Biochimica et Biophysica Acta, 455 (1976) 163–172

BBA 77501

PHYSICO-CHEMICAL CHARACTERIZATION OF THE SPECTRIN TETRAMER FROM BOVINE ERYTHROCYTE MEMBRANES

G. B. RALSTON

Department of Biochemistry, University of Sydney, Sydney, N.S.W., 2006 (Australia)

(Received March 19th, 1976)
(Revised manuscript received June 8th, 1976)

SUMMARY

The tetramer of bovine spectrin has been purified and characterized in terms of its hydrodynamic and optical properties. (1) The molecular weight, from both sedimentation equilibrium and sedimentation velocity/diffusion measurements, is close to one million. (2) The hydrodynamic properties suggest a highly expanded but basically symmetrical molecule of Stokes radius 200 Å. (3) Optical rotatory dispersion measurements indicate a high degree of order in the tertiary structure of the molecule.

These results are not consistent with the assumption that is often made, that the spectrin molecule is a long fibrous rod.

INTRODUCTION

Spectrin, a protein extracted from erythrocyte membranes in low ionic strength buffers, is believed to play an important role in maintaining the shape of the erythrocyte. Extraction of the protein from the membrane results in fragmentation of the ghost [1–3] and allows the intramembranous particles to aggregate under conditions which do not cause their aggregation in the untreated cell [3, 4].

A number of workers have reported "fuzzy" material lining the inner face of the ghost [5, 6] and the loss of this material after removal of spectrin. Furthermore, the presence of fibrillar material, visible in the electron microscope, after addition of salt or ATP+Mg^{2+} to the water-soluble protein, has been taken as evidence that spectrin forms a fibrous network on the cytoplasmic face of the membrane [3, 5, 6].

However, Tilney and Detmers [7] have recently disputed the presence of long fibres in the ghost, and suggest that fibre formation is the result of polymerization of erythrocyte "actin" (component 5 in the nomenclature of Fairbanks et al. [1]) after modification of the spectrin.

An understanding of the mechanism of the function of spectrin in shape maintenance requires an accurate knowledge of the shape and size of the molecules. Models which are inconsistent with the protein structure may then obviously be eliminated. To date, however, the understanding of the structure of spectrin has been confused, partly as a result of the heterogeneity of most preparations.

On the basis of approximate sedimentation coefficient and viscosity measurements, and assuming a rigid rod model, Clarke [8] calculated an axial ratio of 45 and a molecular weight of 600 000 for spectrin which she suggested may be a dimer or tetramer. Elgsaeter and Branton [3], quoting a private communication from Clarke, have used a value of 2100 Å for the length of the spectrin molecule, which they consider to be a double-stranded rod. However, the intrinsic viscosity used by Clarke (139 ml/g) was measured at low ionic strength and is at variance with the value (10 ml/g) determined by Marchesi et al. [2].

The discrepancy in this parameter is almost certainly due to the presence of variable proportions of different oligomeric states of spectrin in the different preparations.

Recently, a method for isolating the water-soluble oligomers of spectrin has been reported [9]. The oligomers are stable over a fairly wide range of salt concentration and pH, and do not aggregate or dissociate further under the conditions used for isolation. The tetramer is the predominant soluble oligomer in bovine preparations made at low temperature, and the properties of this oligomer have been examined in the present study.

The hydrodynamic properties reported in the present study are best explained by a model of a highly expanded but basically symmetrical particle. Optical rotatory dispersion measurements indicate, however, that the molecule has a non-random structure.

MATERIALS AND METHODS

Preparation of erythrocyte membranes. Bovine blood was obtained from the Homebush abattoir, Sydney. The blood was collected with citrate anticoagulant, chilled in ice and transported to the laboratory.

Red cells were washed free of plasma and buffy layer by washing four times with cold 0.95 % NaCl containing 5 mM sodium phosphate buffer, pH 7.5. After each wash, the red cells were centrifuged at $2500 \times g$ for 15 min at 2–4 °C in the GSA rotor of a Sorvall RC-2B refrigerated centrifuge, and the supernatant and buffy coat carefully removed by aspiration.

