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Research Article

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Diminished Spectrin Extraction from ATP-Depleted Human Erythrocytes

EVIDENCE RELATING SPECTRIN TO CHANGES IN ERYTHROCYTE SHAPE AND DEFORMABILITY

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ABSTRACT We measured spectrin "extractability" in erythrocytes which were metabolically depleted by incubation at 37°C in plasma or glucose-free buffers. Membranes were extracted with 1 mM EDTA (pH 8, 40 h, 4°C) and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. This procedure solubilized 85–90% of the spectrin, actin, and residual hemoglobin from ghosts of fresh erythrocytes. In incubated erythrocytes, inextractable spectrin rapidly accumulated when ATP concentrations fell below 0–15% of normal. In severely depleted cells, 60–90% of the total ghost spectrin became inextractable. Inextractability was not abolished by physically disrupting the ghost before extraction, but was reversed when erythrocyte ATP was replenished with adenosine. The accumulation of inextractable spectrin correlated temporally with the increase in apparent membrane deformability and the increases in erythrocyte vicosity, calcium content, sodium gain, and potassium loss characteristic of ATP-depleted erythrocytes. No change in integral membrane protein topography (assessed by the distribution of intramembranous particles and concanavalin A surface-binding sites) was detected in depleted cells. Analogous changes were observed in erythrocytes exposed to extremes of pH and temperature. When the pH in the erythrocyte interior fell below 5.5, a pH where spectrin was aggregated and isoelectrically precipitated, erythrocyte and ghost viscosity increased coincident with a marked decrease in spectrin extractability. Similarly above 49°C, a temperature where spectrin was denatured and precipitated, erythrocyte viscosity rose as inextractable spectrin accumulated. These observations provide direct evidence of a change in the physical state of spectrin associated with a change in erythrocyte shape and deformability. They support the concept that erythrocyte shape and deformability are largely determined by the shape and deformability of the spectrin-actin protein meshwork which laminates the inner membrane surface.

INTRODUCTION

Human erythrocyte membranes contain at least seven major proteins and innumerable minor ones (1). One of the most abundant species is spectrin, a large elongated protein containing two polypeptide chains of 220,000 and 240,000 daltons.[1] Current evidence suggests that spectrin is disposed in a microfibrillar

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[1] Nomenclature: We have adopted the system proposed by Fairbanks et al. (1) and modified by Steck (2) for numbering the polypeptide bands on sodium dodecyl sulfate gels of erythrocyte membranes. In this system of nomenclature, bands 1 and 2 designate the two chains of spectrin. Band 5 is an erythrocyte actin (3–5).

meshwork on the cytoplasmic surface (6–8), which normally inhibits the lateral mobility of integral membrane glycoproteins in the membrane bilayer (6, 9, 10). If true, this spectrin "laminate" would likely impose extrinsic stability to the membrane and be a major determinant of erythrocyte shape, deformability, and integrity.

ATP-depleted erythrocytes leak potassium, accumulate sodium and calcium, become rigid and echinocytic, and thereby risk splenic sequestration (11, 12). It is generally assumed that these alterations are induced by changes in membrane protein structure, but the particular protein or proteins involved and the nature of the implied change have not been investigated. If spectrin is responsible then it must exist in at least two physical states: one associated with the flexible, ATP-replete discocyte, the other with the rigid, metabolically depleted, echinocyte. To test this premise, we are investigating properties of spectrin obtained from fresh and ATP-depleted erythrocytes. In the present study, we compare the solubilization of spectrin from such membranes at low ionic strength, a property termed "spectrin extractability".

METHODS

The usual experimental design is outlined in Fig. 1.

Incubations. Incubations were conducted in plasma (11) or buffered media. Three buffer systems were used: (*a*) glycylglycine buffer: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM glycylglycine, and 1 mg/ml bovine serum albumin, pH 7.4; (*b*) imidazole buffer: 50 mM imidazole-HCl, 100 mM NaCl, 10 mM dextrose, 1 mg/ml bovine serum albumin, pH 7.4; and (*c*) bicarbonate buffer: 20 mM $NaHCO_3$, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM dextrose, 1 mg/ml bovine serum albumin. Defibrinated erythrocytes were washed three times with the chosen buffer and suspended to a hematocrit to 3–5% before incubation. Erythrocytes incubated in the glycylglycine or imidazole buffers were left in contact with air. In the bicarbonate system the erythrocyte suspension was bathed with a humidified mixture of 5% CO_2 and 95% air. All three buffers were sterilized by filtration through a 0.2-μm Nalgene filter (Nalge Co., Inc., Rochester, N. Y.) and contained penicillin and streptomycin (final concentration of each = 100 μg/ml).

When no glucose was added, the pH of the plasma and glycylglycine-buffered incubations always remained between 7.1 and 7.6 and usually remained between 7.25 and 7.5. Slightly better pH control was achieved in the bicarbonate and imidazole buffer systems (pH ranges 7.3–7.5), even with prolonged incubations (up to 40 h in the bicarbonate system) in the presence of glucose.

Hemolysis. Incubated erythrocytes were washed three times with 10 vol of isotonic sodium phosphate buffer, pH 7.4, portions were removed for various studies (Fig. 1), and the remaining cells were hemolyzed at 4°C and washed twice in 10 vol of ice-cold 20 mosmol sodium phosphate, pH 7.4.

This washing procedure produced pink to red ghosts. White ghosts could be prepared with an additional two or three washes, but we elected to use the incompletely washed ghosts to minimize spectrin loss.

Extraction. Immediately after preparations, ghosts were diluted with one or two volumes of 1 mM sodium EDTA, pH 8, and dialyzed against 1,000 vol of the diluent for 40 h (unless otherwise stated) at 4°C. The supernate and ghost pellet were separated by ultracentrifugation (6.8×10^6 *g*-min) and stored in liquid nitrogen for 1–3 days. The spectrin content of the ghosts, extracts, and ghost pellets was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[2] Under these extraction conditions, spectrin remaining in the ghost pellet was classified as inextractable.

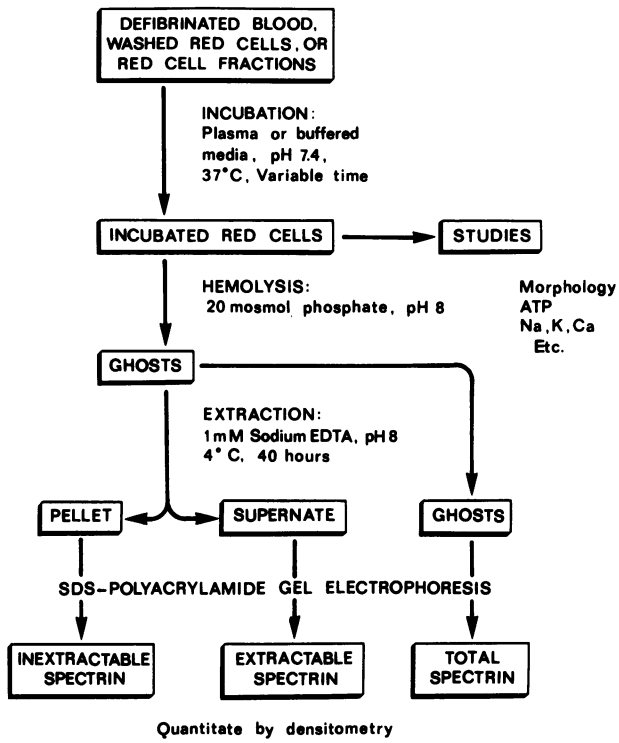

FIGURE 1 The experimental design used in most of the experiments in this study.

