Biochimica et Biophysica Acta, 345 (1974) 448–457

BBA 76623

SEPARATION OF EDTA-EXTRACTABLE ERYTHROCYTE MEMBRANE PROTEINS BY ISOELECTRIC FOCUSSING LINKED TO ELECTROPHORESIS IN SODIUM DODECYL SULFATE

SUCHARIT BHAKDI[a]*, HUBERTUS KNÜFERMANN[a] and DONALD F. HOELZL WALLACH[b]

[a] *Max-Planck-Institut für Immunbiologie, 78 Freiburg, Stübeweg 51 (W. Germany) and* [b] *Department of Therapeutic Radiology, Division of Radiobiology, Tufts New England Medical Center, 136 Harrison Ave., Boston, Mass. 02111 (U.S.A.)*

(Received December 12th, 1973)

SUMMARY

1. EDTA-extractable proteins of human erythrocyte membranes can be separated in 8 M urea by isoelectric focussing in polyacrylamide gels.

2. Subsequent electrophoresis at right angles in sodium dodecyl sulfate-laden polyacrylamide shows that most of the bands hitherto defined by simple sodium dodecyl sulfate polyacrylamide gel electrophoresis do not represent single proteins/peptides, but comprise mixtures of diverse molecular species with different isoelectric points.

3. The high molecular weight sodium dodecyl sulfate bands 1,2,2.1 and 2.2 possess identical isoelectric points. The isoelectric points of Band 5 components also overlap with those of the Bands 1, 2, 2.1 and 2.2. This suggests a structural relationship between these entities.

4. Membranes from different donors show slight variations in the two-dimensional electrophoretic pattern.

5. The separation techniques presented here provide greater resolution than hitherto attained and should prove useful for the detailed analysis and characterisation of membrane proteins.

INTRODUCTION

Efforts to separate and characterize proteins of biological membranes continue to be hampered by the relative insolubility of these substances and the difficulty of fractionating membrane protein mixtures obtained by various solubilisation procedures [1–3].

In order to avoid protein aggregation during electrophoretic separations, detergent-electrophoresis systems have been introduced into the field of membrane

* To whom correspondence should be addressed.

research. Analyses of human erythrocyte membrane proteins using electrophoresis in sodium dodecyl sulfate-laden polyacrylamide are exemplified by the work of Fairbanks et al. [4], whose system and related approaches have established themselves as powerful tools in the analysis of certain membrane preparations. However, simple sodium dodecyl sulfate polyacrylamide gel electrophoresis suffers from serious drawbacks. Thus, protein bands appearing after sodium dodecyl sulfate polyacrylamide gel electrophoresis do not necessarily represent homogeneous molecular species, but may comprise aggregates and/or sets of different proteins/peptides with similar molecular weights. Also, the biochemist is troubled by the often irreversible destruction of biological activity due to sodium dodecyl sulfate, as well as the difficulty of removing the detergent from the proteins after separation. Finally, treatment of membranes with powerful surfactants such as sodium dodecyl sulfate precludes the possibility of defining the neighbor relationships that exist in native membranes.

Isoelectric focussing is a separation technique which has come into increased usage since its introduction into biochemistry by Svensson [5] and Vesterberg and Svensson [6]. Its resolution derives from an extremely sensitive separation principle and the concentrating effect inherent in the system. The method has recently been applied to the separation of urea-extractable membrane proteins of human erythrocytes [7]. However, the results are difficult to interpret because of the lack of a reference system. Moreover, prolonged treatment of proteins with urea introduces the hazard of artifact due to possible carbamylation of proteins by cyanate contaminating the urea.

We therefore choose to selectively elute erythrocyte membrane proteins with EDTA, and subsequently analyse the extract by two-dimensional electrophoresis, following isoelectric focussing in urea by sodium dodecyl sulfate polyacrylamide gel electrophoresis. This allows substantially greater analytical resolution than hitherto achieved and demonstrates the heterogeneity of many bands hitherto defined by sodium dodecyl sulfate polyacrylamide gel electrophoresis alone.

