Red Cell Membranes

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Preface

During the past decade a great deal of new information concerning the structure and function of the red cell membrane has surfaced from many laboratories. One particularly fruitful area has been the development of the concept of the so-called membrane skeleton in an attempt to decipher the biochemical anatomy of the red cell membrane. Many of the techniques involved in this development derive from comparatively disparate disciplines. In an effort to make several of these methods conveniently available to our colleagues, the editors have solicited chapters from leading exponents of these techniques for inclusion in this volume. We asked each author to provide a brief historical perspective on the utility and development of the technique and detailed methods for the application of the technique so that, for the most part, the reader would be able to utilize these chapters in his own experimental program without further referral. For this last purpose we requested that adequate illustrative and methodologic details be provided, either within the chapter itself or as an appendix to each contribution. Beyond these very general guidelines, we did not impose any stylistic limitations since both the subjects and the authors differ appreciably.

The contributions have been organized into related areas. The first of these emphasize analytic methods for directly describing the morphology, biochemistry, and function of red cell membrane skeletal proteins. This group includes Dr. Branton's contribution on protein electron microscopy, Dr. Fairbanks' description of the fundamental methods for protein separation, Dr. Agre's techniques for studying some of the functional interactions, and Dr. Kay's immunologic techniques for obtaining complementary information on protein disposition and function. The second group involves studies of structures that may be influenced by the membrane skeleton, but that are for the most part located in the adjacent membrane bilayer. These methods include Dr. Lubin's measurements of membrane lipids and some of their dynamics, Dr. Chiu's measurements of the oxidative reactions, which especially occur in the lipid bilayer, and Dr. Clark's measurements of permeability, which are probably regulated to a large extent by the bilayer leaflet and its integral proteins. The third and final group of chapters involves more global measurements of cell and membrane deformability, which have emerged as two of the cardinal characteristics of red cells substantially influenced by the membrane skeletal proteins. These include Dr. Evans' contribution on the micropipette techniques for measuring physical properties of the membrane that influence cell deformability and adhesiveness and Dr. Mohandas' analogous studies with the ektacytometer for measuring similar physical properties that regulate cell deformability and related behavior.

We do not pretend that this collection of methods is exhaustive, and we recognize that some areas of growing importance have not been represented. In particular, we have not included applications of molecular biological techniques. We certainly

feel that this burgeoning area will be of great importance in the near future. Indeed, we have personal commitments to these approaches. However, these techniques are amply reviewed elsewhere, and it appears to us that they have limited, unique application to the red cell membrane.

Finally, the editors and contributors recognize that there are many alternative methodologic approaches for solving some of the experimental questions posed in these pages. Although we have selected methods that we personally feel are the most useful, we would be very pleased to hear from our colleagues regarding either alternative methods or successful refinements of the methods presented here.

Stephen B. Shohet, M.D.
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1
Electron Microscopy of Red Cell Cytoskeletal Proteins

Daniel Branton and Don L. Siegel

INTRODUCTION

The set of interconnected proteins responsible for the major structural and rheologic properties of mammalian red blood cells lies within 10 nm of the red cell membrane.[1-6] The coordinated application of biochemical, immunochemical, and electron microscopic methods has been required to understand the properties of this set of proteins that constitutes what has been called the red cell *cytoskeleton* or *membrane skeleton*. This chapter reviews some of the structural information that has emerged from the application of electron microscopic methods.

Efforts to elucidate the structure of the mammalian red cell cytoskeleton have involved electron microscopy of individual, purified cytoskeletal proteins and studies of intact or lipid extracted cells and complexes. The studies of individual proteins have significantly advanced our knowledge of how the proteins may associate to form the membrane skeleton; the studies of intact and lipid-extracted cells and complexes are still in their infancy.

