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Physical and Chemical Properties of a Protein Isolated from Red Cell Membranes*

S. L. Marchesi, E. Steers, V. T. Marchesi, and T. W. Tillack

ABSTRACT: A protein component of erythrocyte membranes has been extracted and purified from human red blood cell ghosts, and has been partially characterized. The protein was solubilized from the membranes in low ionic strength aqueous solutions containing ethylenediaminetetraacetate. Purification was achieved by gel filtration and was established by polyacrylamide gel electrophoresis. In neutral salt solutions, the purified protein polymerizes into two major species with $s_{20,w}$ values between 8 and 11 S. In 6 M guanidine the aggregates are dissociated into a single monomeric unit with an $s_{20,w}$ of 1.9 S. This subunit has a molecular weight of approximately 140,000 as measured by equilibrium ultracentrifugation and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The number of peptides produced by cyanogen bromide cleavage is also consistent with monomeric units of 140,000–150,000. Amino acid analysis shows a predominance of glutamic acid (20 μM %) and the presence of cysteine (1 μM %).

We have described briefly in a recent publication (Marchesi and Steers, 1968) the extraction and purification of a protein from guinea pig red blood cell ghosts. The extraction procedure described consisted of dialysis of the ghosts against ATP[1] and β-mercaptoethanol at low ionic strength. The protein solubilized in this manner accounts for approximately 20% of the total membrane protein, has an ultraviolet absorption maximum at 282 mμ, and is free of lipid and carbohydrate. Other characteristics of this protein include formation of a single broad band on polyacrylamide gel electrophoresis and a single peak in free-boundary electrophoresis over a pH range of 6.5–9.8.

Immune serum from rabbits injected with the purified membrane protein forms a single precipitin band with the protein on Ouchterlony plates. No precipitin line forms when the immune serum is run against guinea pig plasma or red cell hemolysate, indicating that the protein is not derived from plasma or red cell contents, but is specific for the red cell membrane. The suggestion was made that this protein be named spectrin.

We have extended our study of spectrin to other species including horse, sheep, rabbit, and human. A comparison of the physical, chemical, and immunologic characteristics of the spectrin obtained from these species will be reported elsewhere (Tillack *et al.*, 1970). Spectrin obtained from human red cells was studied in more detail and the results are reported here.

Our purpose has been to define some of the properties of a purified membrane protein as a step toward understanding the structural role of proteins within membranes. Some important questions are: (1) the number and size of proteins in the membrane; (2) the characteristics of these proteins which make them especially suited to a structural role; and (3) the particular arrangement of these proteins which, together with associated lipid molecules, gives the membrane its characteristic stability, insolubility, and selective permeability.

Methods

Preparation of Erythrocyte Ghosts. Blood was collected in ACD (Fenwal; 75 ml of 0.8% citric acid, 2.2% sodium citrate, 2.45% dextrose for 500 ml of blood) from normal human

* From the Laboratories of Chemical Biology and Chemical Pathology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received August 28, 1969.*

[1] The use of ATP in this sample is explained in the Discussion.

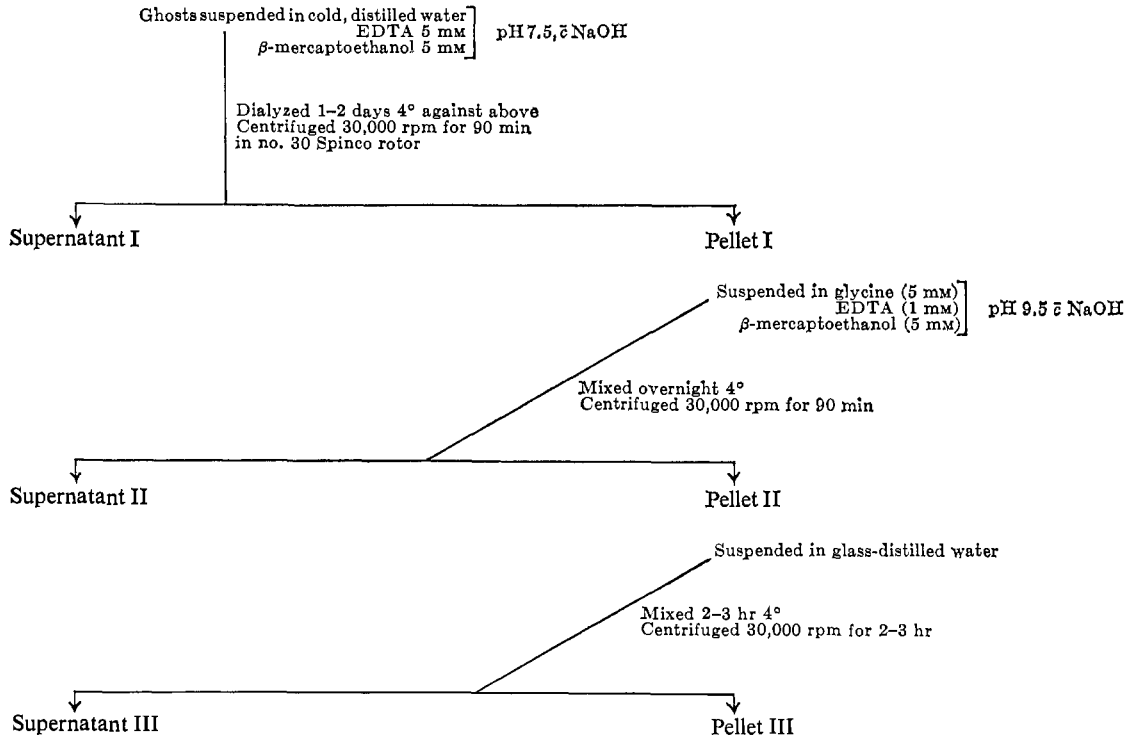

FIGURE 1: Procedure for extraction of soluble protein from red blood cell ghosts.

