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THE PROTEIN COMPONENTS OF THE ERYTHROCYTE MEMBRANE AND THEIR MOLECULAR WEIGHTS

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SUMMARY

Membrane proteins from pig erythrocytes were solubilized by a phenol–formic acid–water solvent. Such treatment yielded monomers which were subjected to electrophoresis across a polyacrylamide gel gradient. The heterogeneity of the protein components was demonstrated by the appearance of 14–16 zones.

The major components comprising about 30 and 13 % of the total protein had molecular weights of 48000 and 27000, respectively. With the exception of one zone, the other components, which each contributed about 5 % or less to the total protein mass, ranged in molecular weights from 26000 to 65000. One zone accounting for about 8 % of the protein may have had a molecular weight as high as 180000, but it could not be proved to be a monomeric protein.

The variation of results reported by others is explained by difficulties in completely dissolving the membrane proteins to monomers rather than by species differences.

INTRODUCTION

Since it is possible to prepare highly purified membranes from erythrocytes, these preparations are suitable for the study of macromolecular plasma membrane components. Variability of results on protein components and their molecular weights reported in the literature suggested that differences in these findings were due in the first place to incomplete solubilization of the membrane proteins. By treatment of membranes with a phenol–formic acid–water (14:3:3, w/v/v) solvent it was possible to dissolve the membrane lipoproteins into their monomer components of proteins and lipids[1]. By polyacrylamide gel gradient electrophoresis with the same solvent, evidence was obtained that the protein zones did not represent polymeric aggregates. In contrast to gel electrophoresis of proteins solubilized in sodium dodecyl sulfate, we were able to analyze the protein components in respect to homogeneity of charge as well as molecular size. Under these conditions, it was possible to define the main components of the erythrocyte membrane, which are thought to be structural proteins.

MATERIALS AND METHODS

Preparation of the erythrocyte membranes

4 vol. of fresh pig blood were mixed with 1 vol. of 3.8 % sodium citrate. The erythrocytes were washed and lysed as described by DODGE, MITCHELL AND HANAHAN[3].

Erythrocyte membranes were collected from the hemolysate by centrifugation with the "Szent-Györgyi and Blum" continuous flow system of the Servall centrifuge (35000 × *g*, flow rate 45 ml/min). The remaining hemoglobin and other water-soluble proteins were removed by resuspending and washing the pellet 10 times in about 3 vol. of 7 mM phosphate buffer (pH 7.4) and recentrifuging at 35000 × *g* for 20 min. The final pellet was resuspended in the above buffer and stored at −20°.

Preparation of polyacrylamide gels

Polyacrylamide gel slabs (12 %) with 4 % spacer gels were prepared as previously described[1]. In addition to these gels, gel gradients ranging from 3.5 to 14 % acrylamide concentration with a constant cross-linkage of 2 % were mixed at 2° by a programmed gradient pump (Beckman Instruments) from two standard solutions (A and B). The gradient was displaced upwards between a pair of plates (10 cm × 10 cm) and two spacers (10 cm × 1 cm × 0.3 cm) made of plexiglass. The gels polymerized within 2 h. 100 ml of solution A adjusted to pH 6.0 with 1 M HCl contained 13.72 g of acrylamide, 0.28 g of *N,N'*-methylene-bisacrylamide and 0.05 ml of *N,N,N',N'*-tetramethylethylenediamine in distilled water. Before the final addition of 40 mg of ammonium persulfate, the solution was brought to 2° and later degassed at 0.3 atm. 100 ml of solution B adjusted to pH 6.0 with 1 M HCl contained 16.5 g sucrose, 0.06 ml of *N,N,N',N'*-tetramethylethylenediamine and 40 mg of ammonium persulfate in distilled water. The solution was cooled to 2° and degassed at 0.2 atm. The polymerized gel slabs were washed in running tap-water for 2 days and then equilibrated against phenol–formic acid–water (14:3:3, w/v/v). The size of the gel slabs increased about 1.3 times during the equilibration.