MATERIALS AND METHODS

Unless otherwise stated, we obtain all chemicals and biochemicals from Serva (Heidelberg), Boehringer (Mannheim) and Merck (Darmstadt). We obtain ampholines from LKB (Uppsala). We obtain freshly drawn human blood (blood group O, Rh positive) in acid citrate dextrose solution.

We have purified analytical grade urea further by passing 8 M solutions over Amberlite MB-3 (column diameter 3.0 cm, height 36 cm), thereafter immediately lyophilizing the eluate. An 8 M solution of purified urea shows a conductivity of 3–5 Ω/cm, compared with 15–25 Ω/cm for an 8 M solution of the commercial product. We omit the purification step in later experiments because the electrophoretic patterns obtained are identical to those with unpurified urea.

Membrane preparation

We wash erythrocytes five times in isotonic saline, taking special care to remove the buffy coat. We prepare membranes as described in ref. 8. After 3–4 washings with 5 mM phosphate buffer, pH 8, we suspend the membranes to a protein con-

centration of about 4 mg/ml. We intentionally do not elute all the hemoglobin, because this serves as a marker in the isoelectric focussing experiments.

EDTA extraction of membrane proteins

We dialyse ghost suspensions for 36 h at 4 °C against 1000 vol. of 0.5 mM EDTA, pH 7.5. We then centrifuge at $9 \cdot 10^6 \times g \cdot \text{min}$ (Beckman Ultracentrifuge L 2-65 B, rotor SW 50.1).

Isoelectric focussing

We use the clear supernatant after EDTA extraction. We make the EDTA extract 8 M in urea, shake vigorously and incubate at 37 °C for 10 min. We employ gels 4% in acylamide cross-linked with 2.5% *N,N'*-methylenebisacrylamide containing 10% sucrose and 1% ampholines of pH range 3.5–10.0. We cast the gels in glass tubes 0.6 cm in diameter and 18 cm in height, using the following stock solutions: (A) 7.8 g acrylamide, 0.2 g *N,N'*-methylenebisacrylamide, 12.5 g sucrose, made up to 50 ml with 8 M urea; (B) ampholine concentrate, pH 3.5–10.0 (40%, w/w); (C) sucrose concentrate: 20 g sucrose made up to 50 ml with 8 M urea; (D) tetramethylethylenediamine; (E) 8 M urea; (F) ammonium persulfate: 50 mg dissolved in 4 ml 8 M urea.

We combine 10 ml Solution A with 1.0 ml Solution B, 5.0 ml Solution C and 22.0 ml Solution E, thoroughly degas this mixture, add 2.0 ml of Solution F and 40 µl Solution D and then immediately cast gels of 15 cm length. We overlay the surfaces with degassed distilled water and allow polymerisation to proceed at room temperature for at least 2 h.

For electrophoresis, we place polymerized gels vertically in a conventional disc electrophoresis apparatus accomodating two sets of six gels in each compartment. We use thoroughly degassed solutions of 0.05 M H_2SO_4 and 0.03 M NaOH as anodal (lower) and cathodal (upper) buffers, respectively. Before sample application, we electrophorese at 1 mA/gel for 15 min to remove residual ammonium persulfate.

We apply 350–450 µl samples in 8 M urea containing approximately 750 µg of protein to the top of the gels using a microsyringe (Hamilton) to underlay the buffer. We keep the current at 0.5 mA/gel during the first 5 h of electrofocussing and then change to a constant voltage of 40 V/cm, continuing for another 12 h at this voltage. At this time the current per gel is about 0.10 mA. The procedure is carried out at room temperature (18 °C).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

We proceed essentially as described in ref. 4, using 5.0% gels cross-linked to 2.5% with *N,N'*-methylenebisacrylamide. We use 0.5 cm × 7.5 cm gel discs for one-directional runs and 0.5 cm × 15 cm gels for two-dimensional studies, employing Pyronin G as tracking dye. The electrophoresis buffer is 0.1 M Tris, 0.2 M sodium acetate, 0.02 M EDTA, pH 7.4, containing 1.0% sodium dodecyl sulfate. We electrophorese at 5 mA per gel at room temperature.

