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Erythrocyte Spectrin. Purification in Deoxycholate and Preliminary Characterization†

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ABSTRACT: Erythrocyte spectrin, isolated by aqueous extraction of erythrocyte ghosts, may be freed from contaminating membrane lipids and small amounts of other proteins by gel chromatography in 5 or 10 mM deoxycholate. The purified protein, in deoxycholate, is a mixture of monomers and dimers, both highly asymmetric molecules. The hydrodynamic properties of the dimer closely resemble those of muscle myosin, and spectrin and myosin also have similar circular dichroism spectra. The proportion of dimer to monomer in the purified protein varies from one preparation to another, an observation for which there is no simple explanation. In the absence of deoxycholate, spectrin associates beyond the dimer stage, possibly by loose end-to-end aggregation involving hydrophobic forces.

The term "spectrin" was originally coined (Marchesi and Steers, 1968) to designate the protein that is readily solubilized from erythrocyte ghosts by dilute EDTA[1] or other aqueous media in which the concentration of inorganic cations is kept very low. Polypeptide chain analysis by sodium dodecyl sulfate gel electrophoresis shows that the principal constituents of this material appear as two bands corresponding to apparent molecular weights of about 220 000 and 200 000 (Trayer et al., 1971), but numerous other polypeptide components are present as well, in amounts depending on the precise conditions of solubilization (Reynolds and Trayer, 1971). More recent usage is to employ the term "spectrin" to refer exclusively to the two major electrophoretic bands (Fairbanks et al., 1971), and this is the definition adopted in this paper, with the understanding that this does not necessarily imply the presence of only two kinds of chemically distinct polypeptide chains. An alternative name for the same protein is "tektin A" (Clarke, 1971). This name derives from the solubilization procedure described by Mazia and Ruby (1968), which is less selective than the aqueous extraction method.

There is strong evidence suggesting that the proteins soluble in aqueous media play a central role in the maintenance of the shape of the erythrocyte membrane (Rosenthal et al., 1970; Steck, 1974; Singer, 1974), and that they may serve as a site of attachment for trans-membrane proteins, limiting their freedom of motion (Nicolson and Painter, 1973; Elgsaeter and Branton, 1974; Pinto da Silva and Nicolson, 1974). It is not known, however, whether this structural function is ascribable

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[1] Abbreviations used are: DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetic acid.

purely to spectrin (as here defined) or whether other soluble proteins are involved as well. Guidotti (1972) has speculated that spectrin may be related to muscle myosin and that it may function in concert with the actin-like 45 000-dalton polypeptide of the erythrocyte (Tilney and Detmers, 1975; Painter et al., 1975). When the fibrous structure that provides the morphological evidence for the idea of a structural framework was first described, it was likened to fibers of actin *alone* (Marchesi and Palade, 1967).

Previous studies of soluble protein containing spectrin as major component have led to contradictory results. Marchesi et al. (1970) reported that the protein is aggregated in aqueous solution, but their results are of questionable significance because the behavior of their sample in concentrated guanidine hydrochloride clearly indicates gross contamination by polypeptides of low molecular weight. Clarke (1971) also found the protein to be aggregated and showed that the aggregates were highly asymmetric. Gratzer and Beaven (1975) on the other hand report that the principal species under physiological conditions is a dimer and observed no tendency for aggregation even in the presence of Ca^{2+}, removal of which ion is thought to be a major factor in the initial solubilization of the protein (Reynolds, 1972). Fuller et al. (1974) also find spectrin to be nonaggregated in aqueous media, but report a molecular weight of 239 000, corresponding roughly to monomeric protein. These discrepancies undoubtedly reflect differences in preparative procedures and/or storage of the solubilized protein, and possibly in the care that was taken to avoid proteolysis.

In our hands freshly solubilized spectrin is a heterogeneous aggregate, with properties similar to those described by Clarke (1971). We find, however, that our freshly solubilized protein is still far from pure and that spectrin of much better purity can be prepared by chromatography after the addition of deoxycholate (DOC). The protein also becomes disaggregated in this solvent, with the formation of monomers and dimers. The dimers obtained in this way have, however, quite different hydrodynamic properties from those reported by Gratzer and Beaven (1975) for dimers in a purely aqueous medium.

Experimental Section

Materials. Deoxycholate (DOC) was obtained from Fisher Scientific Co. and purified as described by Makino et al. (1973). [^{14}C]DOC was purchased from Mallinckrodt Co. and found to be 99% pure by thin-layer chromatography. Sepharose 4B was obtained from Pharmacia, Inc., and columns from the same source were used for chromatography. All other reagents were standard laboratory reagents.

Aqueous Extraction. Erythrocyte ghosts were prepared from 100–500 ml of freshly drawn blood by the method of Dodge et al. (1963). Bicarbonate buffer (pH 8.2) was used instead of phosphate buffer, so as to permit subsequent analysis for organic phosphate as a measure of lipid content. The water-soluble proteins were extracted in 100–500 ml of distilled water at 4 °C, as described by Reynolds and Trayer (1971), for a period of 6–20 h. About 25% of the total membrane protein was solubilized after 20 h.