PAGE. SDS-PAGE was performed by the procedure of Fairbanks et al. (1) as modified by Steck (2). Acrylamide monomer concentrations of 4 and 5% were used. The gels were stained with Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, Mo.) (13) and scanned with an E-C densitometer connected to an integrating recorder (Linear Instruments Corp. Irvine, Calif., model 252A). The accuracy of this procedure was assessed using a purified spectrin standard. The integrated area under the spectrin peaks was linear when 1–20 μg of spectrin was applied to the gels.

Effect of pH on spectrin extractability. Fresh erythrocytes were washed twice in buffers containing 30 mM Tris-maleate, 140 mM NaCl, and 0.1% bovine serum albumin at pH 5, 6, 7, and 8, and then incubated for 1 h at 37°C at a hematocrit of 10%. After the incubation the erythrocytes were collected by centrifugation and divided into four portions. One portion was fixed in 60 vol of phosphate-buffered saline (PBS) containing 2% glutaraldehyde, and

[2] *Abbreviations used in this paper:* PBS, phosphate-buffered saline (20 mM sodium phosphate, 130 mM sodium chloride, pH 7.4); SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

examined by light microscopy. The second portion (1 ml) was washed twice with isotonic saline and hemolyzed by brief sonification. The pH of the hemolysate was taken as a measure of the intracellular pH at the end of the incubation. The third portion was used for erythrocyte viscosity measurements as described below. Spectrin extractability was determined on ghosts prepared from the fourth portion using the procedures for hemolysis and extraction described earlier.

Effect of temperature on spectrin extractability. Fresh erythrocytes were washed three times in PBS. 3-ml portions of these washed erythrocytes were added to 20 ml of the same buffer heated to a temperature of 42.5°, 45.4°, 47.0°, 48.4°, or 49.9°C, and incubated for 10 min. After heating, the erythrocytes were collected by centrifugation, suspended in the incubation buffer plus 1% bovine serum albumin, and then divided into two portions. One portion was fixed in 60 vol of PBS containing 2% glutaraldehyde and examined by light microscopy. The second portion was used for erythrocyte viscosity measurements as described below.

Viscosity measurements. ATP-depleted erythrocytes were washed and suspended in PBS containing 0.5% bovine serum albumin at hematocrits of 65–80%. Fresh erythrocytes incubated in Tris-maleate buffers at various pH's were washed and resuspended in the incubating buffers at hematocrits of 80%. Measurements were made on 1-ml samples at 37°C in a Brookfield model LVT cone-plate viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, Mass.). After a 2-min equilibration at a shear rate of 115/s, successive readings were taken at shear rates of 115, 46, 23, 11.5, 5.75, 2.30, and 1.15/s. This sequence was repeated twice and the values (which differed <10%) were averaged.

Spectrin solubility as a function of temperature. White ghosts were washed once with ice-cold 0.1 mM sodium EDTA, pH 8, and the packed ghosts were heated for 15 min at 37°C. The EDTA extract was collected by centrifugation as above. This procedure provided a concentrated extract (1–2 mg protein/ml) without the need for ultrafiltration. 10 ml of this extract was chromatographed on a 90 × 2.5-cm column of Agarose A15M, 200–400 mesh, (Bio-Rad Laboratories, Richmond, Calif.), equilibrated, and then eluted with 150 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, pH 8. Most of the spectrin eluted with $K_{av} = 0.5$ and was homogeneous (>99% pure) by SDS-PAGE. This spectrin preparation was diluted to 0.3 mg/ml with the column buffer and 1-ml portions were heated for 10 min to temperatures between 46° and 50°C. The heated samples were cooled to 4°C and centrifuged at 100,000 *g* for 60 min. Soluble spectrin was defined as the protein remaining in the supernate after centrifugation.

Electron microscopy. Freeze-cleave electron microscopy was conducted by established procedures (14). For surface labeling erythrocytes were incubated with concanavalin A and hemocyanin as described by Rosenblith et al. (15). The labeled cells were washed and fixed in 1.0% glutaraldehyde. The erythrocytes were then adsorbed onto glass cover slips that had been treated with 1.0% poly-L-lysine to promote cell adhesion. In some experiments the erythrocytes were first fixed with 1.0% paraformaldehyde and adsorbed onto cover slips before labeling. The binding site distribution was the same with either technique. The hemocyanin label was specific for surface-bound concanavalin A since no hemocyanin labeling occurred in the presence of α-D-methyl mannoside, a competitive inhibitor of concanavalin A. Carbon-platinum replicas of the cells were then prepared (15) and examined by transmission electron microscopy.

Other procedures. Protein concentrations (16), acetylcholinesterase activity (17), erythrocyte ATP (18), and erythrocyte sodium, potassium (19), and calcium (11) were measured by published procedures. Accessibility of acetylcholinesterase was determined as described by Kant and Steck (20).

RESULTS

Accumulation of inextractable spectrin in incubated erythrocytes. The extraction procedure used in these experiments selectively solubilized spectrin, actin, and most residual membrane hemoglobin from erythrocyte ghosts (Fig. 2A). The "extra" proteins present in incompletely washed red ghosts (band 8, part of band 7, and a band in the band 4.5 region) were also solubilized by this procedure (e.g., compare gels 1 and 2 in Fig. 2B). The other ghost proteins were largely unaffected. Band 2.1 appeared to be increased in the extracted ghost pellets, but this was not confirmed by densitometry. The proportion of band 2.1 and the other unextracted proteins relative to band 3 (see below) was the same before and after extraction.

88–92% of the spectrin was extracted from ghosts of fresh, unincubated erythrocytes (Fig. 2B, gel 2); however, when erythrocytes were incubated in the absence of glucose, inextractable spectrin[3] appeared (Fig. 2B, gels 3 and 4). This spectrin could not be removed by repetitive washing and was present in ghosts extracted with dilute (0.1 mM, pH 8) sodium phosphate or Tris-HCl as well as EDTA. Inextractable actin and hemoglobin were also observed in some incubations, but, unlike spectrin, the accumulation of these proteins was unpredictable, and they typically appeared only after prolonged metabolic depletion. Occasionally, however, inextractable actin and/or hemoglobin appeared coincidentally with inextractable spectrin. The reason(s) for this variability are unknown. Variable, small increases in the concentrations of bands 4.5, 6, 7, and 8 were also seen in the extracted ghosts after prolonged incubation probably due, in part, to binding of erythrocyte cytoplasmic proteins to the membrane (21, 22).