volunteers. The red cells were collected by centrifugation at 2500 rpm 15 min, and washed three times in 0.9% NaCl. The buffy coat was aspirated after each centrifugation. The red cells were then lysed at 4° in 5 mM Tris-Cl–1 mM EDTA (pH 7.5), at a lysate-packed cell volume ratio of 30:1. It was found that if this ratio exceeds 40:1, protein may be lost from the ghosts into the lysate.

The lysed cells were centrifuged in a Sorvall RC-2B centrifuge at 20,000 rpm, 4°, using a continuous-flow apparatus for large volumes. The ghost pellets were suspended in a small volume of the same lysing buffer to remove trapped hemolysate and centrifuged as above. The ghosts were then light pink and intact when viewed by phase microscopy.

Extraction of Soluble Protein. Figure 1 outlines the series of extractions performed on the ghost pellet.

Pellet III was composed of small vesicles when viewed by phase microscopy. Supernatants I, II, and III were combined and the extracted material was precipitated by addition of an equal volume of cold saturated ammonium sulfate. In some cases the extract was concentrated by Diaflo ultrafiltration (Amicon Corp., Lexington, Mass.) using the UM-10 filter (10,000 molecular weight exclusion) before ammonium sulfate precipitation. The extracted material may be stored as an ammonium sulfate precipitate for several months with no apparent change in properties.

The buffer used for final purification and analysis of the soluble ghost protein was altered as the experiments progressed; the final buffer contained 0.05 M Tris, 0.3 M NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol or dithiothreitol, pH adjusted to 8.6 with HCl. The EDTA, reducing agent, high salt concentration, and alkaline pH were all found to be helpful in reducing aggregation and gel formation by the extracted protein.

Protein Concentration. In earlier studies, protein concentration was determined by the Lowry procedure (Lowry *et al.*, 1951). After the extinction coefficient of the purified spectrin was determined (see Results), subsequent protein determinations were made in a Zeiss PMQII spectrophotometer at 280 mμ.

Carboxymethylation. Protein samples were incubated in 8 M urea (with 0.1 M β-mercaptoethanol unless otherwise indicated) for 3–4 hr at 38–40°. Iodoacetic acid was then added slowly at room temperature to a concentration of 0.2 M. During this time the pH was maintained above 8.0 with NaOH. The reaction was then allowed to proceed an additional 15 min, and was stopped by addition of excess β-mercaptoethanol. Conversion of half-cystine residues into *S*-carboxymethylcysteine was determined by amino acid analysis.

Amino Acid Analysis. Aliquots of lyophilized carboxymethylated protein (approximately 2 mg) were suspended in 1 ml of 6 N HCl, evacuated and sealed, and hydrolyzed for 22 hr at 110°. The hydrolysate was dried under vacuum and analyzed on a Beckman 120 amino acid analyzer by the method of Moore and Stein (1963).

Electrophoresis. Five per cent polyacrylamide gels containing 8 M urea were made using reagents and methods supplied by Canal Industrial Corp., Rockville, Md. Electrophoresis was carried out for approximately 3 hr in 0.05 M Tris–0.38 M glycine (pH 8.4) at 4°. Electrophoresis was also performed in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate according to Shapiro *et al.* (1967). Samples to be electrophoresed in sodium dodecyl sulfate gels were first

