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SPECTRIN LOSS DURING IN VITRO RED CELL LYSIS

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Summary

Spectrin was extracted from washed erythrocyte ghosts in 1 mM EDTA buffer (pH 8.0) and purified to homogeneity by gel filtration. Anti-human spectrin was raised in rabbits. Specificity of the antibody was demonstrated by immunodiffusion, immunoelectrophoresis and immunofluorescent techniques. Membrane-free hemolysate prepared by lysing red cells in 5 mM phosphate buffer (pH 8.0) for variable intervals (5—60 min) at 4°C was found to contain spectrin identifiable by immunodiffusion, immunoelectrophoresis, immunofluorescence and sodium dodecyl sulfate polyacrylamide gel elctrophoresis. Spectrin was demonstrable in ultracentrifuged membrane-free hemolysate and, in progressively decreasing amounts, in membrane washes. Membrane-free hemolysate contained more spectrin when erythrocytes were lysed for 60 min than for 5 min. The data indicate that a significant amount of spectrin is detached from the membrane following lysis in hypotonic buffer for different time intervals. Spectrin lost in this manner might be part of spectrin attached to the lipid bilayer.

Introduction

Spectrin constitutes about 25—30% [1] of the human erythrocyte membrane proteins and is readily extracted in media of low ionic strength [2]. In a previous study it has been shown that membranes isolated from red cells exposed to low pH prior to lysis contain significantly higher amounts of spectrin whereas membranes prepared from erythrocytes heated to 50°C prior to

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lysis contain significantly less spectrin [3]. This suggests that a significant amount of spectrin is lost during the usual processes of preparing washed erythrocyte membranes [4,5] and that total cell spectrin is in reality greater than what is estimated in washed ghosts. The present study confirms immunologically that spectrin is lost by the usual methods of isolating washed erythrocyte membranes.

Methods

Preparation of pure spectrin. Heparinized blood was obtained from healthy human donors and was processed immediately. White membranes washed free of hemoglobin were prepared by the method of Fairbanks et al. [5]. Spectrin was extracted from washed membranes in 1 mM EDTA (pH 8.0) by the method of Marchesi [2]. The crude spectrin containing actin and other minor contaminants was purified by gel filtration on Sepharose 4B (1.2 × 50 cm) eluted at 10 ml/h in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 5 mM 2-mercaptoethanol, pH 8.5. The purity of spectrin was confirmed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate [5]. The peaks containing pure spectrin were retained for further analyses.

Preparation of spectrin antibody. Anti-spectrin antibody was raised in New Zealand white rabbits by the method of Vaitukaitis et al. [6]. Multiple sites were injected on the back with pure spectrin homogenized in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Rabbits were reimmunized with spectrin in incomplete adjuvant as needed. They were then bled from the central ear artery and the antiserum was isolated and the IgG fraction of the spectrin antiserum was prepared by $(NH_4)_2SO_4$ precipitation followed by DE-52 column chromatography [7,8].

Staining of acrylamide gels with fluorescein conjugated antiserum. Red cell membrane proteins and pure spectrin were analyzed by 5.6% polyacrylamide gels in the presence of sodium dodecyl sulfate [5]. In some experiments the gels were fixed in rabbit anti-spectrin IgG diluted 1 : 5 in physiologic buffered saline (pH 7.4) and incubated at room temperature for two days. The gels were then washed for two days in 30 ml of physiologic buffered saline (pH 7.4) with a single change after 24 h. Gels were then incubated at room temperature for two days with 1 : 10 dilution of fluorescein-conjugated IgG fraction of goat anti-rabbit IgG (Cappell Lab, Downingtown, PA). The gels were then washed for two days in physiologic buffered saline, pH 7.4, examined and photographed by time exposure under ultraviolet light [9].

Antibody assays. Ouchterlony double-immunodiffusion assays were performed in petri dishes containing 1.0% agar in 0.9% saline [10,11]. In experiments in which hemoglobin-containing samples were tested, the petri dishes were repeatedly washed with normal saline to elute the red color prior to photography. Fractions and samples containing spectrin were also analyzed by immunoelectrophoresis with rabbit anti-spectrin, rabbit anti-human erythrocyte membrane (DAKO-immunoglobulin, Copenhagen, Denmark), normal human and rabbit sera [12].