Volume Reduction. Several procedures were used to reduce the volume of the aqueous extract. In some preparations the extract was lyophilized and stored frozen. In one preparation the volume was reduced by vacuum evaporation from frozen extract, but the process was not carried to the state of dryness. Precipitations at pH 4.6 (Reynolds and Trayer, 1971) or by $(NH_4)_2SO_4$ (Marchesi et al., 1970) were each used once. One preparation was stored in the cold without volume reduction after dithiothreitol and EDTA had been added and was concentrated by pressure dialysis (Amicon Corp.) after buffered DOC was added in the following step of the preparation.

Gel Chromatography in DOC. Columns (1.5 × 90 cm) of Sepharose 4B were equilibrated and subsequently eluted with 5 or 10 mM DOC, 10 mM Tris buffer (pH 9.0), 0.1 mM EDTA with or without 1.0 mM dithiothreitol, and in one case with the addition of 0.05 M NaCl. About 5 mg of extracted protein was dissolved in 1 ml of the same solution, except that a large excess of DOC was added (final concentration 0.1 M) for solubilization of lipid in the form of small lipid–DOC micelles. This solution was layered on the column and chromatographed at 4 °C or at room temperature. The procedural variations described here had no effect on the results.

Protein Concentrations. Relative protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Absolute concentrations were based on quantitative amino acid analysis of one sample, from which a multiplying factor of 1.08 was determined for the conversion of concentrations obtained by the Lowry method to absolute values. The extinction coefficient at 280 nm in DOC solution was found to be $E_{1cm}^{1\%} = 10.7$.

Sodium Dodecyl Sulfate Gel Electrophoresis. The method of Weber and Osborn (1969) was used. Initial ghost preparations and aqueous extracts contain the two spectrin bands of essentially equal intensity, as originally described by Berg (1969), and purified spectrin contains no other bands. Sometimes there appeared to be a slight shoulder on the faster migrating band when the gel was scanned spectrophotometrically, as illustrated by Figure 3 (below). It is difficult to decide whether this is experimentally significant.

Ultracentrifugation. Sedimentation equilibrium and velocity measurements utilized a Beckman-Spinco Model E analytical ultracentrifuge equipped with a photo-electric scanner. Absorbance at 280 nm was used to measure protein concentration as a function of position in the cell. No fluorocarbon was used at the bottom of the cell because membrane proteins and detergents tend to interact with this material. This prevented concentration measurements to the very bottom of the solution column, which proved not to be a serious inconvenience. The partial specific volume of spectrin, calculated from its amino acid composition by the method of Cohn and Edsall (1943), was found to be 0.737 cm^3/g. Allowance for bound DOC was made as previously described (Tanford et al., 1974).

Circular Dichroism. A Cary Model 60 spectropolarimeter with circular dichroism attachment was employed. A mean residue weight of 115 (based on amino acid composition) was used for calculation of mean residue ellipticity.

Results

Initial Aqueous Extract. The time of extraction was varied from 6 to 20 h without apparent effect on the purified product ultimately obtained. More than 50% of the spectrin was extracted, but no attempt was usually made to approach 100% yield because contamination by other membrane proteins becomes significant as the extraction is prolonged (Reynolds and Trayer, 1971). Sodium dodecyl sulfate gel electrophoresis showed that the product consisted predominantly of the two spectrin polypeptides, but the 45 000-dalton polypeptide was also present together with small amounts of other chains, as has been previously reported (e.g., Steck and Yu, 1973). The initial extract always contained a small amount of phospholipid, at a level of about 0.1 g/g protein. Comparable figures have been reported previously, e.g., 0.1–0.2 g/g (Reynolds and Trayer, 1971) and 0.04 g/g (Clarke, 1971). One preparation

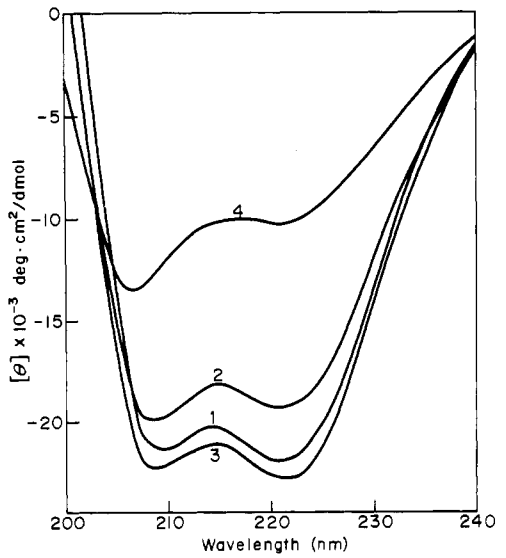


FIGURE 1: Circular dichroism spectra of typical crude aqueous extract (curve 1), the same extract after addition of DOC to a concentration of 10 mM (curve 2), purified spectrin in 10 mM DOC (curve 3) and in 0.5% sodium dodecyl sulfate after removal of DOC (curve 4).