These changes in membrane protein extractability are shown graphically in Fig. 3. The concentration of each protein in this figure is represented in proportion to band 3, an integral membrane glycolipoprotein which is not extracted at low ionic strength (1). The rapid accumulation of inextractable spectrin relative to the other membrane proteins is evident. Compared

[3] It should be noted that although we use the term "inextractable" spectrin to designate the spectrin remaining after our conventional extraction procedure (1 mM sodium EDTA, pH 8, 4°C, 40 h), this material is not completely inextractable. Some additional inextractable spectrin can be solubilized if the conventional extraction is prolonged for a very long time (e.g. 7 or 8 days) and most of the inextractable spectrin is removed if the extraction is conducted at 37°C instead of at 4°C.

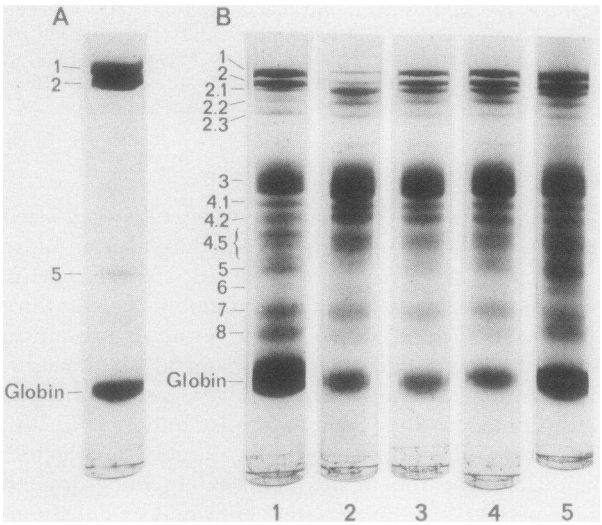

FIGURE 2 SDS-PAGE of erythrocyte (RBC) membrane protein fractions. (A) A typical low ionic strength extract of unincubated pink erythrocyte ghosts. The predominant proteins are spectrin (bands 1 and 2), actin (band 5), and the globin chains of hemoglobin. The protein load is ≅20 μg. (B) Accumulation of inextractable proteins with metabolic depletion. Gel 1: Unextracted red ghosts at 0 time. Gels 2–5: extracted ghost pellets after (2) 0 h, (3) 11.5 h, (4) 18.8 h, and (5) 33.3 h of incubation.

to the total amount of spectrin in the membrane at zero time, the amount of inextractable spectrin was 8.5% (0 h of incubation), 54.5% (11.5 h), 76.5% (18.8 h), and 94.3% (33.3 h).

Relationship of inextractable spectrin to erythrocyte ATP. In plasma, which contains some glucose, erythrocyte ATP levels were maintained for ≅8–10 h and then fell rapidly, reaching zero by about 20 h (Fig. 4A). The proportion of inextractable spectrin rose slightly from 12 to 18% as ATP levels declined. After ATP exhaustion, spectrin accumulation rapidly increased until by 33 h two-thirds of the total spectrin in the membrane was inextractable. In buffered media without added glucose (Fig. 4B), erythrocyte ATP concentrations declined more rapidly and the rate of inextractable spectrin accumulation was correspondingly accelerated. In general, inextractable spectrin accumulated slowly until ATP concentrations reached ≅0–15% of normal. Below this level inextractable spectrin rapidly accumulated (Fig. 4C).

The change in spectrin extractability was not simply an artifact of the incubation procedure. Similar results were obtained in each of the three buffer systems tested (glycylglycine, imidazole, and bicarbonate). Further, when ATP levels were maintained by addition of dextrose (10 mM) to the bicarbonate buffer system, spectrin extractability remained normal, even after incubations as long as 48 h (data not shown).

Control experiments. Initially we were concerned that inextractable spectrin might simply be trapped inside the rigid, ATP-depleted ghost rather than physically associated with the membrane. Three observations suggested this was not so. First, as noted above, hemoglobin and actin were easily extracted from ATP-depleted erythrocytes at times when inextractable spectrin was rapidly accumulating (e.g., Fig. 3). These two proteins serve as internal controls for trapping since they are normally extracted with spectrin and since all three proteins are confined to the cytoplasmic membrane surface (23). Second, both normal and ATP-depleted ghosts were completely vesiculated by the end of the extraction period. Further, assays of acetylcholinesterase, an enzyme which is confined to the outer membrane surface (24), indicated that 70–80% of the extracted vesicles from fresh and ATP-depleted ghosts were inside out (20) so that spectrin could not have been trapped inside. Third, physical or chemical perturbation of the ATP-depleted ghosts (including: repeated freezing and thawing (six times), homogenization in a Dounce homogenizer (100 strokes), or treatment with saponin reagent (Zaponin, Coulter Diagnostics, Inc., Hialeah, Fla.; 0.25 ml/1.5 ml ghosts) before extraction did not improve extractability of either spectrin or actin (data not shown).

We also considered the possibility that the accumula-

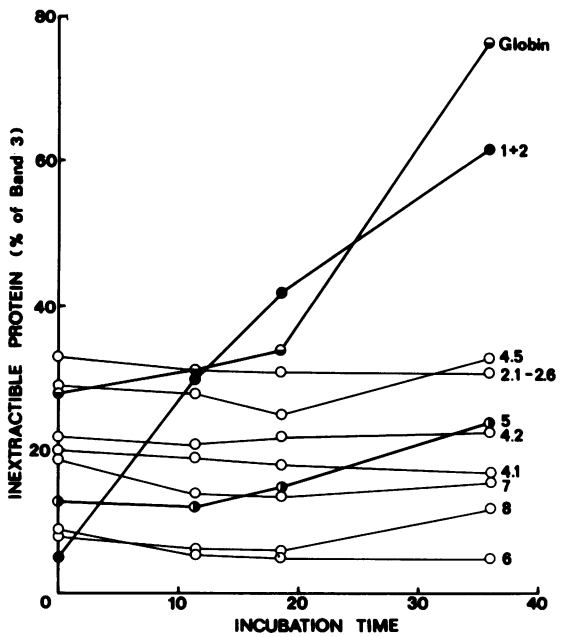

FIGURE 3 Accumulation of inextractable proteins in ghosts of metabolically depleted erythrocytes. The concentrations of the various protein bands in Fig. 2B (gels 2–5) were determined by densitometry and are expressed relative to the concentration of band 3, an integral membrane glycolipoprotein(s) which is not extracted at low ionic strength.