Preparation of membrane-free hemolysate. Washed erythrocytes were lysed in 4 vols. of 5 mM phosphate buffer (pH 8.0) for different time intervals (5—60

min) at 4°C. Membrane-free hemolysate was then isolated by centrifugation at 35—50 000 × *g* for 10—15 min. The supernatant was then similarly recentrifuged twice more. This triply centrifuged membrane-free hemolysate was then concentrated and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by Ouchterlony double-immunodiffusion assays. In some experiments the membrane-free hemolysate was ultracentrifuged at 150 000 × *g* for 1 h prior to further analysis. Membranes were then washed free of hemoglobin in the same buffer and each wash solution was saved, concentrated and tested for the presence of spectrin.

Analytical methods. Membranes or membrane-free hemolysates were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate as described previously [5]. Aliquots were dissolved in 2% SDS and 2% 2-mercaptoethanol, boiled for 3 min at 100°C and electrophoresed on 5.6% polyacrylamide gels containing 0.1% SDS and stained with Coomassie brilliant blue [5]. The bands were numbered according to the system instituted by Fairbanks et al. [5] and extended by Steck [13]. The proportion of the bands of the stained gels was assessed by densitometry with a Gilford spectrophotometer and model 2410 linear transport accessory, scanning through a 0.1-mm slit at 560 nm. The area under each band was measured and expressed as a percentage of the total area of the membrane protein scan. Protein concentration was measured by the method of Lowry et al. [14] using bovine serum albumin as standard. The statistical significance was determined by the Student's paired *t*-test whenever applicable.

Results

Preparation of pure spectrin

Fig. 1 confirms the purity of spectrin prepared as described in Methods. Fig. 1A represents the membrane protein pattern of normal human erythrocytes determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Fig. 1B is the membrane protein pattern of red cell membrane pellet from which spectrin was extracted and Fig. 1C is the pattern of the extracted crude spectrin containing actin (band 5) and other minor contaminants besides spectrin (bands 1 and 2). Fig. 1D is the pattern of spectrin purified by gel filtration showing the successful removal of actin and the other minor contaminants. Gels loaded with up to 76 μg of pure spectrin showed no contaminants indicating the purity of this membrane polypeptide.

Specificity of anti-spectrin antibody

The rabbit anti-spectrin IgG fraction gave single, sharp confluent precipitin lines when tested by double immunodiffusion against purified spectrin but no precipitin lines were observed with pre-immune rabbit serum, normal human serum or with spectrin absorbed with anti-spectrin prior to testing (Fig. 2). Normal human red cell membrane solubilized in 0.1% Triton X-100 gave one precipitin line when tested by immunoelectrophoresis against anti-spectrin but more than one precipitin line when tested against anti-human erythrocyte membrane (Fig. 3A). Purified spectrin gave single sharp identical lines when tested by immunoelectrophoresis against both rabbit anti-human erythrocyte

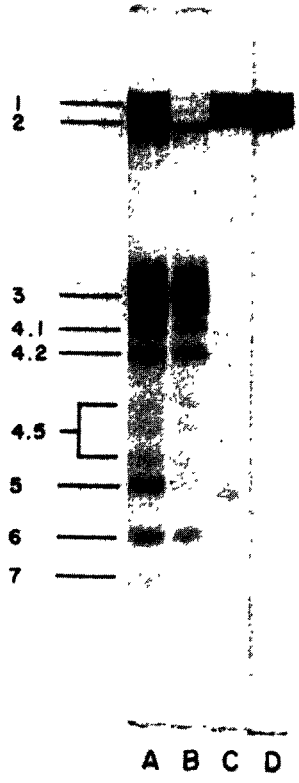


Fig. 1. SDS-polyacrylamide gels of red cell membrane protein and purified spectrin. Gel A, 47 μg of red cell membrane protein; Gel B, 30 μg of EDTA-extracted ghosts; gel C, 25 μg of an EDTA extract of spectrin and actin, and gel D, 32 μg of spectrin purified by gel filtration.

membrane antibody and against rabbit anti-spectrin (Fig. 3B). Finally, further evidence of specificity of the antibody is shown in Fig. 4 by immunofluorescent staining of antigen-antibody reaction in SDS-polyacrylamide gels. Fig. 4 compares three gels, all of which were loaded with red cell membrane protein (44 μg). Gel A was stained with Coomassie blue and shows the expected membrane protein bands. Gel B was placed in rabbit anti-spectrin serum followed by treatment with the fluorescein-conjugated anti-IgC. Fluorescent bands were seen only in the spectrin region of the gel. Gel C was immersed in normal preimmune rabbit serum followed by treatment with the fluorescein-conjugated anti-IgG. No fluorescent bands are seen. Similar results were obtained when gels were loaded with pure spectrin only.