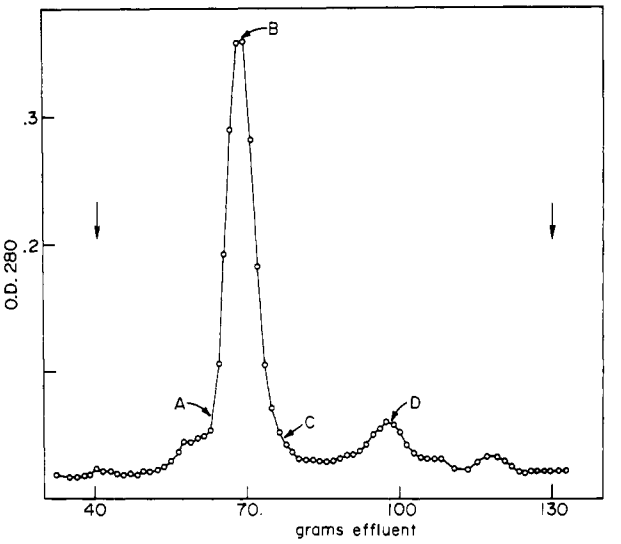


FIGURE 2: Elution profile of aqueous extract dissolved in 0.1 M DOC and chromatographed on Sepharose 4B, equilibrated and eluted with 10 mM DOC, 10 mM Tris buffer (pH 9.0), 50 mM NaCl, 0.1 mM EDTA. The arrows show the positions of void and total volume markers. The letters A to D give the approximate origin of fractions for the electrophoretic scans shown in Figure 3.

was extracted for a longer period (46 h), so as to leave only trace amounts of spectrin associated with the particulate fraction after centrifugation. The final purified product did not differ from that obtained from partial extracts.

The extracted spectrin was heterogeneous on the basis of gel chromatography and sedimentation equilibrium and somewhat aggregated. Average properties were not unlike those reported by Clarke (1971). Because the following steps in our procedure provided us with spectrin of greater purity, the characterization of the initial aqueous extract with regard to size and shape was not pursued. The circular dichroism spectrum was measured (Figure 1) to assess the possibility of major conformational changes in the subsequent treatment.

Several different methods were used to concentrate the aqueous extract, as described in the Experimental Section. It is possible that these different procedures have an effect on the final purified product, as will be noted below, but no systematic investigation was carried out.

Iodoacetamide was added at the very beginning of the extraction in one experiment, so as to protect exposed SH groups and prevent subsequent formation of intermolecular disulfide bonds. This had no significant effect on the character of the initial extract, nor on the results for purified spectrin presented in the remainder of this paper. We concluded that disulfide bond formation does not play an important role in the association between spectrin polypeptides. The same conclusion is reached from the lack of any dependence of the results on whether or not dithiothreitol was present during subsequent purification steps.

Purification in Deoxycholate. Reynolds and Trayer (1971) had previously shown that phospholipid can be removed from the aqueous extract by prolonged dialysis. Addition of DOC to the concentrated, precipitated, or lyophilized extract was initially intended to provide a more convenient method for delipidation since small mixed DOC–lipid micelles are readily separable from relatively large protein molecules by gel chromatography. It was found, however, that DOC exerted two other powerful effects, illustrated by the typical chromatogram shown in Figure 2. (1) Spectrin became disaggregated. The spectrin peak is fairly symmetrical, and significantly more retarded by the gel than the protein in the original extract, which always gave a broad, unsymmetrical elution pattern extending to the void volume marker. (2) Spectrin was clearly separated from the 45 000-dalton protein and from other minor contaminants. Sodium dodecyl sulfate gel electrophoresis showed that the central part of the spectrin peak contains only the two spectrin polypeptides, as illustrated by Figure 3. The trailing edge contained additional weak bands that correspond to apparent molecular weights in the range of 150 000–180 000. The entirely separate elution peak (D) contains principally a single polypeptide of apparent molecular

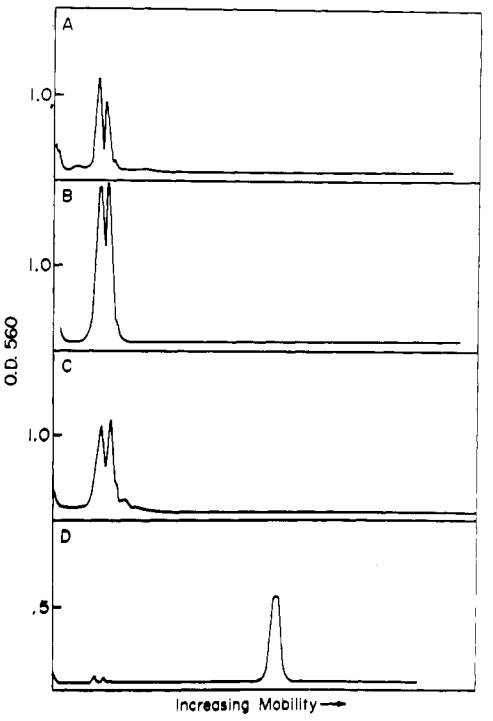
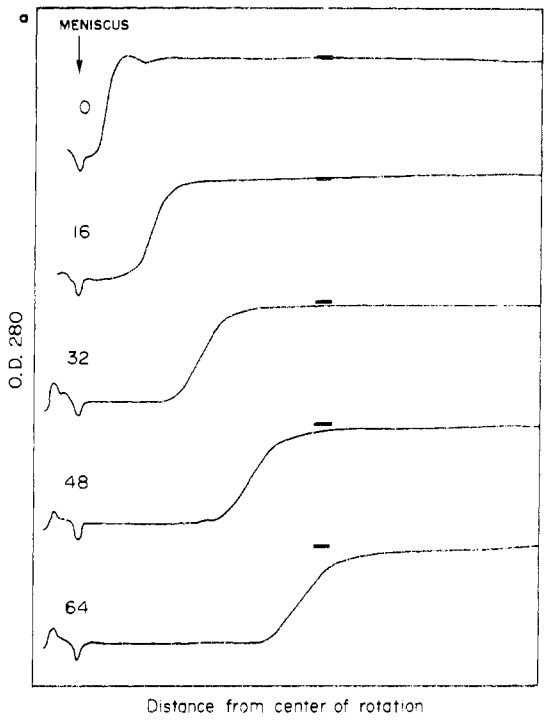