STRUCTURE OF PURIFIED CYTOSKELETON PROTEINS AND THEIR ASSOCIATIONS

SPECTRIN

Electrophoresis on polyacrylamide gels in the presence of sodium dodecylsulfate (SDS) separates two high-molecular-weight (220,000 and 240,000) polypeptides from approximately 10 other major membrane proteins (Figure 1-1). Together, these two polypeptides, which constitute 25 percent of the protein mass of the red cell membrane, are now referred to as the α and β chains (bands 1 and 2, respectively, Figure 1-1) of the protein spectrin. Because this protein is a dominant feature of the "shell" or cytoskeleton that remains after the membrane lipid is extracted with detergent (Figure 1-1) and because removal of this protein is usually accompanied by shape changes and loss of membrane integrity, spectrin has been considered an element of the cytoskeleton that plays a crucial role in determining cell shape.[7] Our understanding of how spectrin plays this crucial role is still developing, but an important step has been the realization that spectrin is an elongated, flexible molecule whose time-averaged end-to-end distance is considerably less than its contour length.[8-12]

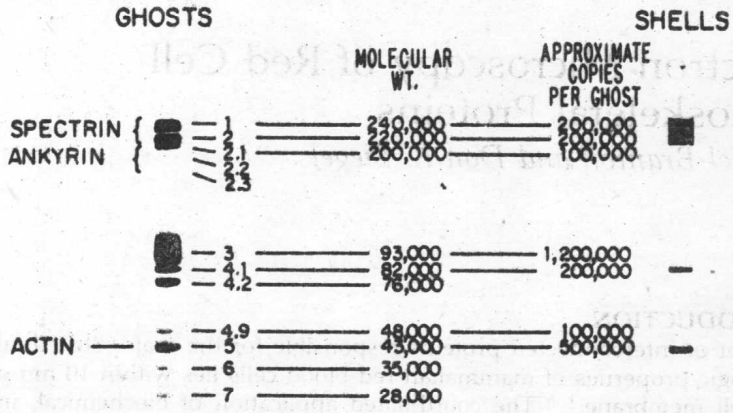

Fig. 1-1. SDS–polyacrylamide gels of the polypeptides of human red cell ghosts and of the shells remaining after the ghosts were extracted with 1 percent Triton X-100 in 5 mM phosphate buffer.

Early attempts to visualize spectrin in negatively stained preparations showed that the molecule is capable of forming aperiodic fibrous aggregates. Such results suggested that spectrin molecules are elongated, but little in the way of specific molecular details emerged from these studies. A significant advance was made by Shotton et al.,[13] who used low-angle shadowing to visualize molecules that were dried out of glycerol solutions onto a mica substrate. These studies were important because they provided direct visual evidence for the flexibility of the α- and β-spectrin heterodimers (Figure 1-2A) and because they demonstrated that the formation of spectrin tetramers[14-16] involved an end-to-end association of two heterodimers without measurable overlap (Figure 1-2B). The electron micrographs showed the individual α and β polypeptide chains of the heterodimer coiled loosely around each other or lying separated, side by side but normally joined at their ends. Where two heterodimers join to form a tetramer, the observed separation of monomer strands indicated that the normal tight binding between sister monomer strands is replaced by interdimer binding of monomers between the paired heterodimers. These structural studies not only showed the many shapes that spectrin could assume but also made it possible to observe the sites at which spectrin associated with other membrane proteins.

The relation between the appearance of molecules dried out of glycerol onto a mica substrate and the appearance of spectrin molecules in solution has been investigated by examining unstained molecules in vitreous water on a cold stage at approximately 110 K in a transmission electron microscope (Figure 1-2D). Although these studies of unstained molecules on a cold stage are still preliminary, the similarity in the appearance of the fully hydrated untreated molecules in solution (Figure 1-2D) and the molecules dried out of

