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TWO-DIMENSIONAL SEPARATION OF ERYTHROCYTE MEMBRANE PROTEINS

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SUMMARY

1). Erythrocyte membrane proteins eluted with Triton *X*-100 or dilute EDTA have been separated two-dimensionally by isoelectric focusing in polyacrylamide gels containing 1 % Triton X-100 plus 8 M urea, followed by electrophoresis using sodium dodecyl sulfate. Characteristic patterns, consistent among 40 healthy donors, were obtained.

2. The resulting patterns contain at least 30 components. The "spectrin" components (sodium dodecyl sulfate Bands 1 and 2) focus in the same pH range. Other membrane components giving single bands in sodium dodecyl sulfate electrophoresis appear to be heterogeneous.

3. Triton X-100, but not EDTA, extracts the principal membrane glycoproteins and the major "intrinsic" protein. Otherwise, proteins preferentially eluted by EDTA extract poorly with Triton X-100 and vice versa.

4. Membrane glycoproteins migrate anodally during electrofocusing and can be purified in a simple, one-step procedure.

INTRODUCTION

Merz et al. [1] and Miner and Heston [2] have previously presented evidence suggesting that one might separate erythrocyte membrane proteins or insoluble brain proteins by electrofocusing in polyacrylamide gels containing 8 M urea plus [2] or minus [1] Triton X-100. Membrane protein analysis can be further improved by combining sodium dodecyl sulfate-polyacrylamide gel electrophoresis with electrofocusing. We have previously described this approach for the analysis of EDTA-extractable membrane proteins of human erythrocytes [3] and have now extended it to proteins requiring detergents for elution. A preliminary report on this topic has been presented [4]. This communication deals with the comparative analysis of the proteins extracted from human erythrocyte ghosts by EDTA or Triton X-100.

We demonstrate that the protein composition of erythrocyte membranes is considerably more complex than suggested by simple polyacrylamide electrophoresis in sodium dodecyl sulfate. We also show that dilute EDTA elutes almost all protein entities from erythrocyte ghosts other than the "intrinsic" and major membrane glycoproteins.

MATERIALS AND METHODS

Unless otherwise stated, we obtained all chemicals and biochemicals from Serva (Heidelberg), Boehringer (Mannheim) and Merck (Darmstadt). Carrier ampholines of pH range 3.5–10.0 were purchased from LKB (Uppsala).

Erythrocyte ghosts and EDTA extracts were prepared as in ref. 3. For Triton extraction, 540-μl aliquots of membrane suspensions (protein concentration 4–5 mg/ml) in 5 mM phosphate buffer, pH 8.0, were mixed with 60 μl 20 % (v/v) Triton X-100 (Serva, scintillation grade), kept at room temperature for 5 min, and then centrifuged at $1.2 \cdot 10^7$ $g \cdot$ min (Spinco ultracentrifuge L2-653, rotor SW 50.1, 0.8-ml adapter). The supernatants, designated the "Triton extracts", were directly utilized for electrofocusing.

Gels for electrofocusing experiments (0.5 cm diameter, 9 cm height), 4 % in acrylamide cross-linked to 2.5 % with *N,N'*-methylenebisacrylamide, containing 1 % Triton X-100, 8 M urea, 10 % sucrose and 2 % ampholines were prepared as in ref. 4. Gel mixtures were filtered through Millipore filters ("Millex" disposable filter unit, Millipore, Buc, France), cast into glass tubes, and immediately overlayered with distilled water. Polymerisation took place within 15 min at room temperature and gels were ready for use immediately thereafter.

The anodal buffer in electrofocusing was 0.05 M H_2SO_4, which was deaerated before use [1,3]. The cathodal (upper) buffer was 0.03 M NaOH. Before application of samples, we generally pre-electrophoresed for 15–30 min at 0.5 mA/gel to remove residual persulfate from the gel origins: omitting this step did not affect our two-dimensional patterns. Electrofocusing was performed in a cold room at 5 °C.

