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# Spontaneous, Reversible Protein Cross-Linking in the Human Erythrocyte Membrane. Temperature and pH Dependence†

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ABSTRACT: Changes in pH significantly affect the morphology and physical properties of red cell membranes. We have explored the molecular basis for these phenomena by characterizing the pattern of protein disulfide cross-linkages formed spontaneously in ghosts exposed to acid pH or elevated temperature (37 °C). Protein aggregation was analyzed by two-dimensional polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Incubation of ghosts at pH 4.0 to 5.5 (0–4 °C) yielded (i) complexes of spectrin and band 3, (ii) complexes of actin and band 3, (iii) band 3 complexes, i.e. dimer and trimer, and (iv) heterogeneous aggregates involving spectrin, band 3, band 4.2, and actin in varying proportions. Aggregation was maximal near the isoelectric points of the major membrane proteins, and appeared to reflect (i) the aggregation of intramembrane particles including band 3 and (ii) more intimate contact between spectrin–actin meshwork and band 3.

Changes in pH significantly affect the morphology and physical properties of the human erythrocyte membrane. Electron microscopic studies on intact red cells and isolated ghosts revealed several reversible pH-dependent phenomena such as changes in shape (Weed and Chailley, 1973; Nicolson, 1973), aggregation of intramembrane or freeze-etch particles (Pinto da Silva, 1972; Elgsaeter and Branton, 1974), clustering of surface anionic sites composed of *N*-acetylneuraminic acid residues (Nicolson, 1973), and clustering of ferritin binding sites (Pinto da Silva et al., 1973). It has been suggested (Elgsaeter et al., 1976) that these rearrangements reflect the isoelectric precipitation of spectrin and actin, the peripheral proteins which form a meshwork on the membrane–cytosol interface (Kirkpatrick, 1976), and are mediated through their interaction with transmembrane proteins.

To elucidate the molecular basis of such interactions, we have investigated spontaneous cross-linking of membrane proteins produced by exposing ghosts to reduced pH. The composition of the cross-linked products has been analyzed by two-dimensional sodium dodecyl sulfate gel electrophoresis, in which resolution in the second dimension is preceded by dithiothreitol reduction to free individual polypeptides from complexes.

The data demonstrate maximal cross-linking in the pH range 4.5–5.0 and provide direct evidence of covalent bond formation between spectrin and the major transmembrane protein, band 3. The correlation between the formation of homologous band 3 complexes and the intramembrane particle aggregation is also discussed. A preliminary report of this study has been published (Liu et al., 1976).

## Materials and Methods

*Preparation of Erythrocyte Ghosts.* Venous blood was collected from healthy volunteers into citrate–phosphate–dextrose anticoagulant and used within 2 weeks of storage at 4 °C. Red cells were isolated by centrifugation at 1000*g* for 15 min. The supernatant and buffy coat were discarded by aspiration, and red cells were washed three times with 3 vol-

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umes of 10 mM Tris[1]-0.15 M NaCl (pH 7.4). Erythrocyte ghosts were prepared by hypotonic lysis of washed cells at 0–4 °C in 30 volumes of 10 mM Tris (pH 7.4) buffer, followed by centrifugation at 29 000*g* for 10 min. After the cream-colored "buttons" were removed (Fairbanks et al., 1971), the membrane pellet was resuspended and washed twice.

*pH-Dependent Cross-Linking.* Buffers containing 10 mM Tris were adjusted to pH 3.5–7.0 (room temperature) by addition of acetic acid. One volume of freshly prepared packed ghosts (about 3 mg/mL) was incubated with 30 volumes of Tris-acetate buffer at 0 or 37 °C. After incubation, 2 mM EDTA was added to terminate the reaction. The membranes were recovered by centrifugation and dissolved in sodium dodecyl sulfate solution for electrophoresis as described below. It should be noted that the pH value that is given in the data is not corrected for the variation (±0.3 unit) with temperature.

For *N*-ethylmaleimide (MalNEt) pretreatment, washed red cells were suspended in isotonic saline (10% hematocrit) containing 10 mM Hepes (pH 7.4) and 4 mM MalNEt and were incubated at 25 °C for 2 h. The red cells were then centrifuged and washed before subsequent lysis.

A suspension of spectrin and actin (extracted by 1 mM Tris (pH 8.0) without EDTA according to Avruch and Fairbanks, 1974) was mixed with an equal volume of 20 mM Tris-acetate (pH 5.0) and incubated at 0–4 °C for 20 min. After adding 2 mM EDTA (pH 5.0) to quench the reaction, the protein precipitate was centrifuged at 1000*g* for 10 min and dissolved in the sodium dodecyl sulfate concentrate (see below).

*Sodium Dodecyl Sulfate–Agarose–Acrylamide Gel Electrophoresis.* High molecular weight complexes were resolved using buffer and catalyst formulations of Steck (1972), with the following modifications. Membrane samples were dissolved for electrophoresis in 0.25 volume of the concentrate: 5% sodium dodecyl sulfate, 250 mM Tris (pH 7.4), 10 mM EDTA, 1.25 M sucrose, and 0.1 mg/mL pyronin Y, and stored frozen at −30 °C. Just prior to gel electrophoresis, samples were heated for 1 min at 100 °C. The composite gels containing 2.5% acrylamide and 0.3% agarose were cast according to Peacock and Dingman (1968). The cylindrical gels were 0.6 cm in diameter and 8 cm long. It was found that the temperature control during the casting of gels was important in relation to the flatness of stained patterns. Best results were obtained by mixing stock solutions of acrylamide, bisacrylamide, sodium dodecyl sulfate, Tris buffer, and hot agarose first and equilibrating to 40 °C in a water bath. After adding ammonium persulfate and Temed, the gel solution was transferred immediately into glass tubes which had been warmed to 37 °C. The gels were overlaid gently with a solution of 0.2% sodium dodecyl sulfate and solidified by immersion in an ice–water bath for 4 min. The gels were then left to stand for at least 12 h at room temperature before electrophoresis. Fractionation was completed in about 2 h applying a current of 5 mA per gel.