Two-dimensional polyacrylamide gel electrophoresis

We proceed essentially as described in ref. 9 with minor apparatus modifications for use with sodium dodecyl sulfate (Knüfermann, H., Bhakdi, S. and Wallach,

D. F. H., in preparation). We use 20 cm × 20 cm × 0.5 cm gel slabs with an acrylamide concentration of 5.0%, cross-linked to 2.5% with *N,N'*-methylenebisacrylamide. We run sodium dodecyl sulfate electrophoreses at 100 mA per gel slab at room temperature for about 6 h. We use the same electrophoresis buffer as in the unidirectional system, but add sodium dodecyl sulfate to the upper cathodal buffer only.

Experimental protocol

For isoelectric focussing we load three gels with identical samples and leave one unloaded. After termination of the run, we cut the unloaded gel and one protein-containing gel into 5-mm slices with a razor blade, soak each slice in 1 ml of degassed, distilled water for 1 h, and subsequently determine the pH gradient. We immediately dialyse one of the remaining gels for 1 h at room temperature against electrophoresis buffer containing 3% sodium dodecyl sulfate. We then polymerize the gel horizontally onto an acrylamide gel slab [10] and proceed with sodium dodecyl sulfate electrophoresis in the second dimension. The last gel is stained with Coomassie Brilliant Blue as given below.

We run parallel samples treated with sodium dodecyl sulfate alone, or with 8 M urea plus 3% sodium dodecyl sulfate on 7.5-cm gels according to ref. 4.

Staining procedures

Staining following ordinary, one-dimensional sodium dodecyl sulfate electrophoresis is as in ref. 4. However, after isoelectric focussing one must thoroughly elute the ampholines prior to staining. We do this by washing for 48 h in isopropanol–acetic acid–water (25:10:65; v/v/v), changing this fixative once after 24 h. We then stain with Coomassie Brilliant Blue [4]. Because ampholines distribute diffusely upon sodium dodecyl sulfate polyacrylamide gel electrophoresis, one must elute these also after two-dimensional runs. Before staining we therefore wash five gel slabs in 10 l of fixative, changing the solution once after 24 h.

Photography of gels

We photograph discs and slabs with a Polaroid Land MP-3 camera using a Polaroid film Type PN 55. Shutter settings are 11/16 and 16/22 for gel discs and slabs, respectively. Exposure time is 1 s.

RESULTS

EDTA extraction of membrane proteins

As found by others [1,12] EDTA extracts membrane proteins somewhat selectively. This is illustrated in Fig. 1, showing the normal sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of human erythrocyte membranes, that of the EDTA extract, and that of the pellet remaining after EDTA extraction. We number the electrophoretic bands essentially as in ref. 4, but designate the two most rapidly moving components with numbers 7 and 8, respectively. The data show that EDTA extracts almost all of the components with high apparent molecular weights (Spectrin) as well as Component 5, and partially elutes Components 4.1–4.3, 6, 7 and 8. Most of Component 3 remains in the pellet, but the EDTA extract reveals several discrete bands in its molecular weight domain (approx. 90 000).