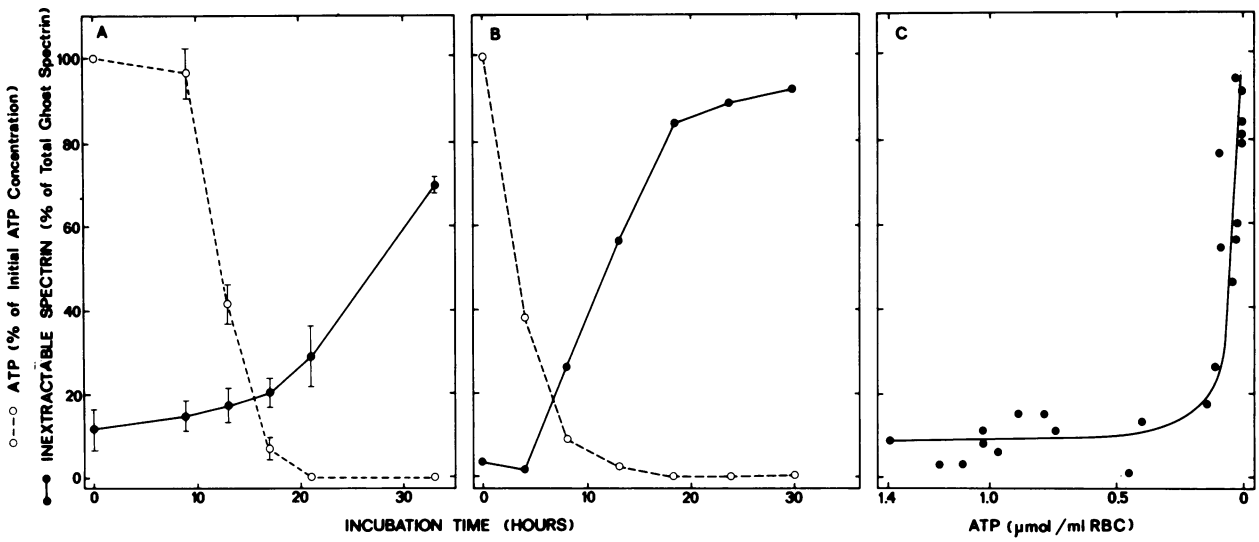

FIGURE 4 Comparison of ATP depletion and inextractable spectrin accumulation. (A) Incubation in plasma. (B) Incubation in imidazole-buffered media. (C) Inextractable spectrin vs. ATP: composite results of four experiments in buffered media. ATP concentrations are expressed as the percent of the value at 0 time. Inextractable spectrin is expressed as the proportion of the total ghost spectrin that was not solubilized by dialysis against 1,000 vol of 1 mM EDTA, pH 8, for 40 h at 4°C. The experiment in panel A shows the mean and range of two independent experiments on different subjects. In panel B the mean and range of duplicate experiments on erythrocytes (RBC) from a third individual are shown.

tion of inextractable spectrin in ATP-depleted ghosts might reflect a slower rate of spectrin extraction rather than an absolute change in the amount of extractable spectrin. But measurements of spectrin extraction from fresh and ATP-depleted ghosts as a function of extraction time showed that the decrease in spectrin extractability in the ATP-depleted sample, compared to normal, was evident throughout the extraction period. Extraction of spectrin from both samples reached a plateau by 20–30 h, well in advance of the time (40 h) when extractions were usually terminated (data not shown).

Comparison of spectrin extractability with changes in erythrocyte cation content and deformability. To place the decrease in spectrin extractability in perspective with the other membrane changes of ATP-depleted erythrocytes, we compared spectrin accumulation to the potassium loss, sodium and calcium gain, increased cellular viscosity, and decreased deformability that accompany ATP depletion. The results are presented in Fig. 5. We measured spectrin extractability and erythrocyte sodium and potassium concentrations in erythrocytes incubated in plasma by the procedure of Weed et al. (11). The data for erythrocyte calcium, erythrocyte viscosity, and apparent erythrocyte membrane deformability were replotted from the data in Weed's report (Figs. 4 and 5 of reference 11). To be certain our results were comparable to theirs we repeated (in a single experiment) their measurements of erythrocyte filterability, calcium, viscosity, and mean corpuscular volume during ATP depletion. Our results conformed closely to their reported measurements (11) (data not shown). Judging from these results and from the rate of ATP decline shown in Fig. 5, our incubations closely duplicated theirs and should be comparable.

In general, the rates of change of erythrocyte potassium (Fig. 5A), viscosity (Fig. 5B), calcium (Fig. 5C), and apparent membrane deformability (Fig. 5B) during ATP depletion are similar to the rate of accumulation of inextractable spectrin. The correlation of spectrin extractability and erythrocyte sodium (Fig. 5A), though similar, is less exact. A notable variation is the decrease in the sodium, potassium, and viscosity curves during the first 12 h of incubation. This is not reflected in the spectrin extractability curve. In addition, the apparent membrane deformability, measured by micropipette, deviates from spectrin extractability near 20 h (Fig. 5B).

Restoration of spectrin extractability in ATP-depleted erythrocytes. Weed and his colleagues (11) also observed that the characteristic changes in erythrocyte shape, viscosity, deformability, and calcium content of ATP-depleted cells were reversible, at least up to 21 h of incubation, if ATP was restored to near normal levels with adenosine. We performed a similar experiment to test the reversibility of spectrin accumulation (Fig. 6). After a 21-h incubation in

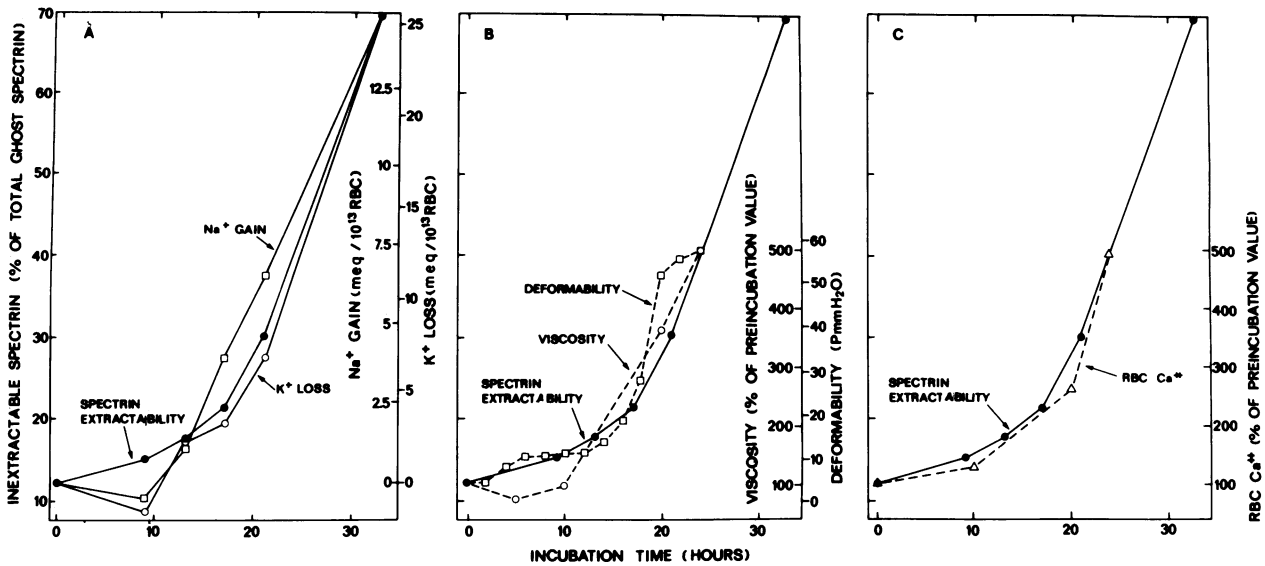

FIGURE 5 Changes in inextractable spectrin, erythrocyte cation content, and apparent membrane deformability during incubation of erythrocytes at 37°C in plasma. The incubation conditions were identical to those of Weed et al. (11). Changes in spectrin extractability (●, panels A, B, and C), sodium (□, panel A), and potassium (○, panel A) were measured directly. The data for erythrocyte viscosity (○, in panel B), apparent erythrocyte membrane deformability (□, panel B), and erythrocyte calcium content (△, panel C) were taken from Weed et al. (Figs. 4 and 5 of reference 11). These workers measured viscosity in a cone-plate viscometer at an 80% hematocrit and a shear rate of 1.15/s. Apparent membrane deformability was measured as the negative pressure (P) required for deformation of the membrane with a 3-μm micropipette (11). The scales for each of the different variables have been normalized to permit direct comparison of the relative rates of change of spectrin extractability with the changes in erythrocyte sodium, potassium, calcium, viscosity, and apparent membrane deformability.

