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Structural Study of Spectrin from Human Erythrocyte Membranes[†]

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ABSTRACT: Human erythrocyte spectrin prepared from fresh blood is a mixture of different association states. Depending on the manner of preparation, the two-chain dimer or the tetramer predominates. These forms are not in rapid thermodynamic equilibrium. The molecular weight of the dimer by sedimentation and diffusion and by light scattering is about 5×10^5 . The frictional properties indicate a low or moderate asymmetry (axial ratio in the range 2–10), and from the angular dependence of light scattering intensity an upper limit of about 80 Å can be set for the radius of gyration. The tetramer similarly has a moderate asymmetry. Electron microscopy

reveals that the dimer is a compact, slightly elongated molecule, and that the tetramer probably consists of two parallel dimers. On increasing the concentration of solutions containing spectrin dimers, oligomers are formed, which are not rapidly dissociated on dilution. At very low protein concentrations (below about 0.05 mg/mL) there is evidence of the onset of a rapid dissociation equilibrium between dimers and single chains. Other physical properties of the spectrin have been measured. The size and shape of the spectrin molecule would seem to rule out any major physical resemblance to myosin.

Spectrin is the major protein of the erythrocyte membrane, and is thought to play a critical role in determining its physical properties. In particular there is now an accumulation of evidence to suggest that it exerts a contractile control over the shape of the cell (Sheetz et al., 1976b). The presumption of a contractile function, as well as the subunit molecular weight, the presence of ATPase activity in the preparations, and the appearance of actin in the aqueous extracts from the mem-

branes have led to the conjecture that spectrin may be structurally and functionally related to myosin (Guidotti, 1972; Brandon, 1975; Schechter et al., 1976). Arguments have been given (Gratzer and Beaven, 1975) against such a view, but there has been poor agreement between results from different laboratories, bearing on the size and shape of the molecule; one study (Schechter et al., 1976) indeed reports, in contrast to earlier work, frictional properties compatible with a very asymmetric, myosin-like particle. In addition Sheetz et al. (1976b) have reported a weak cross-reaction of spectrin with antibodies to smooth-muscle myosin (though not of myosin to antispectrin). We have undertaken a study of spectrin by hydrodynamic methods, light scattering, and electron microscopy

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in an effort to resolve the question of its shape and size. The evidence indicates that the gross structure of spectrin is quite different from that of myosin.

Materials and Methods

Spectrin was prepared from human blood, not more than 1 day after it had been drawn. Most preparations followed the 37 °C extraction procedure of Marchesi (1974), using the conditions previously described (Gratzer and Beaven, 1975). In some cases the 4 °C dialysis procedure (Marchesi et al., 1970) was followed. The resulting preparations were concentrated when desired by vacuum dialysis, or by precipitation, either by addition of an equal volume of saturated ammonium sulfate (Ralston, 1976) or by adjustment of the pH. For the latter purpose 0.1 vol of 0.5 M acetate buffer (pH 4.9) was added to the protein solution. The precipitate was rapidly collected by centrifugation for 5 min at 5000 rpm and was immediately redissolved in the column buffer. The crude spectrin, containing a variable quantity of haemoglobin, as well as actin and other minor contaminants, was chromatographed on a column of Sepharose 4B (1.5 × 90 cm), eluted at 10 mL/h. The buffer was generally 10 mM Tris, containing 25–100 mM sodium chloride (pH 7.8). For some purposes (e.g., samples required for nitrogen determinations) a borate buffer was used. Nitrogen determinations were performed by either the standard Kjeldahl or by a colorimetric micro-Kjeldahl technique (Jaenicke, 1974). These determinations were performed respectively by Mrs. S. Ehrlich-Rogozinski and Dr. J. C. Pinder. The partial specific volume was measured with the aid of a Paar densimeter, thermostated at 25 °C. The refractive index increment was measured in a Beckman Model E analytical ultracentrifuge, using the Rayleigh optical system, and a double sector synthetic boundary cell.