The washed red cells were hemolyzed in 20 vols. of cold 5 mM sodium phosphate, pH 7.5, and the membranes were collected by centrifugation at $15\,000 \times g$ for 40 min at 2–4 °C. The membranes were washed several times by suspension in 5 mM sodium phosphate buffer, pH 7.5, and centrifuged as above, until they were cream coloured. The underlying pink pellet was discarded after careful removal of the loose layer of red cell ghosts [1].

Preparation of the spectrin tetramer. The washed membranes were diluted with an equal volume of cold 0.2 mM EDTA, pH 7.5, and were dialyzed for 24 h against the same buffer at 2–4 °C. Water-soluble proteins were removed by centrifugation at $27\,000 \times g$, and the membrane pellet resuspended and returned for two further extractions.

After pooling the three supernatants, protein was precipitated by the addition of an equal volume of cold, saturated $(NH_4)_2SO_4$, and the precipitate resuspended in a minimum volume of the buffer used for gel filtration, (0.1 M NaCl, 0.01 M sodium phosphate, 5 mM EDTA, and 5 mM mercaptoethanol, pH 7.5). The suspension was

dialyzed for 24 h against the same buffer, turbidity removed by centrifugation at 35 000 $\times g$ for 30 min and the clear supernatant was applied to a column (60 $\times$ 2 cm) of Bio-gel A-15m agarose beads.

The fraction eluting in the position of the tetramer [9] was collected, reconcentrated by dialysis against aquacide IIB, and subjected once again to gel filtration on the same column. Again, the tetramer peak was concentrated and rechromatographed, yielding a single symmetrical peak on the third gel filtration step.

For some experiments, the central fraction of this peak was used directly. For other experiments requiring a higher protein concentration, the central portion of the tetramer peak was pooled and concentrated, either by precipitation with an equal volume of cold, saturated $(NH_4)_2SO_4$, or by dialysis against aquacide IIB.

In order to prevent the contamination of the protein solution with aquacide, which could lead to drastic increases in solution viscosity, this final concentration step was done by placing the dialysis bag containing the protein solution inside a second bag which was then placed in the aquacide. After thoroughly washing the outer bag with the gel filtration buffer at the end of the concentration process, the outer bag was carefully removed, the inner bag washed briefly and the protein contents removed carefully with the aid of a Pasteur pipette.

The hydrodynamic properties of the protein were independent of the method used for concentration, and on dilution of the protein these properties matched closely those of samples that had been examined without prior concentration. However, the ammonium sulphate method of concentration did sometimes produce a trace (usually less than 5 %) of aggregated material which eluted in the void volume of the agarose column on rechromatography.

The purified tetramer showed only the spectrin doublet [1] on acrylamide gel electrophoresis in dodecyl sulphate. Only a single band was seen on acrylamide gradient electrophoresis, and a single boundary on sedimentation in the ultracentrifuge.

The results reported in the present study were obtained with four different preparations of the spectrin tetramer. No systematic differences in properties were noticed between any of the preparations. In most experiments, the protein was dissolved in the buffer used for gel filtration. However, no differences in properties were detectable when this buffer was replaced with 0.1 M NaCl containing 0.01 M sodium phosphate or 0.01 M Tris · HCl.

Sedimentation velocity. Sedimentation velocity was determined in a Beckman-Spinco Model E analytical ultracentrifuge, fitted with RTIC and with both Schlieren and Rayleigh interference optics. Sedimentation coefficients of the tetramer at 20 °C were determined at 40 000 rev./min with the aid of a filled epon double-sector centrepiece. Since the boundary was sharp and symmetrical, the position of maximum ordinate of the Schlieren curve was taken as the position of the boundary.