plasma, erythrocyte ATP levels had declined, inextractable spectrin had increased, and the typical changes in erythrocyte sodium, potassium, and calcium were present. After an additional 3-h incubation in the presence of 30 mM adenosine, ATP was largely replenished (89% of preincubation value), and the erythrocyte sodium, potassium, calcium, and inextractable spectrin, were all markedly improved. We concluded that accumulation of inextractable spectrin is a reversible process, at least during the first 21 h of incubation in plasma.

Effect of pH and temperature on spectrin extractability. One interpretation of the previous results is that spectrin becomes inextractable in ATP-depleted erythrocytes because it aggregates and/or precipitates. Since spectrin also aggregates and precipitates at low pH (6, 25, 26) and at temperatures near 50°C (27, 28), we wondered if such precipitation would affect spectrin extractability and erythrocyte membrane deformability.

To test this we first incubated erythrocytes for 1 h at 37°C in Tris-maleate buffers at pH 5, 6, 7, and 8, and observed the effect of the pH change on erythrocyte shape, intracellular pH, erythrocyte and ghost viscosity, and spectrin extractability. Erythrocyte shape was normal in the samples incubated at pH 7 and 8. At pH 6 approximately one-half of the erythrocytes were stomatocytes. At pH 5, all of the erythrocytes formed microspherocytes. As shown in Fig. 7 erythrocyte viscosity was constant between pH 6 (apparent intracellular pH = 6.30) and pH 8 (apparent intracellular pH = 7.80), but increased dramatically at pH 5 (apparent intracellular pH = 5.32). Since the viscosity of hemoglobin solutions at concentrations approaching those in the erythrocyte are four to five orders of magnitude less (29) than the estimated bulk viscosity of the membrane (30), this increase in erythrocyte viscosity is likely due, in part, to diminished membrane deformability. This conclusion is supported by the observation that unsealed ghosts isolated from erythrocytes exposed to pH 5 show a similar increase in viscosity (Fig. 7). Spectrin extractability correlated closely with these viscosity changes. All of the spectrin was inextractable at an intracellular pH of 5.32, but <8% was inextractable when the intracellular pH was 6.30 or greater (Fig. 7). The relationship to spectrin solubility is notable. At pH 6.30, purified spectrin is mostly soluble and at pH 5.32, spectrin is completely precipitated after 60 min at 100,000 *g* (6, 25, 26). Erythrocyte actin extractability paralleled spectrin.

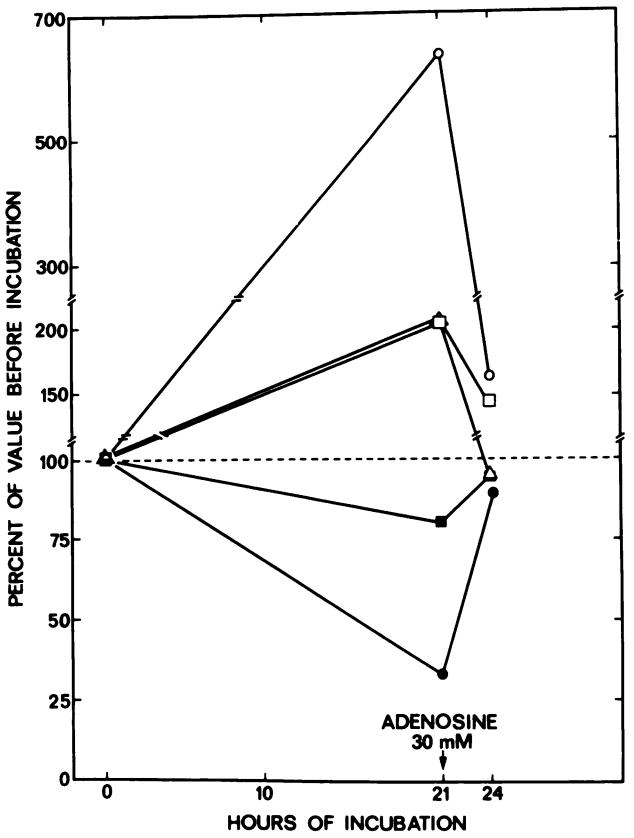

FIGURE 6 Effect of depletion and restoration of erythrocyte ATP on spectrin extractability and erythrocyte cation content. Erythrocytes were incubated in plasma at 37°C for 21 h. At that time adenosine was added (final concentration = 30 mM) and the incubation was continued for 3 more h. Aliquots were removed at 0, 21, and 24 h for measurements of inextractable spectrin (○), and erythrocyte sodium (□), potassium (■), calcium (△) and ATP (●) content. Initial values were: ATP = 1.53 mmol/liter erythrocytes, sodium = 11.7 meq/liter erythrocytes, potassium = 98.1 meq/liter erythrocytes, calcium = 16 μmol/liter erythrocytes, and inextractable spectrin = 7.8% of total ghost spectrin.

Actin was completely extractable in ghosts of erythrocytes incubated between pH 6 and 8 and almost completely inextractable at pH 5 (data not shown). Its solubility at pH 5.32 has not been reported.

An equally dramatic change in erythrocyte shape and spectrin extractability was observed on heating. Erythrocyte shape was not altered by heating for 10 min at 47°C or less. At 48.4°C all of the cells were normal except for a small number (10%) of echinocytes. In contrast, at 49.9°C, 95% of the erythrocytes were spiculated, fragmented, or microspherocytic. The shape changes were identical to those described by Schultze more than 100 yr ago (31) and are well documented (32). Spectrin extractability and erythrocyte viscosity correlated closely with the change in shape. Both increased slightly at 48.4°C and markedly at 49.9°C (Fig. 8). To test the relationship between these observations and spectrin solubility we heated chromatographically pure spectrin in a similar fashion. 57% of the spectrin sample was heat-labile and became insoluble after 10 min at 50°C (Fig. 9). The maximum change in solubility occurred at about 48.8°C. This corresponds closely to the temperatures of the maximum change in spectrin extractability (≅48.9°C) and erythrocyte viscosity (≅48.9°C) (Fig. 8). The remaining 43% of the spectrin was heat-stable to at least 54°C (data not shown). The reason for this interesting heterogeneity in an apparently homogeneous sample remains to be determined.

