IT IS WIDELY believed that the lipid bilayer of the erythrocyte membrane is supported by an elaborate infrastructure of protein molecules that give it viscoelastic properties. The proteins that provide this function are popularly referred to as the "membrane skeleton." This membrane skeleton is considered to be distinct from the so-called "cytoskeleton," a term used by cell biologists to describe the complex array of tubes and fibers that connect different parts of cells to each other and to the membrane. In most cells the cytoskeleton is clearly involved with the maintenance of cell shape and probably also plays a role in cell movement. It is assumed that connections exist between the cytoskeleton and the membrane skeleton in cells that have both, but nonnucleated red cells rely solely on the membrane skeleton to confer shape and stability. Perhaps for this reason, membrane skeletons of red cells are elaborately engineered, multiprotein complexes.

SPECTRIN IS THE PRINCIPAL PROTEIN OF THE MEMBRANE SKELETON

The membrane skeleton of the human erythrocyte is composed of several different proteins, but the predominant species is a water-soluble high molecular weight protein known as spectrin. When red cell ghost membranes are examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), spectrin appears as two discrete high molecular weight bands (Fig. 1). Other components now known to contribute to the membrane skeleton complex include bands 4.1a and b and an erythrocyte form of actin.

The spectrin molecule is a dimer composed of two nonidentical subunits now generally referred to as α and β. Both subunits are extraordinarily large; the alpha subunit has an estimated molecular weight of 250,000 daltons, and the beta is slightly smaller at 225,000 daltons. Both subunits seem to be organized into discrete chemical domains that have been identified by limited proteolytic digestion experiments; specific functional sites have also been assigned to different domains. Since both subunits seem to be extended rod-like structures each approximately 1000 Å long, the dimeric form of the molecule is most likely the result of side-to-side associations of parallel monomers. Recent experiments suggesting that there are multiple noncovalent associations between adjacent segments of contiguous subunits are consistent with this model. Thus, in simple terms, one might describe the spectrin molecule as being composed of two distinct but extremely similar noodle-like forms that are tacked together at multiple points along their lengths. This model was originally conceived on the basis of the appearance of spectrin molecules that were examined by electron microscopy after low angle shadowing techniques were used to outline their structure. An electron micrograph of spectrin molecules prepared in this way is shown in Fig. 2.

Many misleading impressions of the size and shape of the spectrin molecule were generated by earlier electron microscopic studies using conventional shadowing techniques and negative staining, but it is likely that the form of the molecule seen by these newer techniques is reasonably close to the mark, since it is consistent with many other biochemical observations. Nevertheless, given the history of the many artifactual observations reported in this field, one would be wise to wait for further chemical and physical confirmation of this latest model.
Spectrin forms oligomers by self-association

Spectrin molecules have remarkable powers of self-assembly. Although the spectrin dimer composed of single copies of each subunit seems to be the smallest non-denatured form of spectrin, highly purified spectrin molecules can assemble into huge macromolecular aggregates without the collaboration of any other proteins. The first suggestion that spectrin had this unusual property was provided by observations of Gratzer and coworkers and Ralston. Working independently, both groups of investigators discovered that forms of spectrin larger than dimer could be obtained under appropriate conditions. It had been assumed for some time that spectrin molecules could “polymerize” in the presence of high salt solutions and/or divalent cations into what were considered to be nonspecific and unphysiologic aggregates. In contrast to these earlier findings, a careful analysis of dilute solutions of spectrin indicated that spectrin dimers could convert to tetrameric forms, which were later shown to be created by an end-to-end association of dimers, forming tetrameric units twice the original length. Spectrin tetramers were subsequently found to have unusual binding properties, leading many to suggest that the tetrameric form of spectrin is the functional unit of spectrin in the membrane skeleton. The results of chemical crosslinking experiments provided further evidence for the existence of spectrin tetramers in the intact red cell membrane skeleton.