*Two-Dimensional Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis.* The discontinuous gel system described by Laemmli (1970) was employed in a gel slab for resolution in the second dimension. Slab gels were prepared in an apparatus similar to that described by Studier (1973) except that the upper buffer chamber was notched on two sides so that two slabs could be run at once. The stacking and separating gel slabs were formed of 2.67 and 8.0% acrylamide, respectively. The slabs were 15 cm wide, 0.6 cm thick, 1.5 cm high for the stacking gel and 7 cm high for the separation gel. A 2-mm zone of high reducing activity was applied over the stacking gel by adding the melted agarose solution (50 °C) containing 1% agarose, 125 mM Tris (pH 6.8), 1% sodium dodecyl sulfate, 40 mM dithiothreitol, 2 mM EDTA, and 1 μg/mL pyronin Y. After the membrane protein was fractionated in the first-dimensional gel as described above, the gel was transferred and sealed on top of the slab with 8 mL of the dithiothreitol-containing buffered agarose solution. Electrophoresis was carried out with the current about 20 mA per slab for 18 h. After electrophoresis, gel slabs were put into bags made of nylon net and immersed into solutions (approximately 1 L per slab) for staining and destaining according to the procedure of Fairbanks et al. (1971), except that no Coomassie blue was introduced into the destaining solution.

## Results

*Effect of pH and Temperature on Protein Cross-Linking.* The effects of acid pH on membrane proteins of ghosts incubated at 0–4 and 37 °C are illustrated in Figures 1 and 2, respectively. The sodium dodecyl sulfate gel system we used contained only 2.5% acrylamide, so that spectrin components 1 and 2, the major proteins of highest molecular weight, migrated at least one-third of the total gel length. The use of a highly porous gel thus established a zone below the origin in which good separation of slower migrating protein aggregates could be obtained.

Freshly prepared ghosts dissolved in sodium dodecyl sulfate solution without reducing agent showed no detectable cross-linking among major membrane proteins (as in Figure 1A, pH 7.0). However, incubation at acid pH (Figure 1A) or elevated temperature (Figure 2, pH 7.0) produced a number of new bands, including several complexes with apparent molecular weights exceeding that of spectrin. The cross-linking reactions appeared to be derived from oxidation of intrinsic membrane protein SH groups, because they were prevented by prior alkylation with MalNEt (Figure 1B) and were reversed by reduction with dithiothreitol (Figure 1B) or mercaptoethanol (not shown). The membranes from MalNEt-pretreated cells yielded increased staining intensities in the region 4.5 (60 000 daltons), 8 (18 000 daltons), and globin subunits. However, these changes are not related to pH 5.0 incubation, because they appear in membrane samples prepared for electrophoresis without incubation (data not shown). MalNEt treatment appears to alter the retention of cytoplasmic proteins by well-washed membranes.

The presence of 2 mM EDTA prevented cross-linking (Figure 1B), but did not reverse it once the protein aggregate had been formed. A similar observation has been made by others (Steck, 1972; Wang and Richards, 1974) in catalytic oxidation of isolated ghosts at pH 8.0 with a $CuSO_4$/*o*-phenanthroline mixture. In fact, 37 °C incubation of white ghosts in the pH range 6.0–7.0 (Figure 2) yields patterns similar to those produced by catalytic oxidation.

The maximal cross-linking of membrane proteins at either 4 or 37 °C occurred in the pH range 4.5–5.0, as judged from the increased staining intensity of aggregates and the concomitant depletion of monomeric protein bands as follows: (i) in incubations at 4 °C, the relative intensities of discrete bands in the 260 000–450 000-daltons zone were increased (Figure 1A); (ii) in incubations at 37 °C, large aggregates ($>1.5 \times 10^6$ daltons) that remained at the top of the gels were formed (Figure 2); (iii) the amount of diffuse staining above the

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; MalNEt, *N*-ethylmaleimide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Temed, *N,N,N,N*-tetramethylethylenediamine; PAS, periodic acid-Schiff (stain).