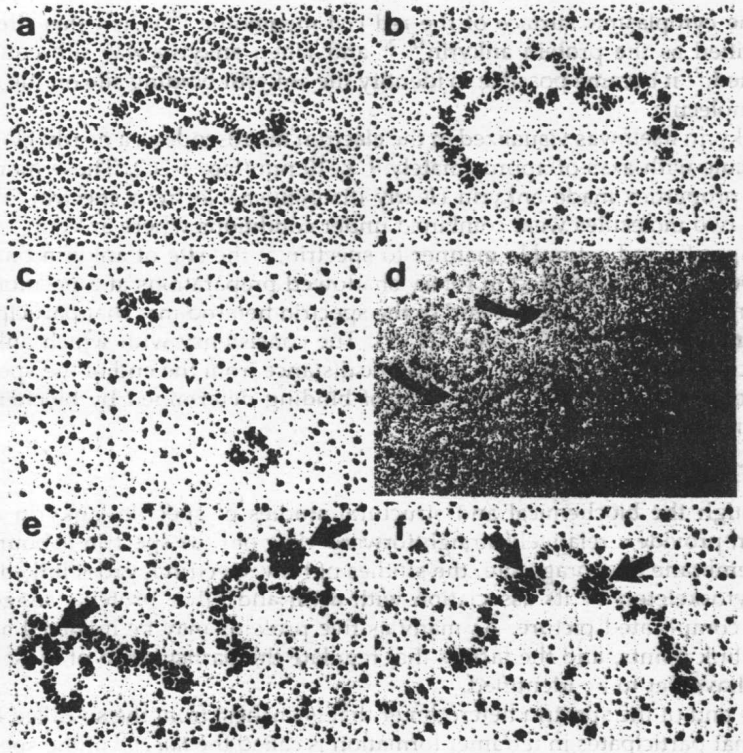

Fig. 1-2. Spectrin and ankyrin visualized in the electron microscope after low-angle rotary replication (A, B, C, E, F) or after vitrification in buffer without heavy metal contrast enhancement (D). (A) one spectrin dimer; (B) one spectrin tetramer; (C) two ankyrin molecules; (D) spectrin dimers and tetramers; (E) ankyrin (arrows) bound to two spectrin dimers; and (F) ankyrin (arrows) bound to one spectrin tetramer. The spectrin in (D) was prepared by plunging into freezing ethane a thin layer of a spectrin solution (in 10 mM phosphate buffer) spread on an electron microscope grid bearing a holey carbon film. Two spectrin tetramers are seen parallel to the opposing pairs of arrows. A spectrin dimer appears to overlie or underlie the upper tetramer. The photograph, taken in collaboration with Ron Milligan and Nigel Unwin at Stanford University, shows a region of vitreous solution suspended across one of the holes in the carbon film. Tetramers can be seen between each of the two sets of arrows. The grid was maintained at less than 110 K on a cold stage, and the area to be photographed was exposed to low electron dosage using a Phillips low-dosage focusing kit. (Original magnifications × 350,000.)

glycerol onto a mica substrate (Figure 1-2A) serves to validate the large number of studies that have used dried molecules.

SPECTRIN–ANKYRIN ASSOCIATION

Biochemical evidence showed that the binding of spectrin to the red cell involved a specific association between spectrin and a protease-sensitive site

on the cytoplasmic surface of the red cell membrane.[17] When this site was identified as the protein ankyrin[18–20] and when ankyrin was subsequently purified,[21] it became possible to identify the ankyrin-binding site on the spectrin molecule.

Ankyrin itself was visualized in the electron microscope as a nearly globular molecule with axes of approximately 9 × 10 nm (Fig. 1-2C). These morphologic properties made ankyrin clearly distinguishable from spectrin. When these two molecules were combined under conditions where ankyrin bound in a specific and saturable manner to spectrin,[21] the site of ankyrin binding on spectrin was identified in rotary shadowed preparations at a position approximately 20 nm from the end of the spectrin heterodimer that participated in tetramer formation (Figure 1-2E, F). The consistency with which ankyrin molecules were observed at this specific distance from the end of the spectrin heterodimer reflected the specificity of binding determined by biochemical techniques.[18,22,23]

SPECTRIN CROSS-LINKS

Although the biochemical and structural studies of spectrin–ankyrin association provide a relatively straightforward view of how spectrin is bound to its membrane anchorage site, the studies of how spectrin is cross-linked into the cytoskeleton by its association with actin and other proteins suggest a more complicated picture. As many as five proteins may be involved at the cross-link points, and the factors that regulate the participation of these proteins have yet to be identified.

