Biochimica et Biophysica Acta, 352 (1974) 327–337

BBA 76666

SELECTIVE SOLUBILIZATION WITH TWEEN 20 OF PROTEINS FROM WATER-EXTRACTED HUMAN ERYTHROCYTE MEMBRANES ANALYSIS BY GEL ELECTROPHORESIS IN DODECYLSULFATE AND IN TWEEN 20.

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(Received December 27th, 1973)

SUMMARY

1. Human erythrocyte membranes (ghosts) were extracted at pH 9.5 (0 °C) with an aqueous solution of low ionic strength (containing EDTA and 2-mercaptoethanol) and then with water. This procedure selectively solubilized 35 % of the membrane protein.

2. The material not solubilized by the above procedure (the membrane residue) was partially solubilized with the non-ionic detergent Tween 20 at an ionic strength of approx. 0.005. The degree of solubilization increased with increasing pH (4.5–9.8) and with increasing detergent concentration.

3. The solubilization of proteins with Tween 20 at pH 9.8 was highly selective at an ionic strength of approx. 0.05 as well as at low concentrations of detergent at an ionic strength of approx. 0.005, as indicated by polyacrylamide-gel electrophoresis in dodecylsulfate, only the two major components of high molecular weight (1,2) and one smaller component (4.1) being solubilized.

4. Membrane residue protein solubilized with Tween 20 separated into 5–6 major zones upon electrophoresis in a polyacrylamide gel containing 0.5 % Tween 20 and 0.02 M glycine–NaOH buffer (pH 9.8), as seen after staining with Coomassie Brilliant Blue.

5. The patterns observed in two-dimensional polyacrylamide-gel electrophoresis using Tween 20 in the first and dodecylsulfate in the second direction suggest that some of the zones obtained by electrophoresis in Tween 20 contained protein complexes which were split by dodecylsulfate.

INTRODUCTION

Selective solubilization of erythrocyte membrane proteins has been achieved, for example, with solutions of low [1–5] or high [1, 4–7] ionic strengths; with non-ionic detergents [8]; with such agents as organic mercurials [9, 10], lithium diiodosalicylate [9, 11], urea or guanidinium · HCl [12–15] and aqueous pyridine [16];

or by reaction with anhydrides of dicarboxylic acids [9] (Lundahl, P. and Hjertén, S., unpublished).

We have extracted certain proteins from human erythrocyte membranes using media of low ionic strength and moderately high pH and have then selectively solubilized proteins of the lipid-rich membrane residue with the non-ionic detergent Tween 20 to permit relatively easy fractionation of some proteins under mild conditions.

The same detergent has been used successfully in this laboratory for the selective solubilization of *Acholeplasma laidlawii* membranes [17].

MATERIALS AND METHODS

Human erythrocyte membranes

The membranes were prepared in sodium phosphate buffer at pH 7.4, 2–5 °C, according to Dodge and co-workers [18].

Water-extractable proteins

The proteins were released at 0–5 °C from the erythrocyte membranes, by a modification of the procedure of Marchesi et al. [2] as described earlier [19]. The unsolubilized material shall be termed the membrane residue.

The solubilized proteins were concentrated by precipitation at 5 °C with $(NH_4)_2SO_4$ (650 g/l of extract, cf. [2]) and collected by centrifugation.

Solubilization of the membrane residue with Tween 20

The membrane residue was suspended in buffer to a final concentration of 2.3 g of protein (about 5 g of membrane residue material) per l, 0.2–5 % (w/v) Tween 20 and 0.010 or 0.10 M of the various buffers. After 15 h at 0 °C the mixture was centrifuged at 30 000 rev./min in a Spinco 75 Ti rotor for 1.5 h (67 000–80 000 × *g*, 1.2 cm sedimentation path). To avoid dilution normally only one extraction was made. The extracts could be analyzed without concentration by polyacrylamide-gel electrophoresis.

Analytical polyacrylamide-gel electrophoresis

Analytical polyacrylamide-gel electrophoresis was performed at room temperature in gel slabs (0.6cm × 10 cm × 10 cm) with nine sample wells [20]. Details are given in the legends to the figures. The gels were stained with Coomassie Brilliant Blue R 250. Two-dimensional polyacrylamide-gel electrophoresis with Tween 20 in the first and dodecylsulfate in the second direction was performed as described in the legend to Fig. 4.

Protein

Protein concentration was estimated by absorbance measurements and expressed as $A^{1\,cm}_{280\,nm} - A^{1\,cm}_{310\,nm}$. Samples with high light scattering were completely solubilized with dodecylsulfate before the absorbance measurements.