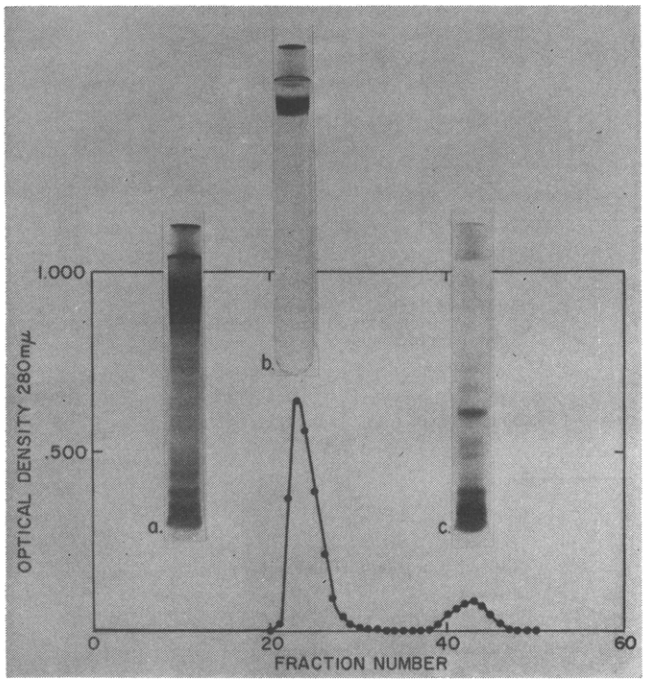

FIGURE 2: Purification of spectrin on Sephadex G-200. Approximately 100 mg of crude ghost extract was applied to a 2.5 × 100 cm column of Sephadex G-200. The column was eluted with 0.025 M Tris, 0.1 M NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol (pH 8.5). The effluent protein peaks, measured by OD_{280}, were pooled and concentrated by ammonium sulfate precipitation. Aliquots of the starting material and the concentrated protein peaks were incubated in 8 M urea at 37° for 1 hr and applied to 5% polyacrylamide–8 M urea disc gels. Electrophoresis and staining were carried out as described in Methods. Gel a = crude extract before chromatography; b = spectrin; c = other small molecular weight proteins.

TABLE IA: Amino Acid Composition of Human Spectrin.[a]

Amino Acid	μM %	Residues/100,000 Mol Wt
Lysine	6.7	59.1
Histidine	2.6	22.9
Arginine	5.8	51.2
S-carboxymethyl-cysteine	1.1	9.7
Aspartic acid	10.9	96.1
Threonine	3.6	31.8
Serine	4.1	36.2
Glutamic acid	20.5	180.8
Proline	2.4	21.2
Glycine	4.9	43.2
Alanine	9.2	81.1
Valine	4.7	41.5
Methionine	1.7	15.0
Isoleucine	4.0	35.3
Leucine	12.4	109.4
Tyrosine	2.0	17.6
Phenylalanine	3.0	26.5

[a] The figures above represent an average of three determinations. Mean residue weight calculated is 113.4.

incubated in 1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer (pH 7.1) for 3 hr at 37°, and then dialyzed overnight at room temperature against 0.1% sodium dodecyl sulfate in the same buffer. Sodium dodecyl sulfate gels were electrophoresed 3 hr at room temperature in 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1% sodium dodecyl sulfate.

The gels were stained with Amido-Schwartz or with coomassie blue after fixation overnight in 20% sulfosalicylic acid in water.

Ultracentrifugation. Analytical ultracentrifugation was performed on a Spinco Model E ultracentrifuge equipped with electronic speed control and RTIC temperature control unit. Sedimentation velocity studies were performed in a standard single-sector cell at 56,000 rpm, 20°, and analyzed with schlieren optics (concentrations over 4 mg/ml) or ultraviolet absorption optics (concentration less than 1 mg/ml). Sedimentation coefficients were calculated according to Schachman (1957) and corrected when required to $s_{20,w}$ using the viscosity and density of 6 M guanidine reported by Kawahara and Tanford (1966). High-speed sedimentation equilibrium studies were performed in a six-channel Yphantis cell at speeds of 16,000–28,000 rpm using interference optics. All photographic plates were measured with a Nikon 6C comparator. Molecular weights were calculated from plots of log dy (y = fringe displacement in μ) *vs.* x^2 (x = distance in centimeters from the center of the rotor) using the equation

$$M = \frac{2RT \times 2.303}{(1 - \bar{v}\rho)\omega^2} \times \frac{\log \mathrm{d}y}{\mathrm{d}x^2}$$

where M = molecular weight, R = gas constant, T = absolute temperature, $\bar{v}$ = partial specific volume of solute, ρ = density of the solution, and ω = angular velocity of the rotor in radians per second. A $\bar{v}$ of 0.730 was calculated from the amino acid composition (Cohn and Edsall, 1943). A value of 1.145 for ρ of 6 M guanidine was obtained from the tables of Kawahara and Tanford (1966).

Cyanogen Bromide Cleavage. The lyophilized protein (25–50 mg) was dissolved in 70% formic acid and a 50-fold molar excess of CNBr was added. The reaction was carried out at room temperature in a stoppered container for 24 hr, and the digest was then diluted tenfold with water and lyophilized. Samples were assayed for completeness of methionine destruction by amino acid analysis.

Results

Purification of Spectrin from Crude Ghost Extract. Polyacrylamide disc gels of the soluble ghost protein (Figure 1, supernatants I–III) show a wide dense band near the origin, as well as numerous minor bands (Figure 2, gel a). The major component, which we have named spectrin, can be purified on a G-200 Sephadex column as shown in the same figure. Spectrin is excluded from the gel and appears in the void volume free of minor contaminants. Carboxymethylated spectrin was also found to be excluded from Sephadex G-200.

Since hemoglobin is separated from spectrin by the Sephadex

TABLE IB: Effect of β-Mercaptoethanol Concentration on Carboxymethylation of Half-Cysteines.[a]

β-Mercaptoethanol (mM)	μM % S-Carboxymethylcysteine
100	1.1, 1.04
5	1.2
0	0.86

[a] Carboxymethylation was carried out as described in Methods, except that the concentration of β-mercaptoethanol in the 8 M urea incubation mixture was varied as shown above. After carboxymethylation, 2-mg samples were prepared for amino acid analysis as described.