FIGURE 3: Spectrophotometric scans of sodium dodecyl sulfate gel electrophoretic patterns of four fractions from chromatography on Sepharose 4B. The samples came from a column similar to the one on which Figure 2 is based.



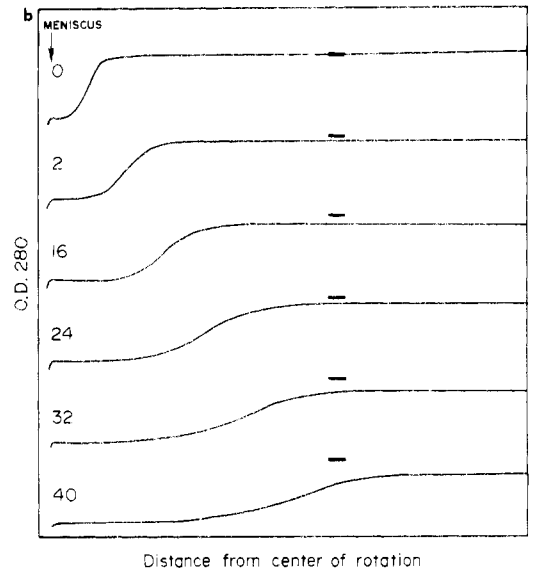


FIGURE 4: Sedimentation boundary of purified spectrin (a) in 10 mM DOC, and (b) after redissolving in aqueous buffer (0.01 M Tris-HCl at pH 8, 0.05 M NaCl, 0.1 mM EDTA, 1 mM dithiothreitol). The numbers for each scan represent time in minutes after the first scan. The heavy bar in the center of each scan is the initial optical density, uncorrected for radial dilution. The meniscus was at 6.3 cm from the center of rotation, and the right-hand edge of each diagram corresponds to 7.1 cm. Both runs were made at room temperature at 48 000 rpm. Sedimentation boundaries of the unpurified aqueous extract were similar to those in b, but not quite as broad.

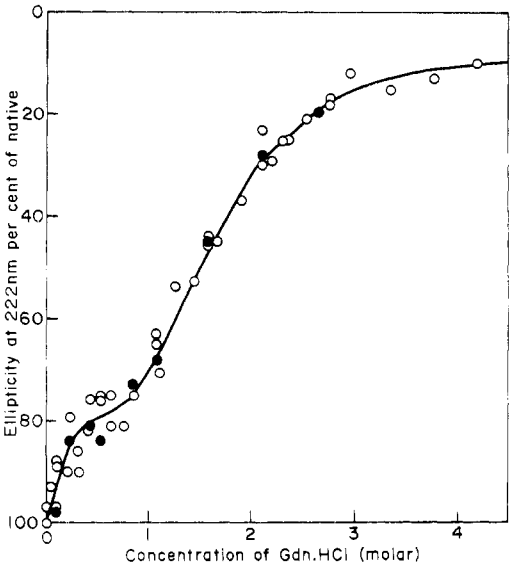


FIGURE 5: Denaturation of spectrin by guanidine hydrochloride at 25 °C. Results of several experiments have been superimposed. Filled circles represent reversed points obtained after equilibration at higher denaturant concentrations (up to two times the final concentrations). Corrections for the effect of denaturant on the refractive index of the solutions were made.

weight 45 000 and presumably represents the actin-like component of the erythrocyte membrane. Hemoglobin, if present in the aqueous extract, elutes after peak D.

Protein from the central portion of the spectrin peak of Figure 2 was used for all subsequent work. Analysis showed that not all of the organic phosphorus (arising primarily from phospholipid) had been removed from the protein: a residue of 2.5 ± 0.5 atoms of P per polypeptide chain remained. This probably reflects the presence of endogenous phosphoserine and/or phosphothreonine. A. D. Roses (personal communication) has found an average of about 2 atoms of bound P per polypeptide chain in spectrin, which can be removed by the action of phosphoprotein phosphatase (Kato and Bishop, 1972).

Figure 1 shows the circular dichroism spectrum of spectrin purified in this manner, and, for comparison, the circular dichroism spectrum of spectrin disaggregated by sodium dodecyl sulfate. It is clear that purification by DOC has not resulted in a major conformational change detectable by this method, such as occurs in sodium dodecyl sulfate.

