

Biosynthesis of Spectrin and Its Assembly into the Cytoskeletal System of Friend Erythroleukemia Cells

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Abstract. Friend erythroleukemia cells, grown in the presence of dimethyl sulfoxide for 3 d, synthesize unequal amounts of the two chains (α and β) of spectrin with ~ 15 – 30% more β than α spectrin. When cells were ruptured by nitrogen cavitation, nascent α and β spectrin were found to be associated with a membranous cell fraction and were not detected in the soluble cytoplasmic cell fraction. Nascent membrane-bound spectrin appeared not to be protected by membranes, since it was susceptible to trypsin degradation in the absence of detergent. On fractionation of cells with 1% Triton X-100, more (1.75-fold) nascent spectrin was found in the Triton-soluble fraction than in the Triton-insoluble fraction (cytoskeleton). In the Triton-soluble fraction, there was 55% more nascent β spectrin than α spectrin, while the cytoskeleton contained nearly equal amounts of α and β spectrin. Cells were pulse-labeled with L-[35 S]methionine for 2 min and chase incubated for varying periods of time from 15 to 90 min with nonradioactive L-methionine. Radioactive spectrin accumulated in the Triton-soluble fraction

for the first 15 min of chase incubation and then dropped by 25% in the next hour. By contrast, the amount of radioactive spectrin in the Triton-insoluble fraction rose gradually for 1 h of the chase period. This indicates that, in Friend erythroleukemia cells, a pool of membrane-bound spectrin containing an excess of the β polypeptide is used to form the cytoskeletal system which is composed of equal molar amounts of α and β spectrin. The location of spectrin was determined by immunoelectron microscopy. Small amounts of spectrin were detected in cells not treated with dimethyl sulfoxide and in these cells it was located on the surface membrane and within the cytoplasm. On treatment with dimethyl sulfoxide, complex vacuolar structures containing viruses appeared in the cells. In cells treated with dimethyl sulfoxide for 3 d 30% of the spectrin was near the outer membrane and 25% was associated with vacuolar structures, whereas in cells treated for 5 and 7 d the majority of spectrin (57–61%) was located in the vacuolar areas.

SPECTRIN is the major component of the erythrocyte membrane, accounting for $\sim 30\%$ of total membrane protein (21). It is made up of two non-identical subunits, α (M_r 240,000) and β (M_r 220,000), which interact noncovalently to form $\alpha\beta$ dimers. Spectrin, together with actin, forms part of the submembranous cytoskeletal network of the erythrocyte and it is connected to the plasma membrane via the protein ankyrin, which binds to the intramembranous protein band 3 (9). Recently, the presence of spectrin and spectrin-like proteins has been reported in numerous non-erythroid cells. In these cells the spectrin-like proteins are also found on the cytoplasmic side of the membrane, suggesting that these proteins probably play a similar structural role in non-erythroid cells (2, 7, 10, 13, 14, 19, 25).

The synthesis of spectrin and its assembly into the cytoskeleton has been studied in chicken erythroid cells during embryo differentiation (3, 4) and in dimethyl sulfoxide (DMSO)-treated¹ Friend erythroleukemia cells (FELC)

(12). In a previous study (24), we showed that DMSO treatment of FELC caused a staggered increase in the synthesis of a number of membrane proteins. Spectrin synthesis was maximal on the third day of DMSO treatment and declined thereafter, whereas the synthesis of bands 3 and 4 was increased on the fourth day and that of bands 6 and 5 on the fifth and sixth day of DMSO treatment, respectively. Glenney and Glenney (12) reported that in uninduced FELC both erythroid spectrin and non-erythroid fodrin are present at low levels and that their syntheses are both induced by treatment with DMSO. In this study it was also shown that FELC produce larger amounts of β spectrin than of α spectrin. By contrast, chicken erythroid cells synthesize two- to three-fold more α spectrin than β spectrin (4). Newly synthesized chicken erythroid α and β spectrin is present in both a Triton-soluble cell fraction and a Triton-insoluble cytoskeleton, but in the Triton-soluble fraction there is a several-fold excess of α spectrin relative to β spectrin, while there are stoichiometric amounts present in the cytoskeleton. In vitro translation of spectrin mRNA results in the synthesis of three times more α than β spectrin. 95% of the in vitro-translated

1. *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide; FELC, Friend erythroleukemia cells; STM, 0.25 M sucrose in 5 mM Tris-HCl, pH 7.4, 1 mM MgCl₂.

β spectrin and an equimolar amount of α spectrin bind post-translationally to spectrin-depleted rabbit red blood cell membranes. The excess nascent α spectrin remains unbound, indicating that the assembly of α spectrin is limited by the availability of β spectrin (22). A comparison of ankyrin synthesis to that of spectrin showed that the kinetics of ankyrin synthesis are similar to that of β spectrin (23). These data suggest that the assembly of spectrin in chicken erythroid cells is regulated by the limited amounts of β spectrin and ankyrin. This may not, however, be the case in all cells, since FELC appear to produce more β than α spectrin (12).

In chicken erythroid cells, excess ankyrin and α and β spectrin, present in the Triton-soluble pool, are rapidly catabolized. This is in contrast to nascent ankyrin and spectrin which is assembled into the cytoskeleton and shows no detectable turnover (23). This has led to the alternate suggestion that the assembly of cytoskeletal proteins is regulated by the availability of protein receptors and that the ultimate receptor is the anion transport protein (band 3) which is an integral component of the plasma membrane (18).

To clarify the mechanisms of spectrin assembly we have studied, at a time of maximal spectrin production by FELC (3 d of DMSO treatment), the rate of synthesis of α and β spectrin, the intracellular location of the nascent spectrin chains, and their insertion into the cytoskeletal network.

Materials and Methods

Cells

Friend erythroleukemia cells (FELC), line 745, were maintained in suspension as described earlier (24). To induce differentiation, cells were seeded at a density of 5×10^4 cells/ml and grown in the presence of 1.8% (vol/vol) DMSO for 3 d. The cells were a kind gift from Dr. A. O. Pogo, New York Blood Center, who obtained them from Dr. C. Friend (Center for Experimental Cell Biology, Mount Sinai School of Medicine, New York).

Preparation of Spectrin and Spectrin Antibodies

Spectrin was purified from mouse erythrocyte membranes by slight modifications to the method of Ungewickell and Gratzer (28). Briefly, mouse red blood cells were lysed in 5 mM sodium phosphate, pH 8.0, extracted with 0.3 mM sodium phosphate, pH 7.6, containing 100 U/ml Trasylol (Aprotinin, FBA Pharmaceuticals, New York, NY) for 20 min at 37°C, followed by centrifugation at 80,000 *g* for 30 min. The supernate, which consists mainly of spectrin and actin, was concentrated by ultrafiltration, and then further purified on a Sepharose 4B column. Purified spectrin was coupled to CNBr-activated Sepharose 4B, and used for the purification of spectrin antibody.

To prepare spectrin antibody, the concentrated proteins in the 80,000 *g* supernate were separated by gradient (5–16%) PAGE. The α and β bands of spectrin were excised from the gels, homogenized with a small amount of water using a Brinkmann Polytron, mixed with an equal volume of Freund's complete adjuvant (Miles Laboratories, Inc., Elkhart, IN) and injected into rabbits subcutaneously for several weeks at weekly intervals. After a positive response was obtained, serum was collected. It was found that sera from both rabbits injected with either the α or the β band reacted with both bands of spectrin. The sera were therefore combined and spectrin antibody was purified by affinity chromatography using spectrin coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The antibody thus purified precipitated only radioactive spectrin (bands α and β) from whole cell homogenates of FELC which have been metabolically labeled with L-[³⁵S]methionine. The amount of antibody required to quantitatively immunoprecipitate 10 μ g of mouse erythrocyte spectrin was determined by titration and based on this a slight excess was used in the immunoprecipitation of spectrin from FELC.