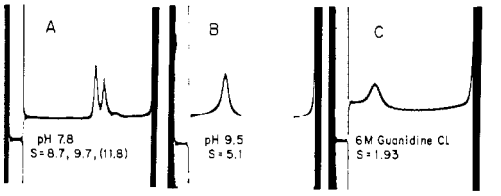

FIGURE 3: Sedimentation velocity ultracentrifugation of purified preparations of human spectrin. Human spectrin, purified as described, was centrifuged in a Spinco Model E ultracentrifuge at 56,000 rpm, 20°, in (A) 0.025 M Tris, 0.1 M NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol (pH 7.8); (B) 0.02 M glycine, 0.3 mM ATP,[1] and 5 mM β-mercaptoethanol (pH 9.5); (C) 6 M guanidine-HCl in 0.05 M Tris, 0.3 M NaCl, 5 mM EDTA, and 5 mM dithiothreitol. Protein concentrations were (A) 12.4 mg/ml, (B) 4.5 mg/ml, and (C) 8.8 mg/ml. Pictures were taken at (A) 59 min, (B) 58 min, and (C) 269 min after reaching speed.

G-200 filtration procedure, the ghosts used for extraction need not be washed completely free of hemoglobin. This is important, because repeated washes of the ghosts with hypotonic solutions may remove significant amounts of spectrin. The yield of pure spectrin is approximately 75–100 mg/500 ml of whole blood.

The ultraviolet spectrum of the purified spectrin shows a typical protein pattern, with a maximum extinction at 282 mμ. Assays for sialic acid (Warren, 1959) and neutral sugars by paper chromatography were negative.

Purified human spectrin contained no detectable lipid as indicated by negative assays for total phosphorus, and no evidence of sterols or fatty acids by gas chromatography.

Aliquots of spectrin with known extinction at 280 mμ were dialyzed against water and dried until the weight was constant. The extinction coefficient calculated was $E^{1\%}_{280}$ 8.8.

Amino Acid Analysis. Table IA shows the amino acid content of carboxymethylated spectrin after 22-hr hydrolysis. The numbers given are the average of three separate determinations, and are expressed both as μM % and as moles/100,000 mol wt. To compare the availability of SH groups, (1) in the presence of excess reducing agent, (2) under the standard reducing conditions, and (3) in the absence of reducing agent, spectrin was carboxymethylated in 100 mM (excess), 5 mM (standard) or in the absence of β-mercaptoethanol. The extent of S carboxymethylation was then determined by amino acid analysis. The results shown in Table IB indicate that at least 8 out of 10 sulfhydryls are reactive even in the absence of reducing agent, and that all are reactive in the 5 mM β-mercaptoethanol used in analytical studies.

Sedimentation Velocity Ultracentrifugation. Sedimentation velocity ultracentrifugation of purified spectrin was carried out under a variety of conditions. The schlieren patterns of the protein at pH 7.8, pH 9.5, and in 6 M guanidine-HCl are compared in Figure 3.

Multiple forms of the spectrin molecule are consistently found in the pH range 7.0–7.8. Their occurrence is not dependent on concentration since sedimentation at concentrations between 0.2 and 12 mg per ml gave the same results: two peaks of approximately equal size with $s_{20,w}$ values between 8 and 11 S. In some cases a shoulder may be seen on one of the major peaks. It should be emphasized that the spectrin used in these analyses forms a single band on 5% polyacrylamide–8 M urea disk gels. Thus, the polydispersity seen at neutral or slightly alkaline pH presumably is due to polymerization to varying degrees of the single monomeric unit.

At pH 9.5, spectrin appears to be monodisperse, with a sedimentation coefficient of 5.1 S. In 6 M guanidine-HCl, with dithiothreitol present in order to achieve maximal dissociation and unfolding, the $s_{20,w}$ drops to 1.93 S.

Equilibrium Ultracentrifugation. High-speed equilibrium ultracentrifugation (Yphantis, 1964) was used to determine the homogeneity and molecular weight of spectrin in 6 M guanidine. It was assumed that this solvent, with the addition of 5 mM dithiothreitol, produces maximum dissociation of polypeptide chains and thus gives a minimum molecular weight.

Numerous ultracentrifugal analyses were performed at protein concentrations ranging from 0.2 to 0.8 mg per ml at speeds ranging from 16,000 to 28,000 rpm. Molecular weights calculated from two separate series of determinations are shown in Table II. Plots (log dy *vs.* x^2) of two sample runs giving

TABLE II: Molecular Weight of Human Spectrin in 6 M Guanidine Determined by High-Speed Equilibrium Ultracentrifugation.[a]

Run	Protein Concn (mg/ml)	Rotor Speed (rpm) 16,000	20,000	24,000	28,000
1	0.45	133,000	115,000	103,000	92,300
	0.23	138,000	104,000	100,500	
2	0.80		150,000	124,000	117,000
	0.40		156,000	134,000	128,500
	0.20		154,000		

[a] Purified human spectrin was dialyzed against 6 M guanidine in 0.05 M Tris-Cl, 0.3 M NaCl, and 5 mM dithiothreitol (pH 8.4), for 48 hr. Dilutions made with the dialysate were centrifuged in the six-chamber Yphantis cell in the Spinco Model E ultracentrifuge. The initial equilibrium was established at the lowest speed (run 1) or highest speed (run 2) in 48 hr. Speeds were changed and pictures were taken at 24-hr intervals thereafter. Protein distribution was followed by Rayleigh optics.