The end of the spectrin heterodimer that is farthest from the so-called head end that participates in tetramer formation is called the *tail end* of the spectrin molecule. This tail end has been visualized as the end that participates in cross-links with other membrane proteins because it is this tail end of spectrin that binds to actin polymers[24] and to band 4.1[23] and because interaction of spectrin tetramers with these two proteins can result in striking increases in the viscosity of a spectrin solution.[25,26] Although it has been difficult to visualize action filaments in the intact red cell cytoskeleton, indirect biochemical[27–30] and structural[31–33] evidence indicates that red cell actin exists as short filaments or oligomers that could cross-link several spectrin molecules together. In vitro studies with the purified components leave no doubt that the tail end of spectrin can bind to actin filaments[24] (Figure 1-3A) and that the presence of band 4.1, which can also bind to the tail end of spectrin[23] (Figure 1-3B), can promote or stabilize the interaction of spectrin with actin.[25,26,34–36]

Studies show that, in addition to actin and band 4.1, two other proteins may participate in the junctions that cross-link the tail ends of spectrin molecules. The first of these proteins is tropomyosin, a molecule that comprises about 1 percent of the red cell membrane protein, being present in a ratio of one M_r 60,000 tropomyosin molecule to six or seven actin monomers. This amount should be sufficient to saturate all of the short actin filaments on the membrane.[37] By analogy to tropomyosin in muscle, red cell tropomyosin

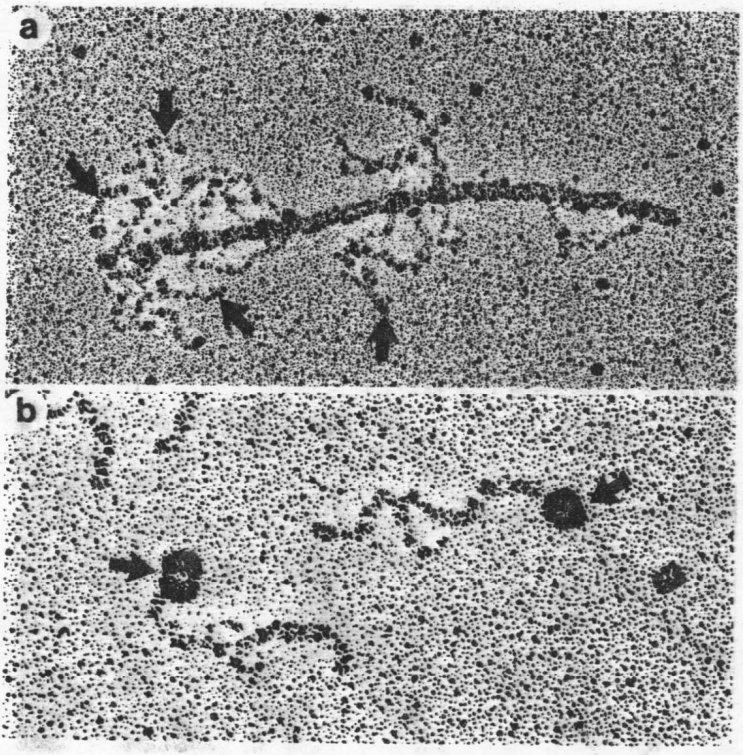

Fig. 1-3. (A) Spectrin dimers (arrows) decorating an actin filament. (Original magnification × 175,000. (B) Spectrin tetramers that had bound band 4.1 conjugated to biotin were incubated with avidin conjugated to ferritin. The site of 4.1 binding at the tail end of the spectrin is labeled with ferritin (arrows). (Original magnification × 300,000.)

could regulate the interactions of red cell actin with other cytoskeletal proteins.