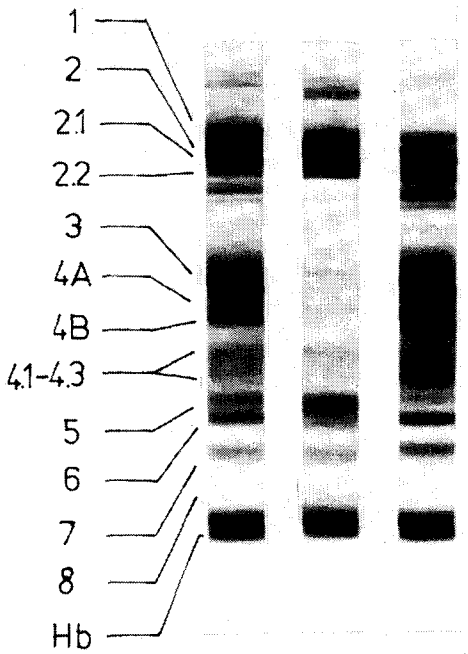

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of human erythrocyte ghosts and their derivatives. Left, whole ghosts; center, EDTA extract (see text); right, residue after EDTA extract (see text). Numbering of components from top to bottom in order of decreasing apparent molecular weight (text). Staining: Coomassie Brilliant Blue.

Isoelectric focussing of the EDTA extract in 8 M urea

We obtain essentially the same pH gradient as did Merz et al. [7]. The gradient is non-linear above pH 8.2, probably due to shrinking of the gel at the alkaline end. We find identical pH gradients in loaded and unloaded gels.

Fig. 2, the photograph of a typical gel stained with Coomassie Brilliant Blue, together with the corresponding pH gradient, shows the largest number of bands around pH 5–6; other bands are distributed over the range of pH 4.5–7.5. Slight variations in the electrophoretic pattern occur with samples from different donors.

Two-dimensional gel electrophoresis

Fig. 3 shows the staining pattern typically obtained by isoelectric focussing of the EDTA extract followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis at right angles. Fig. 4 represents a schematized illustration, which also gives the pH gradient and a reference sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern. By running samples in one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis together with the two-dimensional separation, the different bands found with the latter method can be simply related to conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis components. The following correlations can be established:

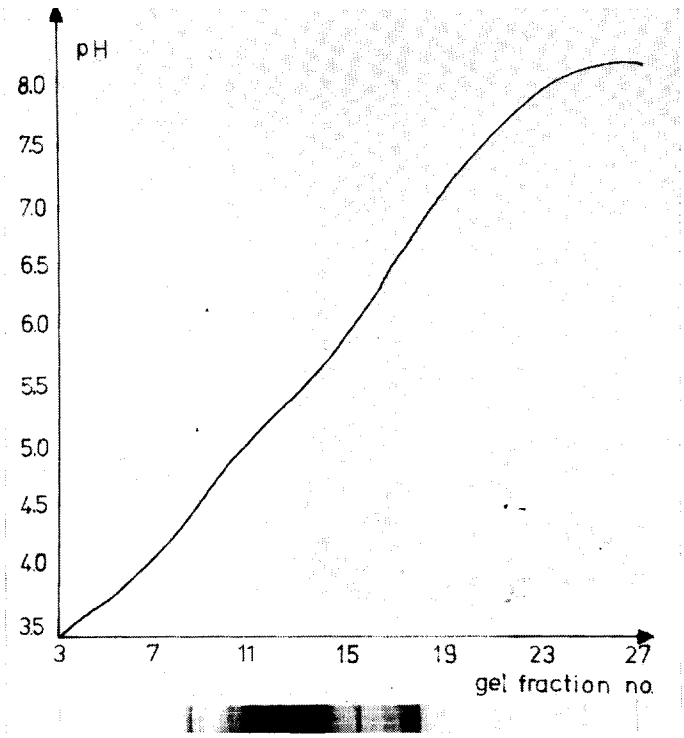

Fig. 2. Isoelectric focussing of EDTA-extractable proteins of human erythrocyte ghosts and the corresponding pH gradient (see text). Coomassie Brilliant Blue.

Bands 1, 2, 2.1 and 2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis Bands 1, 2, 2.1 and 2.2 always arise together from a set of discrete components focussing between pH 4.8–6.0. Each component obtained by isoelectric focussing yields all four bands. What is defined as spectrin [11, 12] by sodium dodecyl sulfate polyacrylamide gel electrophoresis thus seems to comprise a heterogeneous mixture. Interestingly, some of the spectrin components possess the same isoelectric points as sodium dodecyl sulfate polyacrylamide gel electrophoresis Band 5.