Radioactive Labeling

Cells were washed several times with phosphate buffered saline (PBS) (10 mM NaPO₄, pH 7.4, 140 mM NaCl) and then were suspended at 1×10^7 cells/ml in methionine-free minimal essential medium (Flow Laboratories, Inc., McLean, VA) supplemented with 10% fetal calf serum. In continuous labeling experiments, cells were preincubated at 37°C for 15–20 min before the addition of 10–20 μ Ci/ml L-[³⁵S]methionine (>800 Ci/mmol; New England Nuclear, Boston, MA). In pulse-chase incubations, 100 μ Ci/ml of L-[³⁵S]methionine was used and at the end of the pulse period 1 mM of unlabeled methionine was added. All incubations were carried out at 37°C with gentle mixing of cell suspensions. At various times, aliquots were taken and the samples were processed as described below.

Cell Fractionation

All solutions used in cell fractionation contained protease inhibitors at the following concentrations: 200 U/ml Trasylol, 1 mM phenylmethylsulfonyl fluoride (Calbiochem-Behring Corp., San Diego, CA), and 0.1 mM L-1-tosylamide-2-phenylmethylchloromethyl ketone (Sigma Chemical Co., St. Louis, MO). All operations were carried out at 4°C.

(a) *Membranous and Soluble Cytoplasmic Cell Fractions.* After incubation with L-[³⁵S]methionine, cells were washed several times with 0.25 M sucrose in 5 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ (STM) and resuspended in STM at $1-10 \times 10^7$ cells/ml. Cells were lysed by nitrogen cavitation at 400 psi for 10 min. Lysed cells were centrifuged at 105,000 *g* for 30 min in order to obtain a membranous pellet and a soluble cytoplasmic supernate. To obtain other cell fractions, lysed cells were centrifuged at 480 *g* for 10 min, at 9,750 *g* for 10 min, and at 105,000 *g* for 30 min. When membranes were treated with trypsin, protease inhibitors were omitted from the solutions.

(b) *Triton-soluble and -insoluble (Cytoskeletal) Cell Fractions.* Cytoskeletal and Triton-soluble fractions were prepared by treatment of cells with Triton X-100 as described by Cervera et al. (8). After incubation with L-[³⁵S]methionine, cells were washed with PBS, pH 7.4, and gently suspended in extraction buffer (10 mM Pipes, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 0.3 M sucrose). Triton X-100 was added to a final concentration of 1% and the samples were placed on ice for 3 min. The samples were then centrifuged at 1,200 *g* for 3 min, the resulting pellets were washed once with extraction buffer containing 1% Triton X-100, and the supernatant fractions were combined (Triton-soluble fraction). The insoluble material contains the bulk of the cytoskeletal fraction.

Protease Treatment of the Membrane Fraction

The membrane fractions were suspended in STM and aliquots were incubated with and without trypsin (0.5 mg/ml), in the presence and absence of 1% Triton X-100, at room temperature for 20 min. The incubation was stopped by the addition of SDS to a final concentration of 2% and by heating at 100°C for 3 min. The spectrin present in the samples was immunoprecipitated as described below.

Immunoprecipitation

Cells ($2-5 \times 10^7$) or cell fractions were suspended in 200–250 μ l of 2% SDS and heated at 100°C for 3 min to dissolve proteins. The samples were then diluted to 0.1% SDS by the addition of NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.02% sodium azide) containing 0.5% Triton X-100, 300 U/ml Trasylol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM L-1-tosylamide-2-phenylmethylchloromethyl ketone. Particulate material was removed by centrifugation at 12,000 *g* for 10 min and the supernates were carefully decanted and used for the immunoprecipitation of spectrin. Affinity-purified anti-spectrin (60 μ l) was added and samples were incubated overnight at 4°C with gentle shaking. Immunoprecipitation was aided by the addition of 100 μ l of 100 mg/ml Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) and the samples were incubated for an additional 2–3 h at 4°C. Samples were centrifuged and the Protein A-immune complex was washed six times with NET buffer containing 0.1% SDS, 0.5% Triton X-100, and 300 U/ml Trasylol. The immune complex was eluted from Protein A-Sepharose by heating at 100°C for 3 min with electrophoresis sample buffer (0.34 M Tris-phosphoric acid, pH 6.7, containing 6 M urea, 7.1 mM EDTA, 71 mM dithiothreitol, and 2.85% SDS). Protein A-Sepharose was removed by centrifugation and the radioactivity in an aliquot of the supernatant fraction was determined to measure total spectrin

radioactivity. The remainder of the supernatant fraction was subjected to SDS PAGE to obtain the radioactivity of the separated α and β spectrin chains.

Electrophoresis and Fluorography

Proteins were analyzed by electrophoresis on SDS polyacrylamide slab gels according to the method of Maizel (20). Gels contained 5–16% acrylamide gradients with a 3.75% acrylamide stack. Radioactive proteins were located by fluorography (5, 17) and the radioactive areas of the gel were excised and solubilized by heating at 70–75°C for 3 h with 20% perchloric acid and 20% hydrogen peroxide. The samples were cooled to room temperature before the addition of Scinti Verse II (Fisher Scientific Co., Pittsburgh, PA). Radioactivity was determined by liquid scintillation spectrometry.

Amino Acid Analysis

α and β spectrin from mouse red cell membrane extracts were eluted from SDS polyacrylamide gels according to the method of Hager and Burgess (16). The proteins were precipitated with acetone and hydrolyzed in 6 N HCl. Amino acid composition was determined in a Beckman amino acid analyzer 6300 using ninhydrin reaction program No. 1 (Beckman Instrument Co., Palo Alto, CA).

Electron Microscopy

Cells or cell fractions were fixed in suspension with 2% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer, pH 7.4 for 30 min at 4°C, followed by centrifugation. The pellets obtained were postfixed with 1% OsO₄, stained with 2% uranyl acetate, dehydrated in graded ethanol, and embedded in Epon 812. Thin sections were obtained using a Sorvall Porter-Blum MT2-B ultramicrotome. Sections were stained with uranyl acetate and lead citrate and examined in a Philips 410 microscope.

Immunocytochemistry

The cells were processed for electron microscopy, embedded in Lowicryl K4M and immunocytochemically labeled as described by Bendayan (1). Affinity-purified rabbit anti-spectrin, diluted 1:10 or 1:50 with PBS containing 1% bovine serum albumin, was the first antibody used and in the second step goat anti-rabbit IgG coupled to 20-nm colloidal gold particles (Janssen Life Science, Piscataway, NJ) was used. We performed two immunocytochemical controls: (a) incubation of the tissues with non-immune rabbit serum instead of anti-spectrin, and (b) incubation of the tissue sections directly with goat anti-rabbit IgG gold complex.

Other Methods

Western immunoblots, using affinity-purified anti-spectrin or a rabbit antibody against mouse fodrin (generously provided by Dr. J. Glenney, Salk Institute for Biological Sciences, San Diego, CA) were performed according to the method of Burnette (6). [¹²⁵I]-labeled protein A (>30 mCi/mg, Amersham Corp., Arlington Heights, IL) was used as a second reagent. Radioactive areas were detected by autoradiography.

Mouse brain membranes were prepared and demyelinated by the method of Goodman et al. (15).