Effect of ATP-depletion on integral membrane protein topography. Since current evidence suggests that spectrin and/or actin may influence the lateral mobility of integral membrane glycoproteins (6, 9, 10), we wondered if the changes in spectrin produced by ATP depletion might induce a change in integral membrane protein topography. However,

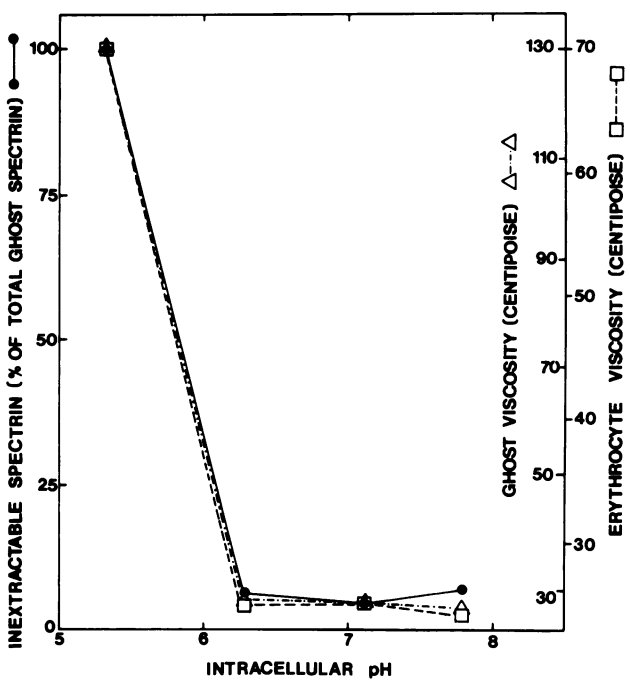

FIGURE 7 Effect of pH on erythrocyte and ghost viscosity and spectrin extractability. Erythrocytes were incubated for 1 h at 37°C in Tris-maleate buffered saline solutions at pH 5, 6, 7, and 8. After the incubation portions were removed for measurements of erythrocyte viscosity, ghost viscosity and inextractable spectrin. The experiment is described in detail in Methods. Erythrocyte and ghost viscosities were measured in a cone-plate viscometer. Erythrocyte studies were performed at a hematocrit of 80%. The results shown were obtained at a shear rate of 1.15/s. Inextractable spectrin is expressed as a percentage of the total ghost spectrin. The viscosity and inextractable spectrin scales are adjusted to permit direct comparison of the relative rates of change of these variables.

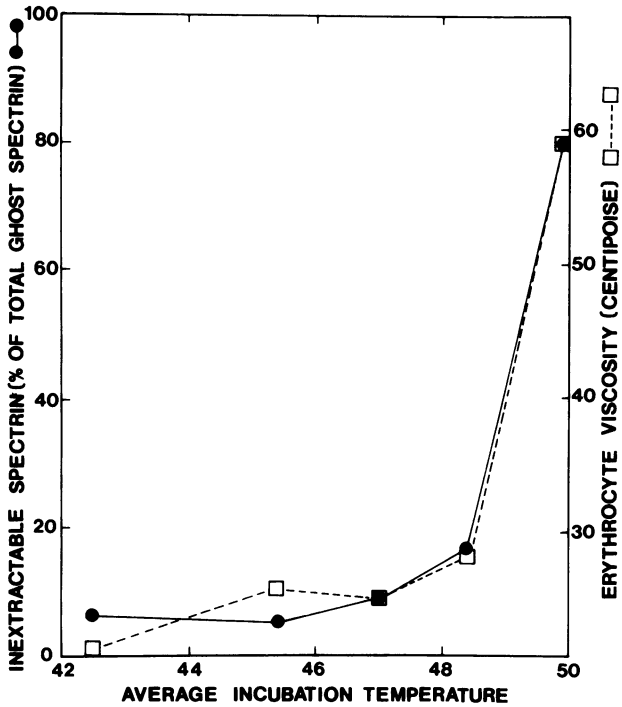

FIGURE 8 Effect of temperature on erythrocyte vicosity and spectrin extractability. Erythrocytes were incubated for 10 min in PBS at various temperatures between 42° and 50°C. After the incubation portions were removed for measurements of erythrocyte viscosity and inextractable spectrin. The experiment is described in detail in Methods. Erythrocyte viscosity was measured in a cone-plate viscometer at a hematocrit of 69%. The results shown were obtained at a shear rate of 2.30/s. Inextractable spectrin is expressed as a percentage of the total ghost spectrin. The viscosity and inextractable spectrin scales are adjusted to permit direct comparison of the relative rates of change of these variables.

freeze-cleave electron microscopy of intramembranous particles and scanning electron microscopy of concanavalin A surface-binding sites showed that the distribution of integral membrane proteins in human erythrocytes was not substantially altered by substrate deprivation and ATP depletion, even if prolonged. The distribution of intramembranous particles (Fig. 10B) and concanavalin A-binding sites (Fig. 11B) was as apparently random in erythrocytes incubated in plasma for 40–48 h at 37°C as it was in fresh, unincubated erythrocytes (Fig. 10A and 11A). Similarly the distribution of intramembranous particles (not shown) and concanavalin A-binding sites (Fig. 11C) was normal in the ATP-deficient (34), rigid (35) echinocytes from a patient with pyruvate kinase deficiency. We concluded that integral membrane protein topography is not substantially altered in metabolically depleted human erythrocytes.

DISCUSSION

Evidence that spectrin is a major determinant of erythrocyte shape and deformability. In a classic study (11), Weed and his co-workers found that apparent membrane deformability deteriorated and spheroechinocytes formed (11, 36) in ATP-depleted erythrocytes. We find that spectrin extractability is similarly altered. Ghosts of ATP-depleted erythrocytes retain spectrin on their membranes under conditions where spectrin is easily extracted from fresh ghosts (Figs. 2 and 3). This change in spectrin extractability is initiated when erythrocyte ATP concentrations reach 0–15% of normal (Fig. 4), is restored to normal when ATP is replenished (Fig. 6), and correlates with the previously reported changes in erythrocyte shape and deformability (11) (Fig. 5).