The phenomenon of spectrin self-assembly into tetrameric forms was investigated further by Morrow...
and coworkers with even more surprising results. Highly purified spectrin containing only trace amounts of contaminating actin could be concentrated in vitro to levels sometimes exceeding 25 mg/ml, and such preparations invariably generated an impressive array of higher oligomeric forms composed entirely of spectrin molecules. The presence of discrete oligomeric species composed solely of spectrin could be demonstrated by analytical ultracentrifugation of sucrose gradients, gel filtration chromatography, and non-denaturing acrylamide gel electrophoresis. Figure 3 shows a typical nondenaturing gel electropherogram illustrating the number and extent of spectrin oligomers generated in vitro in isotonic neutral salt solutions.

Further analysis of factors that regulate spectrin oligomer formation have generated some interesting findings. The individual spectrin dimers self-assemble to the tetrameric species through noncovalent interactions between a segment of an 80,000 dalton domain of the alpha subunit and a 28,000 dalton domain of the beta subunit. This association is presumably due to specific interactions between a terminal piece of the alpha chain with its corresponding "receptor" on the beta chain, and the process is a concentration-dependent reaction. It is likely that larger oligomers of spectrin (hexamer, octamer, etc.) are also generated by the same type of alpha-beta association, although this point has not yet been rigorously demonstrated. It is also important to stress that although the capacity of spectrin to self-assemble into higher oligomeric forms seems to be an inherent property of the spectrin molecule, independent of other membrane proteins, it is certainly conceivable that other components of the membrane skeleton could exert some regulatory effects on this process. It is also possible that spectrin oligomers could be assembled into even larger func-

![Oligomeric forms of spectrin can be analyzed by nondenaturing PAGE. Concentrated solutions of spectrin, composed largely of dimers (αβ) and tetramers (αβ), convert into higher forms during incubation at 30°C for the times shown. (Reproduced by permission of Journal of Supramolecular Structure.)](https://www.bloodjournal.org/content/3/3/537.f1)
tional units through the actions of other proteins. Some ideas along these lines will be described below.

The capacity of purified spectrin to form higher oligomers in vitro is clear and uncontested; what remains to be determined, however, is the true structural-functional unit of the spectrin molecule in situ. So far, evidence on this question is conflicting, and at the present time, the opinions of experts in spectrinology seem to fall into two camps. One group favors the notion that the tetrameric form of spectrin is the functional unit in the membrane. Another view, one held by a distinct minority, argues that the capacity of spectrin to form oligomers is so impressive that it is inconceivable that this remarkable mechanism of self-assembly is not put to physiologic use by the red cell. Since the bulk of the spectrin is plastered up against the inner surface of the red cell membrane, its local concentration is extremely high, estimated to approach 10^4 M, and it is certainly high enough to favor oligomer formation, based on the in vitro experiments described earlier. Furthermore, when spectrin is extracted from ghost membranes in low ionic strength media in the cold, a significant fraction of extracted material appears in the medium in an oligomeric form under conditions and at spectrin concentrations described earlier. When spectrin is extracted from ghost membranes by prolonged incubation in low ionic strength media and at 37°C, we consider it likely that its high local concentration drastically reduces its local concentration. Under these conditions, spectrin would be expected to dissociate into its smallest native unit, a γ-dimer.

If these arguments are valid, one might infer that the 4°C extraction procedure is likely to yield spectrin forms that are more “physiologic” than the 37°C procedure. However, both procedures rely on the use of a low ionic strength medium to separate spectrin from the membrane, and we have no way to evaluate the effects of this on the state of spectrin, since it normally exists in much higher salt solutions inside the cell. A method to extract spectrin from red cell membranes in isotonic salt solutions without denaturing the spectrin is clearly needed to resolve this dilemma.

Given this degree of uncertainty about the significance of low ionic strength extracts at either temperature, it is difficult to evaluate the results of recent attempts to quantitate the amounts of dimeric and tetrameric forms of spectrin extracted from red cells of patients with hereditary hemolytic disorders. Such experiments will be most meaningful if the capacity of such spectrin preparations to oligomerize in vitro is tested using methods capable of providing a quantitative estimate of the capacity of the spectrin to self-associate under appropriate conditions.