Results

Amino Acid Composition of α and β Spectrin

The biosynthesis of the individual chains of spectrin was measured by following the incorporation of L-[³⁵S]methionine into the isolated proteins. It was therefore necessary to determine the L-methionine content of these polypeptides. The amino acid composition of mouse erythrocyte spectrin in mole % is shown in Table I. Both α and β spectrin contain similar amounts of L-methionine: 1.7 moles % in the α chain and 1.4 moles % in the β chain. The composition of the other amino acids in the α and β chains is similar with perhaps β spectrin containing a larger mole % of proline and lysine. In

Table I. Amino Acid Composition of α and β Spectrin (in mole %)

Amino Acid	α Spectrin	β Spectrin	Human Spectrin (21)
Asp	13.0	11.0	10.8
Thr	5.3	5.8	3.9
Ser	10.2	8.0	5.2
Glu	18.0	16.2	20.1
Pro	1.6	4.6	2.1
Ala	9.0	7.8	9.3
Val	4.8	6.0	4.8
Met	1.7	1.4	1.1
Ile	4.2	3.2	3.5
Leu	11.0	11.1	14.2
Tyr	3.0	3.2	2.1
Phe	3.8	4.0	3.4
His	3.0	2.6	3.0
Lys	5.0	9.0	7.1
Arg	6.2	5.8	6.4
Gly	ND*	ND*	4.7

*The content of glycine in α and β spectrin was not determined, because the proteins were eluted from gels run in Tris-glycine buffer. Some of the glycine, present in the buffer, was retained during the isolation of spectrin and yielded artificially high levels of glycine.

addition, the composition of mouse spectrin is strikingly similar to that of human erythrocyte spectrin (21), whose amino acid composition is included in Table I for comparison.

Synthesis of α and β Chains of Spectrin

The studies on the biosynthesis of spectrin were all performed on cells grown in the presence of DMSO for 3 d, since previously we have shown that this is a time of maximal spectrin production (24). To study the rate of synthesis of the individual α and β spectrin polypeptides, the cells were incubated with L-[³⁵S]methionine at 37°C for varying periods, from 15 min to 2 h, and the incorporation of radioactivity into α and β spectrin was measured. During the incubation period there was a near linear, increasing amount of incorporation of radioactivity into both α and β spectrin. At all times, however, there were unequal amounts of radioactivity in the two spectrin chains with a 15–30% excess of β chain radioactivity (Fig. 1). This indicates that there is unequal synthesis of the two spectrin chains with a greater amount of β chain being produced.

Specificity of Spectrin Antibody

FELC contain both spectrin and non-erythroid fodrin (12). To ascertain that the spectrin antibody used in these studies did not react with fodrin, it was tested by Western immunoblotting of mouse brain membrane proteins which contain fodrin but not spectrin. As shown in Fig. 2, the antibody to spectrin reacted with proteins from FELC but not from mouse brain membranes. As a control, antibody against mouse fodrin was also tested in this system and it reacted with fodrin from both FELC and mouse brain membranes. Thus, FELC contain fodrin but the spectrin antibody does not react with it.

Intracellular Location of Nascent Spectrin

To determine the cellular location of nascent spectrin, FELC

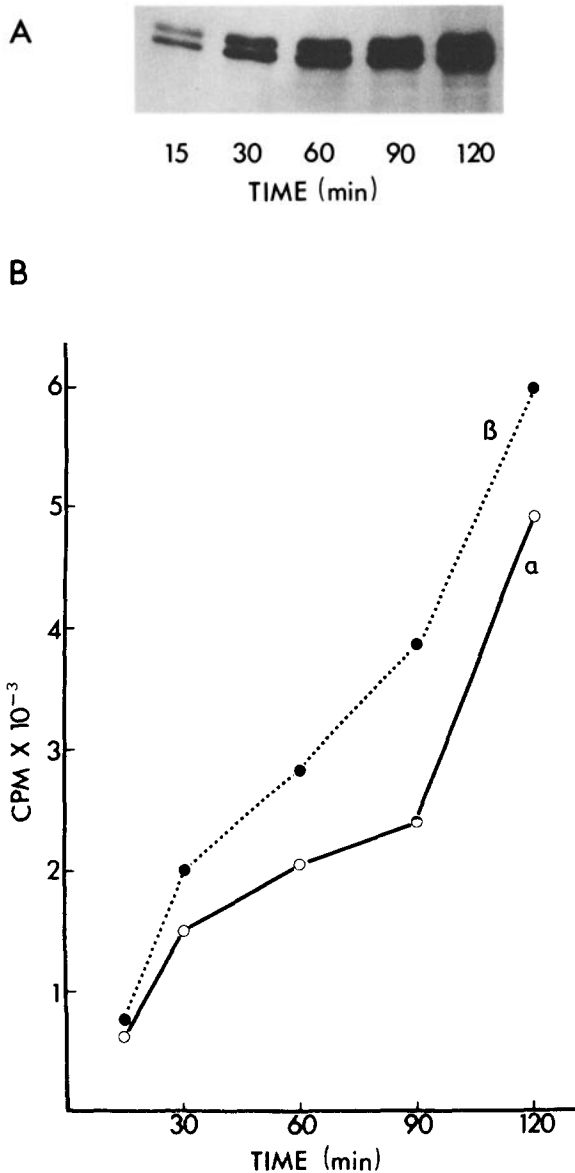


Figure 1. L-[³⁵S]methionine incorporation into α and β spectrin. (A) FELC, at a concentration of 1×10^7 cells/ml in methionine-free medium with 10% fetal calf serum, were incubated with L-[³⁵S]methionine (10 μ Ci/ml) for 2 h at 37°C. Aliquots were taken at 15, 30, 60, 90, and 120 min, cells were washed with PBS, and heated with 2% SDS for 3 min at 100°C. Spectrin was isolated by immunoprecipitation, followed by SDS PAGE and fluorography. A portion of the fluorogram is shown. (B) The α and β bands of spectrin were excised from the gel shown in the above fluorogram. The gel slices were solubilized and the radioactivity in each band was determined. (●) Radioactivity in β spectrin; (○) radioactivity in α spectrin.

were disrupted by nitrogen cavitation and fractionated into a membranous pellet (material which sedimented at 105,000 g) and soluble cytoplasmic cell fraction. Spectrin was isolated from both of these cell fractions by immunoprecipitation, followed by SDS PAGE and fluorography. At all times during a 3-h incubation with L-[³⁵S]methionine, radioactive spectrin was found to be associated only with the 105,000 g pellet, and was not found in the cytoplasmic fraction (Fig. 3).

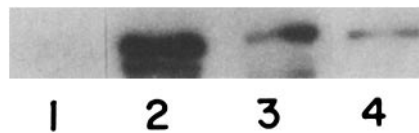


Figure 2. Specificity of spectrin antibody. Proteins from the Triton-insoluble fraction of FELC and from mouse brain membranes were separated by SDS PAGE and analyzed by Western immunoblotting using either spectrin antibody or a rabbit antibody against mouse fodrin, followed by [¹²⁵I]-labeled protein A. An autoradiogram is shown. (Lane 1) Mouse brain membrane proteins probed with anti-spectrin; (lane 2) FELC proteins probed with anti-spectrin; (lane 3) mouse brain membrane proteins probed with anti-fodrin; (lane 4) FELC proteins probed with anti-fodrin.

The membranous material was subfractionated by differential centrifugation into fractions which sedimented at 480 g in 10 min, 9,750 g in 10 min, and at 105,000 g in 30 min. Radioactive spectrin was present in all of these fractions with ~37% of the total spectrin in the homogenate recovered in the 480 g fraction, 24% in the 9,750 g fraction, and 34% in the 105,000 g pellet. Electron microscopy of the isolated cell fractions showed that the 480 g fraction contained unbroken cells, nuclei, and some membranous material, and the 9,750 g fraction was enriched in mitochondria but also contained other membranous organelles. The 105,000 g fraction was the most homogeneous (see Fig. 4) and consisted of a mixture of closed membrane vesicles and vesicles with attached polysomes. Some free polysomes and lysosomes were also apparent.

To determine whether nascent spectrin was contained within the membranous vesicles or was associated with the outer surface of the membrane, the samples were treated with trypsin (0.5 mg/ml) in the presence and absence of 1% Triton X-100. Nascent spectrin was susceptible to trypsin degradation even in the absence of Triton X-100 (Fig. 5), indicating that although it is associated with the membrane vesicles it does not appear to be protected by them; this suggests that spectrin is not found inside the membranous vesicles but is associated on the outer surface.

