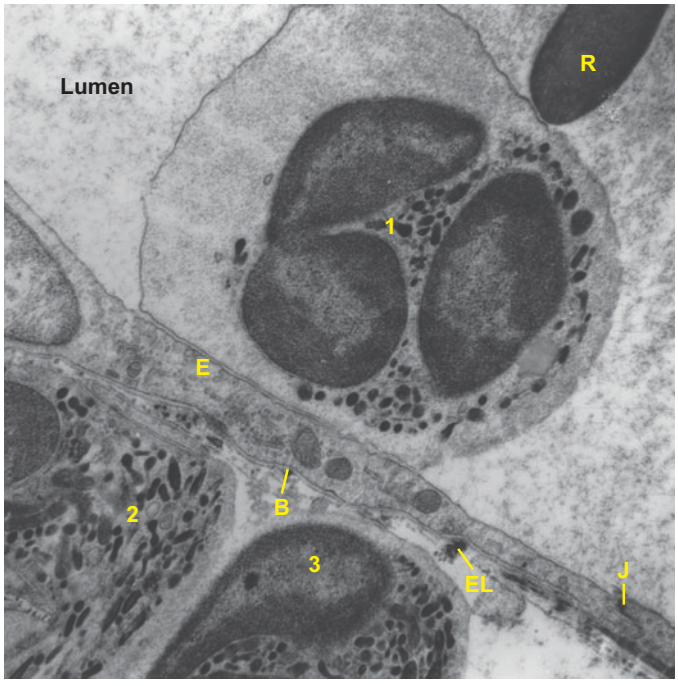

Figure 1

Electron micrograph of a polymorphonuclear leukocyte adherent to the endothelium of an inflamed venule. *J* marks the intercellular junction between two endothelial cells (*E* and *EL*), and *B* and *E* mark the basement membrane and elastin that surrounds the vessel. Two other leukocytes (*2* and *3*) are streaming out into the tissue spaces.

My first encounter with the cell membrane problem began not with the study of RBCs, but with attempts to study the process of leukocyte emigration with the electron microscope (1). In the course of these studies, I produced in 1960 the photograph shown in **Figure 1**. This electron micrograph was taken of a small venule in the mesentery of a rat that was inflamed by mild trauma and examined by the then standard thin sectioning techniques of electron microscopy. I took this picture as a graduate student at the Sir William Dunn School of Pathology at Oxford University, sometime during the 1959–60 academic year. Howard Florey, my mentor, then Sir and later Lord, a man not known for distributing excessive praise freely, was visibly pleased when he saw this photograph.

What did this eye-opening photograph actually show? What information, not known before about inflammation, was now revealed? Well, actually, especially in retrospect, very little. But it was a nice, clear picture of what a leukocyte stuck to the surface of a venular endothelial cell looked like up close. We could identify the surface membranes of both the leukocyte and the underlying endothelial cell by the electron-dense lines, but this was a decade before the first tentative formulation of the fluid-mosaic model of membrane structure by Singer and Nicholson was published, so we really had no idea what we were looking at. However, we did think it important that there was a space between the opposing

membranes that must contain something that formed the tight but reversible bond between the leukocyte and endothelium. The term intercellular cement was constantly referred to by Florey, who was convinced that earlier claims for the presence of this putative bonding substance were ruled out by its apparent absence. We were impressed by the extensive contacts between the membranes of the adherent leukocyte and the underlying endothelial cell; what we did not know at the time was the large number of proteins that link the two membrane surfaces together. Their identity was revealed when it became possible years later to study low-abundance proteins with cloning techniques.

Subsequent studies revealed that the path through leukocytes migrates across the endothelial barrier, something that could not be observed by light microscopy. It became possible to study serial sections of many migrating cells, and the reconstruction of their images showed that migrating inflammatory cells passed through open intercellular junctions. In a later study with Jim Gowans, a pioneer in the study of lymphocyte recirculation, we found that circulating T cells could migrate across the venular endothelium of postcapillary venules by what appeared, by serial sectioning, to be a transcytoplasmic pathway (2). This finding has been both confirmed and denied repeatedly over the past 45 years. I am told by Tim Springer that it may prove to be correct after all.