The issue as to whether spectrin functions either as a tetramer or a higher oligomer is obviously of critical importance to our understanding of the structure and function of the red cell skeleton and the many recent attempts to evaluate abnormalities of spectrin in hemolytic diseases. As will be discussed in detail below, spectrin molecules extracted from red blood cell membranes of patients with the clinical entity called hereditary pyropoikilocytosis (HPP) seem to be defective in their capacity to form higher oligomers in vitro. If the capacity to form oligomers is an important physiologic property of the spectrin molecule, the decreased capacity of spectrin of HPP patients to form oligomers may be of important pathophysiologic significance.
THE BETAN SUBUNIT OF SPECTRIN IS PHOSPHORYLATED

It has been observed repeatedly that the beta subunit of spectrin can be phosphorylated both in intact red cells and when red cell membrane ghosts are incubated with adenosine triphosphate (ATP) in vitro.22,26 The sites of phosphorylated amino acids have recently been localized to one end of the beta subunit, and interestingly enough, within the same domain of the beta subunit that appears to interact with the alpha subunit during the oligomerization reaction.427 This being the case, one might expect phosphorylation to play some role in modulating spectrin–spectrin interactions. Unfortunately, if one had both the time and the interest to study the voluminous literature devoted to this question, the results would be less than satisfying. The state of phosphorylation of spectrin has been found both to have a significant effect on red cell shape28,29 and no effect on red cell shape30,31; similarly, the state of spectrin phosphorylation was found to correlate with hereditary spherocytosis (HS) by one laboratory32 but not by another.33 Conflicting results have also been reported with respect to the effect of phosphorylation on the capacity of spectrin to associate with actin.11,16,34 In short, we have no idea what influence if any the state of phosphorylation has on the function and/or pathophysiologic capacity of the spectrin molecule.

If anything, the most recent findings suggest that attempts to understand the significance of spectrin phosphorylation should be pursued with vigor rather than abandoned in despair. The beta subunits of spectrin are phosphorylated at multiple sites; possibly four or five different amino acids are phosphorylated.27 None of the studies described above has taken into account the possibility that different phosphorylated sites might mediate different reactions. Most of the studies have also followed the effects of spectrin labeled with radioactive phosphorus, assuming that all sites turned over at equal rates. Thus, if any one site was either not exposed to a kinase in the intact cell or inaccessible to phosphatase in a particular membrane preparation, it would not be phosphorylated by the radioactive isotope, and its dephosphorylation would also escape detection. Thus, differences in the state of endogenous phosphorylation could have been responsible for the striking discrepancies between experimental results of different laboratories in their attempts to carry out the same experiment. Finally, one must also consider the types of assays used to measure the effects of spectrin phosphorylation. The presence of a particular phosphorylated amino acid or the absence of one at specific sites on the spectrin molecule could conceivably have modulating effects on the function of the molecule that might regulate in subtle ways the actions of the whole membrane skeletal ensemble. Thus, it is not inconceivable that the phosphorylation of spectrin exerts subtle effects on the reactivity of spectrin with other components that have escaped detection using the experimental parameters explored so far.

Preliminary experiments suggest that the phosphorylation of spectrin does not influence the capacity of spectrin dimers to convert to tetrameric forms, but such studies did not explore the effects of phosphorylation on oligomer formation. However, judging from the results of our studies on the factors that regulate spectrin oligomerization in vitro one might predict that the state of phosphorylation of the beta chain is likely to represent only one of several factors that modify the capacity of spectrin to form oligomers. Although we know there are multiple phosphorylated sites on the beta subunit, only one or two may be really close to the oligomer binding site, thus selective and quantitative changes in the phosphorylation of individual residues may be necessary for this point to be adequately tested. Even if this is achieved, there is no guarantee that either the removal or the addition of phosphates will have enough of an effect on the affinity of the alpha–beta interactions to influence oligomer formation if the concentration of spectrin is high enough to induce associations even at lower affinities. If oligomer formation is an important structural principle, and we believe it is, it is conceivable that there are multiple factors that regulate this process, and changes that reduce the affinity of association might have little measurable effect if other factors that promote oligomer formation are not also modified.