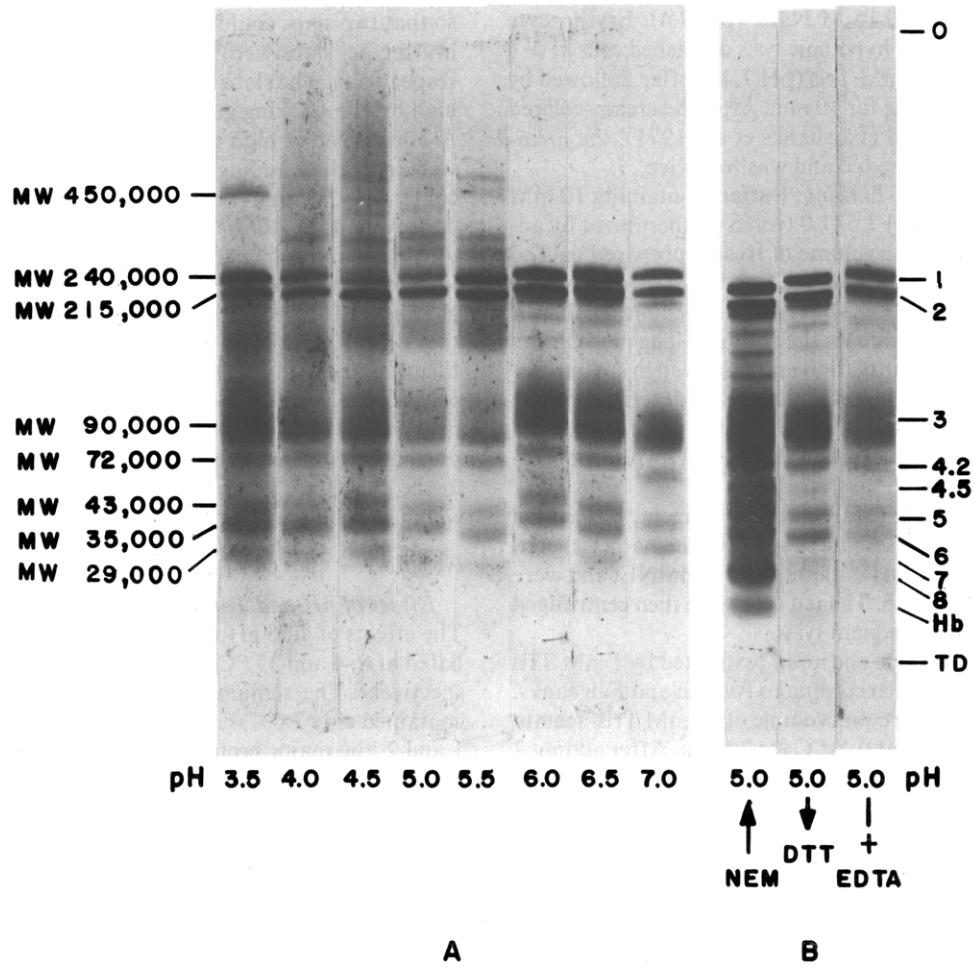


FIGURE 1: pH dependence of membrane protein cross-linking at 0–4 °C. (A) Fresh ghosts were incubated for 20 min in 10 mM Tris-acetate buffer at the indicated pH. Reactions were terminated by addition of 2 mM EDTA. Membranes recovered by centrifugation were dissolved in 0.25 volume of sodium dodecyl sulfate concentrate (see Materials and Methods). Membrane samples (10 μL, or about 25 μg) were electrophoresed in sodium dodecyl sulfate–polyacrylamide–agarose gels without dithiothreitol reduction. (B) Effects of MalNEt, diethiothreitol, and EDTA on results of pH 5.0 incubation: (left) ghosts isolated from MalNEt-treated cells; (center) dithiothreitol reduction before electrophoresis; (right) incubation in the presence of EDTA.

spectrin monomer bands was increased. In this pH range, the preferential depletion of monomeric spectrin component 1 as compared to 2 was also noted.

Some two-dimensional gels (see below) reveal minor bands in the 2.1 to 3 and 4.2 to 5 zones suggesting a slight degree of proteolysis during incubation. However, in gels of incubated samples electrophoresed after dithiothreitol reduction (e.g., Figure 1B), no prominent degradation products appeared in these zones or below band 6 and the relative intensities of major bands were unchanged (densitometric scans, not shown). Thus, the band changes noted primarily reflect cross-linking events.

*Two-Dimensional Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis.* In order to identify the protein constituents in aggregates and to define their spatial arrangements in the membrane, the cross-linked complexes were analyzed by two-dimensional gel electrophoresis. The approach is similar to that of Wang and Richards (1974, 1975) who cross-linked membrane protein with cleavable bifunctional reagents, used gels of low acrylamide concentration to resolve the complexes, and identified their polypeptide constituents as off-diagonal spots in the pattern formed by electrophoresis in the second dimension after reductive cleavage of the cross-links.

In the work reported here, a discontinuous gel system (Laemmli, 1970) was used for resolution in the second dimension. This system focuses the bands as they move laterally out of the first gel, thus enhancing the sharpness of the spots prior to their entry into the separating gel. This improves the sensitivity with which components released from cross-linked complexes can be detected. Figure 3 depicts two-dimensional fractionation of a control membrane sample run without cross-linking in the first dimension. Individual components are resolved in the slab as distinct spots which fall on a sigmoid curve. A separate membrane sample was usually applied directly onto the slab as a reference for spot identification. The off-diagonal spots and streaking encircled by the dotted curve in Figure 3 are not cleaved components, but are glycoproteins, as they could be stained by the periodic acid–Schiff reagent and were not affected by the reduction prior to electrophoresis in the first dimension. Anomalous migration of glycoprotein in sodium dodecyl sulfate gels has been observed by others (Wang and Richards, 1974) and has been ascribed to altered sodium dodecyl sulfate binding to highly glycosylated species.

The designation of the major membrane polypeptides in both first and second dimensions followed that of Steck (1972) and was checked by low ionic strength extraction of components 1, 2, and 5 and salt extraction of band 6 (data not shown). However, in the discontinuous gel system, at least seven discrete bands were resolved between 4.2 (72 000 daltons) and 5 (43 000 daltons), and a doublet of 4.1 (mol wt 78 000) was detected (Figure 3). The diffuse staining background between

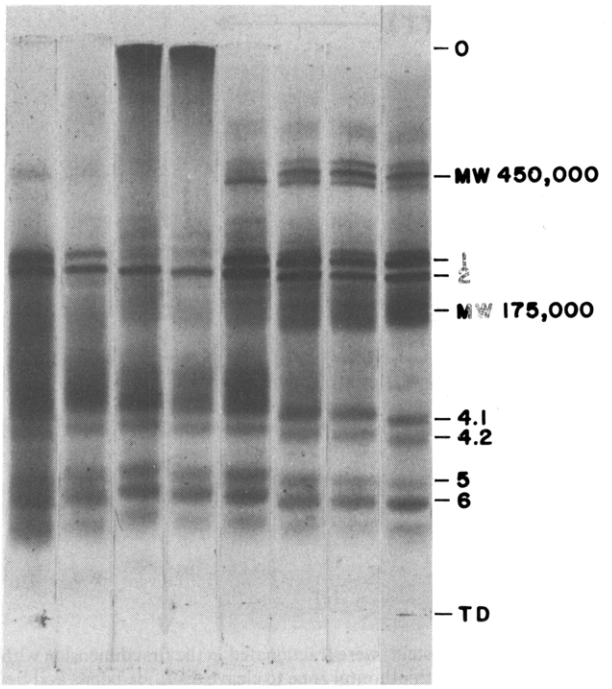


FIGURE 2: pH dependence of membrane protein cross-linking at 37 °C. Samples were prepared as in Figure 1A except for incubation at 37 °C.

4.2 and 5 was probably due to glycoproteins that migrated in this region. We tentatively assigned designations 4.5 (60 000 daltons) and 4.9 (46 000 daltons) to two bands in this region. A recent study (L. M. Snyder, S. C. Liu, J. Palek, L. M. Edelstein, and N. Fortier, unpublished data) showed that 4.5 is a subunit of catalase, which is predominantly cytoplasmic but variably partitioned between the cytosol and the membrane. It should be noted that bands 4.5 and 4.9 in the discontinuous system probably correspond to the bands designated 4.4 and 4.5, respectively, by Wang and Richards (1974), who employed a continuous gel system containing 5.2% polyacrylamide. This is indicated by their similarity in apparent molecular weight and by their behavior in oxidative cross-linking at neutral pH (see below).