Another molecule is band 4.9, a 48,000-dalton polypeptide that also comprises about 1 percent of the red cell membrane protein. Band 4.9 is present in a ratio of approximately one polypeptide for every five actin monomers. By systematically investigating the interactions of band 4.9 with the other purified skeletal components, it was discovered that when actin is polymerized in the presence of band 4.9 bundles of actin filaments are generated that are similar in appearance to those induced by other known actin bundling proteins present in other cell types.[38,39] Negatively stained images of such preparations revealed large fibers up to 0.8 μm in diameter with a banding pattern typical of in-register filaments at intervals of about 36 nm (Figure 1-4). The structural information provided by these studies helped to explain a number of biochemical and biophysical properties of actin–band 4.9 com-

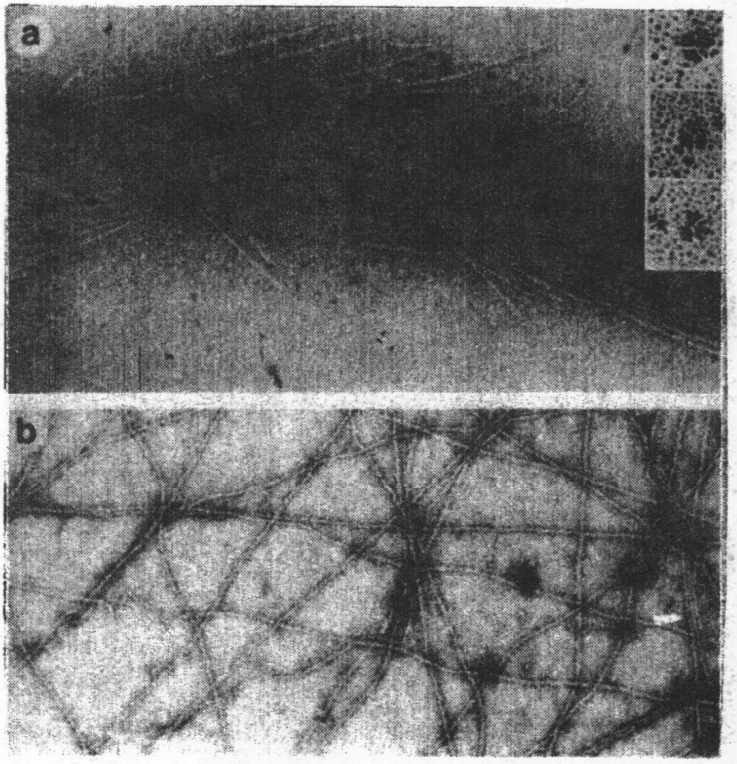

Fig. 1-4. Negatively stained actin (A) bundled by band 4.9. **Inset.** Rotary-replicated band 4.9 molecules exhibiting a tripartite appearance. (B) Actin maintained under conditions similar to those in (A) but without band 4.9. (Original magnifications: A and B × 100,000; inset × 550,000.)

plexes in solution, including the increased low shear viscosity of actin filaments in the presence of stoichiometric amounts of band 4.9.[39]

Band 4.9 itself was visualized by low-angle rotary shadowing as a three-lobed structure with a radius slightly more than 50 Å (Figure 1-4C). This finding was consistent with gel filtration and chemical cross-linking studies that showed that in solution band 4.9 is a trimer with an apparent molecular weight of 145,000 and a Stokes radius of 50 Å.[39] If each band 4.9 subunit contains an actin-binding site, the trimeric structure of band 4.9 may account for its ability to cross-link actin filaments into bundles. The role band 4.9 may play in the red cell, a cell apparently lacking long actin filaments or bundles of long filaments, is not yet clear. We have suggested that band 4.9 molecules may cluster several short actin protofilaments at the junctions that cross-link the tail ends of spectrin molecules.