Band 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis Band 3 shows a diffuse front, followed by a more sharply defined zone. At least two protein entities, the major glycoprotein of human erythrocyte membranes and a non-glycosylated protein of apparent mol. wt 89 000, lie within this band [1, 4, 13]. These components of Band 3 do not appear in EDTA extracts. However, such eluates contain several minor peptides which migrate in the region of Band 3 (Fig. 1). Our two-dimensional separations yield 3–4 defined spots on the gel slab, which correspond to these minor sodium dodecyl sulfate bands. Their isoelectric points lie near pH 5.5. The minor components, which do not resolve in conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis, may in part account for the diffuseness of sodium dodecyl sulfate polyacrylamide gel electrophoresis Band 3.

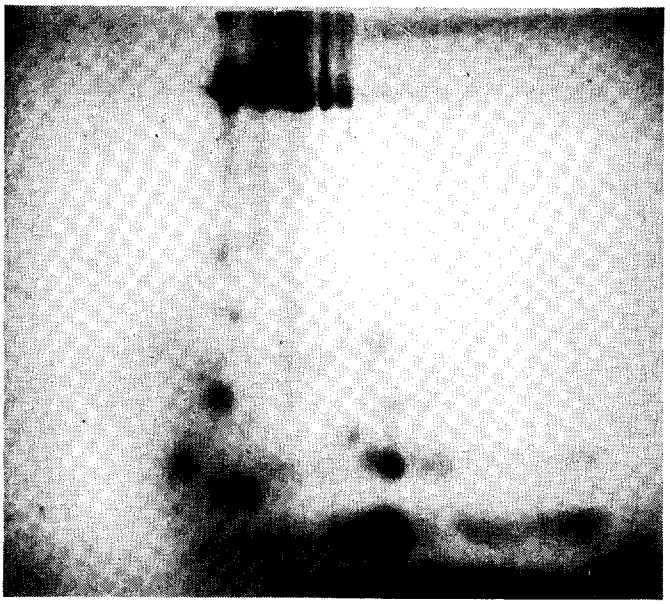

Fig. 3. Two-dimensional electrophoresis of the EDTA extract. Isoelectric focussing, right to left. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, top to bottom. The lake-like material at the sodium dodecyl sulfate polyacrylamide gel electrophoresis front represents ampholines used in isoelectric focussing. Coomassie Brilliant Blue.

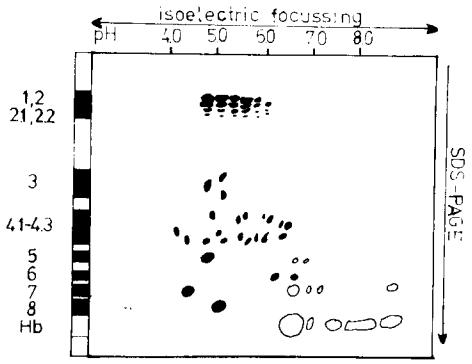

Fig. 4. Two-dimensional electrophoretic pattern of EDTA-extractable human erythrocyte membrane proteins, schematic illustration. Isoelectric focussing, right to left. The corresponding pH gradient is shown on top of the gel slab. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, top to bottom. On the left side a schematized unidirectional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of the separated proteins, numbered as in Fig. 1. Open circles, hemoglobin components.

Bands 4A and 4B. Only very small amounts of these sodium dodecyl sulfate polyacrylamide gel electrophoresis components are extracted with EDTA (Fig. 1), and we have not been able to establish their positions on the two-dimensional plate with certainty.

Bands 4.1–4.3. These components, which always appear as a series of diffuse bands in unidirectional sodium dodecyl sulfate polyacrylamide gel electrophoresis, comprise mixtures of a large number of minor membrane proteins. Thus, this molecular weight region yields at least 15 spots after isoelectric focussing; these scatter over a wide pH range. Although EDTA extracts only a part of these peptides from the membrane, the two-dimensional electrophoretic pattern is highly reproducible.