A typical two-dimensional gel of a membrane sample incubated at pH 5.0, 0–4 °C, is shown in Figure 4A. Several off-diagonal spots representing the released constituents of the cross-linked complexes were seen and easily identified. Among these are the dimers and/or trimers of bands 3, 4.1, 4.2, 4.5, 4.9, and 5. Aggregates that migrated in the 260 000- to 450 000-dalton region were clearly seen to contain components 1, 2, 3, 4.9, and 5. This is best seen in Figure 4B, which shows a slab produced by increasing the running time in the first dimension to improve resolution in the zone of interest. Several discrete off-diagonal spots corresponding to spectrin monomers are superimposed on a continuum of streaking. Several recognizable spots corresponding to bands 3, 4.9, and 5 are localized directly below these off-diagonal spots of spectrin. However, it is difficult to determine the molecular composition and stoichiometry unambiguously for all aggregates. Some tentative assignments are shown in Figures 4B and 4C. These are based on the apparent molecular weights of the aggregates in the first dimension, plus the identification of the individual polypeptides released by reduction. A calibration curve for the estimation of aggregate molecular weights was constructed by extrapolating the curve for the major membrane polypeptides through the point representing the 1 + 2 complex, which is readily identified (e.g., in Figure 6).

Certain other complexes are readily identified in Figures 4B and 4C: (i) the dimer and trimer of band 3; (ii) 1 + 3 and 2 + 3, representing the coupling of single spectrin chains to the band 3 monomer; (iii) 2 + 4.9; (iv) 1 + 5; and (v) 3 + 5. Complexes between single spectrin chains and band 3 dimers (i.e., $1 + (3)_2$, $2 + (3)_2$) are tentatively identified based on the molecular weights and the appearance of "accents" in the spectrin zone on the slab, but the corresponding accents in the band 3 zone are not sharply resolved from the continuum. Other off-diagonal spots in Figure 4 also represent discrete complexes—dimers and/or trimers of 2.1, 2.3, 2.4, 4.1, 4.2, 4.5, 4.9, and 5. The streaks corresponding to bands 1, 2, 3, 4.2, and 5 may result from multiple disulfide aggregation complicated by conformational heterogeneity introduced by intrachain disulfide bonds. Some of this streaking, particularly at high loading, may be an artifact of electrophoresis in the absence of a reducing agent. It is interesting that spectrin component 1 is more extensively aggregated than spectrin component 2. Preferential depletion of 1 relative to 2 is a function of time, temperature, and pH, and thus appears to reflect differences in the behavior of the two chains in the membrane rather than differences in their responses to electrophoresis.

As described above (Figure 2), incubation at pH 5.0, 37 °C, produced a very large aggregate which remained at the origin of the porous agarose–acrylamide gels. Two-dimensional analysis of these samples (Figure 5) revealed that the material trapped at the origin consisted primarily of components 1, 2, 3, 4.2, and 5. This aggregate is enriched in spectrin, with more 1 than 2; it is not clear if any of the polypeptides are present in fixed proportions. Ghosts incubated at pH 3.5 produced a much more restricted pattern of cross-linking (Figure 6). The spectrin heterodimer, 1 + 2, was the major complex and a small amount of band 3 dimer was formed. Under these conditions, large aggregates did not appear and cross-linking of spectrin to band 3 was not detectable.

Incubation at 37 °C in the pH range 6.0–7.0 also results in selective cross-linking with formation of discrete complexes (Figure 2). In a heavily loaded two-dimensional gel (Figure 7) both homopolymers—$(3)_{2,3,4}$; $(4.1)_2$; $(4.2)_{2,3}$—and heteropolymers—2 + 4.9, 1 + 5, 4.5 + ?, 1 + 2 + 4.9, 1 + 2 + 5, and $(1 + 2)_{2,3,4}$—could be readily detected. Although cross-linking is rather extensive under these conditions, cross-linking of spectrin to band 3 does not occur. The pattern is similar to that produced by catalytic oxidation (Steck, 1972; Wang and Richards, 1974) except that the first-dimension gel did not show large aggregates at the origin. The reason for this discrepancy is not clear but may be related to the milder reaction conditions used in our experiments, as well as the use of a much more porous gel for the fractionation in the first dimension.

Because maximal cross-linking was observed in the pH range at which spectrin undergoes isoelectric precipitation, we examined the pattern of cross-linking of a low ionic strength spectrin–actin extract for comparison with that observed in intact ghosts. As shown in Figure 8, incubation at pH 5.0, 0–4 °C, resulted in efficient formation of the spectrin heterodimer, 1 + 2. The gel patterns of the cross-linked proteins are also indicative of the formation of higher oligomers of this dimer unit (probably extending to the hexamer, i.e. $(1 + 2)_6$). These results demonstrate that spectrin is capable of extensive self-association in the absence of constraints imposed by its interactions with other membrane constituents.

## Discussion

*Chemical Nature of Cross-Links.* Because pH-dependent cross-linking of membrane proteins was prevented by blocking