Nascent α and β Spectrin in Triton-soluble and -insoluble Cell Fractions

FELC were also fractionated in the presence of 1% Triton

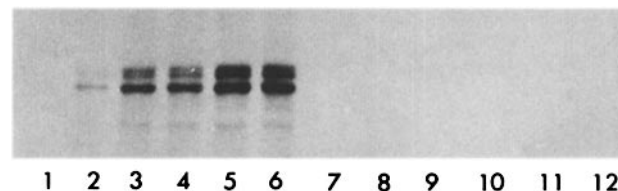


Figure 3. Nascent spectrin in soluble cytoplasmic and membrane cell fractions. FELC were incubated with L-[³⁵S]methionine as described in legend to Fig. 1. At various times from 15 to 180 min, aliquots were taken, cells were washed with STM, and lysed by nitrogen cavitation at 400 psi for 10 min. Lysed cells were centrifuged at 105,000 g for 30 min in order to obtain a soluble cytoplasmic and a membranous cell fraction. Spectrin from these cell fractions was isolated by immunoprecipitation, followed by SDS PAGE and fluorography. (Lanes 1-6) Spectrin in the membrane fraction (105,000 g pellet) at 15, 30, 60, 90, 120, and 180 min. (Lanes 7-12) Spectrin in the soluble cell fraction at the same time points as those described above.

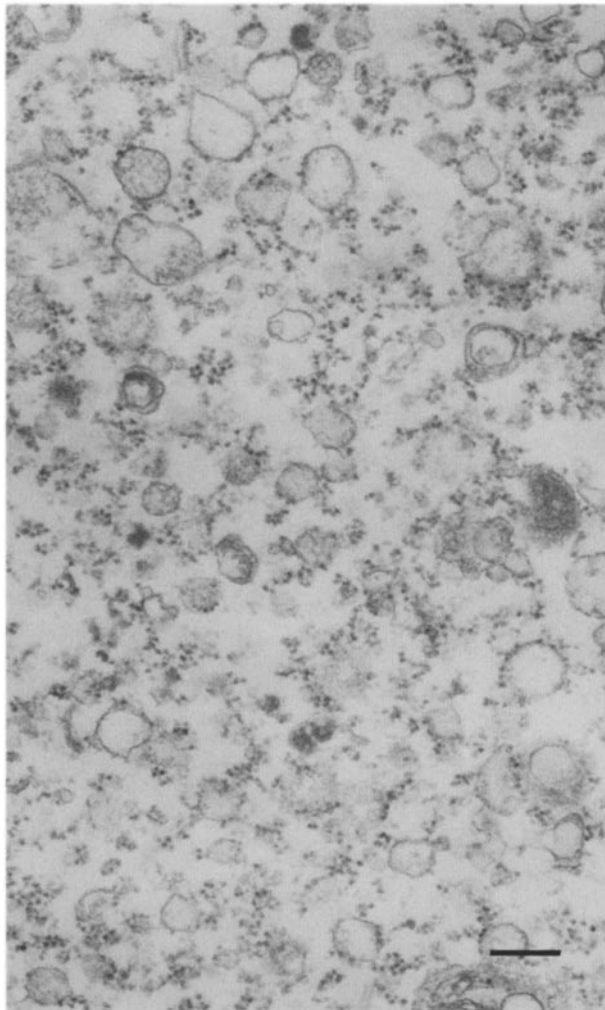
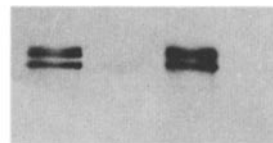


Figure 4. Electron micrograph of the 105,000 g (membrane) pellet. FELC, labeled for 30 min with L-[³⁵S]methionine, were lysed as described in legend to Fig. 2. Lysed cells were centrifuged at 480 g and 9,750 g to eliminate nuclei and mitochondria and the post-mitochondrial supernate was centrifuged at 105,000 g for 30 min. The electron micrograph shows the material in the 105,000 g pellet. Bar, 250 nm.

X-100 in order to study the distribution of spectrin in the cytoskeletal (Triton-insoluble) and Triton-soluble cell fractions. The morphology of untreated cells and cells extracted with 1% Triton X-100 is shown in Fig. 6. The untreated cells contain a typically large nucleus, and the cytoplasm, which is surrounded by an intact plasma membrane, contains well-defined mitochondria, endoplasmic reticulum, and many free ribosomes. Treatment with Triton X-100 depletes the cytoplasm of organelles and an amorphous insoluble network, presumably the cytoskeletal system, accumulates around the nucleus.

Cells were incubated with L-[³⁵S]methionine for various periods of time up to 2 h and then fractionated with Triton X-100 into a cytoskeletal (Triton-insoluble) and a Triton-soluble fraction. At all times, there was 50–75% more radioactive spectrin in the Triton-soluble than in the cytoskeletal fraction (Fig. 7). In addition, the ratios of the two spectrin bands (α and β) were found to be markedly different in these cell fractions. The nascent spectrin in the Triton-soluble



Trypsin	–	+	–	+
Triton	–	–	+	+
Lane	1	2	3	4

Figure 5. Susceptibility of spectrin in the membrane fraction to trypsin. A 105,000 g pellet was prepared as described in legend to Fig. 3 and the material was suspended in STM. Aliquots were incubated with and without trypsin (0.5 mg/ml) in the absence and presence of 1% Triton X-100 for 20 min at room temperature. Incubations were terminated by heating at 100°C for 3 min in the presence of 2% SDS. Spectrin was isolated by immunoprecipitation and SDS PAGE, followed by fluorography. Lane 1 shows the radioactive α and β spectrin bands from samples not treated with trypsin nor detergent; lane 2, that treated with trypsin in the absence of detergent; lane 3, that treated with detergent but not with trypsin; and lane 4, that treated with both trypsin and detergent.

fraction contained more radioactive β spectrin than α spectrin (Fig. 8), while in the cytoskeletal cell fraction the radioactivities in the two spectrin bands were similar (Fig. 9) or contained a slight excess of radioactive α spectrin. The average ratios of radioactive α to β spectrin from several experiments were 0.74 ± 0.16 in the Triton-soluble fraction and 1.17 ± 0.26 in the cytoskeletal fraction.

To determine whether or not nascent spectrin moved, with time, from one cellular compartment to another, FELC were pulse-labeled for 2 min with L-[³⁵S]methionine and then were chase incubated with nonradioactive methionine for various periods of time up to 90 min. At each time point the cells were fractionated into a cytoskeletal and a Triton-soluble fraction and the radioactivity of spectrin in each fraction was determined. At the end of the 2-min pulse incubation, the immunoprecipitate contained radioactive incomplete spectrin polypeptide chains. This was noted especially in the Triton-insoluble fraction. During the chase period, the incomplete polypeptide chains disappeared and radioactivity was detected in completed α and β bands of spectrin. During the first 15 min of the chase period, there was a rapid three-fold increase of radioactive spectrin in the Triton-soluble fraction followed by a 25% decrease in the subsequent chase periods. Radioactive spectrin was also quickly incorporated into the Triton-insoluble fraction, but it did not reach maximal incorporation into this fraction until 60 min of the chase period. At all time points, there was more radioactive spectrin in the Triton-soluble than in the Triton-insoluble fraction. The kinetics suggest that nascent spectrin quickly enters a Triton-soluble fraction and then a portion of it is incorporated into a cytoskeletal fraction. At the end of a 90-min chase period, ~65% of the pulse-labeled spectrin remained in the Triton-soluble fraction (Fig. 10).