STRUCTURE OF INTACT CELLS AND COMPLEXES

Studies with purified cytoskeletal components can identify and characterize the binding sites and modes of association that may be important in maintaining the integrity of the red cell membrane and its skeleton. But establishing that purified components can reassociate in a specific fashion, does not prove that such associations are in fact part of the native red cell membrane structure. Just as the presence and known association of actin, myosin, and other regulatory proteins in the cytoskeleton of most nucleated cells leave open the question of how these components are organized, so too do the presence and association of actin, spectrin, and other proteins in the red cell cytoskeleton leave open the question of how these components are actually distributed in the red cell membrane. For example, the discovery that band 4.9 is an actin bundling protein and the suggestion that trimers of band 4.9 may cluster several short actin protofilaments[39] lead to a different conception of the junctions that cross-link spectrin than was depicted in earlier models.[2] The total number of molecules per cell (Figure 1-1) and the total surface area per cell are fixed; thus if several short actin protofilaments lie at each junction point, the number of junctions per cell would necessarily be less than if only one, similarly short protofilament was at each junction. This change in the model would in turn increase the number of spectrin molecules cross-linked per junction and would also increase the average distance between the junctions. Thus the introduction of one additional element, e.g., band 4.9 as an actin bundling protein, could have multiple consequences for models of the in situ distribution of membrane skeletal components. One way to specify the in situ distribution of components is to use the electron microscope to examine the arrangement of proteins in the intact or partially disassembled red cell.

Early electron microscopic studies of red cells revealed the presence of what appeared to be poorly organized, wispy material on the cytoplasmic surface of the cell membrane,[40–42] and observations of cytoskeletons prepared by extracting the membrane with detergent showed a filamentous, web-like structure containing poorly resolved, variable-sized elements.[42–45] A major problem with many of these initial electron microscopic observations was the absence of well-founded conceptions about the shape and appearance of the individual cytoskeletal elements. Without any ideas of what the individual components looked like and without appropriate reagents to identify individual components in the extraordinarily dense meshwork of the membrane skeleton, these initial observations were nearly impossible to interpret.

With a better understanding of the shape and appearance of spectrin and with the application of tannic acid fixation techniques, Tsukita et al.[33,46,47] have been able to visualize the red cell membrane skeleton in thin sections with a clarity previously unobtainable. Their results are among the first to reveal the molecule details of the skeleton in situ and have in general confirmed the conclusions derived from studies of the isolated components. Obliquely cut membranes reveal a generally distributed meshwork of approximately eight or nine filaments (presumably spectrin) interconnected by

about 25 nm diameter nodes (presumably actin, band 4.1, band 4.9, and any associated tropomyosin). Closer to the lipid bilayer, granular particles 10–13 nm in diameter appear as studs extending between the lipid bilayer and the meshwork of filaments. These granular particles may be ascribed to ankyrin molecules associated with the *N*-terminal cytoplasmic protrusion of band 3, but specific reagents such as antibodies are required to confirm this identification.

Although some of the earliest microscopic images of red cell membrane skeletons were obtained by negative staining,[43,45,48] only in the work by Pinder et al.[49] can one clearly visualize individual spectrin tetramers, the plectonemic winding of their constituent polypeptide chains, and their cross-linked tail ends. The clarity of some of these images suggests that negative staining could be used with colloidal gold-labeled antibodies to learn a great deal about the in situ arrangement of the cytoskeletal molecules.

For a two-dimensional structure such as the red cell membrane skeleton, face views should be particularly revealing. Scanning electron miscroscopy can provide such views, and images of ghosts and Triton shells in the scanning microscope have been published by Hainfeld and Steck.[44] Although their photographs of Triton shells show a reticular pattern, their images are difficult to interpret. The resolution of the scanning electron microscope is probably inadequate to image the individual molecules of the membrane skeleton.