Purified Spectrin in Aqueous Solution. The heterogeneous aggregated state of spectrin in the initial aqueous extract could have resulted from interaction with other components, such as the 45 000-dalton polypeptide or phospholipid. This possibility was eliminated by removal of DOC from purified spectrin by dialysis and examination of the DOC-free solution which contained none of these components. Gel chromatography, sedimentation equilibrium, and sedimentation velocity measurements in various media, including media that can be used to solubilize spectrin from erythrocyte ghosts, all indicated that purified spectrin under these conditions is in fact slightly more aggregated and more heterogeneous than the crude protein of the initial aqueous extract. A typical boundary from a sedimentation velocity experiment is shown in Figure 4. The average sedimentation coefficient was about 8S, similar to that observed in the initial extract.

Denaturation by Guanidine Hydrochloride. It has been shown previously (Gwynne and Tanford, 1969) that spectrin is readily denatured by guanidine hydrochloride, forming randomly coiled polypeptides in which all traces of ordered structure, detectable by circular dichroism, have disappeared. A somewhat more detailed study of this process was carried out to explore the possibility that low concentrations of guanidine hydrochloride might disaggregate the protein without causing denaturation. Progress of denaturation as a function of denaturant concentration was followed by measurement of ellipticity at the 222-nm trough (Figure 1). Equilibrium was attained rapidly, and was found to be reversible. The results

are given in Figure 5. The change in $[\theta]$ occurs in two stages, the first of which represents a small fraction of the total change such as might accompany dissociation of oligomers without denaturation. However, when spectrin in 0.65 M guanidine hydrochloride was examined by sedimentation equilibrium, it was found to be still heterogeneous in molecular weight. Moreover, a precipitate appeared slowly under these conditions.

Preliminary Characterization in Deoxycholate. Since the disaggregation of spectrin by DOC is reversed when DOC is removed, it follows from thermodynamic principles (Wyman, 1964) that DOC must be bound to the disaggregated protein. If the binding is large, it would significantly affect the interpretation of sedimentation data and preliminary binding data were therefore obtained, by carrying out column chromatography as in Figure 2, using radioactive DOC, so that the excess DOC traveling with the protein could be measured. The measurement was made only at the high concentration of DOC that was used for ultracentrifugation, where the background counts are necessarily high and preclude precise measurement of binding. We found that the binding was very small, barely detectable above background: our best estimate is 0.07 ± 0.07 g of DOC bound per g of protein. The probable error in the measurement leads to an uncertainty of ±5% in molecular weight measurements.

Sedimentation equilibrium results obtained from different preparations were not identical, but the plots were always curved, indicating the presence of more than one species, as illustrated by the typical result shown in Figure 6. Local weight-average molecular weights (corrected for DOC binding) approached about 200 000 at the top of the solution column (at relatively high rotor speed), and never exceeded 400 000 at the bottom, suggesting that only monomeric and dimeric species were present. A more convincing demonstration of this feature of the results was obtained by making use of the fact that any sedimentation equilibrium distribution can be represented as the sum of contributions from each species present, regardless of whether these species are independent or interconvertible (Svedberg and Pedersen, 1940). For thermodynamically ideal solutions

$$C = \sum_i C_i = \sum_i A_i e^{B_i(r^2 - r_0^2)} \quad (1)$$

where C is the total concentration at any position (r) in the cell, C_i is the contribution of a given component at that position, and r_0 is a suitable reference point. The term B_i depends on the molecular weight (M_i) and the buoyant density factor of each component

$$B_i = [M_i(1 - \phi_i'\rho)\omega^2]/2RT \quad (2)$$

where ρ is the density of the solvent, ω the rotor speed in radians per seconds, and ϕ_i' the effective specific volume, which includes the influence of bound DOC which we incorporate as described previously (Tanford et al., 1974). The constant A_i is related to the total amount of component i in the cell. By integration of $C_i d(r^2)$ from the meniscus (r_a) to the bottom of the cell (r_b), one obtains

$$A_i = \frac{C_{i,tot} B_i (r_b^2 - r_a^2)}{e^{B_i(r_b^2 - r_0^2)} - e^{B_i(r_a^2 - r_0^2)}} \quad (3)$$

where $C_{i,tot}$ is the total amount of component i at equilibrium, expressed as a concentration in the total solution volume.

When one is dealing with a curved sedimentation plot with contributions from species differing in molecular weight by a factor of two or more, it is not possible experimentally to distinguish between species differing in B_i by 10%, such as the individual spectrin chains or the different dimers, trimers, etc., that can be formed from them. We have thus analyzed the results in terms of species of molecular weights of 220 000 and integral multiples thereof. We have also assumed that ϕ_i' is the same for all species, with the same contribution from bound DOC. Equation 1 then reduces to

$$C = A_1 x + A_2 x^2 + A_3 x^3 + \ldots \quad (4)$$

where x is equal to $e^{B(r^2 - r_0^2)}$ for monomeric spectrin of nominal molecular weight 220 000. Equation 4 has the advantage of being analyzable by standard least-squares analysis. (While this work was in progress, a similar procedure for analysis of sedimentation data was reported by Stevens (1975).)