EDTA extracts were made 2 % in Triton X-100. Sucrose was added to all samples to final concentrations of 15–20 %. It was not necessary to add urea or ampholines to the samples, or to place an ampholine layer over the samples. We applied samples to the cathodal ends of the gels by underlayering the buffer, using a 500-μl syringe (Hamilton). During the first 2 h of electrofocusing, we kept the current at 0.5 mA/gel. The voltage gradually rose during this time. When voltage reached 25 V/cm, electrofocusing was continued at this voltage for another 12-15 h. Lengthening of electrofocusing time to 24 h at 25 V/cm did not change the two-dimensional pattern, but was avoided to minimize decay of pH gradients. Varying the amount of protein applied during electrofocusing from 0.1–1.0 mg did not result in changes in the two-dimensional electropherograms. For optimal staining patterns, approx. 0.5–0.8 mg protein was applied.

Following electrofocusing, we measured pH gradients in two ways: (a) gel rods were sectioned into 5-mm slices, which were soaked for 2 h in 1 ml deaerated, distilled water. The pH of these samples were then determined at room temperature; (b) pH gradients were measured directly with a contact pH electrode at 2-mm intervals and at 4 °C. Both methods yielded similar pH gradients. The pH values are

not absolute because of the presence of urea; it is not our objective to precisely define the p*I* values of diverse membrane proteins.

Two-dimensional electrophoresis was performed as before [3] using a multislab apparatus [5] modified for use with sodium dodecyl sulfate as in ref. 6. The use of shorter (9 cm) gels in electrofocusing doubled the capacity of the apparatus and allowed us to run 10 different two-dimensional separations at a time. We have also shortened the electrophoresis in the second dimension and now terminate sodium dodecyl sulfate electrophoresis after the tracker dye (Pyronin G) has migrated 9 cm into the gel. The time for electrophoresis in the second dimension is about 4.5 h at 100 mA/gel slab (approx. 70 V). Instead of attaching the first-dimension gels to the slabs by polymerisation with 5 % acrylamide [3], we replaced the acrylamide with fluid agarose dissolved in electrophoresis buffer and containing 1 % sodium dodecyl sulfate: this procedure is faster and equally as effective.

Unidirectional sodium dodecyl sulfate gel electrophoresis was performed as in ref. 7. Electrophoresis buffer was 0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.4, containing 1 % sodium dodecyl sulfate.

Staining was as in ref. 3, except that ampholines were removed using 50 % methanol (12 h). Acetic acid in the fixative led to precipitation of ampholines in the gels, which retarded their removal. The first staining solution was 0.05 % Coomassie Brilliant Blue (G-250, Serva) in 25 % isopropanol. After 4–6 h in this solution, gels were transferred to 0.0025 % Coomassie in 10 % isopropanol/10 % methanol for another 3–4 h, and then destained overnight in 20 % methanol/10 % acetic acid and preserved in 10 % acetic acid. Control experiments showed that omitting acetic acid in the fixative and staining solutions did not result in removal of proteins from the gels. Staining for glycoproteins with periodate-Schiff was as in ref. 7.

RESULTS

Fig. 1 shows the electrophoretograms obtained by simple sodium dodecyl sulfate gel electrophoresis of normal, untreated human erythrocyte ghosts (a), their EDTA extracts (b), their Triton extracts (c) and a mixture of (b) and (c) (Gel d). Protein bands are numbered in order of decreasing molecular weight as in ref. 3. As shown before, EDTA extracts principally the more water-soluble membrane proteins, i.e. the "spectrin" Bands 1 and 2, Band 5 and a number of other minor components in the regions of Bands 3, 4.1–4.3, 7 and 8 [3, 7–9]. In contrast, as also recently shown by Yu et al. [10], Triton X-100 selectively extracts primarily the glycoproteins and the major "intrinsic" protein (Band 3, [10–15]) under our conditions, leaving the bulk of water-soluble proteins ("spectrin" and Band 5) in the pellet. Mixtures of the extracts (Gel d) yield electrophoretograms which are almost identical to that of untreated membranes (Gel a). The mixture thus seems to contain most, if not all erythrocyte membrane proteins.

Periodate-Schiff staining showed that all glycoproteins were present in Triton extracts, but were virtually absent in EDTA extracts.

Isoelectric focusing of the same samples (b–d) showed patterns similar to those obtained previously [3]. Most membrane proteins focus between pH 4.5–6.5 in this system. However, the focusing patterns are very complex, with nearly 40 bands in each gel. We find it difficult to interpret data on the basis of electrofocusing alone.