An analysis of the amounts of radioactive α and β spectrin in the cytoskeletal and Triton-soluble fraction at various times of the chase period showed that at all times there was ~14% more radioactive β spectrin than α spectrin in the Triton-soluble fraction. The reverse occurred in the cytoskeletal fraction which contained 35% more radioactive α

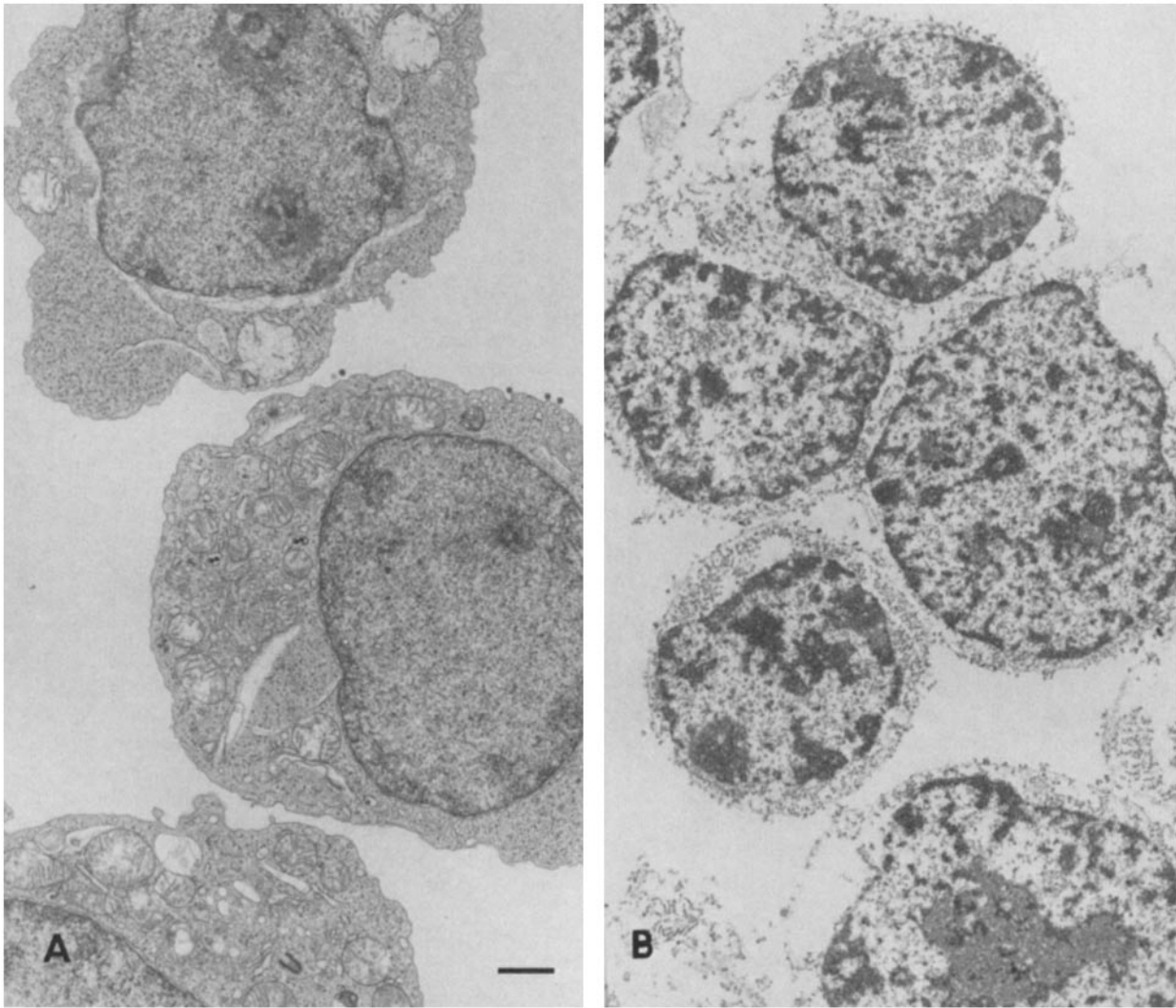


Figure 6. Electron micrograph of cells treated with Triton X-100. FELC were extracted with 1% Triton X-100 and prepared for electron microscopy as described in Materials and Methods. (A) Untreated FELC; (B) Triton-treated FELC. Bar, 1,000 nm.

than β spectrin (Fig. 10). This unequal distribution of radioactive α and β chains, in the Triton-soluble and insoluble fractions in pulse-chase conditions, confirms the existence of a larger amount of β spectrin in the Triton-soluble fraction and also suggests that some of the pool of preformed β spectrin is incorporated in the cytoskeletal system since the specific radioactivity of the β chain is less than that of the α chain.

Intracellular Pools of α and β Spectrin

Further evidence that there is an excess of β spectrin in the Triton-soluble pool was obtained by labeling cells for 18 h with L-[35 S]methionine. Equilibration of protein radioactivity occurs after such a long-term incubation. The amount of radioactive α and β spectrin in the cell homogenate and in Triton-soluble and -insoluble fractions was determined (Fig. 11). There was more β than α spectrin in the cell homogenate and the Triton-soluble fraction but there were near equal amounts in the Triton-insoluble fraction. The ratio of α to β spectrin in the cell homogenate was 0.85, in the Triton-

soluble fraction 0.74, and in the Triton-insoluble fraction 1.06.

Immunocytochemical Location of Spectrin in Untreated and DMSO-treated FELC

As previously described by others, using indirect immunofluorescent (11, 26) and biochemical (24) methods, small amounts of spectrin are detectable in uninduced cells and DMSO treatment causes an accumulation of spectrin. This was confirmed by immunocytochemistry (Table II). After 3 d of DMSO treatment there is a 3.5-fold increase in the amount of spectrin and the amounts decline by almost 35% after 5 to 7 d (Table II). At 3 d of DMSO treatment, 30% of the spectrin is localized near the outer plasma membrane of the cell and the majority (26%) of the remaining spectrin is found throughout the cytoplasm but preferentially concentrated (25%) in complex vacuolar structures which contain virus particles. These vacuolar structures are induced by DMSO treatment and do not appear in untreated cells (27). After 5 to 7 d of DMSO treatment, there is a

marked increased amount of spectrin (57–61%) in these vacuolar structures (see Table II and Fig. 12) but substantial amounts (17–21%) also remain associated with the surface membrane.

Discussion

The two polypeptides of spectrin, α and β , are synthesized by FELC in unequal amounts, with more (15–30%) radioactive methionine being incorporated into the β chain as compared to the α chain. Similar results were recently reported by Glenney and Glenney (12) with uninduced and DMSO-induced FELC incubated overnight with L-[³⁵S]methionine. Thus spectrin synthesis by FELC is different from that of