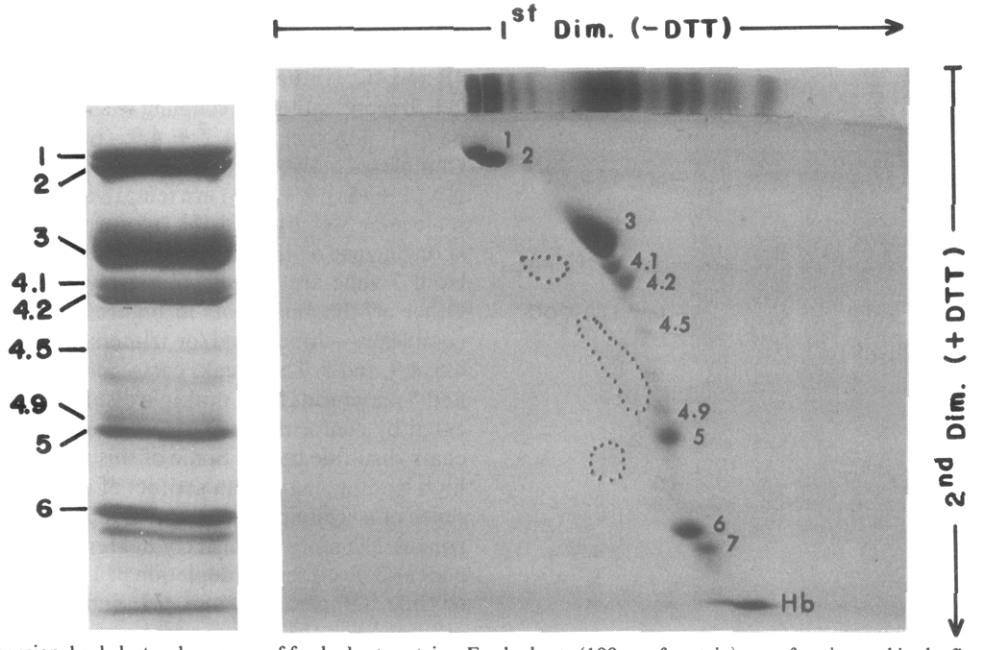


FIGURE 3: Two-dimensional gel electrophorogram of fresh ghost proteins. Fresh ghosts (100 μg of protein) were fractionated in the first dimension without reducing agent. Electrophoresis in the second dimension was performed in a slab, incorporating a dithiothreitol zone to cleave disulfide bonds and buffer discontinuities that form sharp starting zones (see Materials and Methods). The pattern of a membrane sample applied directly is shown at the left for reference. The pattern obtained by electrophoresis in the first dimension is shown in a stained replicate gel placed at the top of the slab. Off-diagonal elements encircled by dotted curves probably represent glycoproteins (see text and Figure 6).

sulfhydryl groups with MalNEt, and was reversed by treatment with dithiothreitol, we infer that the bonds formed are disulfides. The total number of sulfhydryl groups in the membrane has been estimated as $3 \times 10^6$ per ghost (Rothstein, 1971). In the intact cell, these SH groups are normally maintained in the reduced state by glutathione (2.0–2.5 mM) or other unidentified cytoplasmic reducing agents (Jacob and Jandl, 1962). Sulfhydryl groups in isolated membranes and in intact cells with defective glutathione production become susceptible to oxidation (Beutler, 1969; Yawata et al., 1973). We have observed sulfhydryl-mediated cross-linking in membranes of intact normal cells subjected to ATP depletion; the reaction occurs in cells incubated in an air atmosphere, but not in the presence of pure nitrogen (Palek and Liu, 1976; Palek et al., 1976). Thus, cross-linking in the intact cell is correlated with the presence of molecular oxygen and is responsive to changes in levels of reduced glutathione.

*Cross-Linking of Spectrin or Actin to Band 3.* Spectrin and actin (band 5) formed cross-linked complexes with band 3 in isolated membranes incubated at pH 4.0 to 5.5, which coincides with their isoelectric points as defined by isoelectric focusing (Bhakdi et al., 1974) and precipitation (Elgsaeter et al., 1976; Gratzer and Beaven, 1975; Yu and Branton, 1976). We interpret the appearance of these complexes—1 + 3, 2 + 3, and 3 + 5—as reflecting the dramatic reduction of electrostatic repulsion and more intimate contact among these protein molecules at pH 4.0 to 5.5. Since shape changes, aggregation of intramembrane particles, and the clustering of anionic sites of isolated membranes at acid pH are mostly reversible (Nicolson, 1973; Pinto da Silva, 1972) it is believed that extensive derangement of membrane structures does not occur. However, the possibility that minor rearrangement occurs during incubation at pH 4.0–5.5 cannot be excluded totally, because at pH 7–8, extensive disulfide cross-linking induced by $CuSO_4$/*o*-phenanthroline (Steck, 1972; Palek et al., 1976) or exposure to elevated temperature (Figures 2 and 7) did not produce complexes 1 + 3 or 3 + 5. On the other hand, negative results in the cross-linking experiments cannot be used to rule out such interactions or physical contacts (Steck, 1972; Wang and Richards, 1974). Recent electron microscopic studies also suggested that spectrin and integral protein interactions which limit the lateral motion of intramembrane particles (Elgsaeter et al., 1976; Nicolson and Painter, 1973) play a role in the aggregation of intramembrane particles at pH 5 (Yu and Branton, 1976) and in several transmembrane phenomena (Nicolson and Painter, 1973; Ji and Nicolson, 1974). Our cross-linking results clearly indicate that these interactions are enhanced at pH 4.0 to 5.5. Whether this results from subtle conformational changes in the vicinity of critical SH residues or a shift in the position of the spectrin–actin meshwork relative to the membrane matrix remains to be established.

The observation of extensive disulfide linkage in isolated spectrin at pH 5.0 contrasts with the restricted pattern in membranes, and gives added weight to the suggestion that self-association of spectrin is restricted by its interaction with other membrane components. However, the differences in cross-linking of spectrin in these two states may involve other restraints, such as interaction with lipid components or the organization of the spectrin–actin lattice itself.

Our observations reveal differences in the behavior of the two spectrin chains at pH 4.0 to 5.5: (i) preferential depletion of 1 relative to 2 and (ii) differential cross-linking in discrete complexes (e.g., 2 + 4.9, 1 + 5). The depletion of 1 faster than 2 does not appear to be due to the formation of different amounts of the complexes 2 + 4.9 and 1 + 5, but rather reflects preferential involvement of 1 in diffuse, nondiscrete complexes (Figure 4B) and in the very large aggregates with other proteins, i.e. bands 3, 4.2, and 5 (Figure 5). Presumably these results reflect differences in detailed structure or localization of the two chains. As yet there is little information on these points. The two chains have virtually identical amino acid composition (Fuller et al., 1974) and coprecipitate at pH 5.0. One potentially significant difference is that only spectrin 2 is phosphorylated (Gurthrow et al., 1972; Rubin and Rosen, 1973; Avruch and Fairbanks, 1974). The number and the position of protein-bound phosphate groups are not known. A.