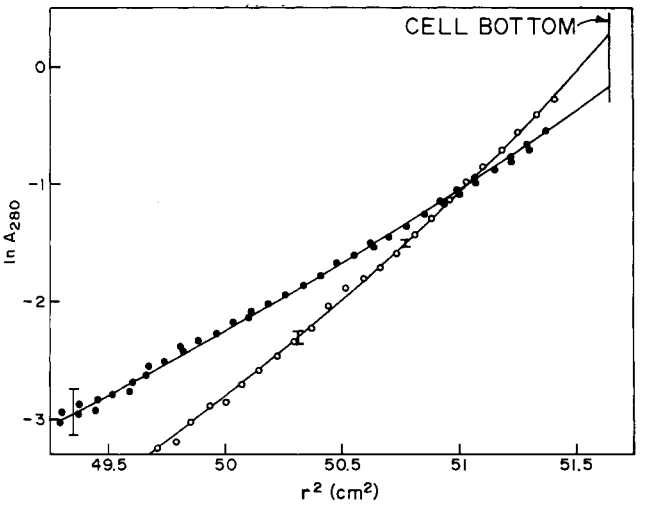


FIGURE 6: Typical sedimentation equilibrium plots. The initial protein concentration was 0.13 mg/ml, and data points represent one scan at 10 000 rpm (open circles) and two scans 10 h apart at 8000 rpm (filled circles). Error bars show the nominal experimental uncertainty at three absorbance values. The curves are theoretical, using eq 1 with the same parameters (54.5% monomer, 45.5% dimer) at each speed. The least-squares analysis gave 53 and 56% monomer, respectively, at 10 000 and 8000 rpm.

We have found that all our data can be fitted with high statistical confidence by eq 4 using only two terms, corresponding to monomer and dimer. An example is provided by Figure 6. When a third term was introduced into the analysis, the value of $C_{3,tot}$ derived from A_3 was zero within experimental error for most of the runs examined. When nonzero values were obtained (generally under conditions where the experimental error in the prime data was large), negative and positive values of A_3 were obtained with equal frequency, indicating that they could not be interpreted as reflecting the presence of trimers or higher oligomers. The presence of a few percent of highly aggregated material is not excluded because concentrations could not be read to the very bottom of the solution for the reason given in the Experimental Section. If present, the amount must be small since the sum of the monomer and dimer concentrations determined from the least-squares analysis was always in reasonable agreement with the total amount of spectrin initially placed into the cell.

The distinction between interconvertible monomer and dimer (i.e., an equilibrium of the form $2S \rightleftarrows S_2$) and noninterconvertible species can be made by examining the same sample at different initial concentrations and different rotor speeds. If the species are interconvertible, dimer will be formed at the expense of monomer as the concentration of protein is increased either initially or by compression of the same amount

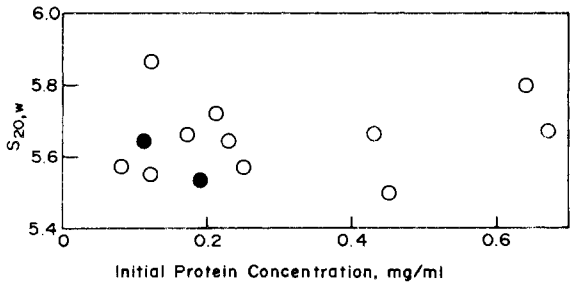


FIGURE 7: Summary of sedimentation velocity results in DOC. Open circles designate protein purified from lyophilized extracts; filled circles designate protein purified from an extract concentrated by pressure dialysis after the addition of DOC. The experimental error at the low concentrations employed here is probably about ±0.2S, and the concentration dependence is expected to be negligible: e.g., see comparable data for myosin by Holtzer and Lowey (1959).

Table I: Sedimentation Equilibrium Results for One Preparation of Spectrin.[a]

Initial Protein Concn (mg/ml)	Rotor Speed (rpm)	% Monomer	% Dimer
0.12	8000	26	74
0.065	5200	17	83
	8000	23	77
0.06	8000	21	79

[a] At room temperature, in 15 mM DOC, 10 mM Tris buffer (pH 9.0), 1 mM dithiothreitol, 0.1 mM EDTA, 0.05 M NaCl. The data were analyzed by least-squares, using eq 1 with two terms.

of protein into a smaller volume by an increase in rotor speed. Application of this test to one sample is shown in Table I. The results favor the interpretation that the species were not interconvertible within the time of the experiments since the monomer/dimer ratio remains unchanged within the accuracy of the measurements. If the results had been under the influence of monomer ⇄ dimer equilibration, the percentage of monomer in the experiment at an initial concentration of 0.065 mg/ml at 5200 rpm should have been about 1.5 times *greater* than at 0.12 mg/ml and 8000 rpm. The results also suggest that the monomer/dimer ratio is not appreciably time dependent: the experiments in Table I cover a time span of 2 weeks.

As noted earlier, the sedimentation equilibrium results were not reproducible from one preparation to another. The preparation shown in Table I had the largest amount of dimer (77 ± 8%) we have observed. The percentages of dimer in other preparations range from 25 to 65%. The preparations derived from aqueous extracts that had been concentrated by lyophilization had the largest percentage of dimer.