The inherent difficulty of fixing a structure composed of elongated, flexible, hydrated, charged molecules (spectrin) cross-linked into a meshwork may be a serious problem in all attempts to visualize the in situ arrangement of the red cell cytoskeleton. Such a cross-linked structure is an ionic gel,[50] and ionic gels are extraordinarily sensitive to modifications of their charge or to small changes in their environment. Chemical fixatives and dehydration are therefore likely to induce substantial changes in the arrangement of the red cell cytoskeleton. Techniques that rely on ultrarapid freezing and vacuum sublimation instead of chemical fixation and dehydration may ultimately be the best way of visualizing the red cell cytoskeleton, but thus far only exploratory studies have been completed. Using ghosts rapidly frozen on impact with a liquid helium-cooled copper block, Heuser has examined red cell membranes after they were freeze-fractured, deeply etched, and rotary-replicated. His results (Figure 1-5) show a dense meshwork of elongated twisted structures on the cytoplasmic surface of the red cell membrane. These structures probably represent spectrin and closely associated molecules because they disappear from the membrane when the ghosts are treated with the low ionic strength solutions known to elute spectrin and actin. Because of the density of molecules, much work is required before these complex images of the membrane skeleton can be interpreted to reveal the distribution and arrangement of individual components.

Images of the membrane skeleton can be greatly simplified if the networks are fragmented without totally destroying the in situ association of proteins. The oligomeric state of spectrin has been examined in such fragments formed by partially dissociating the skeleton at 0°C with low ionic strength buffers

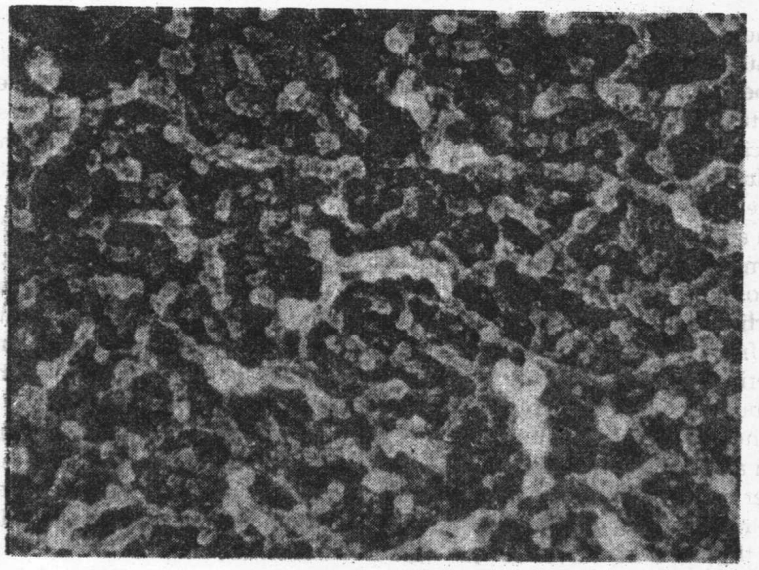

Fig. 1-5. Spectrin meshwork visualized by rotary replication of a deeply etched, rapidly frozen human red cell ghost. (Preparation and photograph by John Heuser, Department of Physiology, Washington University School of Medicine, St. Louis, MO.)

and mild urea treatment under conditions where the spectrin dimers, tetramers, and higher-order oligomers are kinetically frozen, i.e., no interconversion between the various oligomeric forms.[51] Electrophoresis on nondenaturing gels showed that spectrin dimers, tetramers, and medium-sized oligomers were found to be the prominent species, accounting for 5–10, 45–55, and 25–35 percent of the spectrin, respectively. When examined by low-angle rotary shadowing, the oligomers (hexamers, octamers, decamers, dodecamers, and quadecamers) appeared as polyskelions formed by head-to-head association of three to seven dimers. The data suggest that spectrin tetramers and medium-sized oligomers coexist in the normal red cell membrane as the primary native spectrin species.

Given the knowledge that a simple equilibrium determines the association between dimers and tetramers[16] and that higher-order oligomers occur at high spectrin concentrations,[52] it is not surprising that tetramers and medium-sized oligomers are the major species in the membrane skeleton. At the high spectrin concentrations within the thin layer that constitutes the membrane skeleton, the slow association–dissociation equilibrium between the spectrin subunits would make it possible for the individual α and β chains of one heterodimer to participate in two oligomers. The consequent higher-order oligomer formation would be detected when the network is partially dissociated under conditions that kinetically trap the α–β association.