Somewhat analogous changes in shape, deformability, and spectrin extractability are observed in erythrocytes exposed to extremes of pH and temperature. When the pH in the erythrocyte interior falls below 5.5,

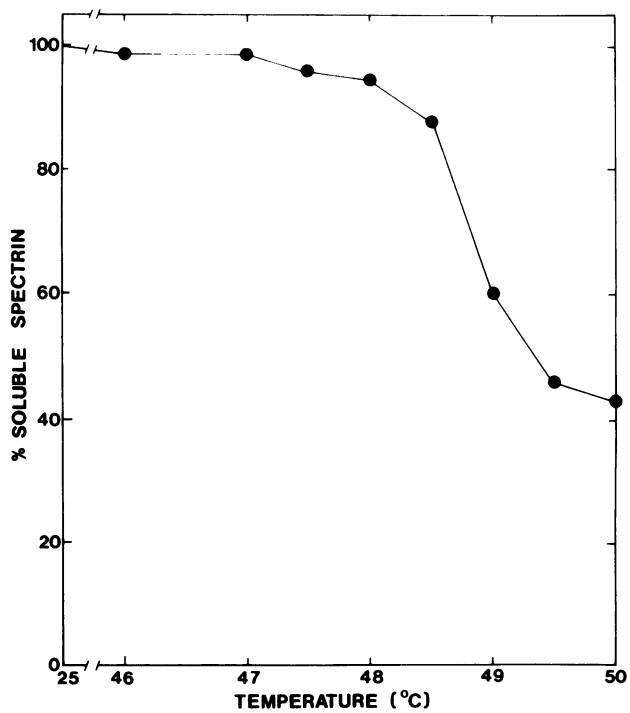

FIGURE 9 Heat denaturation of spectrin. Electrophoretically (SDS-PAGE) pure spectrin (0.3 mg/ml) in 150 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, pH 8, was heated at the temperatures shown (±0.05°C) for 10 min and then centrifuged for 60 min at 100,000 g. The percent of the original spectrin remaining in the supernate = the percent of soluble spectrin. Note that the electrophoretically homogeneous spectrin contains two subcomponents: one is heat labile at 50°C (57%), the other is heat stable (43%).

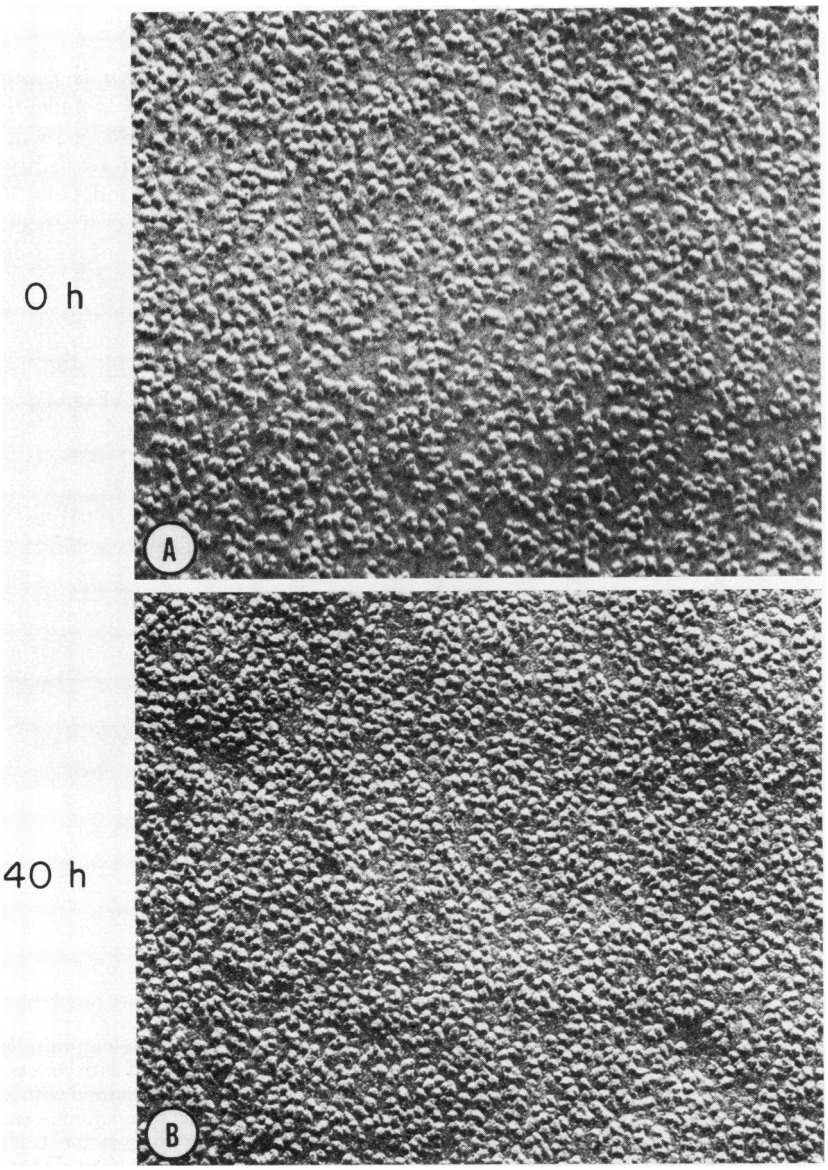

FIGURE 10 Freeze-cleave electron microscopy of fresh normal erythrocytes before (A) and after (B) 40 h of incubation at 37°C in plasma. Intramembranous particles of the metabolically depleted erythrocytes show the normal random distribution.

a pH where spectrin is aggregated and isoelectrically precipitated (6, 25, 26), erythrocyte deformability decreases and microspherocytes form coincident with a marked decrease in spectrin extractability (Fig. 7). Similarly when erythrocytes are heated to 49°C, a temperature where a subcomponent of purified spectrin is heat denatured and precipitated (Fig. 9), the erythrocytes fragment, microspherocytes form, and erythrocyte viscosity rises as inextractable spectrin accumulates (Fig. 8).[4]

These observations bolster the concept that spectrin is a major determinant of erythrocyte membrane shape and deformability. However, they are limited by the fact that the viscosity studies presented here and the micropipet techniques of Weed and co-

[4] Numerous investigators have observed that erythrocytes fragment and become less deformable than normal when heated to 48°–50°C (27, 31, 32, 37–40), but the correlation of these events with changes in the physical properties of spectrin has only recently been appreciated (27, 30, 40–42).