SPECTRIN IS ANCHORED TO THE LIPID BILAYER

All the spectrin of the human red cell seems to be confined to a rather narrow zone immediately beneath the cytoplasmic surface of the lipid bilayer.35,36 Purified spectrin can reassociate to the exposed surfaces of inside-out vesicles depleted of spectrin,37,38 and it seems to do so through a relatively high affinity association between a segment of the beta subunit of spectrin and a membrane-bound protein that has been identified as band 2.1 and is now called ankyrin.39,40 or syndein.41 Ankyrin/syndein is a reasonably large (estimated molecular weight 210,000) protein that is attached to the inner surface of the red cell membrane in a somewhat complex manner. The associations between spectrin and ankyrin are disrupted by suspending red cell membranes in low ionic strength media containing chelating agents, but none of the ankyrin is detached from the membrane under these conditions. The associations of ankyrin with the membrane can be dis-
ruptured if membranes are exposed to concentrated (∼1.0 M) neutral salt solutions. This treatment appears to solubilize intact ankyrin molecules from the membrane. Recent studies by Bennett and coworkers suggest that ankyrin may be attached to red cell ghost membranes via a high affinity association (estimated $k_0 \sim 10^{\text{9}}$) to a segment of the cytoplasmic portion of the band 3 transmembrane glycoprotein. The idea that spectrin may be linked to the membrane through a protein cofactor that binds to band 3 is especially intriguing, since it implies that the membrane skeleton (spectrin and its coworkers) confers its stabilizing effects on the lipid bilayer through its contact with the transmembrane glycoproteins, rather than through direct contacts among spectrin, ankyrin, and the exposed segments of lipid molecules. The story becomes even more complex when one considers how many other proteins also appear to bind to the cytoplasmic segment of band 3. So far the list includes at least two glycolytic enzymes, and under certain circumstances, some hemoglobin molecules. Since band 3 is also the site of anion flux across the red cell membrane and may be related in some as yet undefined ways with other transmembrane glycoproteins, including the glycoporins, one is confronted with an extremely complex set of interactions between the membrane skeleton and the other protein components at the cell surface.

This recent evidence, showing potential links between spectrin and the transmembrane glycoproteins, also allows us to make sense out of many earlier observations that hinted at some direct connections between transmembrane glycoproteins and the spectrin network. Nicolson and coworkers showed some years ago that antispectrin antibodies modify the distribution of externally disposed segments of glycoforin molecules, an observation that correlated with the rearrangements of intramembranous particles induced by subjecting ghost members to pH 5 conditions. Both sets of observations implied direct links between the spectrin network and the two major transmembrane glycoproteins glycoforin A and band 3, although there were also a number of other less intriguing explanations for these results.

The significance of the actin-binding capacity of spectrin is still unclear. Many observers attribute a spectrin-binding function to actin and propose that short segments of actin serve to join together spectrin tetramers to form a lattice-like arrangement that buttresses the lipid bilayer. According to this conception, spectrin would be tethered to the membrane by at least two sites: (1) directly to transmembrane proteins.
glycoproteins by ankyrin, and (2) to another attachment site, possibly via actin. Evidence that the other attachment site might be the protein known as band 3 by other membrane-associated components.

SPECTRIN MOLECULES MAY BE DEFECTIVE IN CERTAIN HEMOLYTIC DISEASES

Since spectrin is the most abundant protein of the red cell membrane skeleton, it is logical to assume that it may be modified in some of the hemolytic anemias associated with fragile cell membranes. One imagines that the total absence of spectrin would not be compatible with red cell survival of any duration, and this seems to be true for red cells of rare strains of mice that lack normal amounts of spectrin. Red cells that have less than normal amounts of spectrin could arrive at that state through reduced synthesis of one or both subunits of the molecule, or there could be increased degradation of spectrin either during red cell maturation or even within the mature red cell itself. There is now good evidence that red cell membranes contain proteolytic enzymes that are inherent within the red cells themselves and not due to proteases derived from contaminating leukocytes or platelets. Mature red cells also contain an acid-activated protease that readily digests spectrin and other membrane-associated proteins. Red cells also seem to contain significant amounts of ubiquitin-activating enzymes as well as ubiquitin, and it is conceivable that spectrin molecules containing covalently bound ubiquitin may be sensitive to proteolysis as are other ubiquitinated proteins.