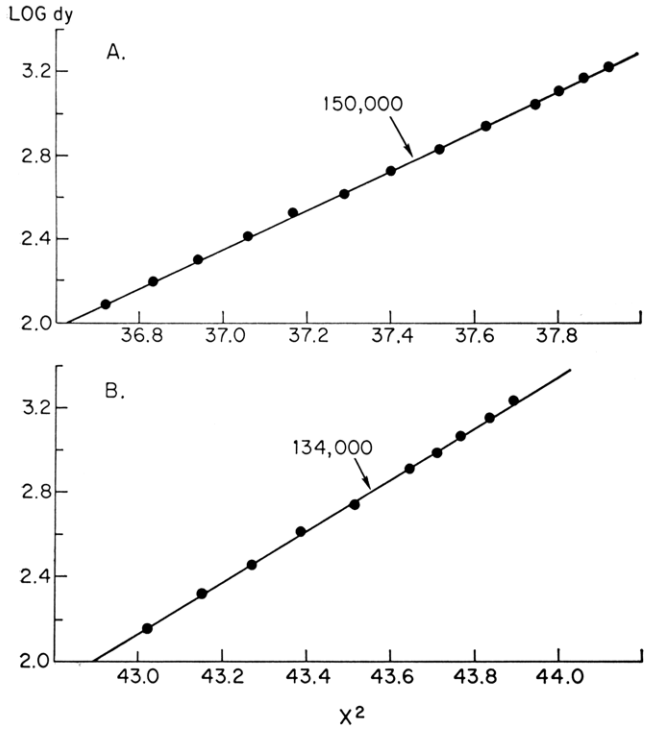

FIGURE 4: Plots of high-speed ultracentrifugation of spectrin in 6 M guanidine–5 mM dithiothreitol performed as described in Table II. Molecular weights were calculated from the slope log dy/x^2 as described in Methods; (A) 20,000 rpm, protein concentration 0.8 mg/ml. (B) 24,000 rpm, protein concentration 0.4 mg/ml.

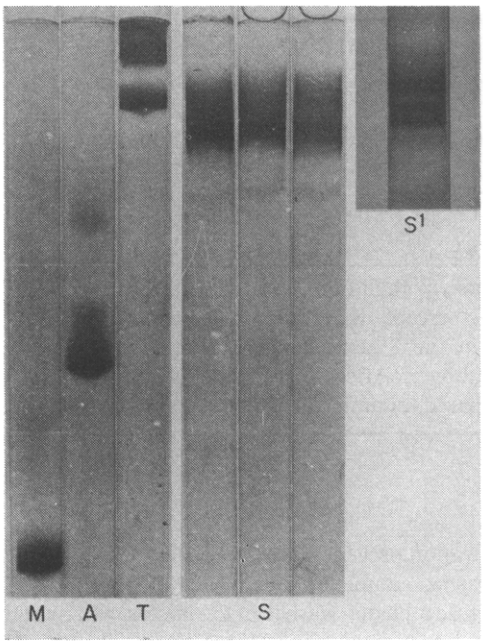

FIGURE 5: Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. Protein samples of 100–150 μg were applied to 5% acrylamide–0.1% sodium dodecyl sulfate gels 15 cm in length. Electrophoresis and staining are described in Methods; (M) myoglobin, mol wt 17,000; (A) bovine serum albumin, mol wt 64,000; (T) thyroglobulin, mol wt 165,000 (de Crombrugghe *et al.*, 1966); (S and S¹) spectrin (see text).

molecular weights of 150,000 and 134,000 are shown in Figure 4.

Viscosity. The hydrodynamic properties of a randomly coiled polymer in a thermodynamically ideal solvent should depend on the length of the polymer (Tanford, 1961). Tanford *et al.* (1967) have shown that a number of proteins in 6 M guanidine–100 mM β-mercaptoethanol behave as random coils; that is, the intrinsic viscosities and sedimentation coefficients are directly related to polypeptide length. In plotting the log $[\eta]$ of twelve proteins in 6 M guanidine against log n (number of residues in the polypeptide chain, determined by other means), Tanford finds a linear relationship defined by $[\eta] = 0.716\, n^{0.66}$.

The viscosities of native spectrin and spectrin in 6 M guanidine were measured in a capillary-type viscometer with a water outflow time of 180 sec over concentration ranges of 2–10 mg/ml of protein. The buffer used for native spectrin was 0.025 M Tris, 0.1 M NaCl, and 5 mM EDTA (pH 8.0). The reduced viscosity was markedly concentration dependent, extrapolating from a value of 0.35 dl/g at 8 mg/ml to 0.10 dl/g at infinite dilution. The reduced viscosity of spectrin in 6 M guanidine–5 mM dithiothreitol, extrapolates to a value of 0.60 dl/g at infinite dilution. Using this value in the equation given above, we obtain a molecular weight of 90,000–100,000. This is to be compared with the range of 100,000–150,000 obtained by equilibrium ultracentrifugation.