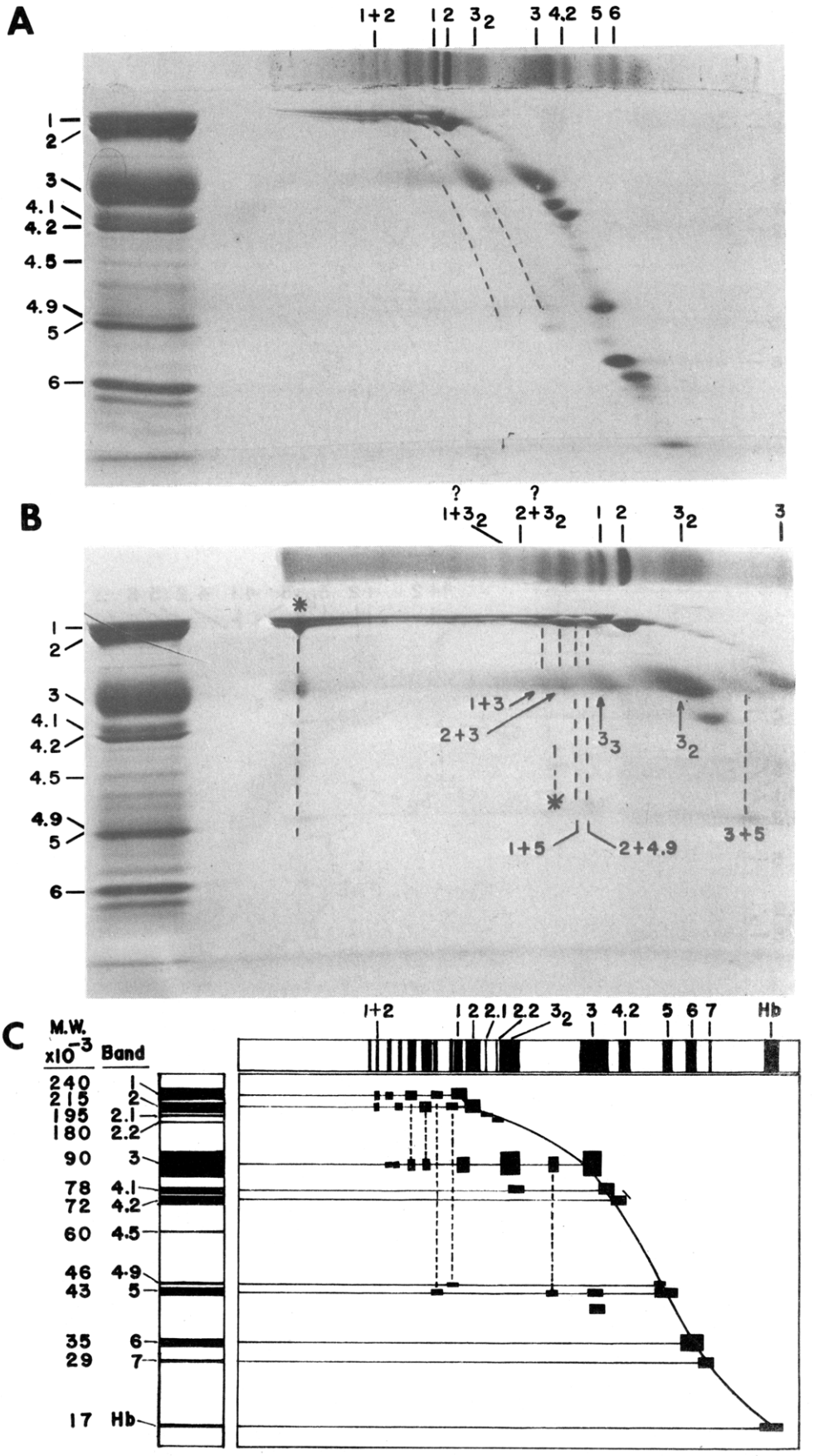


FIGURE 4: Two-dimensional analysis of cross-linking produced by incubation at pH 5.0, 0–4 °C. (A) Cross-linked samples were electrophoresed as in Figure 3. Dashed lines indicate alignments of spots corresponding to polypeptides released after cleavage of homopolymers (dimers or trimers) of membrane proteins. (B) Same as in A, except that the cross-linked sample was subjected to prolonged electrophoresis in the first dimension. Tentative identification of the cross-linked complexes giving rise to off-diagonal spots is indicated. Asterisks denote artifacts. (C) A schematic composite diagram of cross-linking patterns. Dashed lines indicate aligments of spots corresponding to polypeptides released after cleavage of heterocomplexes of membrane proteins.

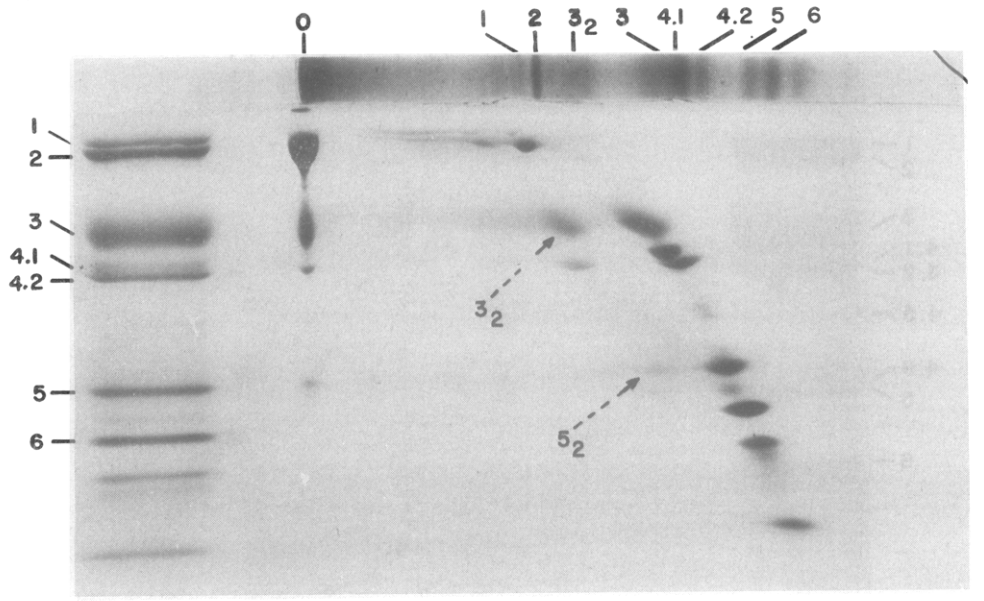


FIGURE 5: Two-dimensional fractionation of membrane incubated at pH 5.0, 37 °C. A large aggregate appears at the origin, O. Preferential depletion of 1 relative to 2 is evident both in the slab and the replicate first-dimension gel.