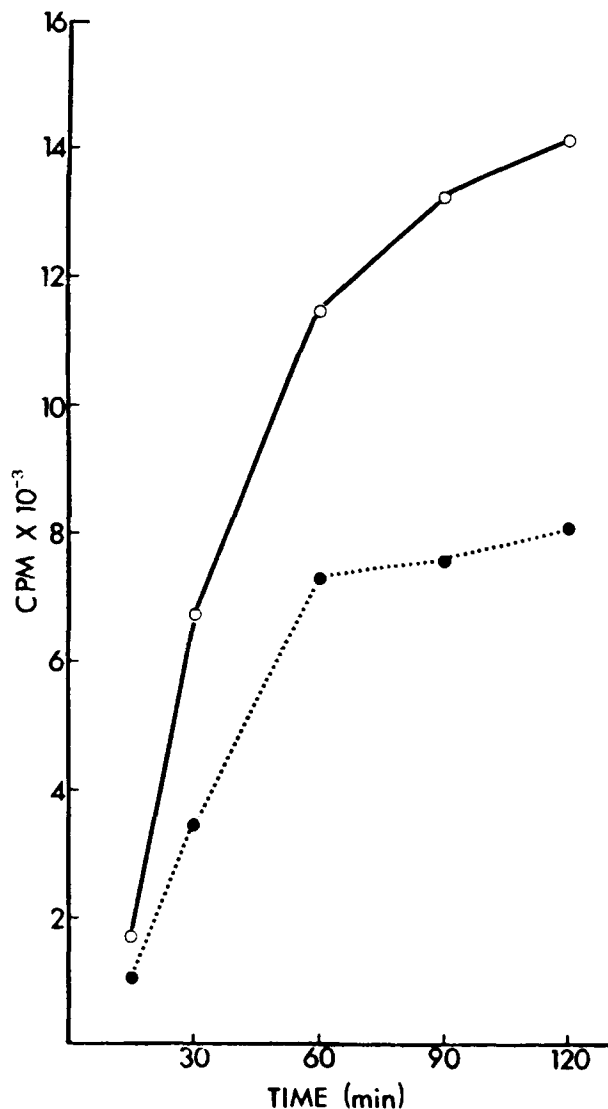


Figure 7. Time course of L-[³⁵S]methionine incorporation into Triton-soluble and Triton-insoluble spectrin. FELC were incubated with L-[³⁵S]methionine as described in legend to Fig. 1. At various times cells were fractionated into a Triton-soluble and -insoluble (cytoskeletal) fraction (see Materials and Methods). Radiolabeled spectrin was isolated from both fractions by immunoprecipitation followed by SDS PAGE. (○) Radioactivity of spectrin in Triton-soluble fraction. (●) Radioactivity of spectrin in Triton-insoluble fraction.

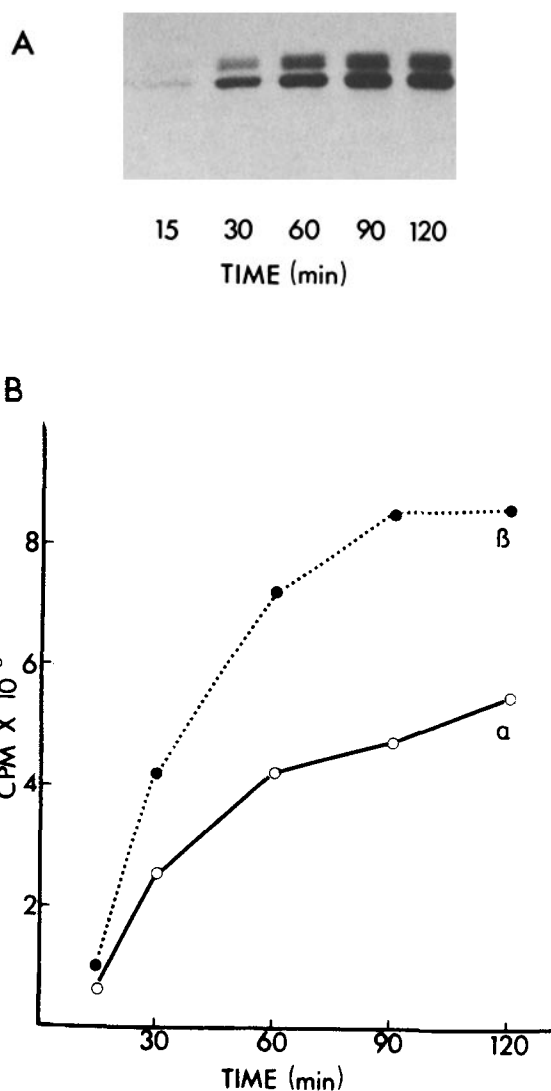


Figure 8. Time course of incorporation of L-[³⁵S]methionine into α and β spectrin of the Triton-soluble fraction. FELC were incubated with L-[³⁵S]methionine for 15, 30, 60, 90, and 120 min and then fractionated into Triton-soluble and Triton-insoluble (cytoskeletal) fractions as in Fig. 6. The radioactivity of spectrin isolated from the Triton-soluble fraction is presented. (A) Fluorogram of radiolabeled spectrin obtained at 15, 30, 60, 90, and 120 min from the Triton-soluble compartment. (B) The α and β bands of spectrin were excised from the gel shown in the above fluorogram. Gel slices were solubilized and the radioactivity in each band was determined. (○) Radioactivity of α spectrin; (●) radioactivity of β spectrin.

avian erythroid cells. In avian erythroid cells, there is also unequal synthesis of the two spectrin chains, but several-fold more α than β chains are produced (4).

Both avian erythroid cells and FELC contain a larger amount of newly synthesized spectrin in the Triton-soluble fraction than in the cytoskeletal (Triton-insoluble) cell fraction. However, the Triton-soluble fraction of FELC contains more radioactive β than α spectrin, while that of the avian erythroid cells contains more α than β chain (4). A common feature is that in the cytoskeletal fraction of both FELC and avian erythroid cells, the two spectrin chains are present in near equal amounts.

The FELC appears to contain a large pool of both α and

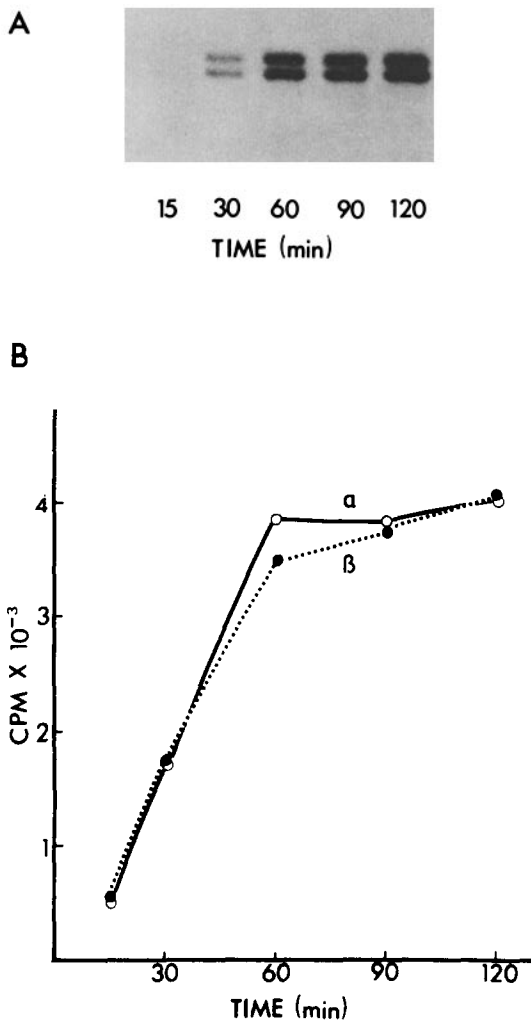


Figure 9. Time course of incorporation of L-[³⁵S]methionine into α and β spectrin of the Triton-insoluble fraction. FELC were incubated with L-[³⁵S]methionine for various times from 15 to 120 min and fractionated into Triton-soluble and Triton-insoluble fractions as described in legend to Fig. 6. The radioactivity of spectrin in the Triton-insoluble (cytoskeletal) fraction is presented. (A) Fluorogram of radiolabeled spectrin at 15, 30, 60, 90, and 120 min. (B) The α and β bands of spectrin were excised from the gel shown in the above fluorogram. Gel slices were solubilized and the radioactivity in each band was determined. (○) Radioactivity of α spectrin; (●) radioactivity of β spectrin.