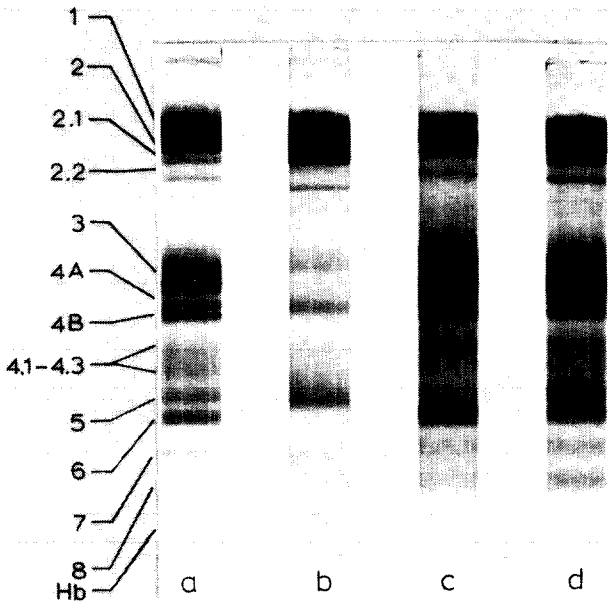


Fig. 1. Sodium dodecyl sulfate gel electrophoretograms of human erythrocyte membranes and their derivatives. (a) Whole ghosts. (b) EDTA extract. (c) Triton extract. (d) Mixture of EDTA and Triton extract. Numbering of components from top to bottom in order of decreasing apparent molecular weight. Staining: Coomassie Brilliant Blue.

Two-dimensional electrophoresis of EDTA extracts (Fig. 2a) yields patterns similar to those described previously [3] using urea only. The present experiments, where urea was not added to samples for electrofocusing, improved the reproducibility of electrophoretograms. In particular, Bands 1 and 2 focus over a narrower pH range (pH 5–6) and Band 5 consistently separates into three to four entities.

Two-dimensional electrophoresis of Triton extracts (Fig. 2b) separates the non-glycosylated component of Band 3, which has an apparent p*I* of around 4.8–5.0 from the major membrane glycoprotein, which migrates to the anodal gel end during electrofocusing (Fig. 2c). There are obvious qualitative similarities between the two-dimensional electrophoretograms of the EDTA and Triton extracts, and experiments performed with mixtures of these samples indeed show that virtually all corresponding protein spots in the two extracts represent identical entities. A typical separation pattern is shown in Fig. 2d. The following points deserve comment. First, the "spectrin" Bands 1 and 2 which stem from the EDTA extract now tend to smear out at pH regions slightly higher than seen in the EDTA extract alone (compare with Fig. 2a). Second, the major "intrinsic" protein and the glycoproteins resolve as in the pure Triton extract. Third, all protein spots in sodium dodecylsulfate regions 4.1–8 overlap to give basically the same electrophoretogram as the EDTA or Triton extract alone. This shows that, with the exception of the "spectrins", our electrofocusing system functions identically for the different applied samples. With regard to the "spectrins", artefacts may be caused by (a) an intrinsic tendency of these proteins to aggregate near their isoelectric points and/or (b) by the presence of lipids in a Triton extract [10] which might potentiate this tendency. However, resolution