In the determination of the concentration dependence of the sedimentation coefficient, the protein concentration was corrected for radial dilution by means of the relationship:

$$C_t/C_0 = r_0^2/r_t^2 \qquad (1)$$

where C_0 is the initial protein concentration, C_t is the concentration of the plateau region in the cell at a time, t, at which the sedimentation coefficient was determined,

and r_0 and r_t are the radial positions of the boundary at the start of the experiment and at time t, respectively.

Diffusion. The diffusion coefficient was measured at 20 °C in the ultracentrifuge with the aid of a capillary type synthetic boundary cell. The rotor was accelerated to a speed of 6000 rev./min and photographs were taken at different times by means of either Schlieren or interference optics. The diffusion coefficient was determined from enlarger tracings of the Schlieren pattern by means of the area/height method [10], or from the width of the Schlieren curve at a height equal to $1/\sqrt{e}$ times the maximum height [11]. In some experiments, the diffusion coefficient was determined from the interference fringes [10].

Viscosity. The viscosity of solutions of spectrin was measured in an Ostwald type capillary viscometer of capacity 2 ml and a flow time for water of 80 s. The temperature was maintained constant within 0.05 °C for each experiment, and at least 10 measurements of the flow time were made for each solution. Kinetic energy and end effect corrections were less than 1 % of the absolute solvent viscosity, and have been considered negligible. The limiting viscosity was determined by extrapolation by means of an unweighted least squares procedure.

Optical rotatory dispersion. Optical rotation was measured in a Perkin-Elmer photoelectric polarimeter, model 241, at the wavelength of the sodium D line, and at several lines of the mercury arc spectrum. The data were plotted in terms of both the Drude equation and the Moffitt-Yang equation [12]. Both plots were closely linear, with correlation coefficients of 0.999.

Absorption coefficient. The absorption spectrum of the purified tetramer was measured in a Zeiss PMQ II spectrophotometer at concentrations between 1 and 3 mg/ml. The protein solutions were dialyzed against two changes of 0.1 M NaCl containing 0.01 M sodium phosphate, and the final dialyzate was used as a blank. The extinction coefficient was calculated from the fringe count in a synthetic boundary cell and the absorbance at 280 nm (corrected for scattering), using the relationship reported by Babul and Stellwagen [13]:

$$4.04 \text{ fringes} = 1 \text{ mg/ml} \qquad (2)$$

The value obtained at 280 nm was ${A_{1\,\text{cm}}}^{1\%} = 11.5$. With the use of this value, protein concentration in all experiments was determined from the absorbance at 280 nm.

Partial specific volume. A value of 0.73 ml/g was used for the partial specific volume of spectrin in the calculation of molecular weight. This value was calculated by Marchesi et al. [2] from the amino acid composition. Although the presence of associated lipid would strongly influence the value of the partial specific volume, we have been unable to detect the presence of lipid in any of our preparations of the purified tetramer.

RESULTS

Absorption spectrum. The absorption spectrum of the tetramer of bovine spectrin in buffered salt solution is typical of a normal protein absorption spectrum with a peak at 281 nm, a shoulder near 290 nm, and a trough at 250 nm. The ratio $A_{280\,\text{nm}}/A_{250\,\text{nm}}$ is 2.105, and the ratio $A_{280\,\text{nm}}/A_{240\,\text{nm}}$ was approx. 1.0.