Band 5. This entity always focusses at around pH 4.8–5.2, as do several spectrin components. Some differences occur from one donor to the next; in some cases, as shown in Fig. 3, only one spot can be visualized, whereas in other cases up to three spots are observed. Such variations are always coupled to similar variations of the spectrin bands, but the relationships between the components of spectrin and of Band 5 do not appear quantitatively constant.

Band 6. Only small amounts of this component are extracted with EDTA. They focus at 2–3 isoelectric points between pH 6.7–8.0.

Bands 7 and 8. Both the EDTA-extractable components of these sodium dodecyl sulfate bands focus as single spots at around pH 4.7 and 5.4, respectively. They thus appear the most homogeneous material in the EDTA extract.

Hemoglobin. The major components of hemoglobin focus around pH 6.8. We observe that sodium dodecyl sulfate does not monomerize this molecule when it is present above a critical concentration in the extract. Fig. 3 shows the appearance of monomeric, dimeric and tetrameric hemoglobin components in the gel slab.

DISCUSSION

Up to now, sodium dodecyl sulfate polyacrylamide gel electrophoresis has been the most potent technique for membrane protein analysis. Unfortunately, the method suffers from serious inadequacies.

First, separations proceed only according to (a) particle size and (b) sodium dodecyl sulfate binding by the proteins/peptides in a mixture. However, not all oligomeric proteins monomerize in sodium dodecyl sulfate [1, 14, 15] and not all proteins/peptides bind sodium dodecyl sulfate in strict proportion to molecular weight [1, 16]. Second, since sodium dodecyl sulfate polyacrylamide gel electrophoresis separations involve primarily particle size, peptides of similar molecular weight but different charge will remain unresolved.

By linking sodium dodecyl sulfate polyacrylamide gel electrophoresis to a preceding, charge-dependent separation, i.e. isoelectric focussing in 8 M urea, we can partially resolve the second dilemma and add a new dimension to membrane protein fractionation. We find it necessary to precede sodium dodecyl sulfate polyacrylamide gel electrophoresis with isoelectric focussing, since sodium dodecyl sulfate cannot easily be eluted from membrane proteins and interferes with isoelectric focussing because of its charge. It is also convenient to utilize an EDTA-extract initially, since EDTA solubilizes almost the same proteins as urea, but avoids the danger of protein modification during prolonged treatment with urea containing traces of cyanate.

Although EDTA extracts membrane proteins selectively and fails to solubilize certain major membrane protein components (e.g. sodium dodecyl sulfate polyacrylamide gel electrophoresis Bands 3, 4A and 4B), our two-dimensional analysis of such extracts yields important information about erythrocyte membrane proteins.

In particular, all of the major sodium dodecyl sulfate polyacrylamide gel electrophoresis components generally thought to be unique polypeptides except Bands 7 and 8, actually comprise heterogeneous mixtures of components with dissimilar isoelectric points, but similar size. The proteins of erythrocyte membranes thus contain many more than the 6–7 major polypeptides assumed on the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. For example, Bands 4.1–4.3 constitute perhaps 15 different molecular species. Also, although the main components of Band 3 cannot be studied in this system, we can demonstrate that a number of minor peptides run in this sodium dodecyl sulfate polyacrylamide gel electrophoresis region, particularly in the diffuse front zone of Band 3.