Sedimentation velocities were determined in the Beckman Model E ultracentrifuge using the Schlieren optical system and ultraviolet scanner optics for low protein concentrations. Speeds between 42 000 and 60 000 rpm and centerpieces of 12 and 30 mm were used. Diffusion coefficients were measured by laser autocorrelation spectroscopy using a Malvern instrument (Malvern 4300, Precision Devices and Systems (UK) Ltd., Malvern), modified as described elsewhere (Jolly and Eisenberg, 1976). Light scattering intensity measurements were made in the same instrument over the angular range 20–160°. Solutions were clarified by centrifugation at 80 000g for 30 min.

Electron microscopy of spectrin was performed on ultrathin carbon films as described by Huxley and Zubay (1960). Protein solutions at different concentrations between 0.1 and 2 mg/mL were negatively stained with uranyl acetate, and examined in a Philips 300 electron microscope at 80 kV and an electron optical magnification of 50 000. Because of the pH-dependent solubility characteristics of the protein, the grid was washed with the buffer before staining. If this step was omitted copious quantities of small aggregates were always observed on the grid.

Results

All spectrin preparations, even when fresh and undisturbed by concentrating procedures, are appreciably heterogeneous. Apart from extraneous proteins (especially hemoglobin and actin), soluble aggregates of spectrin are always present, and separate readily on the Sepharose 4B column. Sedimentation velocity shows these to be mostly relatively small oligomers, sedimenting at about 15 S. This leading (excluded) component is also found by electron microscopy to contain a small amount of actin in the form of short filaments of the characteristic

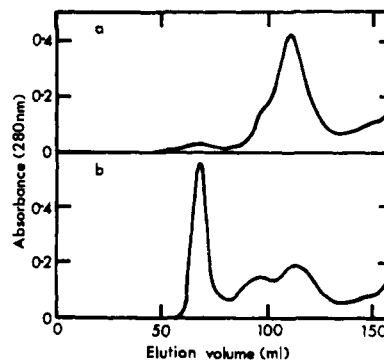


FIGURE 1: (a) Elution profile from Sepharose 4B column of typical spectrin preparation (37 °C extract), showing major dimer component with small proportions of tetramer (shoulder) and higher aggregates. (b) Elution profile after concentration of spectrin dimer preparation by precipitation with ammonium sulfate.

double-helical form (Hanson and Lowy, 1963). When the spectrin is prepared by brief extraction at 37 °C the bulk of it elutes in a single peak (Figure 1) with a sedimentation coefficient of 9.5 S. This is in general the only important component seen in the analytical ultracentrifuge in crude preparations (Gratzer and Beaven, 1975), and was found by sedimentation equilibrium to have a molecular weight of ca. 430 000, corresponding to a dimer of two polypeptide chains. Small proportions of a presumed tetramer (sedimenting at 12.5 S) and of a monomer of 4.4 S are seen in some preparations (Figure 1). When the protein solution is concentrated by precipitation, or by vacuum dialysis to a concentration approaching 1 mg/mL, or perhaps less, the proportion of spectrin in the aggregated state increases (Figure 1). The dimer component reapplied without a concentration step to the column elutes entirely as a single dimer band. If on the other hand it is first concentrated by any of the above procedures it generates new aggregates. This occurs whether or not reducing agents, such as β -mercaptoethanol or dithiothreitol, are present. Thus, except for measurements of partial specific volume and refractive index increment, column-purified spectrin was subjected to no further concentration procedures before use. In substantial agreement with Ralston (1975, 1976), we find that spectrin prepared by 24-h dialysis in the cold contains much more of the 12.5S species, which he has identified as tetramer. The elution volumes and sedimentation coefficients of the dimer and tetramer fractions in the two types of preparation are identical.

The diffusion coefficient of the dimer was determined by the photon correlation spectroscopy technique to be $1.55 \times 10^{-7} \text{ cm}^2/\text{s}^{-1}$. There was no measurable dependence on protein concentration over a tenfold range, but below 0.1 mg/mL (at which the precision of measurement is relatively poor) the diffusion coefficient appeared to increase, suggesting the onset of dissociation.