Molecular Weight Estimation by Polyacrylamide Disc Gel Electrophoresis in Sodium Dodecyl Sulfate. Acrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate has been developed as a means of estimating molecular size (Shapiro *et al.*, 1967). It is assumed that charge and shape differences between proteins are minimized by complex formation of the protein with sodium dodecyl sulfate, and that distance migrated into the gel should reflect polypeptide chain length only. An approximately linear relationship has been found between log molecular weight and distance of migration for a number of proteins of known molecular weight.

Figure 5 shows gels resulting from electrophoresis of thyroglobulin, albumin, myoglobin, and spectrin on 5% acrylamide gels containing 0.1% sodium dodecyl sulfate. Before electrophoresis all protein samples were incubated in 1% sodium dodecyl sulfate as described in Methods. On a plot relating distance migrated to log molecular weight of the known proteins, spectrin has an approximate molecular weight of 140,000. It should be pointed out that on occasional sodium dodecyl sulfate gels, spectrin splits into two distinct bands (Figure 5,S′), raising the possibility that the molecule consists of two unique polypeptide chains of equal (or nearly equal) size.

Cyanogen Bromide Cleavage. Peptides of human spectrin produced by cleavage with cyanogen bromide were chromatographed on Bio-Gel P-150 as shown in Figure 6a. Individual peaks A–D were pooled, treated as described in Figure 6b, and electrophoresed on standard 12% polyacrylamide disc gels (Figure 6b). Although the banding pattern shows that the peaks overlap, identical patterns were produced from three separate preparations. Careful count of unique bands (see arrows, Figure 6b) gives a total of 23, which would result from complete CNBr cleavage of a polypeptide chain with 22 methionines. Since spectrin contains 15 methionine residues/100,000 mol wt (see Table IA), the results of the cyanogen

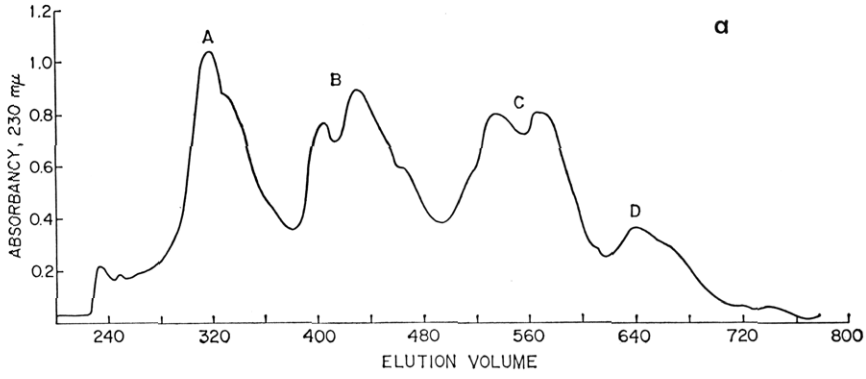

FIGURE 6a: Chromatography of cyanogen bromide peptides of human spectrin. CNBr peptides of spectrin (see Methods) were dissolved in 6 M guanidine-HCl, and diluted to a final concentration of 4 M guanidine in 0.025 M Tris-HCl (pH 7.5). The peptides were applied to a Bio-Gel P-150 column (2.5 × 240 cm) and eluted with the same guanidine-Tris solvent. Appearance of peptides was detected by measuring OD_{230}.

bromide studies indicate a minimal chemical molecular weight of 146,000.

Discussion

Methods which have been reported for the preparation of soluble erythrocyte membrane protein include treatment of the membrane with organic solvents (Maddy, 1966; Zwaal and Van Deenan, 1968; Rega *et al.*, 1967; Rosenberg and Guidotti, 1968) followed by detergents, urea, or other denaturants, and in some cases by sonication (Schneiderman and Junga, 1968). These methods are not designed to distinguish different proteins within the membrane, but to separate lipid from protein and to solubilize all of the membrane protein.

The proteins thus obtained are highly insoluble in aqueous solvents and are extremely heterogeneous by ultracentrifugal and electrophoretic analysis.

The insolubility of total ghost protein preparations is well illustrated by the experience of Rosenberg and Guidotti (1968). In a study of ethanol–ether-extracted erythrocyte ghosts, they found that the ghost protein required solvents such as 88% formic acid, 1% sodium dodecyl sulfate, or phenol, and was soluble in aqueous solutions only after succinylation. It was insoluble initially in 8 M urea and 8 M guanidine, but could be dialyzed into these solvents from phenol. In this case, solubility was defined as failure to sediment in 1 hr at 50,000*g*. In another example, erythrocyte membrane protein extracted in Triton X-100 and urea (Schneiderman and Junga, 1968) was found to be soluble only at pH extremes or in the presence of detergents.

Recently, Mazia and Ruby (1968) have reported success in solubilizing erythrocyte ghost protein in water at pH 9–9.5. Their method involves lysis of the erythrocytes in the presence of Triton X-100 and the maintenance of low electrolyte concentrations throughout the procedure. If detergent is not present during lysis, protein recovery is less than 50%. It is likely that the detergent remains and aids in the solubility of the final preparation.

Blumenfeld (1968) has separated a fraction of erythrocyte ghost protein containing all of the sialic acid of the membrane by treating the ghosts with pyridine. When the pyridine is removed from the solubilized membranes by dialysis against water, a sialoprotein remains in solution, which accounts for 35–40% of the total membrane protein, while the remaining lipoprotein complex precipitates. The sialoprotein aggregates and forms gels during concentration by pressure dialysis and during ultracentrifugation at concentrations greater than 5 mg/ml.