For erythrocyte membranes, the aqueous extract of the membranes and the membrane residue $A^{1\,cm}_{280\,nm} - A^{1\,cm}_{310\,nm}$ was 1.1, 1.3 and 1.0, respectively, for a solution containing 1 g of protein/l. The protein concentrations were determined by total amino acid analysis.

TABLE I

AMINO ACID COMPOSITION OF HUMAN ERYTHROCYTE MEMBRANES, MEMBRANE PROTEINS EXTRACTED WITH AQUEOUS SOLUTIONS AND THE MEMBRANE RESIDUE

The protein samples, prepared as described in Materials and Methods, were hydrolyzed for 24, 36 and 48 h with 3 M *p*-toluenesulfonic acid [29] at 115 °C and the amino acid content was determined [30] with a Biocal BC 200 amino acid analyzer by Dr David Eaker and co-workers. The average or extrapolated values for the three hydrolyses are given. The values for tryptophan, threonine and serine are extrapolated to 0 h, assuming first order kinetics. The samples were taken from one membrane preparation.

Amino acid	Mole fraction (%)		
	(A) Human erythrocyte membranes	(B) Aqueous extract at low ionic strength	(C) Membrane residue
Try	0.4	0.3	0.5
Lys	4.7	6.5	4.0
His	2.5	2.9	2.4
Arg	4.8	5.9	4.7
Asp	9.0	10.7	7.3
Thr	5.8	5.0	6.1
Ser	8.4* (7.0)**	6.6	8.0* (7.4)***
Glu†	12.6	15.7	10.4
Pro	4.9	2.9	5.4
Gly	6.8	5.2	7.2
Ala	8.2	8.7	7.8
Cys††	— (1.1)	— (1.1)	— (1.1)
Val	6.4	5.1	7.5
Met	1.9	1.8	2.4
Ile	4.7	4.0	5.5
Leu	12.2	12.2	12.8
Tyr	2.4	2.3	2.5
Phe	4.5	3.3	5.1
Ammonia†††	20.6	15.8	21.8
Amino acids with charged side groups (Lys, His, Arg, Asp, Glu) including amides (Asn, Gln)	33.6	41.5	28.8
Amino acids with hydrophobic side groups (Try, Val, Met, Ile, Leu, Tyr, Phe)	32.6	29.0	36.3

* Includes phosphatidylserine.

** Value obtained by Bakerman and Wasemiller [31] for lipid-extracted membranes. This value was used here for calculation of the mole fractions.

*** Calculated from the value 7.0 % for Sample A and our value for Sample B assuming that the membrane residue protein (C) comprises 64 % of the membrane protein (A).

† The content of glutamic acid differed sufficiently between Samples A, B, and C to permit an estimate of the weight fraction of the total membrane protein (A) represented by the aqueous extract (B). Estimating the uncertainty in the values for the mole fractions of glutamic acid at less than 1 % of the given values, B/A = 43±5 %, in good agreement with the estimate (41 %) based on absorbance measurements.

†† For the purpose of calculating the tabulated mole fractions the value for cysteine was assumed to be the same in the three samples and equal to the value 1.1 % of Bakerman and Wasemiller [31] for unfractionated membranes.

††† In moles per 100 mole amino acid residues.

The degree of protein solubilization (in percent of the total protein in the suspension) was calculated from the absorbance values neglecting differences in extinction coefficient among the fractions.

Phospholipids

Phospholipids were determined as phosphate according to Bartlett [21]. Determinations in gel zones were made after slicing the gel and eluting the phospholipids with 2-ml portions of buffer containing dodecylsulfate.