In order to evaluate other molecular properties it was necessary to redetermine the ultraviolet specific absorptivity of the protein, so as to provide a firm basis for concentration determinations. Published values vary between $E_{1\text{cm}}^{1\%}$ of 8.8 (Marchesi et al., 1970) and 11.5 (Ralston, 1976). Both conventional and colorimetric Kjeldahl nitrogen determination gave a value of 10.7 for the specific absorptivity. The internal consistency of the data given below suggests that this value is correct. Thus, refractive index measurement using the Rayleigh optical system of the Model E ultracentrifuge, with the isolated mercury emission line at 546.3 nm, gave 4.10 fringes $\text{mg}^{-1} \text{ mL}$. This is precisely the average for unconjugated

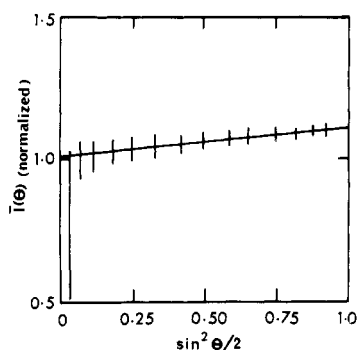


FIGURE 2: Angular dependence of light scattering from spectrin dimer solution. The data are based on 200 intensity measurements; the ordinate is the concentration-normalized scattered intensity relative to benzene. Error bars show total spread of values.

globular proteins, according to Babul and Stellwagen (1968), who found 4.10 ± 0.13 fringes $\text{mg}^{-1} \text{mL}$. The corresponding refractive index increment is 0.186. Moreover, density measurements gave a partial specific volume for spectrin of 0.733, which agrees with the value of 0.73 calculated from the amino acid composition. We are thus in a position to determine the molecular weight by use of the Svedberg equation, from which we obtain for the main homogeneous component of spectrin a value of 540 000.

A value for the molecular weight was also obtained by light scattering. The apparent weight-average molecular weight (Eisenberg, 1971) is:

$$\overline{M}_w = \left[\frac{4\pi^2 n_B^2}{\lambda_0^4 N_{Av} R_B} \left(\frac{\partial n}{\partial c} \right)_\mu \frac{2 \{c I_B(90^\circ)\}}{\Delta I(\theta) \sin \theta} \right]^{-1}$$

where n_B is the refractive index of benzene, 1.511, and R_0 is the Rayleigh ratio, $29.9 \times 10^{-6} \text{ cm}^{-1}$, of benzene, at the wavelength, λ_0 , of 514.5 nm, for vertically polarized light. N_{Av} is the Avogadro number, $(\partial n / \partial c)_\mu$ is the refractive index increment, c is the protein concentration, $I_B(90^\circ)$ is the scattered intensity for benzene at 90° and $I(\theta)$ is that for the sample at an angle θ . Extrapolating to $\theta = 0$, the molecular weight emerges as $470\,000 \pm 30\,000$. The concentration dependence is negligible, and the angular dependence of scattering decreases by less than 10% with change of scattering angle from 30 to 150° (Figure 2). This enables us to set an upper limit to the radius of gyration, R_G , of the molecule. From the extrapolated particle scattering factor, we obtain $R_G \lesssim 80 \text{ \AA}$, assuming a monodisperse system. The upper limits of the principal dimensions for the simplest models then become (Tanford, 1961): 100 \AA for the radius of a sphere, 280 \AA for the length of a thin rod, and 200 \AA for the root-mean-square end-to-end distance of a coil. It is clear, therefore, that the spectrin dimer is a molecule of low to moderate asymmetry, which can be regarded as a globular protein. It has already been noted (Gratzer and Beaven, 1975) that on the basis of the frictional coefficient derived from sedimentation velocity an axial ratio in the range 2–10 and most probably about 5 can be expected. Similarly, the diffusion coefficient corresponds to a frictional ratio of 2.4 leading to a similar conclusion. For the usually assumed values for the hydration of a protein the axial ratio for an equivalent prolate hydrodynamic ellipsoid should be in the range 4–8.