Sedimentation velocity measurements are shown in Figure 7. In contrast to the sedimentation equilibrium experiments there is little variation between preparations, from which it is clear that the spectrin dimer and monomer must have similar sedimentation coefficients. The same conclusion is reached from analysis of the sedimentation boundaries from individual runs, which were always very sharp, as illustrated by Figure 4. If the boundary curves are considered to be superpositions of symmetrical curves from individual components, it is not possible that any one component present in significant amount can have an s value differing by more than about 5% from the average derived from the rate of sedimentation of the center of the boundary. In some of the runs the presence of a small amount (~5%) of faster sedimenting material is indicated by the shape of the boundary at the upper end, but even the sedimentation velocity of this material can only be about 10–15% above the average.

The average sedimentation coefficient derived from Figure 7 is $s_{20,w}^0$ = 5.5 to 6.0S, which is very small for such high molecular weight species, and indicates that the sedimenting particles are very asymmetric. This asymmetry can also account for the small dependence of the sedimentation coefficient on molecular weight since the frictional coefficients of rod-shaped molecules of constant diameter increase almost in proportion to molecular weight and thus make the sedimentation coefficient insensitive to molecular weight. For example, if the spectrin monomer (including 0.07 g/g bound DOC and about 25% hydration) were a cylindrical rod with diameter 24 Å, its length would be 830 Å and the sedimentation coefficient calculated by standard procedure (Tanford, 1961) would be 5.3S. If the spectrin dimer has the same diameter and twice the length, the calculated sedimentation coefficient would be 6.2S.

Discussion

It is evident from this and previous investigations that the properties of erythrocyte spectrin are complicated by self-association and by interaction with other erythrocyte membrane components. We have shown that the residual lipid and contaminating proteins that are present in the aqueous extract of erythrocyte ghosts can be separated from spectrin by gel chromatography in DOC solutions. At the same time DOC prevents unlimited self-association: only monomers and dimers are present in the solution. Monomers and dimers appear not to be readily interconvertible, and we have found that the fraction of each in the final product varies from one preparation to another. The highest percentage of dimer was observed in two samples for which DOC solutions were prepared from lyophilized aqueous extract, which suggests that the initial treatment may be an important factor, but we have not examined enough samples that were concentrated by other methods to be confident about this.

In seeking an explanation for the variable monomer/dimer ratio, it must be kept in mind that spectrin has only two polypeptide chains (which we shall call A and B) that are distinguishable by sodium dodecyl sulfate gel electrophoresis, and that no preparation, in our hands or in other laboratories, has ever exhibited appreciable enrichment of one of these components at the expense of the other. The cross-linking experiments of Clarke (1971) are especially striking in that both electrophoretic components diminished at an equal rate when species of higher molecular weight were formed. While our physical measurements cannot distinguish between the dimeric species A_2, B_2, and AB, these observations suggest that AB is the natural dimeric species, and that neither of the electrophoretic bands contains a significantly large fraction of chemically distinct polypeptides, inherently unrelated to the principal species. It must also be kept in mind, however, that the erythrocyte is a dying cell, with no new synthesis of protein. It is possible therefore that degradation of initially unique A and B polypeptides may occur during the lifetime of the cell, sufficient to make them incompetent to exist as dimers, but not sufficient to lead to distinct products by the criterion of sodium dodecyl sulfate gel electrophoresis. Similar degradation could also occur in the course of preparative procedures, especially if other types of blood cells (which are rich in proteolytic enzymes) are not completely eliminated in the preparation of erythrocyte ghosts. A number of reports purport to show that the two spectrin bands do in fact contain multiple polypeptide

chains by the criterion of end group analysis (Knüfermann et al., 1973; Green et al., 1974; Fuller et al., 1974), but they are by no means conclusive. N-Terminal amino acids were either not quantitated or were recovered in a yield exceeding one per polypeptide chain. Immunoelectrophoretic studies (Bjerrum et al., 1974, 1975) provide stronger evidence, though it should be noted that some of the distinct species originally reported were later found to be artifactual (Bjerrum et al., 1975).

In the light of the foregoing considerations there are two possible explanations for the variable dimer content of our preparations, and there is no compelling evidence for choosing one over the other. (1) Undegraded spectrin may always be an AB dimer. Monomeric molecules would then consist of slightly degraded polypeptides and excess undegraded chains that have lost their partners. The fraction of monomers might be variable in the erythrocyte itself or because of variation in preparative procedures. In addition, different extraction procedures or treatment after extraction (e.g., precipitation) may lead to selective enrichment with respect to degraded or undegraded molecules. (2) Degradation may not be important, and the phenomenon may result from the effect of unknown factors on the equilibration between undegraded chains to form AB. To account for our results we would have to postulate that interconversion ($A + B \rightleftarrows AB$) can occur in the initial extract and during concentration, but that its rate becomes negligibly slow after purification, so that the initial equilibrium composition is maintained unchanged for at least several days.

The question of what causes the variable degree of dimerization does not affect our conclusions about the hydrodynamic properties of spectrin. Both monomer and dimer must be highly asymmetric: dimensions of 24×830 Å and 24×1660 Å, respectively, lead to calculated sedimentation coefficients in approximate agreement with experimental observation. We do not want to suggest, however, that the spectrin dimer is necessarily formed by end-to-end association of monomers. In fact, the sedimentation coefficients for monomer and dimer based on the dimensions we have given (5.3 and 6.2S, respectively) lead to a difference between monomer and dimer that is slightly greater than the experimental data permit: thus the dimer may in reality have more than double the axial ratio of the monomer. Because of uncertainties both in the calculation and in the resolution of the observed sedimentation boundary for the mixture, this discrepancy should probably not be taken seriously. However, the resistance to dimer–monomer interconversion also suggests that dimerization involves more than simple end-to-end association.