The spectrum gives no indication of strongly absorbing prosthetic groups in

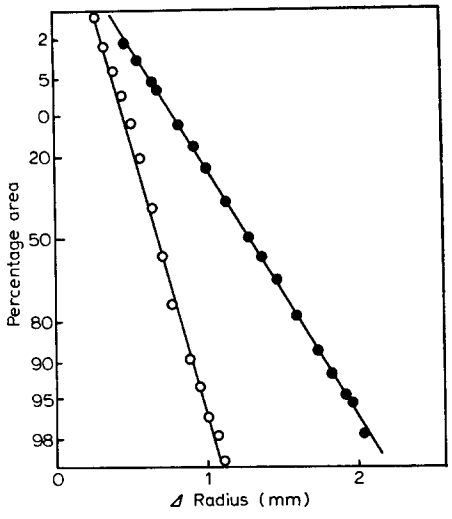

Fig. 1. Gaussian plot of the distribution of protein concentration across the sedimentation and diffusion boundaries. The open circles show the distribution across the sedimentation boundary 100 min after reaching a speed of 40 000 rev./min. The solid circles represent the distribution across the diffusion boundary 130 min after reaching a speed of 6000 rev./min. Protein concentration in each case was 2 mg/ml.

the range 230–600 nm. In the absence of absorbing prosthetic groups, the method of Babul and Stellwagen [13] may be used to determine the absorbance coefficient as described in Materials and Methods.

Sedimentation velocity. The purified tetramer showed a single, shapr boundary in the ultracentrifuge, with sedimentation coefficient near 12 S. Some degree of hypersharpening probably occurs, as indicated by the curvature of the Gaussian distribution plot shown in Fig. 1. With increasing concentration from 0.3 to 5 mg/ml, the sedimentation coefficient decreased from 12 S to 9 S. Plots of sedimentation coefficient versus protein concentration showed slight curvature. However, curvature was not obvious in the plot of the reciprocal of sedimentation coefficient versus concentration (Fig. 2) and this plot was used to determine the concentration-dependence coefficient, K_s, of the reciprocal sedimentation coefficient:

$$1/s = 1/s_0(1+K_s c) \tag{3}$$

The slope of the plot in Fig. 3 is K_s/s_0, and the intercept is $1/s_0$, from which the limiting sedimentation coefficient, s_0, was found to be 12.2 S, and K_s was found to be 96.2 ml/g.

Diffusion coefficient. The diffusion coefficient is plotted against protein concentration in Fig. 3. From this plot, the limiting diffusion coefficient at infinite dilution was found to be $1.1 \cdot 10^{-7}$ cm^2/s. The shape of the diffusion boundary was much more closely Gaussian than that of the sedimentation boundary (Fig. 1), probably because of the greater concentration dependence of the sedimentation coefficient.

Molecular weight. From the limiting sedimentation and diffusion coefficients, a molecular weight of 990 000 was calculated, compared with a value of 960 000 obtained from sedimentation equilibrium studies [9].

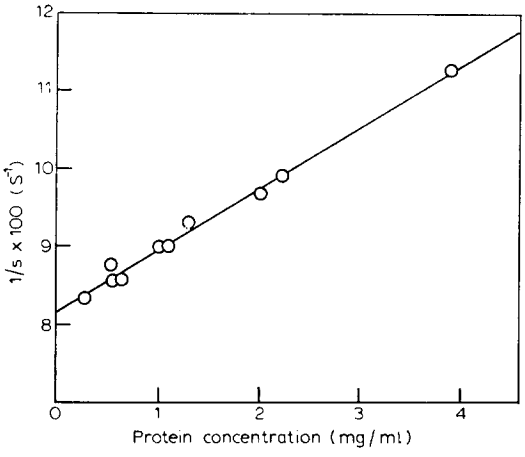

Fig. 2. Concentration dependence of the reciprocal of sedimentation coefficient for the purified tetramer. The slope and intercept were estimated by means of an unweighted least squares procedure.

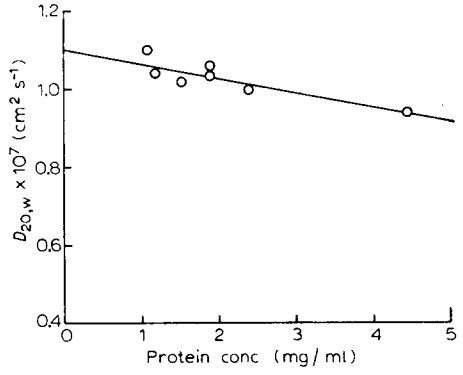

Fig. 3. Concentration dependence of diffusion coefficient for the purified tetramer. The limiting diffusion coefficient was determined by an unweighted least squares procedure.