β spectrin in the noncytoskeletal (Triton-soluble) fraction. L-[³⁵S]methionine continues to be incorporated into the Triton-soluble fraction for 90 to 120 min (Figs. 7 and 8). On the other hand, the incorporation of nascent spectrin into the cytoskeletal fraction is only linear for 1 h (Figs. 7 and 9). The pulse-chase experiments also indicate that FELC contain a large pool of noncytoskeletal spectrin. At 90 min of the chase period, 65% of the pulse-labeled spectrin remains in the Triton-soluble fraction and it is comprised of both α and β spectrin (Fig. 10). This is different from the avian erythroid cell which has a large (threefold) molar excess of α to β spectrin in the Triton-soluble fraction and thus during a chase incubation all of the nascent β spectrin is incorporated into the cytoskeletal fraction and the excess α spectrin remains in the Triton-soluble fraction (23). The Triton-soluble fraction of

FELC does not have a several-fold excess of one chain and thus during the chase period this fraction retains a substantial amount of both α and β spectrin. That there is a greater pool of β spectrin in the Triton-soluble fraction and that the cytoskeleton contains equimolar amounts of α and β spectrin is reflected in the radioactive composition of spectrin in the cytoskeleton. In a continuous labeling experiment (Fig. 9) there are near equal amounts of radioactive α and β chains in the cytoskeleton, yet in a pulse-chase experiment, the chase-labeled cytoskeletal fraction contains more α spectrin

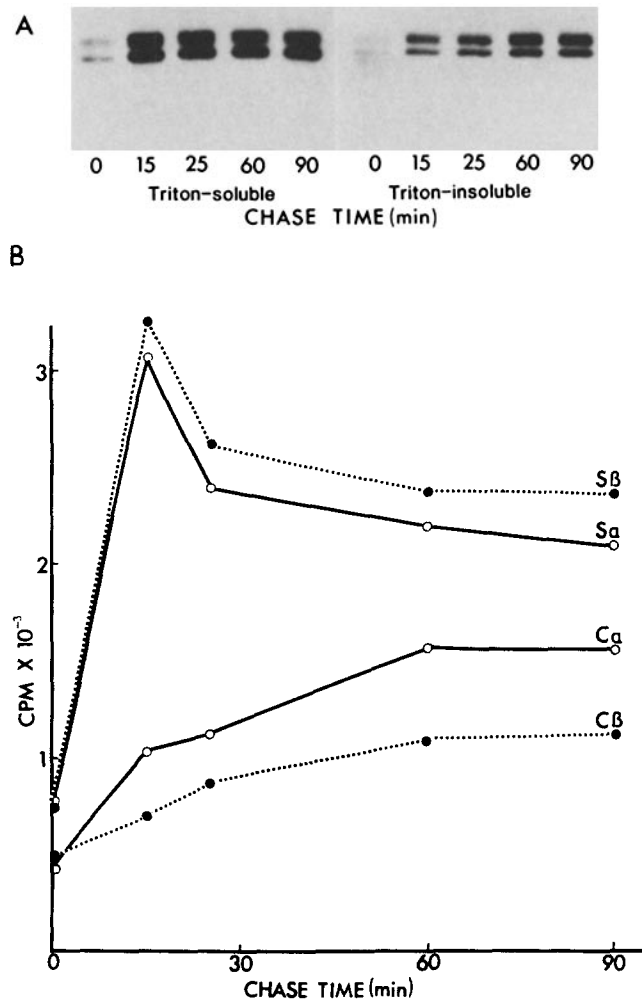


Figure 10. Pulse-chase incubation: appearance of radiolabeled spectrin in the Triton-soluble and Triton-insoluble fractions. FELC were pulse-labeled with L-[³⁵S]methionine (100 μ Ci/ml) for 2 min, followed by a chase incubation in the presence of unlabeled methionine (1 mM). Cells were taken at 0, 15, 25, 60, and 90 min of chase incubation, fractionated with 1% Triton X-100 into Triton-soluble and Triton-insoluble fractions, and spectrin was isolated as described in Materials and Methods. (A) A fluorogram of radioactive spectrin in the Triton-soluble and -insoluble fraction at the end of the pulse period (0) and at various chase intervals from 15 to 90 min. (B) Spectrin was excised from the gel shown in the above fluorogram and the amount of radioactivity in each chain from both cell fractions was determined by liquid scintillation spectrometry. (S α) Radioactivity of α spectrin from the Triton-soluble fraction; (S β) radioactivity of β spectrin from the Triton-soluble fraction; (C α) radioactivity of α spectrin from the cytoskeletal (Triton-insoluble) fraction; (C β) radioactivity of β spectrin from the cytoskeletal fraction.

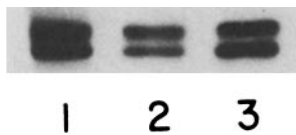


Figure 11. Intracellular pools of α and β spectrin. FELC were incubated with L-[35 S] methionine (10 μ Ci/ml) for 18 h. Radioactive spectrin was isolated from the cell homoge-

nate, Triton-soluble, and Triton-insoluble fractions. An autoradiogram of α and β spectrin is shown. (Lane 1) Spectrin in homogenate; (lane 2) spectrin in Triton-insoluble fraction; (lane 3) spectrin in Triton-soluble fraction.

radioactivity (Fig. 10). This is to be expected if there are stoichiometric amounts of α and β spectrin in the cytoskeleton and the precursor pool in the Triton-soluble fraction contains an α spectrin of higher specific radioactivity. A higher α spectrin-specific radioactivity in the Triton-soluble pool is the result of the unequal and excessive production of β spectrin which leads to an accumulation, in the Triton-soluble fraction, of unlabeled β spectrin before the pulse period and during the chase incubation (see Figs. 1 and 8). The occurrence of a greater pool of β spectrin in the Triton-soluble fraction was confirmed by incubating cells for 18 h with L-[35 S]methionine, a time at which a steady state of protein radioactivity has been achieved, and determining that there was an excess of β spectrin in the Triton-soluble fraction and equal amounts of α and β spectrin in the cytoskeletal fraction (Fig. 11).

Since the amount of α and β spectrin was determined by measuring the radioactivity in the protein bands separated by SDS PAGE, there is a danger that proteolytic products from α spectrin may co-electrophorese with β spectrin. This is possible, but doubtful, since equal amounts of α and β spectrin radioactivity are obtained in the Triton-insoluble fraction. For proteolysis to distort the measured ratio of α to β spectrin, there would have to be more proteolysis in the Triton-soluble fraction than in the Triton-insoluble fraction.

The nature of the pool of spectrin in the Triton-soluble fraction is unclear. It is not soluble in the absence of detergents, but appears to be bound to a variety of different membranous organelles. Yet this pool of spectrin does not appear to be enclosed by the membranous organelles since treatment with trypsin in the absence of detergents digests the nascent spectrin. We do not know, however, whether in the intact cell this pool of spectrin is attached to the membranes or whether it is part of a cytoplasmic structure that is disrupted by homogenization, is insoluble in aqueous solutions, and binds nonspecifically to the outer surface of membranous organelles. If the noncytoskeletal spectrin is part of a cytoplas-

mic structure, it is distinct from the cytoskeleton since it is disrupted by 1% Triton X-100 and its composition of radioactive α and β spectrin differs from that of the cytoskeleton. Whatever the nature of spectrin in the Triton-soluble fraction, our studies clearly indicate that it is not a pool of "soluble" spectrin since disruption of the cells in the absence of detergents shows that the nascent spectrin is attached to sedimentable cell structures.

Nascent incomplete spectrin chains are noted at the end of a 2-min pulse incubation in both the Triton-soluble and the Triton-insoluble fractions. These incomplete chains are more prominent in the Triton-insoluble fraction (Fig. 10). Polysomes fractionate with both Triton-soluble and -insoluble fractions, with the majority of the polysomes sedimenting with the Triton-insoluble fraction. The Triton-insoluble fraction, while enriched in cytoskeletal proteins, is also composed of other cellular structures (Fig. 4) and has been shown, in another cell, to contain polysomes (8). The heterogeneity of the Triton-soluble and -insoluble fractions probably contributes to blurring the measurement of the movement of nascent spectrin from one compartment to the other. The results of the pulse-chase experiments, however, are consistent with nascent α and β spectrin leaving polysomes, which may be present either in the Triton-soluble or -insoluble fraction, mixing with a pool of preformed spectrin in the Triton-soluble fraction and later being incorporated, in equimolar amounts, into the cytoskeletal framework.