Reduced amounts of spectrin associated with the membranes of red cells could also be due to decreased binding of spectrin to the membrane. From what we presently know about the spectrin molecule and its mode of binding to the membrane, it is possible to conceive of beta subunits of spectrin that lack the binding site for ankyrin, or what is perhaps more likely, beta subunits that have a modified binding site with greatly reduced affinity. There is also the possibility of defective red cells that either lack ankyrin or have decreased amounts of it; the latter could be due to decreased or defective ankyrin binding sites. If the ankyrin binding site does turn out to be the cytoplasmic segment of the band 3 molecule, parts of that molecule could be altered or there could be increased competition for binding to a common binding site on band 3 by other membrane-associated components.

In most of the commonly encountered hemolytic anemias that are thought to have an inherent membrane defect, the red cells do not seem to have a decreased amount of spectrin, and when analyzed by SDS gels, both subunits appear to be of normal size and present in equivalent amounts. These findings tell us that the membrane instability in these disorders is not likely to be due to gross changes in spectrin synthesis or to imperfections in the membrane attachment mechanisms; but this type of analysis could not possibly detect subtle differences in the structure of either subunit.

How does one go about identifying structural changes in such mammoth polypeptide chains? Both spectrin subunits are far too large to analyze by conventional peptide mapping techniques, such as those used to identify abnormal hemoglobins, and even modern high performance liquid chromatography methods are inadequate for the task. Attempts to identify variant N-terminal amino acids of spectrin molecule are not likely to bear fruit, since both subunits seem to have blocked N-termini, and previous attempts at analyzing spectrin by isoelectric focusing were also unrewarding.

We have approached this problem by attempting to devise a series of analytical approaches based on partial cleavage of such spectrin subunit into specific chemical and functional domains, followed by analysis of specific domains using both conventional thin-layer electrophoresis-chromatography procedures and HPLC analysis of limit peptides by reverse phase chromatography.

Limited proteolytic digestion of both subunits of the spectrin molecule produces a bewildering set of intermediate-sized peptides when the latter are analyzed by isoelectric focusing and SDS gel electrophoresis. A typical pattern obtained from a normal spectrin sample is shown in Fig. 4. The number of peptides generated by this procedure is far greater than the number expected from the digestion of two single polypeptide chains of approximately 450,000 daltons in size. We now know that many of the peptides generated by this technique represent partially degraded fragments derived from the same chemical domain. By comparing peptide maps of all the peptides illustrated here and other larger precursor forms, we have identified each of the peptides as being derived from specific domains of either one or the other subunit. The evidence supporting this claim has been provided elsewhere.

A significant feature of these findings is that the digest pattern illustrated here is remarkably constant from donor to donor, allowing one to use this approach to identify differences in primary structure of the spectrin molecule, provided that the difference produces either a charge or a size difference in one of the many proteolytic fragments. The power of this approach has been demonstrated recently by showing that the digest pattern of soluble spectrin samples obtained from 85 normal white donors is essentially
A STRUCTURAL CHANGE HAS BEEN DETECTED IN THE ALPHA SUBUNIT OF SPECTRIN FROM HPP RED CELLS

The above techniques are now being applied to the study of spectrin samples from a variety of abnormal red cells with encouraging results. Among the most spectacular is the finding of a modified domain in the alpha subunit of spectrin samples obtained from patients with hereditary pyropoikilocytosis (HPP). Limited proteolytic digestion of normal human spectrin at 0°C invariably generates an 80,000 dalton peptide that is derived from the N-terminal end of the alpha subunit. When HPP spectrin is subjected to digestion under identical conditions, varying amounts of the 80K fragment are generated, depending on
whether HPP patients are homo- or heterozygous for this state. In place of a normal 80K fragment, HPP spectrin samples produce two smaller fragments, and peptide maps of these fragments indicate that they are derived from the 80K domain of the alpha subunit. These findings are particularly intriguing, since the 80K region of the alpha subunit seems to be involved in spectrin–spectrin interactions that are responsible for oligomer formation. The finding of a structural change in the alpha subunit derived further weight from the fact that HPP spectrin has a reduced capacity to form oligomers in vitro.20,21