Polyacrylamide gel electrophoresis

Erythrocyte membranes were dissolved in phenol–formic acid–water (14:3:3, w/v/v) at a concentration of 2 mg protein per ml. For molecular weight determination the following calibrating proteins (collection MS-1 from Serva, Heidelberg) were dissolved in the same solvent mixture at a concentration of 1 mg per ml: cytochrome *c*, chymotrypsinogen A, ovalbumin, bovine serum albumin, transferrin and human γ-globulin. Blocks of filter paper (Schleicher and Schuell, No. 5703) of 0.5 mm thickness, 3.2 mm height and 10 mm length were loaded with 20 µl of protein solution and aligned along one edge of the polyacrylamide gel. Horizontal electrophoresis was carried out as previously described[1] at a current of 10 mA for 3 h.

After electrophoresis the gels were stained with a 1 % solution of amido black in 7 % acetic acid, and subsequently destained electrophoretically with a current of 60 mA in the presence of 7 % acetic acid. By this procedure the gel slabs were found to be reduced in size by one-tenth.

Protein determination

Proteins were determined according to LOWRY *et al.*[4].

Densitometric measurements of the protein zones in the 12 % gels were performed with the Chromoscan (Joyce). The peak areas were cut out from the tracings and weighed. Under the assumption of equal, specific-dye-binding capacity of the protein components, the relative amounts of the individual components present could be estimated.

RESULTS

From 1 l pig blood 945 mg of erythrocyte membrane protein were obtained. Fig. 1 shows the 14 zones of an electrophoretogram of membrane proteins in 12 % polyacrylamide gel. The main component (No. 7) accounted for about 30 % of the total protein, as indicated by densitometry. The second major component (No. 13) represented about 13 % of the protein. Zone 14 was located at the boundary between 4 % and 12 % gel comprising about 8 % of the total protein. All the other components were in the range of 5 % each or less. Two trace zones not numbered were located between zones 10 and 11. Hemoglobin was dissociated into its subunits under these conditions and behaved like a highly basic protein immediately following cytochrome *c* (Fig. 2). Hemoglobin amounted to less than 2 % of the total protein, representing about 0.01 % of the original hemoglobin content of the erythrocyte.

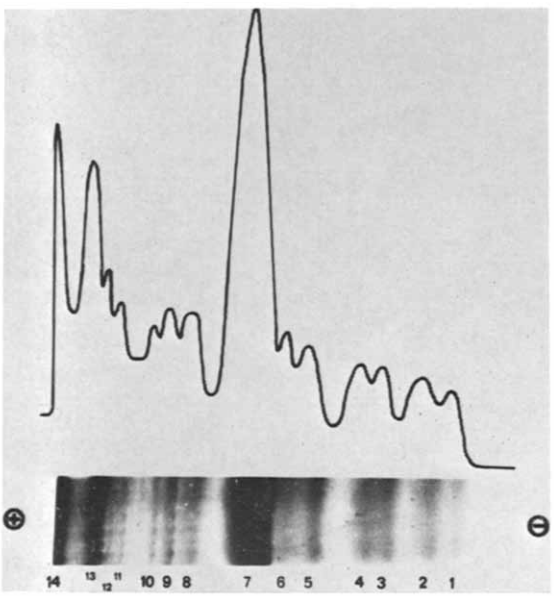

Fig. 1. Electrophoretogram and densitogram of erythrocyte membrane proteins from the pig in 12% polyacrylamide gel with phenol–formic acid–water (14:3:3,w/v/v) as solvent. Main components are zones 7 and 13. Protein zone 14 is located at the boundary between 4 and 12% gel.

Fig. 2 documents the results of an electrophoresis of erythrocyte membrane proteins (1–14) and calibrating proteins (A–F) across a 3.5 to 14 % gel gradient. The figure shows that there was a linear relationship between protein mobility and gel concentration in the middle range of 6 to 12.5 % gel concentration (T_1–T_6), but not in the range of lower or higher gel concentrations. Only γ-globulin (F) and zone 14 showed no linearity in the middle range of gel concentration. In order to obtain mean values, which are more independent of deviations from linearity, the friction ratios were calculated for several constant ranges. All these ranges between T_1 and T_4, T_2 and T_5, T_3 and T_6 included a relative increase in gel concentration of 4 %. For improved measurement, 2.5-fold magnified photographs were taken of the gel slabs.