Presence of spectrin in membrane-free hemolysate

Concentrated membrane-free hemolysate gave single precipitin lines when tested by double-immunodiffusion against anti-spectrin identical to the lines given by purified spectrin (Fig. 5). Concentrated membrane washed contained progressively decreasing amounts of spectrin, the last 2—3 washes containing none as determined by double immunodiffusion against anti-spectrin (Fig. 6).

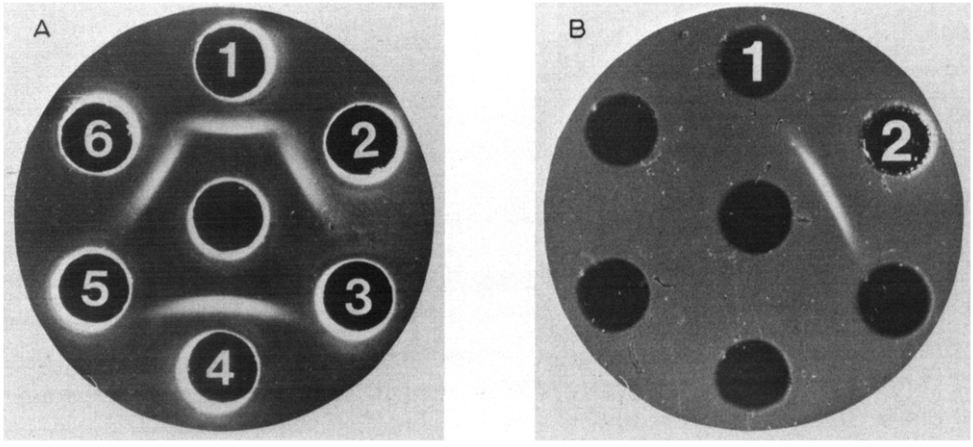


Fig. 2. Double immunodiffusion demonstrating the specific reaction between spectrin and anti-spectrin raised in rabbits. (A) Center well, anti-spectrin (IgG fraction): 1, 2, 4 and 6, purified spectrin; 3, pre-immune rabbit serum; 5, normal human serum. (B) Center well, anti-spectrin: 1, purified spectrin aborbed with anti-spectrin; 2, purified spectrin.

Moreover membrane-free hemolysate gave one precipitin line identical to that given by spectrin when tested by immunoelectrophoresis agains anti-spectrin (Fig. 7). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of membrane-free hemolysate obtained from erythrocytes lysed in hypotonic buffer for 5 min, has faint spectrin bands totalling 0.34% ± 0.10 (mean ± S.D. of seven determinations) of total protein applied to the gel determined by scanning the stained gels, whereas membrane-free hemolysate prepared from red cells lysed for 60 min has denser spectrin bands constituting 1.19% ± 0.62

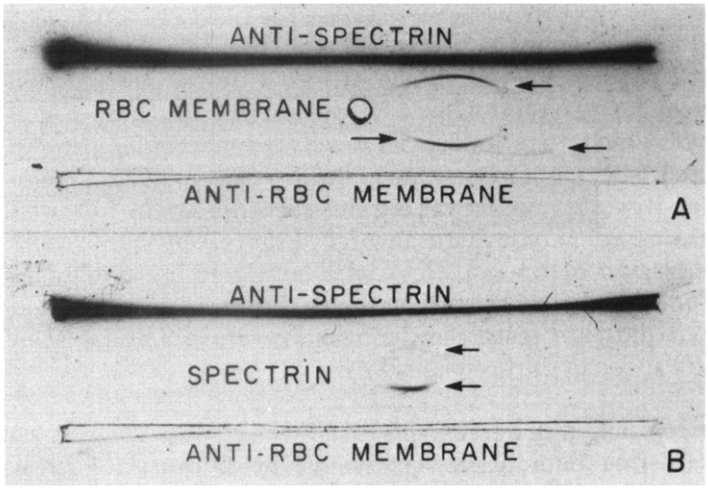
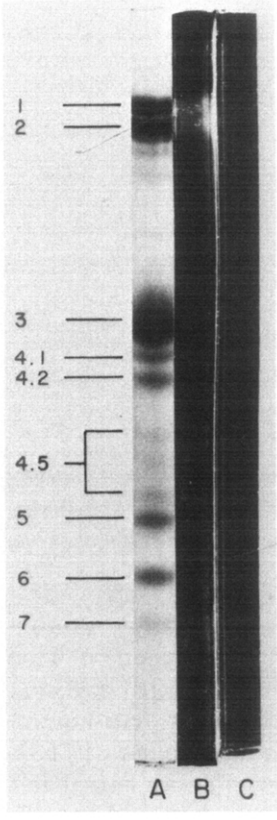