THE ARRANGEMENT OF THE MEMBRANE SKELETON IN SITU

Although we now know a great deal about the structure and functional properties of some of the major components of the membrane skeleton, we still are at the guessing game stage when it comes to figuring out how the parts fit together to form the skeleton in situ. Much has been made of the fact that spectrin binds to a number of other proteins (i.e., 2, 1, 4, 1, actin), and since at least one of these also binds to band 3, it has been assumed that all these components play a role in determining the three-dimensional arrangement of the native skeleton. A highly schematic model of the membrane skeleton that relies on these assumptions has been provided by Lux15 and has been widely popularized. This model incorporates most of the recent in vitro studies on spectrin and has a number of attractive features.

The model's principal weaknesses include the fact that there is no direct evidence that such complexes exist in intact membranes, nor have investigators succeeded in reconstituting fragments of membrane skeletons using purified components. This model also does not take into account the remarkable capacity of

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**Fig. 5.** The spectrin tetramer, composed of two alpha and two beta subunits, is one of the major functional units of the spectrin molecule. A highly schematic diagram of this form of the molecule is illustrated above. Based on recent studies in several different laboratories, it appears that under appropriate conditions spectrin tetramers can bind to short oligomeric units of actin. The figure shows (in a highly schematic way) how five spectrin tetramers might associate with a short actin oligomer to form radiating spoke-like structures. If each spectrin tetramer was completely extended, these complexes could have diameters approaching 4000 Å.

**Fig. 6.** Multimeric forms of spectrin appear to form by the same head-to-head association between dimers that has been proposed for the tetrameric form illustrated in Fig. 5. A hypothetical octimer is depicted. The addition of spectrin dimers to the spectrin–actin complex depicted in Fig. 5 could result in the formation of large "islands" of spectrin, as suggested below.
spectrin to form higher oligomers, a fact that was recently discovered and has not yet been accepted by all investigators in this field.

An alternative model that we would like to propose, rests on the notion that the basic structural unit of the membrane skeleton may be a spectrin–actin complex that is tethered together at appropriate sites by linking proteins, such as bands 2.1 and 4.1, and possibly other as yet undefined elements. All conditions that result in the extraction of spectrin from the red cell membrane invariably coextract actin, and all treatments of the membrane that extract actin also result in solubilization of spectrin and the vesiculation of the red cell ghost membrane. Doesn’t this imply that the two proteins form a functional complex that is essential for the integrity of the lipid bilayer of the red cell membrane? Purified spectrin tetramers are able to link F-actin strands together, while dimeric spectrin is less able to associate with F-actin, and G-actin is probably unable to bind to spectrin to any significant extent. Electron microscopic studies of complexes of short segments of actin with tetrameric spectrin by Branton and coworkers show some fascinating results. Spectrin tetramers have the capacity to bind to filaments of actin at multiple points, producing structures similar to those shown diagrammatically in Fig. 5.

This model becomes even more complex if the capacity of spectrin to form higher oligomers is taken into account. One way in which spectrin dimers could join together to form higher oligomers is illustrated in Fig. 6. Spectrin oligomers appear to be formed through the same mechanism proposed for the dimer–tetramer association; this involves noncovalent interactions between one end of the alpha subunit and the phosphorylated end of the beta subunit. Based on this principle, one might propose that extensive networks of spectrin could be generated by the addition of spectrin dimers to preexisting spectrin–actin complexes, as illustrated in Fig. 6.

This model relies heavily on the notion that spectrin oligomers exist in situ and can interact with actin and other linking proteins. When joined together at specific sites, such “islands” of spectrin and actin could modify the viscoelastic properties of membranes at specific points, and they might also attach to the overlying lipid bilayer using the mechanisms described above or through others yet undiscovered.

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