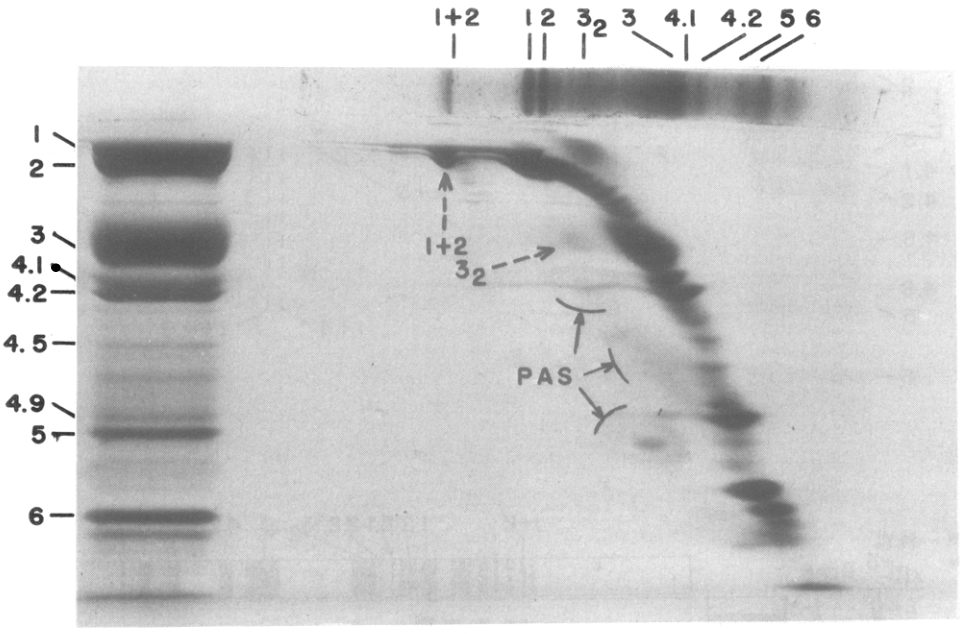


FIGURE 6: Two-dimensional fractionation of membranes incubated at pH 3.5, 0–4 °C. The spectrin heterodimer, 1 + 2, appears as a major cross-linked product. Arcs indicate pink rims that encircled faint off-diagonal zones. These were produced by residual PAS reagent contaminating the staining tray and serve to identify the positions of glycoproteins faintly stained by Coomassie blue (cf. Figure 3).

D. Roses (cited in Schechter et al., 1976) reported the presence of about two phosphate groups per spectrin chain, based on direct chemical analysis. Wolfe and Lux (1976), on the other hand, obtained a value less than one (per spectrin monomer) from equilibrium labeling in intact cells incubated with $^{32}P_i$.

*Formation of Homologous Band 3 Complexes.* Band 3 (mol wt ~90 000) represents approximately 25% of the erythrocyte protein. It has been shown to span the membrane and probably constitutes, at least in part, intramembrane particles visualized by freeze-fracture electron microscopy (Pinto da Silva and Nicolson, 1974). Steck and associates have demonstrated noncovalent self-association of band 3 as a dimer as well as specific interactions with red cell enzymes (Yu and Steck, 1975; Strapazon and Steck, 1976). Cross-linking by catalytic oxidation (Steck, 1972; Wang and Richards, 1974) or incubation at elevated temperature, 37 °C (Figure 2), produces covalent dimerization via a disulfide bond. At 37 °C, tetramer formation of band 3 in the membrane is also detectable. This suggests that incubation at 37 °C increases lateral mobility and results in more frequent collisions between band 3 dimers in the plane of the membrane. In fact, increased aggregation of intramembrane particles has been observed in ghosts incubated at 37 °C, as compared to 0–4 °C (Figure 8 of Elgsaeter and Branton, 1974). The premise that the unit of band 3 organization is the noncovalent dimer is well supported by the results that dilution of band 3 with Triton X-100 (Yu and Steck, 1975) or the cross-linking of ghosts at 0–4 °C, pH 7.4, with dithiobis(succinimidyl propionate) (S.-C. Liu, G. Fairbanks, and J. Palek, unpublished data) effectively diminished trimer and tetramer formation by band 3. On the same basis, formation of band 3 trimer after incubation (0–4 °C) of fresh

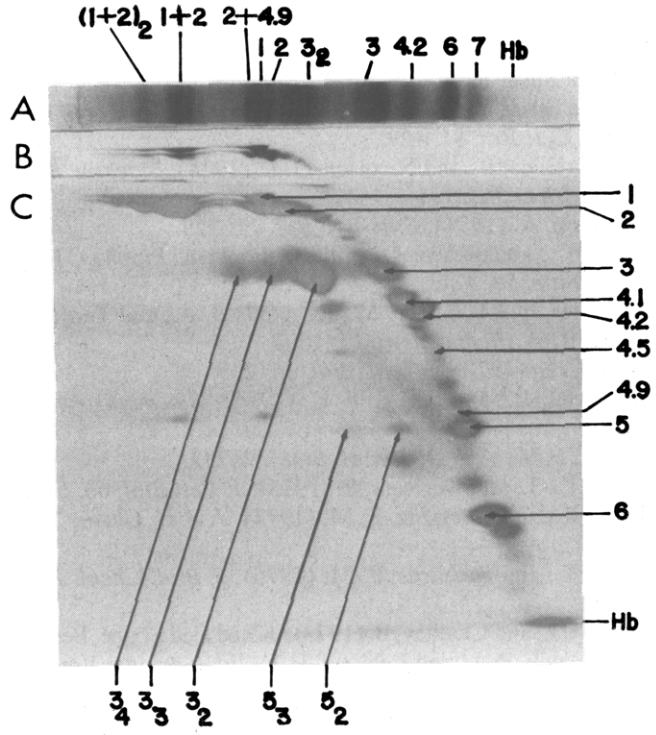


FIGURE 7: Two-dimensional gel electrophorograms of ghosts incubated in pH 7.0 buffer at 37 °C. (A) A replicate gel of first dimension. (B) Spectrin zone of two-dimensional pattern with low loading (50 μg of protein). (C) 150 μg of protein applied. Constituents of some complexes are labeled.