Fig. 3. Immunoelectrophoresis of purified spectrin and anti-spectrin. (A) Center well, normal human red cell membrane solubilized in 0.1% Triton X-100; upper through, anti-spectrin; lower through, rabbit anti-human red cell membrane antibody. (B) Center well, purified spectrin; upper and lower troughs same as in (A). RBC, red blood cell.



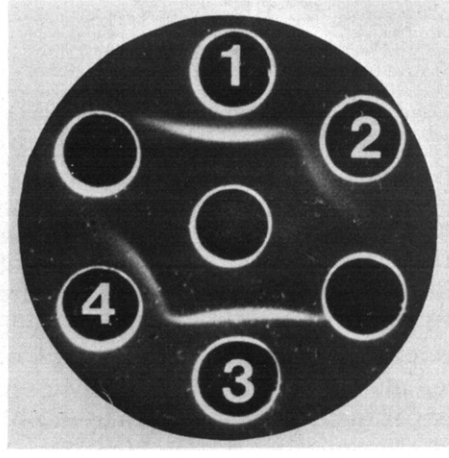


Fig. 4. Antigen-antibody reactions in SDS-polyacrylamide gel electrophoresis. 44 μg of red cell membrane protein was applied to each of the three gels. Gel A, stained with Coomassie brilliant blue; gel B, was immersed in rabbit anti-spectrin serum and then treated with fluorescein-conjugated anti-rabbit IgG; gel C was immersed in normal rabbit serum prior to treatment with fluorescein-conjugated anti-rabbit IgG.

Fig. 5. Double immunodiffusion demonstrating the presence of spectrin in membrane-free hemolysate. Red cells were lysed in 4 vols. of 5 mM phosphate buffer (pH 8.0) for 60 min and the hemolysate was separated by centrifugation at 48 000 × *g* for 10 min. Membrane-free hemolysate was similarly recentrifuged twice and concentrated by ultrafiltration prior to testing. Center well, anti-spectrin; 1 and 3, purified spectrin; 2 and 4, concentrated membrane-free hemolysate. Hemoglobin was eluted from the agar dishes by repeated washing with normal saline.

(mean ± S.D. of five determinations) of total protein applied to the gels. This indicates that the amount of spectrin in the hemolysate is significantly greater ($P < 0.025$) the longer erythrocytes are lysed in hypotonic buffer. Results were similar when erythrocytes were lysed in water or in 5 mM phosphate buffer, pH 8.0. Membrane-free hemolysate containing spectrin was ultracentrifuged as described in Methods and tested by double immunodiffusion and SDS-polyacrylamide gel electrophoresis. Results after ultracentrifugation were identical to those before this procedure indicating that the spectrin present in membrane-free hemolysate is due to release into the supernatant after cell lysis and not to contamination with membrane components. Further evidence that the observed results are not due to the presence of small membrane vesicles in the hemolysate was proven by the following two procedures. First rabbit anti-

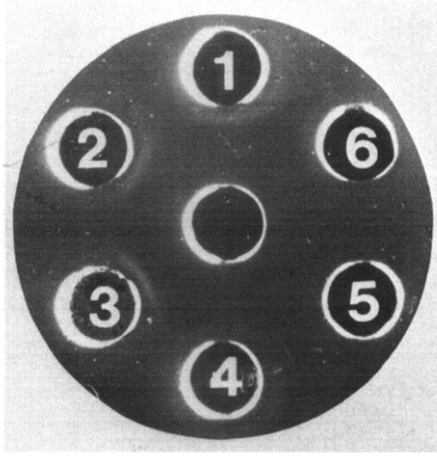


Fig. 6. Double immunodiffusion demonstrating the presence of decreasing amounts of spectrin in membrane washes. Center well, anti-spectrin; 1, membrane-free hemolysate; 2—6, membrane wash 1—5, respectively.