All of the erythrocyte membrane protein preparations described above have been found to be heterogeneous when analyzed by polyacrylamide disc gel electrophoresis and sedimentation velocity ultracentrifugation. The number of bands seen in the gels varies with conditions used, but frequently as many as 8–13 bands are seen. Other analytic studies reported on membrane proteins prepared by the methods described above are seriously hindered by the problems of insolubility, aggregation, and heterogeneity.

Our approach to the study of membrane proteins differs from that of previous work in that the aim was not to extract a

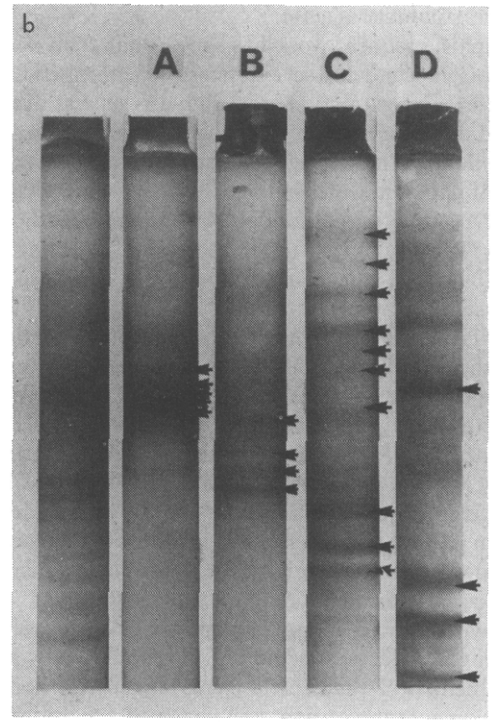

FIGURE 6b: Fractions A–D (Figure 6a) from Bio-Gel chromatography of CNBr digest were pooled, desalted on Bio-Gel P-2 in 0.05 M NH_4HCO_3, lyophilized, taken up in 4 M urea, and electrophoresed on standard 12% polyacrylamide discs. The gels were fixed and strained with coomassie blue as described. Gel on left is unfractioned CNBr digest. Arrows indicate unique bands.

maximum quantity of all membrane proteins, but to solubilize selectively a major membrane protein which could be readily purified and characterized. Mild, nondenaturing conditions were used in the extraction procedure in order to obtain the protein in its native state. Since our yield of spectrin accounts for less than one-quarter of the total membrane protein, it is probable that there is more than one structural protein in membranes. However, it is possible that some spectrin is bound within the membrane in such a way that it is not available for extraction under the mild conditions of the method. For instance, although the extracted spectrin contains no sialic acid or other carbohydrate moieties, it is possible that some of the spectrin within the membrane is conjugated with carbohydrate moieties, altering its solubility and electrophoretic properties. Some spectrin might also be bound to lipid in such a way that it is not extracted by the water–EDTA–β-mercaptoethanol method.

Whether or not spectrin accounts for more than 20% of the total protein of the ghost membrane, it is evident that spectrin is indeed an important structural component, since the intact ghosts become fragmented during the removal of spectrin, and lose a coating of filamentous material normally present on the inner surface of the membrane (Marchesi *et al.*, 1969).

Spectrin was initially extracted in soluble form from guinea pig erythrocyte ghosts which had been subjected to mild hydrolysis with trypsin. The protein extracted in this manner was shown to form fibers identical with muscle actin fibers seen under the same conditions, *viz.*, incubation with divalent cation at 37°. Because of this, extraction of protein from erythrocyte ghosts was attempted without trypsin, using for the extraction low ionic strength buffer in order to reproduce the conditions of maximal solubility of actin.

Although subsequent work has shown that actin and spectrin differ significantly by several physicochemical criteria, the low ionic strength extraction procedure was successful and has continued to be the essential factor in the preparation of spectrin. The final method developed consists of a three-stage extraction of ghosts in water in the presence of a reducing agent and EDTA at neutral pH and at pH 9.5. In the early experiments, ATP was thought to be necessary for extraction of spectrin, but it was subsequently found that ATP was acting primarily as a chelating agent and could be replaced by EDTA. Both EDTA and β-mercaptoethanol reduce aggregation and improve the yield of spectrin in the extraction procedure.

Although spectrin is soluble in water at neutral pH, the tendency toward aggregation is reduced at alkaline pH and in the presence of salt. Thus the optimal buffer developed for use in analytical studies consisted of 0.05 M Tris–0.3 M NaCl at pH 8.6. This is in contrast to the water-soluble protein preparations from ghost membranes reported from other laboratories which show aggregation at ionic strengths greater than 0.05–0.1 M (Mazia and Ruby, 1968; Blumenfeld, 1968).

We have repeated in our laboratory the detergent–low ionic strength extraction method of Mazia and Ruby and the pyridine method of Blumenfeld using human erythrocytes in order to compare these water-soluble membrane protein preparations with spectrin. Both preparations cross react on Ouchterlony plates with rabbit anti-human spectrin. Thus spectrin appears to be one of the components of these heterogeneous protein extracts.