I had a chance to work with Howard Florey and Jim Gowans because I was lucky enough to attend Yale University Medical School at a time when students were encouraged to take time off during medical school to work in a research lab. Those were the days before the existence of MD PhD programs, which now offer medical students serious research experience. There was a tradition at Yale for students to spend time abroad, and in my case I was able to join Florey's lab because I was recommended to him by John Fulton, a professor of physiology at Yale and a close friend of Florey's. Florey's children lived with the Fultons in New Haven during World War II, and it was this connection, I believe, that led to penicillin being administered to a New Haven policeman during the early 1940s. I met John Fulton through a medical student friend named Donald Miller, now a prominent psychiatrist in New York. Florey's last edition of his textbook *General Pathology* included many of my electron micrographs illustrating the stages of leukocyte emigration, and this publicity led to my recruitment to the Department of Pathology at Washington University, with Paul Lacy's strong backing. After two years there, George Palade offered me a postdoctoral position at Rockefeller University. Palade was the first to study blood vessels by electron microscopy in a serious way. His work describing pinocytic vesicles of capillary endothelial made a big impact on Florey, and the fact that I worked with Florey on endothelium also impressed Palade, so I spent a year under his guidance at Rockefeller University, and began there my studies of erythrocyte membranes that lasted for two decades.

Having spent time in two rich scientific settings working closely with two accomplished investigators, one a Nobel laureate and the other who received the prize a decade later, made a big impact on me for many reasons, but the ways they provided guidance stand out as the most pivotal. Both men told me what I should work on, but neither told me how to do it. The other thing both provided was the scientific neighborhood. The laboratories of Florey at Oxford and of Palade at Rockefeller were populated by talented investigators of all ages and skills who worked on their own projects yet contributed to the intellectual activity of the whole group. I am impressed by how different research is organized now compared to then. Many present-day research units at Yale, and I presume at other comparable places, are made up of junior and senior investigators who work largely within their own laboratory environments, surrounded by students and fellows but largely shielded from other peer investigators. The larger the individual research group, the

thicker the shield. A number of factors contribute to this relative isolation, among them the need to demonstrate originality and independence. These are often weighed heavily in the evaluation of faculty for tenured positions and for external awards such as election to national academies, and, in some cases, one suspects, even research grant awards. Collaborative efforts between research groups do exist, but the main focus, where most of the intellectual energy is spent, is devoted to the studies of individual primary investigators. Thus, most research neighborhoods that trainees now experience do not match the depth and diversity that I enjoyed. I spent six years at the National Institutes of Health, where I began studying the proteins of the human erythrocyte membrane, in effect continuing the studies that were initiated under the direction of Palade. In 1972 I moved to Yale at the invitation of the late Lewis Thomas and joined the Department of Pathology. A year later, Thomas left Yale to become president of Memorial Hospital in New York, and I was appointed to succeed him as the chairman of the department.

The success I enjoyed as a graduate student at Oxford persuaded me that my medical career, which I had decided to pursue at a very early age, lay more in basic science than clinical medicine, although I thought at the time, and still do, that academic pathology has the potential to satisfy both aims, the desire to support medical care and the opportunity to study human disease at a fundamental level. My studies of leukocyte emigration with the electron microscope sensitized me to the importance of understanding how cells interacted with each other through their surface membranes, but it was clear then that progress would come only after we knew how membranes were organized at the molecular level. Much was known of the properties of the lipid bilayer, common to all cell membranes, but membrane proteins were a complete unknown.