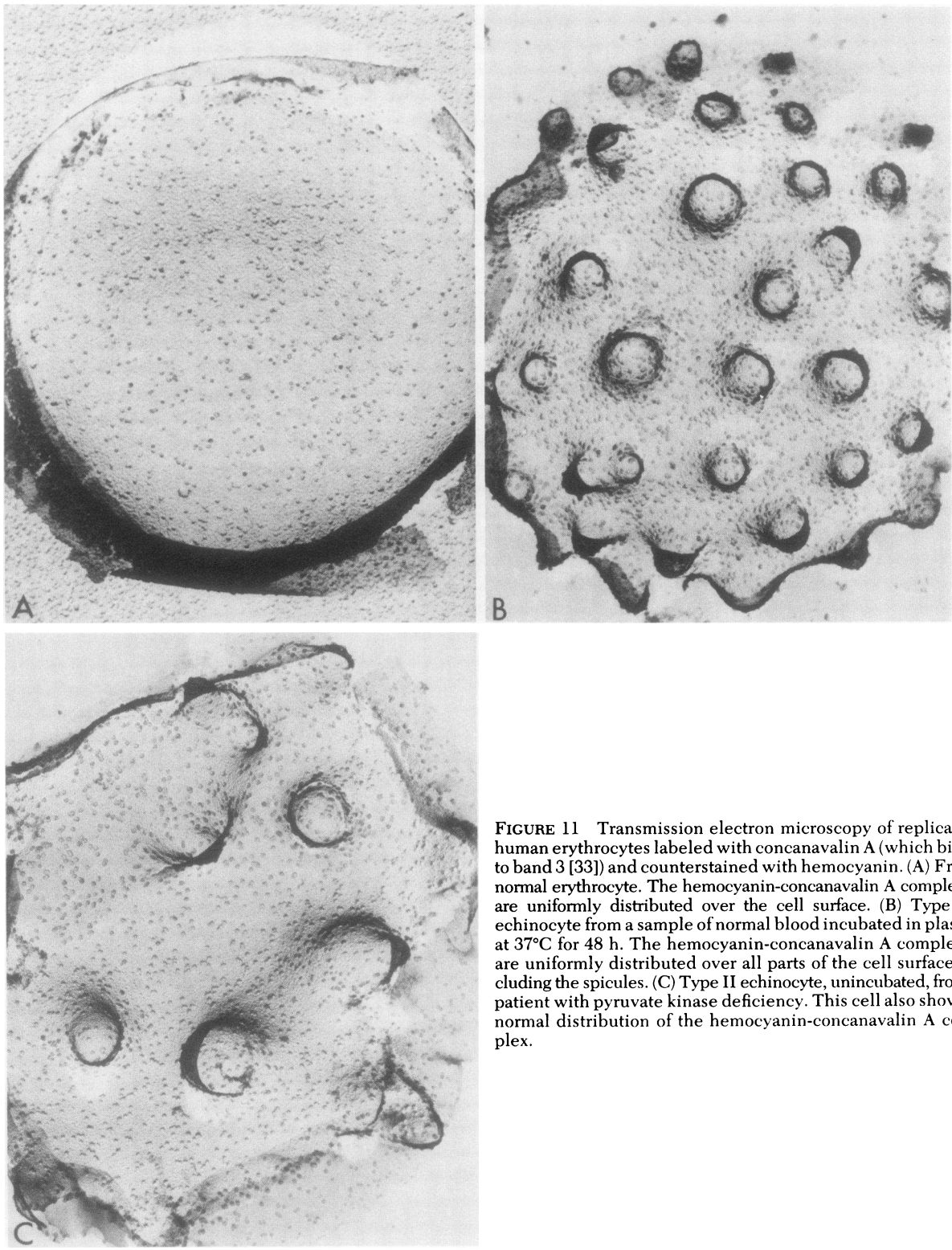

FIGURE 11 Transmission electron microscopy of replicas of human erythrocytes labeled with concanavalin A (which binds to band 3 [33]) and counterstained with hemocyanin. (A) Fresh normal erythrocyte. The hemocyanin-concanavalin A complexes are uniformly distributed over the cell surface. (B) Type III echinocyte from a sample of normal blood incubated in plasma at 37°C for 48 h. The hemocyanin-concanavalin A complexes are uniformly distributed over all parts of the cell surface including the spicules. (C) Type II echinocyte, unincubated, from a patient with pyruvate kinase deficiency. This cell also shows a normal distribution of the hemocyanin-concanavalin A complex.

workers (using 3.0–3.5-μm diameter micropipets) (11) do not clearly measure intrinsic membrane deformability, since the shape change from a biconcave disk to a microspherocyte or spheroechinocyte induced by low pH, heat, and ATP-depletion contributes to an unknown degree to the measured increase in viscosity or micropipete aspiration pressure (43). We have used the term "apparent membrane deformability" in this study to indicate this uncertainty. It appears likely that a true decrease in intrinsic membrane deformability does occur in metabolically depleted erythrocytes since LaCelle has shown that: (*a*) sphering alone accounts for less than half the measured cellular deformability of erythrocytes incubated for 24 h at 37°C in plasma (44) and (*b*) membrane deformability decreases during erythrocyte storage (a form of metabolic depletion) before any discernable change in cell shape (45). However, direct proof is lacking.

The reason why spectrin becomes inextractable in ATP-depleted cells remains to be determined. As noted earlier there is increasing evidence that spectrin and actin form a protein meshwork at the cytoplasmic membrane surface (6–8, 27) (although this is not uniformly accepted [4]), but virtually nothing is known about how this meshwork is organized or how it interacts with the lipid bilayer and its integral membrane protein constituents. Decreased spectrin extractability could result from increased spectrin-spectrin or spectrin-actin interactions within the two-dimensional protein meshwork or it could result from increased binding of spectrin to integral membrane proteins or lipids. Either process would logically reduce membrane flexibility and might alter cell shape. At present there is insufficient evidence to choose between these alternatives. We recently observed that low ionic strength extracts of ATP-depleted erythrocytes contain high molecular weight aggregates of spectrin and actin which are not found in similar extracts from fresh erythrocytes.[5] This suggests that decreased extractability and deformability might be due to increased cross-linking of the spectrin-actin network. On the other hand, Palek and his co-workers (46, 47) find disulfide cross-linked aggregates of spectrin, actin, and band 3 in membranes of ATP-depleted erythrocytes and in fresh erythrocyte membranes treated with calcium; implying that increased interaction of spectrin with band 3 may be a primary defect in these cells. Of course, these processes are not mutually exclusive and both may be operative.

Role of the spectrin-actin skeleton in erythrocyte survival. The combined evidence of these and other recent studies (5–9, 27, 30, 40–43, 46–56) supports the concept that the strength, shape, and flexibility of the erythrocyte membrane are normally determined by the meshwork of spectrin and actin which laminates the inner membrane surface. We suggest that this membrane skeleton is a dynamic structure which must be carefully regulated for the erythrocyte to survive in the circulation. Conditions such as ATP depletion that foster interactions of membrane skeletal components with each other or with integral membrane "anchoring" proteins or lipids would lead to membrane rigidity and splenic sequestration. Conditions that interfere with these interactions would diminish the erythrocytes inability to tolerate circulatory shearing forces and would promote membrane fragmentation and hemolysis. The mechanism for such regulation is unknown. Weed and his co-workers (11, 49) have suggested that control of membrane calcium concentrations by formation of inactive calcium-ATP complexes may be critical. Alternatively ATP may preserve membrane deformability through a protein kinase-phosphoprotein phosphatase system (54, 56–62) that regulates the proportion of spectrin in the phosphorylated (i.e., deformable and biconcave) state. Others have proposed a contractile spectrin-actin system analogous to actomyosin (5, 53, 63–65) in which ATP would maintain normal organization and fuel the contraction process. Various combinations of these hypotheses are also possible in which divalent cations modulate spectrin phosphorylation, phosphorylation modifies the sensitivity of spectrin to precipitation by calcium, or phosphorylation and divalent cations influence "actospectrin" interactions. Any of these ATP-dependent regulatory mechanisms could conceivably generate inextractable spectrin in the absence of ATP. In addition, inextractable spectrin could arise from oxidation-induced cross-linking (47) since ATP-depleted erythrocytes lack substrate for the hexosemonophosphate shunt and are oxidant sensitive. Studies comparing these alternatives are currently in progress.

[5] Lux, S. E., B. Pease, and K. M. John. Unpublished observations.

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