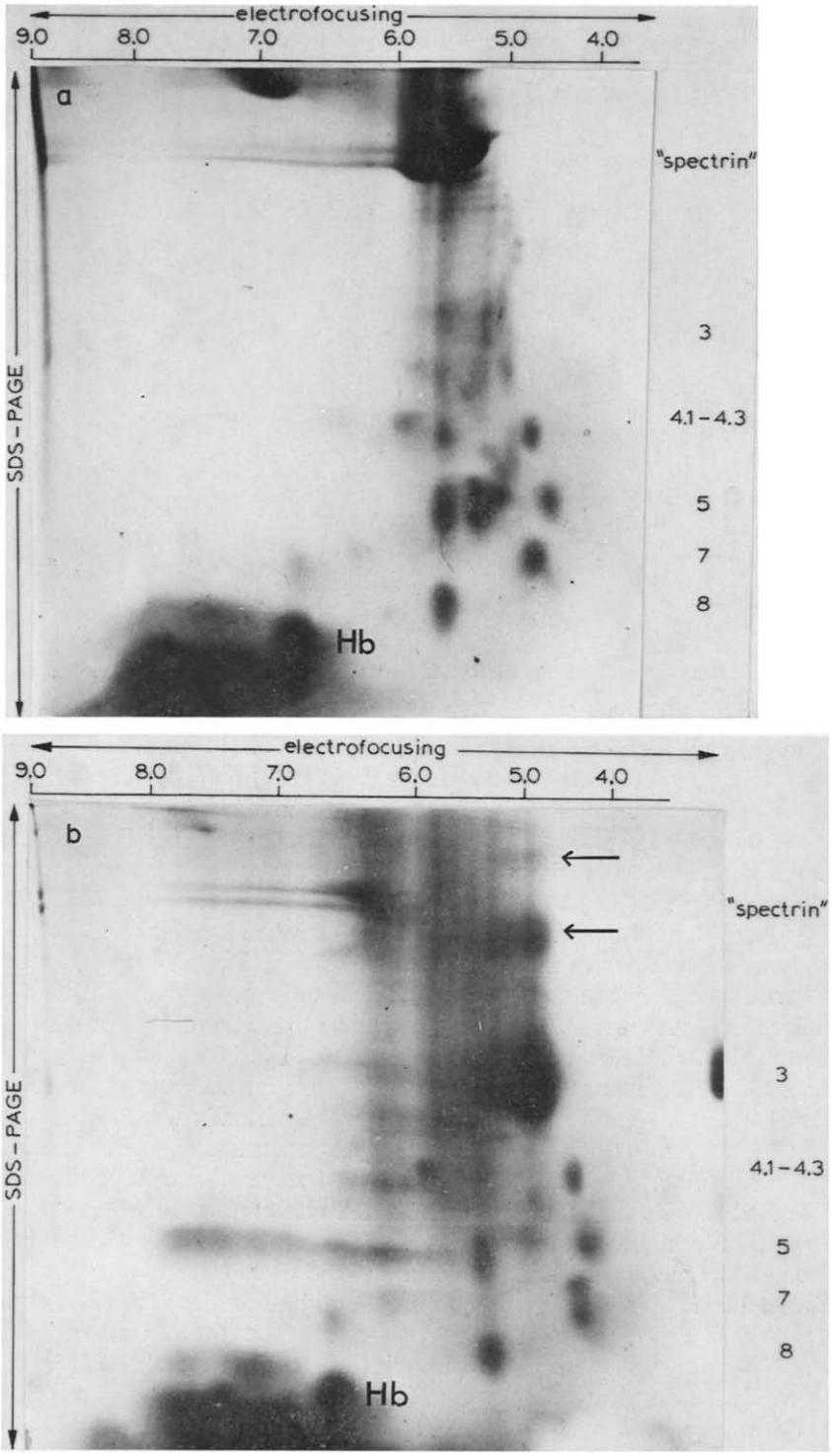
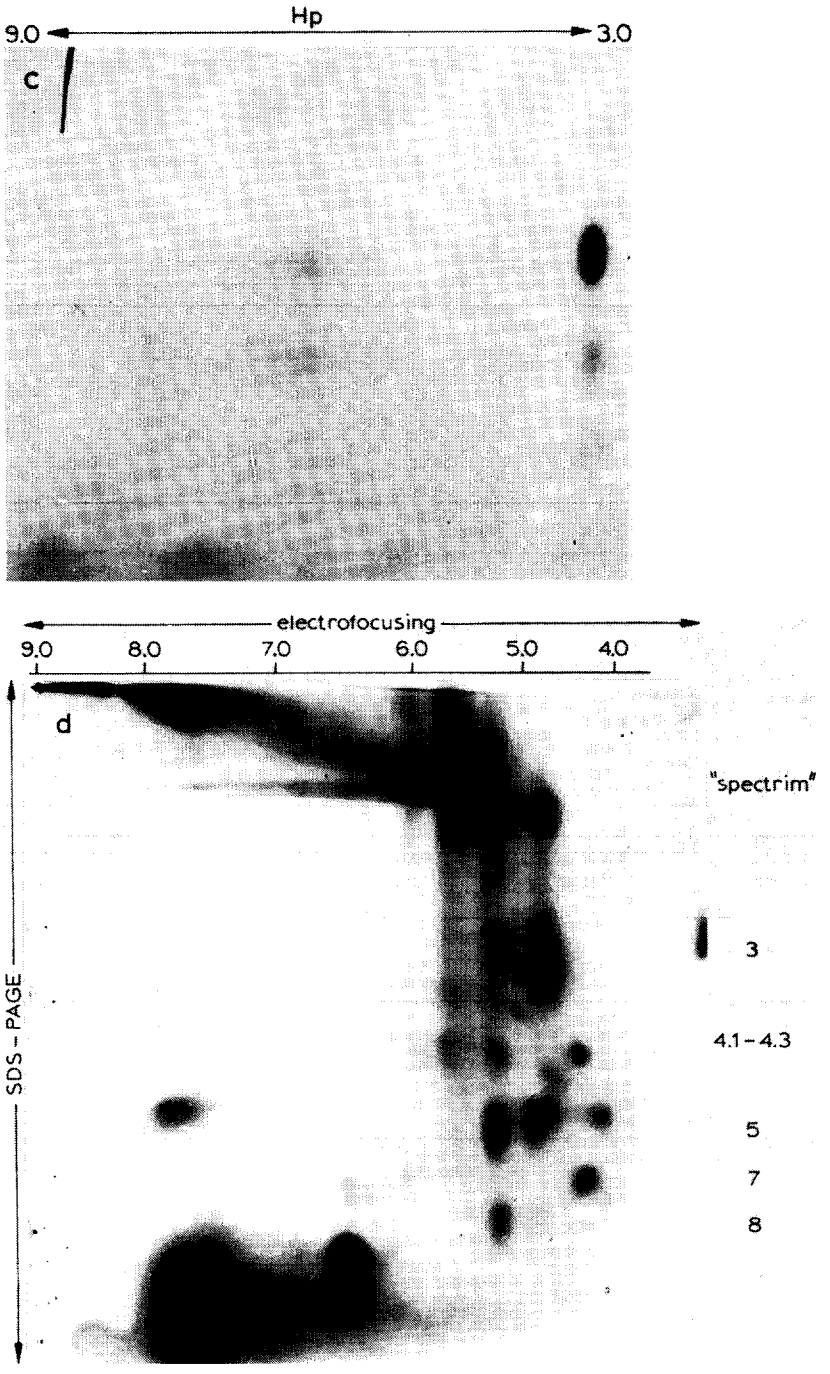