human red cell membrane antibody was absorbed with pure spectrin. The absorbed serum did not show precipitin bands when tested by double immunodiffusion against membrane-free hemolysate or pure spectrin. This absorbed serum, however, showed precipitin lines when tested against erythrocyte membranes solubilized in 0.1% Triton X-100. Since the rabbit anti-human red cell membrane contains anti-spectrin, and anti-(glycophorin + band 3) [15], this experiment indicates the absence of intrinsic membrane components, band 3 and glycophorin, in membrane-free hyemolysate. Second, gels loaded with membrane-free hemolysate were immersed in rabbit-anti-human red cell membrane antibody followed by treatment with fluorescein-conjugated IgG fraction of goat anti-rabbit IgG. Fluorescent bands were seen in the spectrin region of the gel but not in the band 3 or glycophorin regions. Thus the observed results cannot be ascribed to the presence of membrane vesicles in the hemolysate.

Discussion

Our data indicate that spectrin can be purified to apparent homogeneity by relatively simple techniques. Anti-human spectrin antibody raised in rabbits

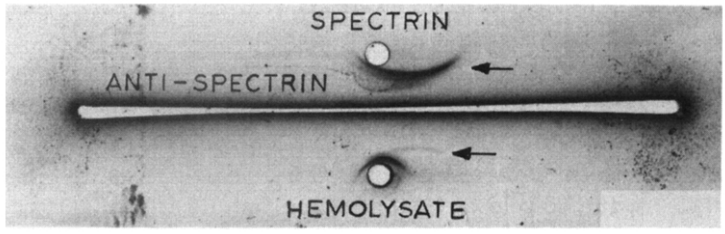


Fig. 7. Immunoelectrophoresis demonstrating the presence of spectrin in membrane-free hemolysate prepared as described for Fig. 5. Center through, anti-spectrin; upper well, purified spectrin; lower cell, membrane free hemolysate.

was found to be specific by double-immunodiffusion, immunoelectrophoresis and immunofluorescent techniques. These same techniques were used to demonstrate the presence of spectrin in membrane-free hemolysate. That the spectrin present in membrane-free hemolysate is not due to the contamination by membrane fragments but is due to detachment of spectrin from the membrane during the process of in vitro lysis is supported by four lines of evidence: first, spectrin was present in membrane-free hemolysate before and after ultracentrifugation; second, the longer the red cells were lysed in hypotonic buffer the more the spectrin was present in membrane-free hemolysate; third, there was progressively decreasing amounts of spectrin in membrane washes performed under similar conditions and, fourth, band 3 and glycophorin could not be demonstrated in membrane-free hemolysate. Thus the content of spectrin in red cells must be more that what is customarily estimated from examining membranes free of hemoglobin. This may also be true of other peripheral red cell membrane proteins such as actin, but these were not examined in this study.

A number of investigators have indicated that spectrin and actin form a protein cytoskeleton or fibrillar meshwork at the cytoplasmic surface of the red cell membrane [16—18]. This view is not uniformly accepted and some investigators believe that a non-filamentous form of spectrin and actin occurs at the cytoplasmic membrane surface [19]. In either case very little is known about how spectrin or spectrin-actin meshwork is organized or how it interacts with the integral membrane proteins or with the lipid bilayer. Lux [20] has recently described two forms of extractable spectrin: polymerized and non-polymerized. Polymerized spectrin is a high molecular weight complex with actin and with band 4.1 whereas the non-polymerized form is composed of solitary spectrin dimers and tetramers. Marinetti and Crain [21] have recently suggested that spectrin may interact with clusters of phosphatidylserine or phosphatidylethanolamine on the inner surface of the membrane via Ca^{2+} bridges that are presumed to occur between the carboxyl groups of phosphatidylserine and the carboxyl groups of spectrin. Our data support this hypothesis according to the following reaction:

phospholipid-Ca^{2+}-spectrin + phosphate → phospholipid-Ca^{2+}-phosphate + spectrin