Immunocytochemical analysis, at the level of the electron microscope, shows that the majority of the spectrin in DMSO-treated cells occurs in two locations; near the outer plasma membrane and associated with complex vacuolar structures which contain virus particles. This method does not distinguish between the pool of spectrin (that which occurs in the Triton-soluble fraction) and spectrin which is a component of the cytoskeletal system. Nor does it detect nascent spectrin which is newly incorporated into the cytoskeletal system as opposed to preformed spectrin. The location of spectrin near the outer plasma membrane is consistent with its role in erythrocyte structure. The marked accumulation of spectrin in the vacuolar structures which contain virus is interesting, since it suggests that spectrin may be involved in the segregation and/or the budding of virus particles from the cell.

In chicken erythroid cells, α and β spectrin and ankyrin, are synthesized in greater amounts than are needed for assembly into the cytoskeleton (23), and this is particularly evident for α spectrin which is produced in threefold excess over β spectrin (4, 22). This has led to the conclusion that

Table II. Immunocytochemical Analysis of Spectrin in Untreated and DMSO-treated Cells

DMSO treatment (days)	Total number particles	Percent particles					
		Nucleus	Endoplasmic reticulum and Golgi region	Other cytoplasmic areas	Mitochondria	Complex vacuoles	Plasma membranes
0	305	4	26	32	3	3	32
3	1,093	7	7	26	5	25	30
5	766	4	6	8	4	57	21
7	629	4	7	10	1	61	17

The spectrin in untreated and DMSO-treated FELC was localized by immunocytochemistry as described in Materials and Methods. In each sample, the number of gold particles in 40 cell profiles was counted. The number of particles in control samples, which were treated with preimmune rabbit IgG instead of antibody, was subtracted from the above figures.

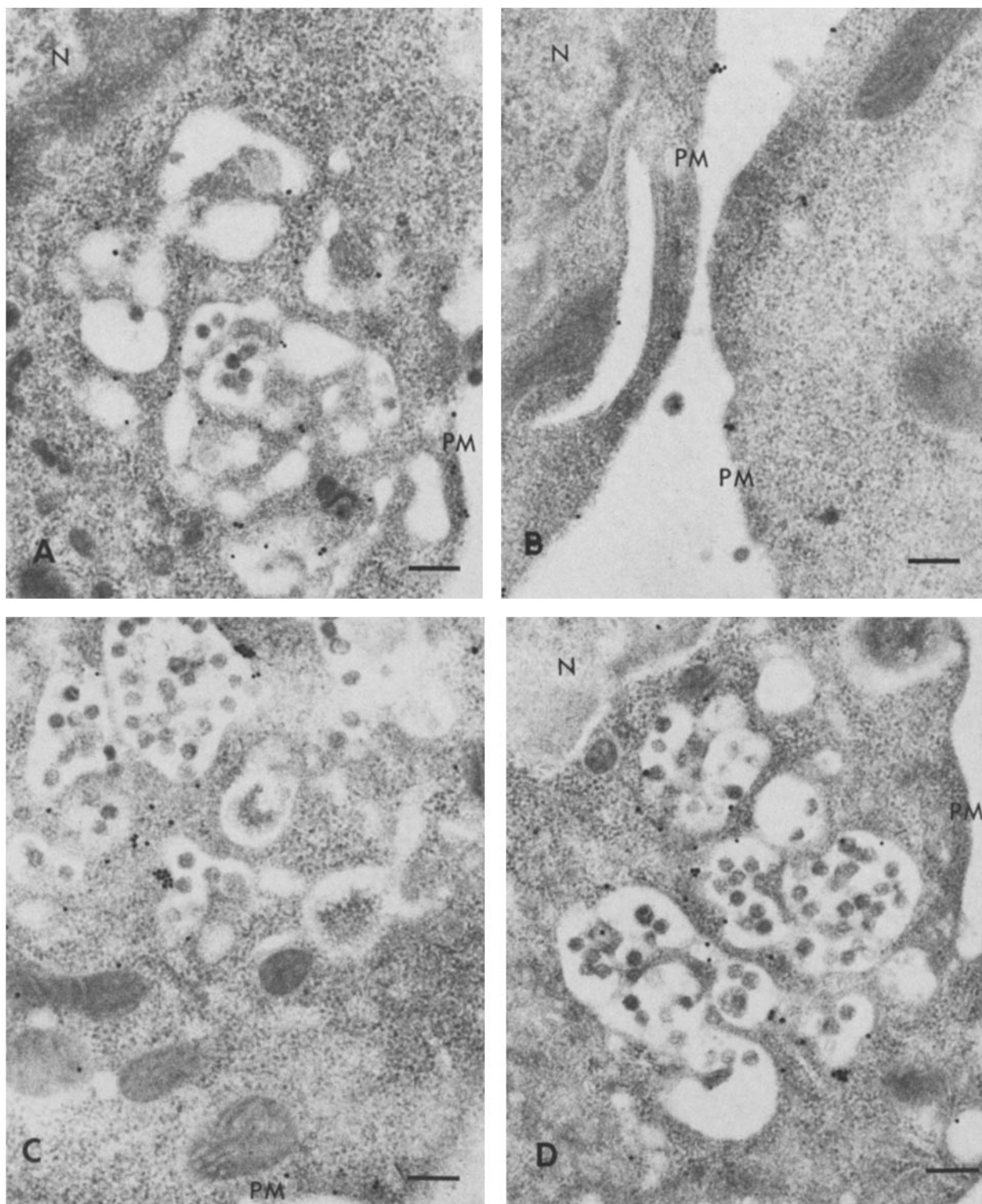


Figure 12. Immunocytochemical localization of spectrin in DMSO-treated cells. FELC were immunochemically labeled using affinity-purified rabbit antibody to human spectrin and goat anti-rabbit IgG coupled to 20-nm colloidal gold particles. *A* and *B* show representative fields of FELC treated for 3 d with DMSO; *C* shows cells treated for 5 d and *D* shows cells treated for 7 d. *N*, nucleus; *PM*, plasma membrane. The complex vacuolar structures containing viruses are apparent in *A*, *C*, and *D*. Bar, 250 nm.

the molar ratios of the component proteins present in the cytoskeleton are determined posttranslationally by the specific attachment of proteins to each other and to the membrane skeleton, rather than by the relative amounts of the component proteins which are synthesized (18). Band 3, an

integral membrane protein, is thought to be a prerequisite for the attachment of cytoskeletal proteins to the plasma membrane (18, 23). The finding that FELC and avian erythroid cells contain a pool of α and β spectrin in the Triton-soluble fraction, but that the ratios of α and β spectrin differ greatly

(in the avian erythroid cells there is three times more α than β spectrin and in FELC there is 25% more β than α spectrin) is consistent with the view that the relative amounts of α and β spectrin synthesized do not regulate their assembly into the cytoskeletal system. FELC, when treated with DMSO, show a staggered increase in the synthesis of various red cell membrane components, with peak synthesis of band 3 occurring 1 d after that of spectrin. However, there is some synthesis of these proteins throughout the entire period of DMSO-induced differentiation, and in fact, synthesis of both band 3 and spectrin can be noted in untreated FELC (24). Thus, the protein components necessary for proper cytoskeletal assembly are probably always present in FELC. We do not know however, what percent of the spectrin, synthesized by FELC, is assembled into a cytoskeletal system similar to that which occurs in erythrocytes. Our immunocytochemical studies only note that the cellular location of spectrin shifts, during DMSO-induced differentiation, and that after 5-7 d of DMSO treatment most of the spectrin is localized near complex vacuoles which are associated with virus.

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