Turning to the spectrin Bands 1, 2, 2.1 and 2.2 in the sodium dodecyl sulfate polyacrylamide gel electrophoresis system, we have recently demonstrated that sodium dodecyl sulfate polyacrylamide gel electrophoresis Bands 1 and 2 contain at least five identical N-terminal acids [17]. By itself, this finding could be interpreted in two, non-exclusive ways. First, there could be multiple polypeptide chains, each of high molecular weight (about 310 000 for Band 1, 290 000 for Band 2), all migrating identically in sodium dodecyl sulfate polyacrylamide gel electrophoresis. Second, there may exist at least five polypeptide chains of lower molecular weight which aggregate to form high molecular weight entities, i.e. the spectrin bands. The information presented here, plus knowledge of the N-terminal identity between Bands 1 and 2 [17], appears consistent with the idea of molecular aggregates comprised of lower molecular weight subunits. We therefore tentatively suggest that sodium dodecyl sulfate does not disaggregate the spectrin bands into their true subunits, and that the sodium dodecyl sulfate polyacrylamide gel electrophoresis Bands 1, 2, and most probably also 2.1 and 2.2 contain the same subunits. It is also possible that urea does not dissociate all spectrin components (e.g. sodium dodecyl sulfate polyacrylamide gel electrophoresis components 1, 2, 2.1, 2.2). These would then focus together in urea, but separate in sodium dodecyl sulfate (second dimension) to form a spectrin pattern. This association may then represent a near-neighbour relationship.

The separation technique presented here should prove to be useful in detecting subtle differences in membrane proteins. Such might be primary in nature, as in hereditary membrane diseases, or secondary, such as in lymphocyte activation or neoplastic conversion. Indeed, we already discern certain discrete variations in the two-dimensional electrophoretic picture amongst preparations from different donors, and we plan to gradually define the normal polymorphism of membrane proteins.

ACKNOWLEDGEMENTS

We thank Dr B. Kickhöfen, Dr M. Modolell and Prof. H. Fischer for their encouragement and advice. This investigation was supported by the Alexander von Humboldt Stiftung (S.B.), the Max-Planck-Gesellschaft zur Forderung der Wissenschaften (H. K.) by Grant Ca 12178 of the United States Public Health Service and Award PRA-78 of the American Cancer Society (D.F.H.W.).

REFERENCES

1 Wallach, D. F. H. and Winzler, R. (1974) Evolving Strategies and Tactics in Membrane Research, Chapter 3, Springer Verlag, New York

2 Rosenberg, S. A. and Guidotti, G. (1969) in Red Cell Membrane (Jamieson, G. A. and Greenwalt, T. J., eds), pp. 93–109, Lippincott, Philadelphia, Toronto
3 Maddy, A. H., Dunn, H. J. and Kelly, P. G. (1972) Biochim. Biophys. Acta 288, 263–276
4 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
5 Svensson, H. (1962) Arch. Biochem. Biophys. Suppl. 1, 132–141
6 Vesterberg, O. and Svenson, H. (1966) Acta Chem. Scand. 20, 820–834
7 Merz, D. C., Good, R. A. and Litman, G. W. (1972) Biochem. Biophys. Res. Commun. 49, 84–91
8 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–134
9 Kaltschmidt, E. and Wittmann, H. G. (1970) Anal. Biochem. 36, 401–412
10 Kaltschmidt, E. and Wittmann, H. G. (1969) Anal. Biochem. 30, 132–141
11 Furthmayr, H. and Timpl, R. (1970) Eur. J. Biochem. 15, 301–310
12 Marchesi, S. L., Steers, E., Marchesi, V. T. and Tillack, T. W. (1970) Biochemistry 9, 50–57
13 Marchesi, V. T. and Andrews, E. P. (1971) Science 174, 1247–1248
14 Wallach, D. F. H. and Weidekamm, E. (1973) in Erythrocytes, Thrombocytes, Leukocytes (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds), pp. 2–9, Thieme Publishers, Stuttgart
15 Moss, B. and Rosenblum, E. H. (1972) J. Biol. Chem. 247, 5194–5198
16 Weber, K., Pringle, J. R. and Osborne, M. (1972) in Methods of Enzymology (Hirs, C. H. W. and Timasheff, S. N., eds), Vol. XXVI, pp. 3–47, Academic Press, New York
17 Knüfermann, H., Bhakdi, S. and Wallach, D. F. H. (1973) Biochim. Biophys. Acta 330, 356–361