The big challenge, when I entered the field in the mid 1960s, was to learn how to extract membrane proteins from membrane lipids in a water-soluble form so they could be studied by conventional techniques of protein chemistry. A second problem, related to the first, was the inability to determine how many different proteins there were in any individual membrane preparation. Because of their insolubility in aqueous media, conventional electrophoretic or chromatographic separation techniques were completely ineffective. The development of SDS PAGE in the 1960s was the major technical achievement that opened up the field then, and to this day it remains the single most useful analytical tool to study protein molecules of all types. SDS PAGE analysis of isolated RBC membranes, so-called ghosts, revealed a relatively small number of stainable bands, consistent with the RBC being an uncomplicated enucleate cell with few movable parts. Once the ghosts were isolated after osmotic lysis, one had the impression, which turned out to be true, that every major band visible on the gel was derived from the cell membrane, because an analysis of the hemolysate showed that none of the major membrane proteins were released in significant amounts during hemolysis. Because they were suspended in SDS, a negatively charged detergent strong enough to strip them from the lipid bilayer, one assumed that the separated polypeptide chains were biologically inert. Polypeptides dissolved in SDS and electrophoresed in the presence of the detergent had another unusual and extremely useful property. They were dissociated into their constituent subunits, and each individual polypeptide migrated through the acrylamide gel roughly proportional to its chain length, a surprising result that provided a reasonably accurate estimation of its molecular weight.

However, seeing bands of proteins on SDS gels did not mean they were ready to be studied by conventional methods. Rendering them water soluble was still an elusive goal. It turned out, however, that a sizable fraction of the total membrane-bound proteins of the RBC membrane could be separated from the lipids by simple manipulations of the ionic

strength of the solubilizing media. Spectrin was the first major protein to be isolated from erythrocyte ghosts by suspending the membranes in a very low-ionic-strength solution (3). By exposing ghost membranes to first low- and then high-ionic-strength buffers, all but the most tightly bound proteins could be released as native, functionally active molecules. Spectrin was found to be composed of two large polypeptides chains (4) that exist as stable dimers with the capacity to assemble into higher forms (5). Spectrin tetramers were subsequently found to be linked together by short actin filaments, and the two together make up the bulk of the membrane skeleton of the mature erythrocyte. These findings were the first clear indication that an elaborate protein framework buttressed the lipid bilayer of the erythrocyte, creating what is now believed to be present in all cells, a membrane skeleton linked to but distinct from the larger cytoskeleton. This is shown schematically in **Figure 2**.

Many studies of nucleated cells carried out since this model was formulated confirm that this general pattern is a feature of all mammalian cells, with each cell type having variations of the RBC forms. Spectrins now comprise a large family of structural proteins with diverse functions. In the course of extracting spectrin and actin from RBC ghosts, the membranes fragmented into small vesicles, suggesting that a spectrin/actin lattice might be necessary for membrane stability, and later studies proved this to be true. This led to a search for defective spectrins in RBCs of patients with hereditary hemolytic anemias, and several have been identified. RBCs from patients with hereditary elliptocytosis and hereditary pyropoikilocytosis both have changes in their spectrin sequences, which correlate with membrane fragility (6).

How the spectrin/actin lattice was linked physically to the overlying lipid bilayer was a hotly pursued question, and work from many laboratories, including those of Dan Branton and Vann Bennett, showed that RBCs contained two critical linking proteins, termed ankyrin and protein 4.1, that were the attachment points. Ankyrin joined spectrin to protein band 3, a prominent anion channel protein, and protein 4.1 connected actin to the glycophorins. This ensemble established the principle, which has been amply