Viscosity. The reduced viscosity (viscosity number) of the purified tetramer was considerably higher than that of most globular proteins, and enabled measurements to be made at protein concentrations as low as 0.2 mg/ml. The concentration dependence of the reduced viscosity is shown in Fig. 4, from which the intrinsic viscosity (limiting viscosity number) was determined as 70.4 ml/g.

Particle shape and size. From the sedimentation or diffusion coefficient, and the molecular weight, the frictional ratio was calculated to be 2.9, suggesting that the tetramer molecule is not a compact, globular molecule. The intrinsic viscosity of 70.4 ml/g is much greater than the value near 3.5 ml/g determined for most globular proteins. Therefore, the protein is either highly asymmetric, highly expanded, or a combination of both.

An indication of the asymmetry may be obtained from the ratio $K_s/[\eta]$, a dimensionless parameter which is expected to be near 1.6 for a spherical particle,

whether compact or expanded, and which is much lower for an asymmetric particle [14].

For the spectrin tetramer, a value of 1.37 was determined for $K_s/[\eta]$, suggesting a molecule no more asymmetric than serum albumin. From this value, an axial ratio of 3.2 was calculated using the relationship given by Creeth and Knight [14].

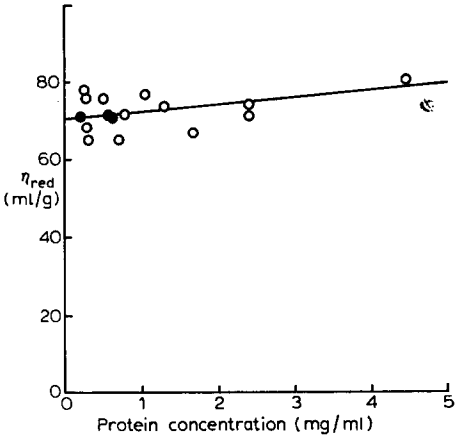

Fig. 4. Concentration dependence of the reduced viscosity of the purified tetramer. Open circles represent experiments with solutions concentrated as described in Materials and Methods, and then diluted to the appropriate concentration. Closed symbols represent results from three preparations which were not concentrated after elution from the agarose column. The straight line is the line of best fit calculated from all the plotted data by means of an unweighted least squares procedure.

TABLE I

PHYSICOCHEMICAL PROPERTIES OF THE TETRAMER OF BOVINE SPECTRIN

(A) Hydrodynamic properties	
$M_{r,eq}$	960 000
$M_{r,sD}$	990 000
$s^0_{20,w}$	12.2 S
$D^0_{20,w}$	$1.1 \cdot 10^{-7}$ cm²/s
$[\eta]$	70.4 ml/g
K_s	96.2 ml/g
$K_s/[\eta]$	1.37
Stokes radius	195 Å (diffusion) 228 Å (viscosity) 201 Å (gel filtration)

(B) Optical rotatory dispersion parameters	
$[\alpha]_D$	−24.3°
λ_c	300 nm
a_0	−98°
b_0	−300.9°
α-helix (%)	45 %

Gel filtration. The column of Bio-gel A-15m was calibrated with hemoglobin, ferritin, and immunoglobulin G, by means of the method of Ackers [15]. Values of r, the gel parameter, were 530, 490 and 510 for the three proteins, respectively. From the average of these values and the K_d value for the spectrin tetramer (0.11), a value of 201 Å was determined for the Stokes radius of the tetramer. This value of the Stokes radius is in good agreement with the value obtained from sedimentation and diffusion measurements, and is in reasonable agreement with that calculated from the intrinsic viscosity. The values of the Stokes radius are compared in Table I.