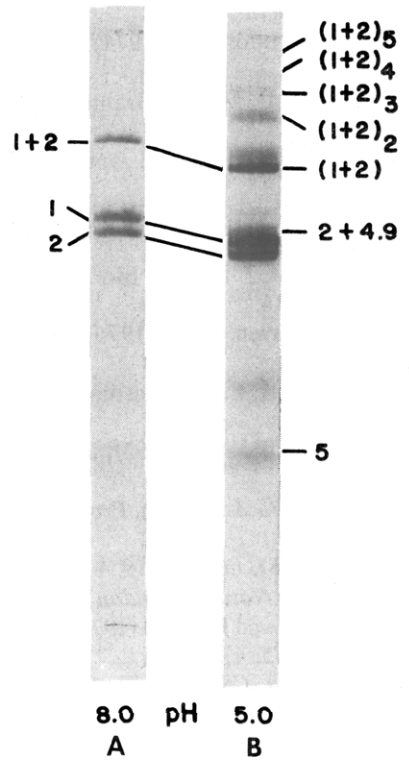


FIGURE 8: Cross-linking of spectrin–actin extracts induced by incubation at pH 5.0. (A) Fresh spectrin–actin extracts before incubation at pH 5.0. Some heterodimers of spectrin, 1 + 2, were present. (B) Extracts incubated at pH 5.0, 0–4 °C for 20 min. Higher polymers of spectrin, i.e. $(1 + 2)_{2,3,4,5}$, are indicated.

ghosts at pH 4.0 to 5.5 is suggestive of a pH-dependent aggregation of band 3 dimer. The result correlates very well with the electron microscopic observations of Elgsaeter and Branton (1974) that, at 0–4 °C, increased aggregation of intramembrane particles occurs when the pH is lowered to 4.0–5.0. However, the intramembrane particle aggregation in ghosts has not been studied at pH 3.5, in which we showed a decrease of band 3 cross-linking (Figure 6). Therefore, it is not known if the particle aggregation exhibits a pH optimum like that shown in Figure 1.

*Cross-Linking of 1 to 5 and 2 to 4.9.* The aggregates, 1 + 5 and 2 + 4.9, appeared in samples from pH 4.0 to 5.5 incubation of isolated ghosts (Figures 1 and 4). Coupling of 2 to 4.9 is also induced by catalytic oxidation or dimethyl-3,3′-dithiobispropionimidate dihydrochloride cross-linking (Wang and Richards, 1974) and occurs as well in ATP depletion of intact cells in air (Palek et al., 1976; Palek and Liu, 1976). The cross-linking of actin (band 5) to spectrin is of importance because it has been suspected that these two proteins interact to regulate viscoelastic properties of the membrane (Kirkpatrick, 1976). This is supported by observation of spectrin effects on polymerization of skeletal muscle actin in cell-free systems (Tilney and Detmers, 1975; Pinder et al., 1975).

Band 4.9 is a minor component (<1% of Coomassie blue stain, data not shown) with a molecular weight of 46 000 based on its mobility in the discontinuous gel system. Spectrin chain 2 is phosphorylated by a cAMP-independent protein kinase of approximately the same molecular size (Fairbanks et al., 1976). The localization and size of 4.9 are then consistent with the possibility that it is spectrin kinase; this question is under investigation.

## Conclusion

We have described cross-linking in red cell membranes yielding heteropolymers of spectrin and actin with band 3 and homopolymers of band 3. Protein cross-linking occurs in isolated membranes in the presence of molecular oxygen but without addition of other oxidants. The conditions that promote it have been shown by others to cause isoelectric precipitation of major membrane proteins and intramembrane particle aggregation. However, the molecular details of the interactions within the spectrin–actin network and between it and band 3 remain to be elucidated. Ultimately, a thorough description of the arrangement of the proteins under various cross-linking conditions will require identification and localization of the specific amino acid residues involved. In the interim, cross-linking studies of interactions between the major components in isolation and in reconstituted model systems (e.g., Yu and Branton, 1976) should be helpful in further defining the specificity of the interactions and their relationship to the organization of the membranes under physiologic conditions. In this connection, we observed recently (S.-C. Liu and J. Palek, in preparation) that mild oxidation of isolated ghosts by incubation in oxygen-saturated buffer at pH 7.4, 0–4 °C, produces complexes of 1 + 3, 2 + 3, 3 + 5, and 1 + 5 as reported here. Thus, the protein contacts implied by cross-linking pattern at acid pH are also detectable at physiological pH. This is consistent with the reversibility of the shape changes, and aggregations of intramembrane particles or anionic sites induced at acid pH, which was taken as indirect evidence that extensive derangement of membrane organization does not occur under these extreme conditions (Nicolson, 1973; Pinto da Silva, 1972).

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# Mouse Sperm Chromatin Proteins: Quantitative Isolation and Partial Characterization†

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ABSTRACT: Conditions are described that permit the quantitative extraction of chromatin proteins from the epididymal sperm of the mouse. These proteins have been isolated free of contaminating tail proteins following removal of the tails with cetyltrimethylammonium bromide (CTAB). Without this treatment, numerous acid-soluble tail proteins coextract with the nuclear proteins isolated from partially purified heads. The proteins isolated in this manner do not require prior modification with iodoacetamide and show no evidence of proteolytic degradation. In acid-urea polyacrylamide gels, 99% of the sperm protein migrates as one electrophoretic band. Evidence is presented that suggests that this single band contains two protamine-like proteins.

During spermatogenesis in most higher animals, differentiation of spermatogonia into mature sperm is accompanied by the replacement of the normal somatic histones and nonhistone proteins in chromatin by an extremely arginine- and cysteine-rich, protamine-like protein (Marushige and Dixon, 1969; Kumaroo et al., 1975). Extensive studies by Dixon (1972) show that the maturation and associated condensation of chromatin in trout sperm are directly correlated with the replacement of the histones on DNA by protamine. Although this process of repackaging may be more complex in mammals than fish, it appears to proceed in a similar fashion. Prior to the first meiotic division as the mammalian primary spermatocyte replicates its DNA, three new "meiotic histones" are synthesized (rat, mouse, rabbit, and monkey: Shires et al., 1975; Kistler and Geroch, 1975; Branson et al., 1975). These proteins are chemically and structurally similar to the H1, H3, and H2b

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