Fig. 2. Two-dimensional electrophoretograms of (a) EDTA extract; (b) and (c) Triton extract; (d) mixture of EDTA and Triton extract. Isoelectric focusing, left to right; sodium dodecyl sulfate gel



electrophoresis, top to bottom. The approximate corresponding pH gradient and the protein bands are given. The lake-like material at the sodium dodecyl sulfate gel electrophoresis front represents residual ampholines used in electrofocusing. Arrows point to dimeric and tetrameric aggregates of protein component 3. Staining, Coomassie Brilliant Blue (Gels a, b and d); periodate-Schiff (Gel c). Hb, Hemoglobin. SDS-PAGE, sodium dodecyl sulfate gel electrophoresis. Note the anodal migration of all membrane glycoproteins during electrofocusing.

of other proteins is not affected. We therefore believe that the separation system is suitable for analysis of all erythrocyte membrane proteins with the possible exception of the "spectrins".

We have also electrophoresed human serum albumin, bovine panreas DNA-ase, glucose oxidase, horse skeletal muscle myoglobin and horseradish peroxidase two-dimensionally. All five proteins yielded single major spots, but addition of urea to the samples sometimes led to formation of two spots in the case of human serum albumin and glucose oxidase. Moreover, we constantly observed incomplete monomerisation of glucose oxidase and serum albumin in second-dimension electrophoresis. This led to the appearance of di-, tetra- and polymeric aggregates upon electrophoresis in the second dimension. Such incomplete monomerisation should always be suspected when a series of spots situated directly over each other appears in the electrophoretograms. This is in fact the case for the major "intrinsic" protein (sodium dodecyl sulfate Band 3, arrows in Fig. 2b), as well as for hemoglobin, when present in too high amounts [3].

DISCUSSION

Whereas smearing artefacts are sometimes seen with "spectrin" Bands 1 and 2 during electrofocusing, artefactual multiple banding leading to formation of two distinct, neighboring entities in two-dimensional patterns has not been observed when urea is omitted from the samples. Cyanate stemming from the urea present in the gels probably has no influence on the proteins to be separated because, as pointed out by Vesterberg [16], the ampholines assert a protective influence by competing with cyanate-reactable sites. At this point, we mention that Triton plus urea in electrofocusing gels is necessary for optimal separations. Increasing the Triton concentration to 3 % does not affect the two-dimensional electrophoretograms.