On the other hand, association beyond the dimer, as occurs in the absence of DOC, is a process that is quite distinct from the formation of dimers, and may well involve loose end-to-end association. Higher aggregates are readily and reversibly disrupted by DOC, which strongly suggests that hydrophobic interactions are responsible for the aggregation. The amount of bound DOC in the disaggregated state is very small, indicating that the hydrophobic areas responsible for aggregation are also small. Because of heterogeneity, as seen for example in Figure 4, precise characterization is not possible, but average sedimentation coefficients have always been about 8S or slightly greater, while average molecular weights, for purified protein in aqueous media, have been near 1 million. If we use the same model as before, a cylindrical rod of diameter 24 Å with length proportional to molecular weight, the predicted sedimentation coefficient for a tetramer would be 7.1S; if we assume side-by-side association of dimers, the predicted value for a tetramer would be 13.3S. End-to-end association with some flexibility at the point of association is compatible with the observed result.

An important conclusion from our results is that the hydrodynamic properties of the spectrin dimer in DOC are very similar to those of muscle myosin, which has a molecular weight of about 500 000 and a sedimentation coefficient of 6.4S (Holtzer and Lowey, 1959; subsequently confirmed by several other laboratories). The circular dichroism spectrum shown in Figure 1 is also very close to that reported for myosin (Oikawa et al., 1968). Our data thus tend to support the speculative suggestion of Guidotti (1972), based on similarities in amino acid composition and polypeptide chain molecular weight, that spectrin and myosin are related chemically and possibly functionally. There are of course equally striking differences between spectrin and myosin. If spectrin is indeed intrinsically an AB dimer, then the constituent chains (unlike the heavy chains of myosin) differ from each other not only in size, but also in some of their chemical properties (Guthrow et al., 1972; Roses and Appel, 1973). Moreover, the solubility characteristics of spectrin are quite different from those of myosin (Bretscher, 1973), as are its antigenic properties (Painter et al., 1975).

It should be noted in conclusion that the results presented here must be considered preliminary and that they leave many questions unresolved. We should like to believe that the DOC-purified protein is the intrinsic spectrin molecule, representative of the spectrin molecule that exists in aggregated form in the cell, but there is of course no proof that this is so. Even if it is true, the question of the origin of what appear to be distinct polypeptides, respectively able and unable to form dimers, remains to be answered. An approach to this latter question might lie in fractionation of the initial aqueous extract by gel chromatography: the broad elution patterns we have observed for a few preparations suggest the possible presence of two populations of particles in the aggregated state. Finally, we have no simple explanation for the discrepant observations on the behavior of spectrin in the initial aqueous extract that were summarized in the introductory section.

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Hydrolysis of Tri- and Monoacylglycerol by Lipoprotein Lipase: Evidence for a Common Active Site†

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ABSTRACT: The relationship between triacylglycerol and monoacylglycerol hydrolyzing activities of purified rat heart lipoprotein lipase was studied using emulsified trioleoylglycerol and micellar or albumin-bound monooleoylglycerol as substrates. The maximal reaction rates obtained with the two substrates were similar (650 and 550 nmol of fatty acid released per min per mg of protein, respectively). Addition of apolipoprotein C-II or serum increased the maximal reaction rate for the trioleoylglycerol hydrolyzing activity about fourfold, but had no effect on the monooleoylglycerol hydrolyzing activity. Hydrolysis of the two substrates apparently takes place at the same active site of the enzyme since (1) mutual competitive inhibition between the substrates could be demonstrated; (2) the rate of inactivation of enzymatic activity with the two substrates in 1.2 M NaCl was the same; (3) similar losses of hydrolytic activity with tri- and monooleoylglycerol were observed in the presence of low concentrations of *n*-butyl(*p*-nitrophenyl)carbamide; (4) inhibition of both hydrolytic activities by this compound could be prevented by prior exposure of lipoprotein lipase to either substrate.

Lipoprotein lipases (EC 3.1.1.34) have a comparatively low substrate specificity, both with regard to the chemical structure and the physicochemical nature of the substrate. In contrast to pancreatic lipase (Desnuelle and Savary, 1963; Morgan et al., 1968), lipoprotein lipases readily act upon emulsified tri- and diacylglycerol as well as micellar or albumin-bound monoacylglycerol substrates (Egelrud and Olivecrona, 1973; Nilsson-Ehle et al., 1973), and also catalyze the hydrolysis of truly water-soluble esters (Egelrud and Olivecrona, 1973). The reaction of lipoprotein lipase (LPL[1]) with these substrates has been shown to have certain different properties. Most notably,

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[1] Abbreviations used are LPL, lipoprotein lipase; Ol_3Gro, trioleoylglycerol; OlGro, monooleoylglycerol; V_{max}, maximal reaction rate; K_m, Michaelis constant.