The multiple peaks seen by sedimentation velocity ultracentrifugation of spectrin at neutral pH most probably represent polymerization of varying degree. The single species with a lower sedimentation coefficient (5.1) seen at high pH may represent partial or complete dissociation into subunits and/or unfolding of the polypeptide chain. Further dissociation and/or unfolding occurs in 6 M guanidine and dithiothreitol such that the sedimentation coefficient drops to 1.9. The reducing agent appears to be necessary to achieve dissociation and unfolding, since the sedimentation coefficient in 6 M guanidine without reducing agent was 2.9.

The wide range of observed molecular weights obtained by equilibrium ultracentrifugation at different speeds and concentrations of protein is not clearly explained. Linearity of the plots (log dy *vs.* x^2) indicates a high degree of homogeneity, but microheterogeneity (particularly if masked by nonideality) might not be detectable. At initial concentrations above 0.4 mg/ml, the apparent molecular weight at a given speed is seen to decrease. Concentration dependence may also explain the decrease of molecular weight seen with increasing speed. However, the molecular weight range obtained for spectrin in 6 M guanidine–5 mM dithiothreitol by this method (100,000–150,000) is supported by molecular weight estimations made by other methods. Viscosity studies under the same conditions give a value of 90,000–100,000 (method of Tanford *et al.*, 1967), and sodium dodecyl sulfate polyacrylamide gel electrophoresis gives 140,000 (method of Shapiro *et al.*, 1967). A count of peptides produced by cyanogen bromide cleavage of carboxymethylated spectrin (23) coupled with methionine content from amino acid analysis gives a value of 146,000.

Combining these various measurements, we estimate the minimum molecular weight of spectrin to be 140,000. Individual polypeptide chains of most well-characterized proteins are found to have molecular weights well under 100,000. Although a value of 140,000 seems high for a single polypeptide chain, it is not unprecedented. Jovin *et al.* (1969) report that *Escherichia coli* DNA polymerase is a single chain of molecular weight 109,000.

The possibility must be considered that the 140,000 molecular weight subunit of spectrin consists of more than one polypeptide chain cross-linked by bonds not cleaved in 6 M guanidine or sodium dodecyl sulfate. This possibility is particularly relevant because of the covalent cross-linking found in other structural proteins such as elastin, collagen, and fibrin. In these cases, however, cross-linking produces large, extremely insoluble polymers. Such cross-linking in spectrin would have to be confined to the 140,000 unit, since the larger polymers are soluble in aqueous solutions and dissociate readily in denaturants.

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The Synthesis of Long-Chain Fatty Acids by a Cell-Free System from *Mycoplasma laidlawii* A*

Shlomo Rottem and Charles Panos

ABSTRACT: A soluble system from *Mycoplasma laidlawii* A for the synthesis of long-chain saturated fatty acids from acetate is described. In addition to partial requirements for adenosine triphosphate, $MgCl_2$, and reduced nicotinamide–adenine dinucleotide phosphate, this system exhibited an absolute need for malonyl coenzyme A. This soluble system contained a high level of acyl carrier protein activity but a very low acetyl carboxylase activity. Highest fatty acid synthetase and "acyl carrier protein" activities were found in cells harvested during the stationary phase of growth. The products of this synthetase system were only saturated fatty acids, mainly stearic (83.2%). Palmitic (6.6%) and arachidic (10.1%) acids were also formed. Likewise, only saturated fatty acids were formed when β-hydroxydecanoic acid replaced acetate in this synthetase system. The addition of a β-hydroxy thioester dehydrase preparation from *Escherichia coli* to this mycoplasma-soluble system resulted in the formation of both saturated and unsaturated acids. Of the unsaturated fatty acids formed, hexadecenoic acid predominated.

Growing cells and washed suspensions of the nonsterol-requiring *Mycoplasma laidlawii* are capable of incorporating [^{14}C]acetate into long-chain saturated fatty acids (Rottem and Razin, 1967a; Pollack and Tourtellotte, 1967). However, the inability to detect any radioactivity in its unsaturated fatty acid content was supported by nutritional experiments showing a need for an octadecenoic acid for growth of this organism (Razin and Rottem, 1963; Razin *et al.*, 1966). Recent elongation studies have shown that while a closely related mycoplasma, *Mycoplasma* sp. KHS, was able to form octadecenoic acids from short-chain monoenoic acid precursors (Panos and Henrikson, 1969), *M. laidawii* A elongated such precursors mainly to hexadecenoic acids (Panos and Rottem, 1970).

These studies represent the first report of information concerning the *de novo* synthesis of saturated and unsaturated fatty acids by a mycoplasmal cell-free system. These results have been presented in preliminary form (Rottem and Panos, 1969).

Experimental Section

Organism and Growth Conditions. Mycoplasma laidlawii (oral strain) was obtained from S. Razin (The Hebrew University, Hadassah Medical School, Jerusalem, Israel) and is related to *Mycoplasma laidlawii* A (Rottem and Razin, 1967b). Cells were grown in 1–3 l. of a modified Edward medium (Razin, 1963) containing 2% PPLO serum fraction (Difco Laboratories, Detroit, Mich.). Growth was estimated by turbidity at 540 mμ. Cells were harvested after 22–30-hr

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