The friction ratios of the calibrating proteins are listed in Table I. Plotting the

mean values of these friction ratios in relation to the molecular weights of the proteins resulted in the calibrating line of Fig. 3. Linearity was maintained between molecular weights of 25000 and 90000. Low (cytochrome *c*) and high (human γ-globulin) molecular weights are scattered around the calibrating line. This indicates an unfavorable range of the gel concentrations applied. In Table II are listed the friction ratios of the

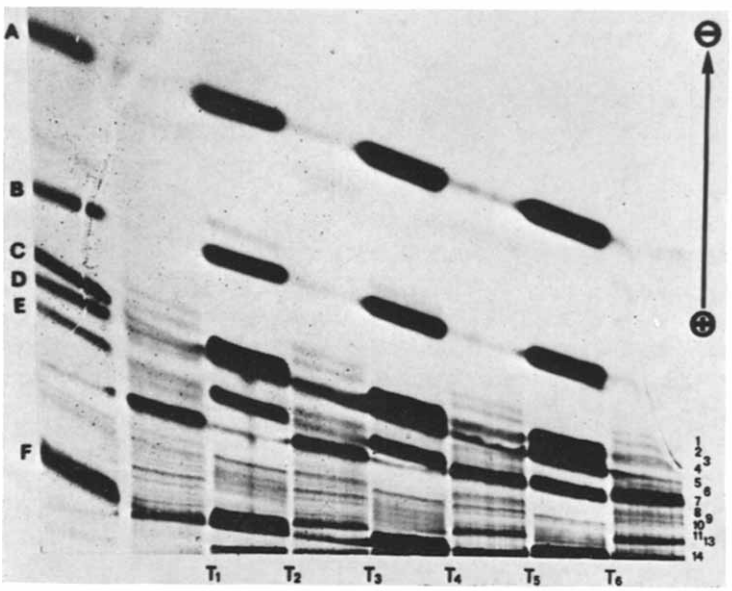

Fig. 2. Electrophoretogram of erythrocyte membrane proteins from the pig (1–14) and marker proteins (A–F) in a 3.5 to 14% polyacrylamide gel gradient with phenol–formic acid–water (14 : 3 : 3, w/v/v) as solvent. T_1–T_6 represent gel concentrations selected for measuring the migration distances of the proteins. Abbreviations: A, cytochrome *c*; B, chymotrypsinogen A; C, ovalbumin; D, bovine serum albumin; E, transferrin; F, human γ-globulin. Some intermixture of samples occurred.

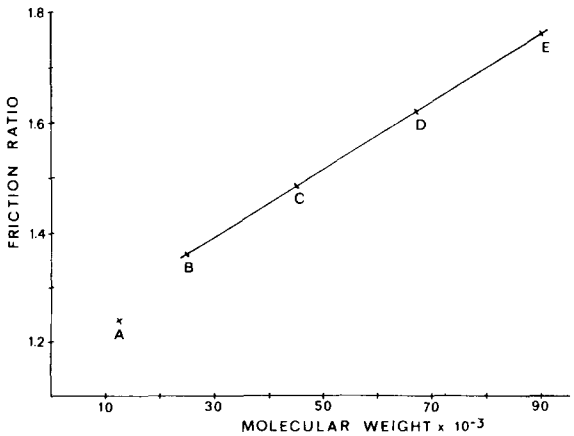

Fig. 3. Calibrating line of friction ratio *versus* molecular weight of standard proteins (A–E). The mean values of friction ratios are listed in Table I. Standard proteins: A, cytochrome *c*; B, chymotrypsinogen A; C, ovalbumin; D, bovine serum albumin; E, transferrin.

TABLE I

FRICTION RATIOS OF CALIBRATING PROTEINS TAKEN AT DIFFERENT GEL CONCENTRATIONS (T_1–T_6) OF THE 3.5 TO 14% POLYACRYLAMIDE GEL GRADIENT

Friction ratios of γ-globulin could not be measured in the higher gel concentrations, because the mobility of this protein was too much restricted.