We have previously mentioned [4] that, although Triton X-100 plus urea can totally solubilize erythrocyte membranes, such samples cannot be electrofocused in our gels because some material aggregates out at the gel origins, and two-dimensional separations are not optimally reproducible. We do not know the precise reason for this. However, the experiments where EDTA and Triton extracts were mixed indicate that the underlying cause is probably not due to protein-protein interaction. We have reasoned that if EDTA preferably extracts hydrophilic entities, and Triton X-100 preferably the hydrophobic moieties, a mixture of the extracts should contain practically all membrane proteins. Such samples are readily accessible to two-dimensional analyses.

The present data show that caution is needed when homogeneity of a protein is judged on the basis of sodium dodecyl sulfate gel electrophoresis alone. Indeed, we can demonstrate heterogeneity of most of the sodium dodecyl sulfate components of human erythrocyte membranes, consistent with findings obtained by immunochemical methods [17–19]. The resolution obtained in our system further allows us to make definite conclusions concerning molecular entities elutable with various agents such as EDTA and Triton X-100. The extracting potency of these agents is somewhat reciprocal. However, virtually all membrane proteins appear to be present in EDTA extracts except the major "intrinsic" protein and the glycoproteins. These are concentrated in Triton extracts, which moreover qualita-

tively appear to contain all other membrane proteins as well. Examination of Triton-extracts may therefore provide a useful tool for routine analysis of erythrocyte membrane proteins.

The separation procedure yields extremely reproducible results. We have examined 40 healthy donors to date, without discerning any significant variation in the two-dimensional electrophoretograms of the Triton extracts. However, the intensities of various spots may vary somewhat from sample to sample. Conceivably, this system will be sensitive enough to detect membrane-protein abnormalities which have hitherto escaped detection with sodium dodecyl sulfate gel electrophoresis alone. The extension of this and similar separation systems should prove useful in other fields of membrane protein chemistry.

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REFERENCES

1 Merz, D. C., Good, R. A. and Litman, G. W. (1972) Biochem. Biophys. Res. Commun. 49, 84–91
2 Miner, G. D. and Heston, L. L. (1972) Anal. Biochem. 50, 313–316
3 Bhakdi, S., Knüfermann, H. and Wallach, D. F. H. (1974) Biochim. Biophys. Acta 345, 448–457
4 Bhakdi, S., Knüfermann, H. and Wallach, D. F. H. (1975) in Progress in Isoelectric Focusing and Isotachophoresis (Righetti, P., ed.), Elsevier Scientific Publishing Co., Amsterdam, in the press
5 Kaltschmidt, E. and Wittmann, H. G. (1970) Anal. Biochem. 36, 401–412
6 Knüfermann, H., Bhakdi, S. and Wallach, D. F. H. (1975) Biochim. Biophys. Acta 389, 464–476
7 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
8 Marchesi, S. L., Steers, E., Marchesi, V. T. and Tillack, T. W. (1970) Biochemistry 9, 50–57
9 Reynolds, J. A. and Trayers, H. (1971) J. Biol. Chem. 246, 7337–7342
10 Yu, J., Fishman, D. A. and Steck, T. L. (1973) J. Supramol. Struct. 1, 233–248
11 Steck, T. L., Fairbanks, G. and Wallach, D. F. H. (1971) Biochemistry 10, 2618–2626
12 Marchesi, V. T. and Andrews, E. P. (1971) Science 174, 1247–1248
13 Bretscher, M. S. (1971) J. Mol. Biol. 59, 351–357
14 Juliano, R. L. and Rothstein, A. (1971) Biochim. Biophys. Acta 249, 227–235
15 Juliano, R. L. (1972) Biochem. Biophys. Acta 266, 301–306
16 Vesterberg, O. (1969) Protein Biol. Fluids, 17th Coll, pp. 417–422, Pergamon Press, Oxford
17 Bjerrum, O. J., Bhakdi, S., Knüfermann, H. and Bøg-Hansen, T. C. (1974) Biochim. Biophys. Acta 373, 44–50
18 Green, J. R., Dunn, M. J., Spooner, R. L. and Maddy, A. H. (1974) Biochim. Biophys. Acta 373, 51–58
19 Bjerrum, O. J., Bhakdi, S., Bøg-Hansen, T. C., Knüfermann, H. and Wallach, D. F. H. (1975) Biochim. Biophys. Acta, submitted for publication