The tetramer has a molecular weight of 10^6 , as already established by Ralston (1975, 1976). This is in accord with our hydrodynamic data, viz. a sedimentation coefficient of 12.2 S and a diffusion coefficient of $1.16 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. The frictional ratio is fairly similar to that of the dimer (2.9 as against 2.1). The tetramer is again therefore not a very

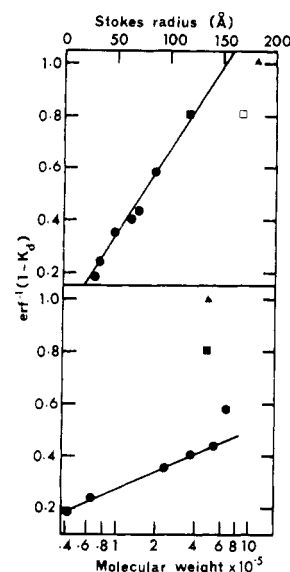


FIGURE 3: Gel filtration on Sepharose 4B of globular proteins and of myosin and spectrin. (Lower panel) Relation of partition coefficient on column and molecular weight. The circles represent the series of globular proteins: G-actin, hemoglobin catalase, glycogen phosphorylase α , β -galactosidase, and thyroglobulin, the triangle rabbit skeletal muscle myosin and the square spectrin dimer. (Upper panel) Relation of partition coefficient on column and Stokes radius, calculated from sedimentation coefficients. Symbols are as above. The open square represents spectrin if the Stokes radius is calculated from the sedimentation coefficient after treatment with deoxycholate (Schechter et al., 1976).

asymmetric molecule. As with the dimer there is little angular dependence of light scattering.

In an attempt to resolve the apparent conflict between our data and those of Schechter et al. (1976), preparations based on extracts with deoxycholate were chromatographed on the Sepharose 4B column. The presence of this detergent in high concentration is the only respect in which their preparative method differs from the procedure used in other laboratories. In agreement with their observations, apart from a small amount of material in the excluded volume, essentially all the protein eluted at the position corresponding to the dimer. Again, in agreement with Schechter et al., the sedimentation coefficient was greatly diminished, with a value of 6.9 S. The diffusion coefficient was found to be $1.9 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. Thus, with the same value of the partial specific volume the weight-average molecular weight emerges as 320 000, using the Svedberg equation. If the partial specific volume, \bar{v} , were the source of the error, it would need to be as high as 0.84 mL g^{-1} , i.e. higher than that of deoxycholate (Tanford et al., 1974) to bring the molecular weight up to 5×10^5 . Thus, the anomaly cannot be explained in terms of binding of deoxycholate. The possibility that the diffusion measurements were vitiated by the presence of deoxycholate micelles can probably be excluded, since no excess light scattering was observed from column fractions devoid of protein.

Additional evidence of a semiquantitative kind in favor of only moderate asymmetry is provided by the retardation of spectrin on the Sepharose 4B columns. It is well established (for a review see Ackers, 1970) that the elution volumes of native globular proteins in gel filtration, that is to say the partition coefficients between the matrix and the mobile phase, are a smooth function of molecular weight, as long as the shape does not deviate substantially from spherical. An asymmetric molecule is recognized, if its molecular weight is known, by an anomalously low elution volume. In principle the elution law can be extended to encompass also species of appreciable

asymmetry by referring the calibration curve to the Stokes radius, R_e . For a partition coefficient, K_d , the following equation (Ackers, 1970), with a_0 and b_0 constants, has been widely used:

$$R_e = a_0 + b_0 \operatorname{erf}^{-1}(1 - K_d)$$

More recently, however, it has been found (Nozaki et al., 1976) that for particles of large asymmetry, this equation fails, apparently in consequence of end-on penetration of the molecules into the matrix. Thus, Nozaki et al. have shown that native fibrinogen, $\phi 2$ bacteriophage, and especially myosin deviate grossly from the linear relation. Our results for spectrin are shown in Figure 3. In the first place the qualitative observation that the spectrin dimer elutes at a substantially higher volume from the Sepharose column than myosin indicates that it is much less asymmetric. As expected from the frictional properties there is at the same time evidence of some asymmetry, for the elution volume, and therefore the function $\operatorname{erf}^{-1}(1 - K_d)$ falls appreciably below the calibration, based on globular proteins (Figure 3). This of course applies equally to spectrin prepared and eluted, according to Schechter et al. (1976), in the presence of deoxycholate, the K_d being identical with that for the standard preparations.

Secondly, when the data are plotted in terms of the Stokes radius, derived from the sedimentation coefficients by way of the relation:

$$R_e = M(1 - \bar{v}\rho)/6\pi\eta_0Ns$$

(where η_0 is the solvent viscosity), the spectrin dimer with a sedimentation coefficient of 9.5 S falls, within experimental error, on or at least near the calibration. By comparison, myosin is grossly anomalous. One may therefore again conclude that spectrin is much less asymmetric than myosin. It may be noted that the value of the Stokes radius corresponding to the very much lower sedimentation coefficient obtained for spectrin in deoxycholate shifts the spectrin dimer far away from the calibration, though not of course to the position for myosin, since this elutes at a much lower K_d .

Electron microscopy of spectrin dimers, tetramers, and oligomers confirmed the identification of spectrin as compact molecules of moderate or low asymmetry (axial ratio not greater than about 5 for the dimer). The dimensions are compatible with the volume of $6 \times 10^5 \text{ \AA}^3$ that corresponds to a molecular weight of 5×10^5 and partial specific volume of $0.73 \text{ cm}^3 \text{ g}^{-1}$. The various forms are shown in Figure 4.

At very low concentrations (in the range of $20 \text{ }\mu\text{g/mL}$) many of the particles appear to be smaller, suggesting that dissociation into single chains has occurred. This is consistent with observations of diffusion coefficients, and is borne out by sedimentation velocity, performed at very low protein concentrations, using ultraviolet scanner absorption optics, and a centerpiece of 30-mm optical path. At low concentrations there is a downward trend in sedimentation coefficient. Only one boundary is apparent, reflecting a rapid association-dissociation equilibrium (Gilbert, 1959), in contrast with the slow transition observed in the course of unfolding at higher protein concentration (Gratzer and Beaven, 1975).

Discussion

The results presented above confirm that spectrin as extracted from the erythrocyte membrane is a mixture of dimer and tetramer, the former predominating in preparations made at 37°C , the latter in preparations produced by prolonged dialysis at 4°C . In polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate both types of preparation show an equimolar mixture of chains of about 2.2 and $2.4 \times$

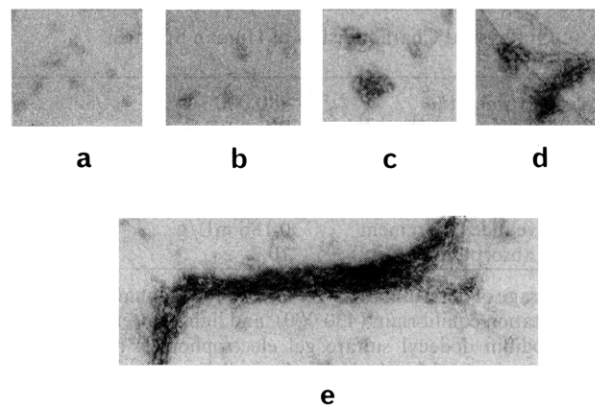


FIGURE 4: Electron microscopy of spectrin fractions: all $\times 90,000$; $1 \text{ mm } 50 \text{ \AA}$: (a) spectrin dimers; (b) spectrin tetramers; (c) oligomers from excluded peak of elution profile of concentrated purified spectrin; (d) oligomers from excluded peak of crude spectrin preparation, showing the presence of F-actin filaments; (e) fibrous aggregates of purified spectrin made by isoelectric precipitation of dimers on the grid, by exposure to the acidic staining solution without prior wash to remove excess protein.