	Molecular weight	T_1/T_4	T_2/T_5	T_3/T_6	*Mean value* ± *S.D.*
Cytochrome *c*	12400	1.22	1.23	1.26	1.24 ± 0.02
Chymotrypsinogen A	25000	1.34	1.36	1.38	1.36 ± 0.02
Ovalbumin	45000	1.49	1.48	1.49	1.49 ± 0.01
Bovine serum albumin	67000	1.60	1.63	1.63	1.62 ± 0.01
Transferrin	90000	1.74	1.78	1.78	1.77 ± 0.02
Human γ-globulin	160000	3.41	—	—	— —

TABLE II

MOLECULAR WEIGHTS OF ERYTHROCYTE MEMBRANE PROTEINS AND THEIR FRICTION RATIOS

The latter were taken at different gel concentrations (T_1–T_6) of the 3.5 to 14% polyacrylamide gel gradient. The corresponding molecular weights were read off from the calibrating line (Fig. 3). Measurement of friction ratios of zone 14 was precluded at higher gel concentrations because of too slight penetration.

Protein zone No.	T_1/T_4	T_2/T_5	T_3/T_6	*Mean value* ± *S.D.*	*Molecular weight*
1	1.43	1.44	1.44	1.44 ± 0.01	37 000
2	1.44	1.47	1.47	1.46 ± 0.01	41 000
3	1.51	1.54	1.52	1.52 ± 0.01	51 000
4	1.60	1.62	1.61	1.61 ± 0.01	65 000
5	1.47	1.48	1.49	1.48 ± 0.01	44 000
6	1.51	1.51	1.51	1.51 ± 0.00	50 000
7	1.49	1.51	1.50	1.50 ± 0.01	48 000
8	1.53	1.53	1.51	1.52 ± 0.01	51 000
9	1.42	1.41	1.41	1.41 ± 0.00	33 000
10	1.42	1.43	1.44	1.43 ± 0.01	36 000
11	1.42	1.43	1.42	1.42 ± 0.00	35 000
12	1.36	1.38	1.37	1.37 ± 0.01	26 000
13	1.36	1.38	1.39	1.38 ± 0.01	27 000 approx.
14	3.93	—	—	— —	180 000

erythrocyte membrane proteins as well as their molecular weights, read off from the calibrating line of Fig. 3. The molecular weight of the main component (No. 7) was 48000, that of the second major component (No. 13) was 27000. Zone 14 behaved similarly to γ-globulin. Its friction ratio is somewhat higher indicating an estimated molecular weight of the order of 180000. It could not be decided whether this component represents aggregated material or a monomeric protein. The other molecular weights ranged from 26000 to 65000.

DISCUSSION

Because of the variable results of reported electrophoretic studies of erythrocyte membrane proteins[5–7], we used a phenol–formic acid–water solvent which was found

to solubilize biological membranes almost completely into their individual lipid and protein molecules[1]. In contrast to 14–16 protein zones demonstrable with this phenolic solvent, only 9 zones could be detected in pig erythrocyte membranes, when butanol-extracted membranes were electrophoresed on a 7.5 % polyacrylamide gel with 6 M urea in 3 % acetic acid as solvent[5]. Under the latter conditions, 16 protein zones were formed in the erythrocyte membrane of the rat. Polyacrylamide gel electrophoresis of human erythrocyte membrane proteins with 6–8 M urea various conditions resulted in 8–12 zones[5,7], but in 15–25 zones with 0.1 % sodium dodecyl sulfate[7]. It is not yet certain to what extent the variable results depend on differences in species, on variety of preparation and monomerization, or on the conditions of electrophoresis.

Molecular weight determinations by electrophoresis across a polyacrylamide gel gradient were reported by ZWAAN[8] for water-soluble proteins in Tris buffer, and by THORUN and MEHL[2] for water-insoluble proteins in phenol–acetic acid–water (2:1:1, w/v/v). By these experiments it became apparent that, on appropriate conditions, a linear relationship exists on the one hand between friction ratio and the logarithm of molecular weight of proteins in the water system, but on the other hand between friction ratio and molecular weight in the phenolic system. In both solvent systems, the effects of electric charges of the proteins are cancelled by formation of friction ratios. In the water system, the uncertain influence of shape and specific volume of the proteins only allows the comparison of spherical proteins in contrast to the phenolic system, where a random-coiled state of all proteins is achieved. To obtain reliable results, the concentration gradient of the gel has to be constant over the whole range. Moreover, the protein size to be determined and the gel concentration used must be in appropriate proportion. Small molecules, such as cytochrome *c*, require higher percentage gels; bigger molecules, such as γ-globulin, need lower percentage gels. The friction ratios of our calibrating line (Fig. 3) apparently level off at 1.2 for very small molecules, a value comparable with that of ZWAAN[8].