Optical rotatory dispersion. The optical rotatory dispersion of the purified tetramer was plotted according to both the Drude relationship [12] and the Moffitt-Yang equation [12]. The parameters from these relationships are shown in Table I, and all indicate a structure different from a random coil. The λ_c value indicates a structure considerably different from that of a random coil, and from the b_0 parameter, a value of 45 % α-helix was computed, assuming that the structure was comprised only of disordered and α-helical regions.

DISCUSSION

The tetramer of spectrin, purified by repeated gel filtration, appeared to be homogeneous on gel filtration, sedimentation velocity, diffusion and gel electrophoresis. Furthermore, the plots of log (concentration) vs. radius^2 in the sedimentation equilibrium experiment [9] were linear, also indicating homogeneity.

The molecular weight calculated from sedimentation/diffusion measurements is in good agreement with the value from sedimentation equilibrium and both values suggest a tetramer of subunits having molecular weight of about 240 000, the average value of the subunit molecular weight determined by means of electrophoresis in dodecyl sulphate [1].

The hydrodynamic properties of the purified tetramer (Table I) indicate a molecule which is either highly expanded or highly asymmetrical. From three independent experimental measurements, the Stokes radius was found to be near 200 Å, a value approximately three times greater than expected for a compact globular protein of 10^6 daltons. The value of the Stokes radius from viscosity is somewhat higher than the values from gel filtration and diffusion, but perhaps this reflects the greater uncertainty and scatter in the individual measurements of viscosity.

It is generally recognised that precise measurement of both shape and size of macromolecules is unattainable from hydrodynamic measurements alone. Determination of the shape and size from the frictional coefficient, for instance, requires the assumption of a model. In previous work on spectrin, the model of a solid prolate ellipsoid has often been assumed [8]. From the data of Table I, the β parameter of Scheraga and Mandelkern [16] was calculated to be $2.45 \cdot 10^6$ and would indicate an axial ratio of about 11 for a solid prolate ellipsoid. However, there has been no evidence presented to suggest that the molecule is, in fact, impenetrable to solvent.

Creeth and Knight [14] have discussed the application of various hydrodynamic approaches to the determination of macromolecular shape, and concluded that the ratio $K_s/[\eta]$ is a good indication of symmetry or asymmetry for both rigid and expanded, solvent-penetrated particles. On the basis of the value 1.37 for the $K_s/[\eta]$ ratio for spectrin tetramer, the shape of the molecule would appear to be no

less symmetrical than bovine serum albumin (1.38) or lactate dehydrogenase (1.33). Rabbit myosin, on the other hand, is a long rigid, rod-like molecule, and has a $K_s/[\eta]$ ratio of 0.34 [14]. Thus, although it must be realised that the $K_s/[\eta]$ ratio is only a semi-quantitative measure of symmetry, it is evident that spectrin behaves in a manner quite different from that of the rod-like structure of myosin.

The high frictional ratio and Stokes radius, therefore, are probably better explained, not on the basis of asymmetry, but rather in terms of the expansion of the molecule. Support for this interpretation comes from the consistent failure to observe recognisable structures in electron microscopy of the purified spectrin tetramer (Ralston, G., unpublished results). A molecule which is permeable to solvent would not be expected to be detectable by means of negative contrast.

The high degree of expansion of the molecule may be considered as indicating an aggregated, denatured molecule. However, there are several reasons for believing that this is not the case. (1) The dimer and tetramer are the predominant water-soluble oligomers. No trimer or pentamer has yet been detected in a range of experiments including gel filtration on Bio-gel A-15m and on Bio-gel A-50m (on which the tetramer elutes well away from the void volume), acrylamide gradient electrophoresis, sedimentation velocity, and meniscus-depletion sedimentation equilibrium. Preferential formation of even-numbered aggregation states by denatured protein is hard to explain. (2) The optical rotation results indicate a partially ordered structure. The value of 300 nm for λ_c is far from the value near 200 nm expected for unfolded, denatured protein. The b_0 parameter indicates about 45 % α-helix, and this value is not consistent with disordered protein [12]. (3) The optical rotatory dispersion parameters were independent of salt concentration, and were identical for three different preparations. Decreasing the ionic environment of a denatured protein would be expected to induce changes in the magnitude of the optical rotation, due to expansion of the molecule.