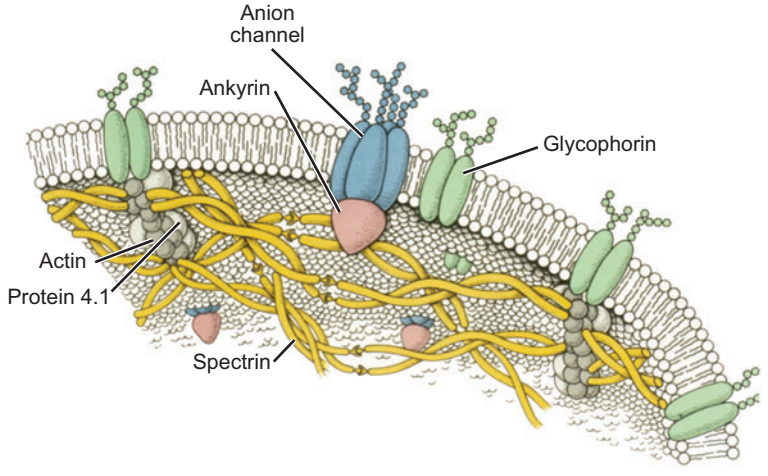

Figure 2

This highly schematic diagram shows spectrin polymers linked together in tetrameric units attached to the overlying lipid bilayer through linking proteins, two of which are ankyrin and protein 4.1. Spectrin attaches directly to ankyrin, which links it to the anion channel protein band 3, and short oligomers of actin link another domain of spectrin to the glycophorins through the protein 4.1 linker.

confirmed, that submembranous protein skeletons of all cells can attach to different classes of transmembrane proteins, which in the case of nucleated cells represent either receptors or channels. Work on spectrin at Yale was carried out by many collaborators, including Jon Morrow, Bill Knowles, and Dave Speicher.

The concept that defective membrane skeletons is responsible for instability of surface membranes in other human diseases has been amply confirmed by studies of muscle membranes of patients with different forms of muscular dystrophy. One of the first defective proteins linked to muscular dystrophy is the molecule known as dystrophin, which is strikingly similar to erythrocyte spectrin. Like spectrin, dystrophin connects to other cytoskeletal proteins including actin, but it also shares with spectrin the capacity to bind tightly to a number of transmembrane proteins of muscle cells, some of which resemble both glycophorin and band 3. Mutant forms of both dystrophin and many of the proteins that interact with it account for the wide variety of muscular dystrophies that affect the human population. In a sense, skeletal muscle membrane instability and the cause of debilitating muscular dystrophy is the muscle counterpart to hereditary spherocytosis, eliptocytosis, and pyropoikilocytosis, which have as their defects an unstable membrane skeleton, with spectrin-like proteins and spectrin-binding proteins as the defective elements.

Roughly half the membrane-bound proteins of the RBC membrane can be extracted from membrane ghosts by simple buffer solutions, but the other half remain tightly bound to the fragmented lipid vesicles, an observation that led Jon Singer to propose the useful generalization that membrane proteins were either peripheral (easily extractable) or integral (tightly associated with lipids). We found that the chaotropic salt lithium diiodosalicylate (7) was remarkably effective in isolating the major sialoglycoprotein of RBCs, now known as glycophorin A, in quantities large enough for Mototwo Tomita to sequence by traditional methods (8). A distinctive feature of the sequence was a stretch of 22 amino acids rich in hydrophobic residues that represented the membrane-spanning segment. A cluster of positively charged amino acids (lysine or arginine) were immediately C terminal to the hydrophobic segment, on the cytoplasmic side of the lipid bilayer, and this is an invariant feature of all known single-pass transmembrane proteins, so constant that it is used routinely to identify single-pass transmembrane segments in newly discovered membrane proteins.

Glycophorin A was found to be a dimer when solubilized in SDS, and Heinz Furthmay found that interactions between the transmembrane domains accounted for its unusual stability (9). This observation was confirmed by Don Engelman and coworkers, who attributed this tight association to the amino acid sequence of the hydrophobic segment. The notion that noncovalent associations between segments of single-pass transmembrane proteins play important regulatory roles has been widely applied to the analysis of many critical receptors involved in signal transduction and neoplastic transformation. The experiment illustrating this principle is depicted in **Figure 3**.

Whereas the pathogenesis of the muscular dystrophies mimicked in many respects the pathogenesis of some hereditary hemolytic anemias, advances in our understanding of more complex medical problems, like Alzheimer's disease, benefited more indirectly from RBC studies. One hundred years ago, Alois Alzheimer reported the autopsy findings of Auguste D., a 54-year-old woman from Frankfurt, Germany, who presented him with incapacitating dementia four years earlier. Histological sections of her brain revealed the presence of dense, argyrophilic deposits scattered throughout the cortical regions, which Alzheimer identified as plaque-like structures. These, he thought, were characteristic of a specific clinical syndrome not previously detected. During the

ensuing years, these deposits were referred to as senile plaques and the clinical syndrome became known as Alzheimer's disease.