When examined by this technique, the molecular weights of erythrocyte membrane proteins of the pig fall in the range between 26000 and 65000. For the one exception having an estimated molecular weight in the order of 180000 (zone 14) the monomeric state cannot by assured. The molecular range is both narrow and low when compared with other results in the literature. After butanol extraction, molecular weights of 300000 (ref. 9) and 4.6-S to 32-S components[5,9,10] were obtained. Extraction with 33 % pyridine resulted in a fraction of the molecular weight at 500000 (ref. 11). The protein moiety of an isolated lipoprotein had a weight of 163000 (ref. 12). An extracted component called spectrin was reported[13] to be between 130000 and 150000. When human erythrocyte ghosts were dissolved in 0.5 % sodium dodecyl sulfate[14], 50 % of the membrane constituents were found to be in a molecular weight class of 40400, in accordance with our results. Assuming these components to be lipid–protein complexes, those authors calculated a molecular weight of 22 200 for the protein moiety. However, lipoproteins were found to be dissociated in this solvent into protein–sodium dodecyl sulfate and lipid–sodium dodecyl sulfate complexes[15].

After ethanol–ether extraction of human erythrocyte membranes, ROSENBERG AND GUIDOTTI[7] found a protein component with a molecular weight of 49000 ± 7000 in 1 % sodium dodecyl sulfate and of 53000 ± 7000 in 5.8 M guanidine·HCl, respectively. 70 % of the total protein was eluted on gel filtration with 1 % sodium dodecyl sulfate between bovine serum albumin (67000) and lysozyme (14400). Our findings

agree well with these values. They indicate that differences in protein composition, of erythrocyte membranes from mammalian species as far as investigated may not concern the molecular size in the first place. The different results may have originated from the difficulty of dissolving the membranes into monomers. However, the phenolic system used by us dissociates complexes into monomers and subunits, respectively.

It seems that the two main components, corresponding to about 43 % of the total protein, represent structural proteins of the erythrocyte membrane. The denaturing effect of phenol precludes a direct examination of the minor protein zones to determine whether some of them are homogeneous enzyme proteins from the membrane-bound enzymes of the erythrocyte. With isolated enzyme proteins as reference for electrophoresis, some indications would be obtainable. At least on the basis of a molecular weight of 65000, for example, component 4 could be a candidate as a subunit of acetylcholinesterase[16]. Moreover, the contribution of glycoproteins to the protein pattern is not yet clear. At present, a complex pattern of protein constituents can be demonstrated with some possible implications on the supramolecular structure of a plasma membrane.

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REFERENCES

1 E. MEHL, *Proc. Intern. Symp. Macromolecules and Function of the Neuron, Castle Liblice, Prague, 1967*, Excerpta Medica, Amsterdam, 1968, p. 22.
2 W. THORUN AND E. MEHL, *Biochim. Biophys. Acta*, 160 (1968) 132.
3 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
4 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
5 R. F. A. ZWAAL AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 163 (1968) 44.
6 L. J. SCHNEIDERMAN, *Biochem. Biophys. Res. Commun.*, 20 (1965) 763.
7 S. A. ROSENBERG AND G. GUIDOTTI, *J. Biol. Chem.*, 243 (1968) 1985.
8 J. ZWAAN, *Anal. Biochem.*, 21 (1967) 155.
9 A. H. MADDY, *Biochim. Biophys. Acta*, 117 (1966) 193.
10 A. F. REGA, R. I. WEED, C. F. REED, G. G. BERG AND A. ROTHSTEIN, *Biochim. Biophys. Acta*, 147 (1967) 297.
11 O. O. BLUMENFELD, *Biochem. Biophys. Res. Commun.*, 30 (1968) 200.
12 TH. E. MORGAN AND D. J. HANAHAN, *Biochemistry*, 5 (1966) 1050.
13 V. T. MARCHESI, E. STEERS, T. W. TILLACK AND S. L. MARCHESI, *Federation Proc.*, 28 (1969) 567.
14 S. BAKERMAN AND G. WASEMILLER, *Biochemistry*, 6 (1967) 1100.
15 S. ROTTEM, O. STEIN AND S. RAZIN, *Arch. Biochem. Biophys.*, 125 (1968) 46.
16 D. NACHMANSOHN, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 1034.