The phosphate anions found in the lysing buffer, 5 mM phosphate, pH 8.0, may react preferentially with calcium releasing spectrin, probably non-polymerized. This is in keeping with the fact that the extraction of spectrin seems to be facilitated by EDTA or other chelating agents. The fact that similar results were obtained when red cells were lysed in water, however, argues against this mechnism. Moreover spectrin released when red cells were lysed in 4 vols. of 5 mM phosphate buffer (pH 8.0) was greater at 60 min than at 5 min (Fig. 8). This indicates that more of the immediately soluble or non-polymerized spectrin is being extracted or that more spectrin is being solubilized by the lower ionic strength. The latter mechanism is unlikely since dilution of 1 vol. of erythrocytes in 0.15 M NaCl with 4 vols. of 5 mM phosphate buffer gives a final ionic strength of 0.03 M which is considerably higher than the 1 mM or 0.1 mM solutions usually used to extract spectrin [2].

This study indicates that a significant amount of spectrin is lost when

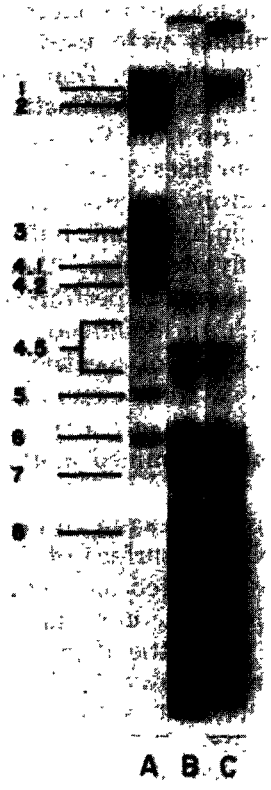


Fig. 8. SDS-polyacrylamide gels demonstrating the presence of spectrin in membrane-free hemolysate prepared as described for Fig. 5. Gel A, 40 μg of red cell membrane protein; gels B and C were each loaded with about 1.5 mg of membrane-free hemolysate obtained from erythrocytes lysed in hypotonic buffer for 5 min and 60 min, respectively. Spectrin constitutes 0.2% of the stained proteins in gel B and 1.83% in gel C.

hemoglobin-free membranes are prepared by standard methods. Details of the experimental procedure including any modification must be taken into account when analyzing membrane research data.

Acknowledgement

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References

1 Kirkpatrick, F.H. (1976) Life Sci. 19, 1—18
2 Marchesi, V.T. (1974) Methods Enzymol. 32, 275—277
3 Ballas, S.K. (1979) Clin. Res. 27, 287A
4 Gratzer, W.B. and Beaven, G.H. (1975) Eur. J. Biochem. 58, 403—409
5 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
6 Vaitukaitis, J., Robbins, J.B., Nieschlag, E. and Ross, G.I. (1971) J. Clin. Endocrinol. Metab. 33, 988—991
7 Peterson, E.A. and Sober, H.A. (1956) J. Am. Chem. Soc. 78, 751—755
8 Williams, C.A. and Chase, M.W. (eds.) (1967) Methods in Immunology and Immunochemistry Vol. 1, p. 321, Academic Press, New York, NY

9 Stumph, W.E., Elgin, S.C. and Hood, L. (1974) J. Immunol. 113, 1752—1756
10 Ouchterlony, O. (1967) in Handbook of Experimental Immunology (Weir, D.M., ed.) p. 655, Blackwell, Oxford
11 Williams, C.A. and Chase, M.W. (eds.) (1971) Methods in Immunology and Immunochemistry, Vol. 3, p. 146, Academic Press, New York, NY
12 Williams, C.A. and Chase, M.W. (eds.) (1971) Methods in Immunology and Immunochemistry, Vol. 3, p. 234, Academic Press, New York, NY
13 Steck, T.L. (1972) J. Mol. Biol. 66, 295—305
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Kirkpatrick, F.H., Rose, D.J. and LaCelle, P. (1978) Arch. Biochem. Biophys. 186, 1—8
16 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101—122
17 Lux, S.E., John, K.M. and Karnovsky, M.J. (1976) J. Clin. Invest. 58, 955—963
18 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233—248
19 Tilney, L.G. and Detmers, P. (1975) J. Cell Biol. 66, 508—520
20 Lux, S.E. (1979) Semin. Hematol. 16, 21—51
21 Marinetti, G.V. and Crain, R.C. (1978) J. Supramol. Struct. 8, 191—213