In 1984, roughly 77 years after Alzheimer's description, George Glenner reported that material extracted from brain blood vessels of dementia patients was composed of small peptides that had the capacity to polymerize into thread-like structures, which he referred to as amyloid fibers. He speculated that they were components of the senile plaques found in the brains of patients with Alzheimer's disease. On the basis of the amino acid sequences of the peptides that Glenner isolated, others determined that the peptides were derived from a large receptor-like molecule that had the structure of a transmembrane protein. They deduced this after sequencing cDNA clones that coded for polypeptides containing the amino acid sequences described by Glenner. The predicted sequence of the complete molecule contained a short 22-amino stretch of peptide that was strikingly similar to peptides of similar length and composition that were parts of known transmembrane proteins, one of which was erythrocyte glycophorin A, one the first mammalian transmembrane proteins to be sequenced. Interestingly, the 131 amino acids of the glycophorin backbone were sequenced by classical protein sequencing methods and took almost three years be completed in 1975. Using recombinant DNA technology, the sequence of the amyloid precursor protein (APP) was determined in a fraction of that time, but its transmembrane orientation was deduced on the basis of principles arrived at decades earlier. Within a few years after this discovery, the enzymes that cleave the APP, the so-called secretases, were identified and characterized, and mutant forms of proteins involved in Aβ production were found in patients with familial Alzheimer's disease, confirming that the Aβ peptides play a pivotal role in the pathogenesis of Alzheimer's disease. All these advances came on the heels of two decades of study of simple membrane systems, with RBC studies leading the way.

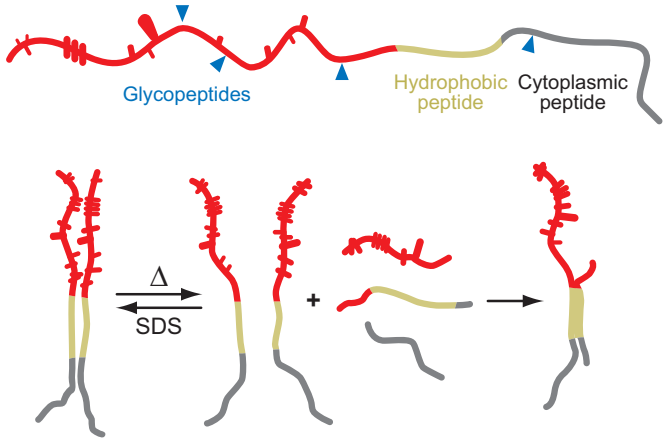

Figure 3

This diagram depicts the results obtained when purified red blood cell glycophorin A, which is normally a stable dimer, is mixed with peptides derived from native glycophorin by tryptic digestion and then heated in the presence of SDS. Under these conditions, peptide fragments generated by proteolytic cleavage of the transmembrane domain can associate with glycophorin monomers, creating chimeric pseudodimers. The ability of an isolated transmembrane peptide to substitute for an intact glycophorin monomer is believed to be due to the tight packing of contiguous, α-helical, transmembrane domains, and it is now believed that a similar mechanism may operate in surface membrane proteins involved in the transmembrane signaling of all cells.

LESSONS LEARNED

The RBC membrane was in retrospect a good experimental system to try to extract and isolate membrane proteins. Two factors favored it: availability and simplicity. Results from its study have been replicated in every other mammalian cell type, and in some crucial points, the patterns outlined by RBC proteins led the way to the interpretations of later studies. The best example is glycophorin A, whose primary sequence satisfied all the earlier expectations based on cell surface labeling, and whose hallmark, a stretch of 22 nonpolar amino acids punctuated by basic residues, turned out to be the landmark that facilitated the ordering of hundreds of other proteins whose structures were deduced by DNA sequences alone.

As good as the RBC was as a model membrane system to study (it helped us enter the modern era of membrane proteins studies), it also held us back in an important ways.