In the present study, a value of 11.5 was used for the absorbance coefficient, $A_{1cm}{}^{1\%}$, at 280 nm. This is somewhat higher than the value 8.8 determined by Marchesi et al. [2]. The presence of prosthetic groups with strong absorbance in the far ultraviolet would be expected to decrease the measured value of the absorbance coefficient, and therefore cannot account for the difference. It is possible that the material used by Marchesi et al. [2] contained some contaminating proteins with lower absorbance coefficients and that these were removed in the present study.

However, the value used for the absorbance coefficient does not alter the ratio $K_s/[\eta]$, which is dimensionless.

It was suggested in a previous report [9] that spectrin may be identical with the "hollow cylinder" protein isolated by Harris [17]. However, the purified tetramer showed no structure in the electron microscope, similar to that seen by Harris [17]. In addition, sedimentation coefficients have been computed for the torus and hollow cylinder proteins described by Harris [17]. Frictional coefficients were calculated by the method of Bloomfield et al. [18], assuming a structure made up from globular units 40 Å in diameter, each with individual frictional ratios of 1.2. The calculated sedimentation coefficients are: single torus, 8.8 S; stack of two tori (dimer), 16 S; stack of four tori (tetramer, a hollow cylinder), 26 S. These values are far too large to be compared with the sedimentation coefficients of the spectrin monomer (approx. 7 S), dimer (approx. 9.5 S) and tetramer (12.2 S), reported by Ralston [9] and in the present study.

ACKNOWLEDGEMENT

The award of a University of Sydney Postdoctoral Fellowship during the period of this research is gratefully acknowledged.

REFERENCES

1 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2616
2 Marchesi, S. L., Steers, E., Marchesi, V. T. and Tillack, T. W. (1970) Biochemistry 9, 50–57
3 Elgsaeter, A. and Branton, D. (1974) J. Cell Biol. 63, 1018–1036
4 Singer, S. J. (1974) Annu. Rev. Biochem. 43, 805–833
5 Rosenthal, A. S., Kregenow, F. M. and Moses, H. L. (1970) Biochim. Biophys. Acta 196, 254–262
6 Marchesi, V. T., Steers, E., Tillack, T. W. and Marchesi, S. L. (1969) in The Red Cell Membrane, Structure and Function (Jamieson, G. A. and Greenwald, T. J., eds.), pp. 117–130, Lippincott, Philadelphia, Pa.
7 Tilney, L. G. and Detmers (1975) J. Cell Biol. 66, 508–520
8 Clarke, M. (1971) Biochem. Biophys. Res. Commun. 45, 1063–1070
9 Ralston, G. B. (1975) Aust. J. Biol. Sci. 28, 259–266
10 Chervenka, C. H. (1973) A Manual of Methods for the Ultracentrifuge, pp. 83–86, Beckman-Spinco, Palo Alto, Calif.
11 Neurath, H. (1942) Chem. Rev. 30, 357–394
12 Urnes, P. and Doty, P. (1961) Adv. Protein Chem. 16, 401–544
13 Babul, J. and Stellwagen, E. (1969) Anal. Biochem. 28, 216–221
14 Creeth, J. M. and Knight, C. G. (1965) Biochim. Biophys. Acta 102, 549–558
15 Ackers, G. K. (1964) Biochemistry 3, 723–730
16 Scheraga, H. A. and Mandelkern, L. (1953) J. Am. Chem. Soc. 75, 179–184
17 Harris, J. R. (1969) J. Mol. Biol. 46, 329–335
18 Bloomfield, V., Dalton, W. O. and Van Holde, K. E. (1967) Biopolymers 5, 135–148