10^5 mol wt, and are not perceptibly different. The dimer and tetramer components elute as single peaks in their original positions when rechromatographed on Sepharose 4B columns, and do not interconvert within the time scale of the experiment. When warmed to 37°C (Ralston et al., 1977; J. C. Pinder and W. B. Gratzer, unpublished results) the tetramer progressively gives place to the dimer. The latter is a stable species, and dissociates or aggregates only on aging, or on treatment with denaturants (Gratzer and Beaven, 1975), though at very low protein concentrations ($50 \text{ }\mu\text{g/mL}$ downwards) a rapid dissociation equilibrium appears to supervene. The concentration range in which this occurs corresponds to a dissociation constant of the order of 10^{-8} – 10^{-9} M . When the spectrin solutions as extracted are subjected to concentrating procedures, the proportion of oligomeric material increases; if this is an equilibrium process, then the equilibrium is established slowly compared with the hydrodynamic time scale. A consequence is that attempts to concentrate a homogeneous dimer isolated by column chromatography must be expected to result in the appearance of a new heterogeneity.

The relation of our observations to those of Schechter et al. (1976) remains unclear. The molecular weights measured by these authors and the identity of the elution volume of their material with that of dimer, prepared by either of the conventional methods, employing no detergent, suggest that it should be similar in size and shape to the dimers observed in our electron micrographs, yet its hydrodynamic characteristics appear to be quite different. It is difficult to see how the observed changes could be the consequence of the attachment of residual deoxycholate. If one assumes, following Schechter et al., that any bound deoxycholate can be disregarded, then the molecular weight from the Svedberg equation emerges as halfway to that of single chains, and indeed Schechter et al. find by sedimentation equilibrium a sizable proportion (up to 26%) of monomeric material. It would not be surprising if the rapid monomer-dimer equilibrium evinced in aqueous solution at very low protein concentration were displaced toward higher concentration in the presence of any deoxycholate. In such a case sedimentation velocity, depending, for example, on the pressure dependence of the equilibrium, would not necessarily give an interpretable value of the sedimentation coefficient. If on the other hand no deoxycholate remains after passage through the column, an explanation for the discrepancy between the results of Schechter et al. and those of ourselves and others might have to be sought in nonequilibrium terms.

TABLE I: Physical Characteristics of Human Spectrin Heterodimer.

Molecular weight ^a	480 000
Sedimentation coefficient	9.5 ± 0.2 S
Diffusion coefficient	(1.55 ± 0.05) × 10 ⁻⁷ cm ² s ⁻¹
Radius of gyration	≤ 80 Å
Partial specific volume	0.73 ₃ g/mL
Refractive index increment	0.186 mL/g
Specific absorptivity ($E_{1\%}^{1\text{cm}}$)	10.7

^a Average of determinations by the Svedberg equation (540 000), sedimentation equilibrium (430 000), and light scattering (470 000). From sodium dodecyl sulfate gel electrophoresis an estimate of 460 000 is obtained for the heterodimer.

The spectrin in the dimer and tetramer peaks from the Sepharose column is very pure. Gel electrophoresis in the presence of sodium dodecyl sulfate shows only very faint traces of actin, the bulk of this contaminant appearing in the void volume as polymers. It seems clear that the toroidal forms first seen by Harris (1971) are not related to spectrin, as Kirkpatrick et al. (1976) state them to be. The shapes of both the dimer and tetramer as seen in the electron microscope are compatible with the inferences from frictional properties. The salient characteristics are summarized in Table I. It seems safe to conclude that spectrin is not in any physical sense similar to myosin.

Acknowledgments

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