Being a cell without a functioning nucleus or active biosynthetic synthetic machinery, it prevented us from using many of the powerful tools of cell physiology and recombinant DNA technology. Human erythrocytes are necessary for our survival, and they have the capacity to last in the circulation for 120 days without replenishing their membrane proteins, surely an astonishing feat, but they cannot do much else. So membrane studies, fueled by the power of cloning, are now the province of nucleated cells of all types, and as a result, many more membrane proteins have been discovered and analyzed.

However, it is premature to pronounce the demise of RBC membranes as experimental tissues worthy of study. Ion transport studies continue to be fruitful. The spectrin/actin/protein 4.1 complex is still the best characterized membrane skeleton, yet we hardly understand how it works. More than that, it is now clear that homologs of the same proteins are essential components in nearly all cell types. Recent studies show that they are instrumental in linking functional ion channels together in neurons, and they may modulate the trafficking of neurotransmitter receptors in postsynaptic terminals of dendrites.

The properties of glycophorin A continue to be intriguing. The stability of the glycophorin dimer in SDS has attracted the attention of biophysicists who are trying to understand how interactions between the intramembranous segments of transmembrane proteins are regulated. It is still a mystery how low-affinity interactions between hydrophobic peptides maintain specificity. It appears that the amino acid sequence of the glycophorin intramembranous segment confers remarkable stability to the glycophorin dimer. What role this plays in the physiology of the RBC is unknown. The activation of tumor cells by growth factors also appears to depend upon changes in the noncovalent interactions between transmembrane domains, as does the activation of T cell receptors by antigen.

The unusual stability of the glycophorin intramembranous segment is also shared by the transmembrane domain of the APP, a prominent player in the pathogenesis of Alzheimer's disease. The amyloid Aβ peptide is a 40- or 42-amino-acid peptide generated by sequential cleavage of two secretases, as described earlier, and it contains a 14-amino-acid segment of the transmembrane domain of APP. Like the transmembrane segment of glycophorin, the Aβ peptides can form stable oligomers in the presence of SDS, and they have a GxxxG sequence motif that is believed to contribute to the stability of the glycophorin dimer. The similarity of these two transmembrane segments raises the possibility that APP molecules may also exist as stable dimers in neuronal membranes. It is also conceivable that Aβ peptides that remain within the lipid bilayer after secretase cleavage may pair up with APP monomers, creating chimeric forms of the type depicted in the glycophorin experiment described above. Clearly, the RBC membrane has proved to be an extraordinarily useful source of membrane proteins for bulk isolation and analysis, and the lessons learned have wide applications.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

LITERATURE CITED

1. Marchesi VT, Florey HW. 1960. Electron micrographic observations on the emigration of leukocytes. *Q. J. Exp. Physiol.* 45:343–48
2. Marchesi VT, Gowans JL. 1964. The migration of lymphocytes across the walls of venules of lymph nodes. *Proc. R. Soc. B Biol. Sci.* 159:283–90

3. Marchesi VT, Steers E Jr. 1968. Selective solubilization of a protein component of the red cell membrane. *Science* 159:203–4
4. Speicher DW, Marchesi VT. 1984. Erythrocyte spectrin is comprised of many homologous triple helical segments. *Nature* 311:177–80
5. Morrow JS, Marchesi VT. 1981. Self-assembly of spectrin oligomers in vitro: a basis for a dynamic cytoskeleton. *J. Cell Biol.* 88:463–68
6. Marchesi SL, Letsinger JT, Speicher DM, Agre P, Hyun B, et al. 1987. Mutant forms of spectrin alpha subunits in hereditary elliptocytosis. *J. Clin. Invest.* 80:191–98
7. Marchesi VT, Andrews EP. 1971. Glycoproteins: isolation from cell membranes with lithium diiodosalicylate. *Science* 174:1247–48
8. Tomita M, Marchesi VT. 1975. Amino acid sequence and oligosaccharide attachment sites of human erythrocyte glycophorin. *Proc. Natl. Acad. Sci. USA* 72:2964–68
9. Furthmayr H, Galardy R, Tomita M, Marchesi VT. 1978. The intramembranous segment of human erythrocyte glycophorin A. *Biochem. Biophys.* 185:21–29