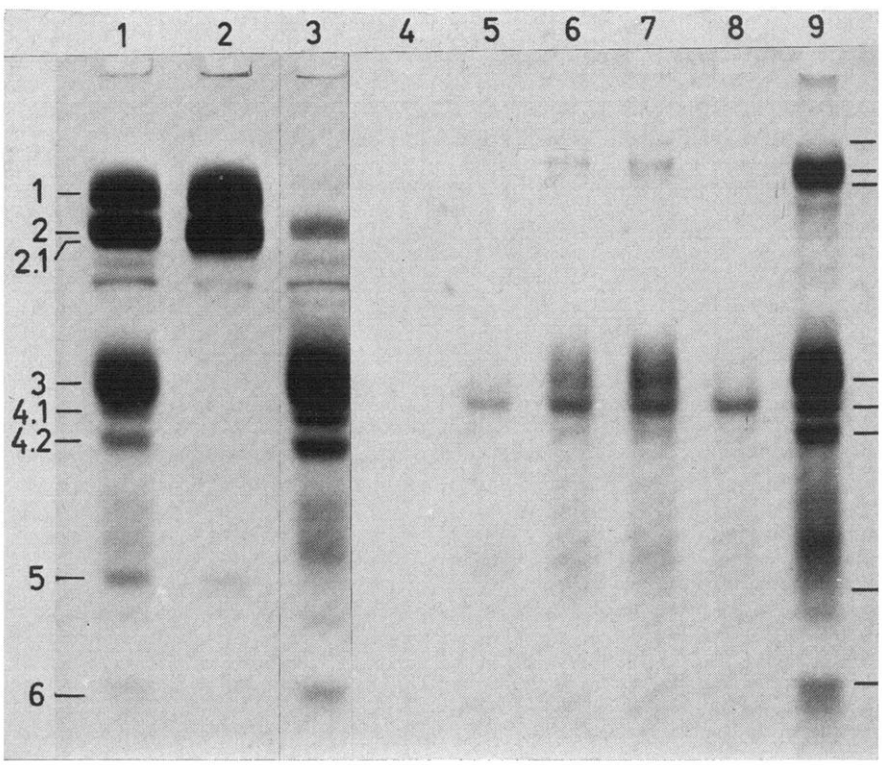

Fig. 1. Solubilization of human erythrocyte membrane proteins with aqueous solutions of low ionic strength and solubilization of the membrane residue with Tween 20 at various pH values. Polyacrylamide-gel electrophoresis in dodecylsulfate. Section 1, erythrocyte membranes; Section 2, combined aqueous extracts; Sections 3 and 9, the membrane residue. Sections 4–7, supernatants after solubilization of the membrane residue with 1 % of Tween 20 in 0.010 M buffers of the following pH values: 4, 4.5 (acetate–NaOH); 5, 6.7 (imidazole–acetic acid); 6, 8.5 (Tris–acetic acid); 7, 9.8 (glycine–NaOH). Section 8 supernatant after solubilization with 1 % Tween in 0.10 M glycine–NaOH buffer (pH 9.8). Sections 1–3 and 4–9 represent two different experiments. The same volume was applied in Wells 4–8, to facilitate comparison of the amounts of the components solubilized under different conditions. All samples applied contained 0.1 M dodecylsulfate, 0.05 M 2-mercaptoethanol, 0.04 M Tris–acetic acid (pH 8.5) and 4 % (w/v) of sucrose. The gel composition was $T = 6\,\%$, $C = 3\,\%$ (When a g of acrylamide and b g of N,N′-methylenebisacrylamide are dissolved in buffer to a volume of m ml, $T = 100(a+b)m^{-1}\,\%$ and $C = 100b(a+b)^{-1}\,\%$ (cf. [32]).) and the buffer (included in the gel) was 0.080 M Tris–acetic acid (pH 8.5) with 0.02 M sodium dodecylsulfate. The cathode buffer, in addition, contained 0.05 M 2-mercaptoethanol. Voltage, 60 V; current, 0.05 A; time, 4.5 h.

RESULTS

Degree of solubilization of human erythrocyte membrane proteins by extractions with aqueous solutions of low ionic strengths

Repeated extraction of human erythrocyte membranes at low ionic strength released 35±5 (S.E.) % of the protein (8 experiments). When the extractions were done in the absence of EDTA about the same amount of protein was solubilized as by the normal procedure (two experiments).

Selectivity of extractions with aqueous solutions of low ionic strength of erythrocyte membranes

The erythrocyte membrane, the aqueous extract of the membrane and the membrane residue contain 34, 42 and 29 %, respectively, of amino acids with charged side groups, and 33, 29 and 36 %, respectively of hydrophobic residues (Table I). The selectivity of the aqueous extractions is also indicated by polyacrylamide-gel electrophoresis (Fig. 1, Sections 1–3, denotations according to Steck and Yu [9]). Components 1, 2 and 5 were largely removed from the membrane, whereas very little of Components 3, 4.1 and 4.2 was extracted. The same components were also removed when the series of extractions was done in the absence of EDTA.

Degree of solubilization of the membrane residue by extractions with Tween 20

Dependence on pH. When the final concentration of detergent was 1 % and the ionic strength was approx. 0.005 the amount of solubilized protein increased steadily with increasing pH from 2 % at pH 4.5 to about 34 % of the total membrane residue protein at pH 9.8 (Table II, cf. also Fig. 1, Sections 4–7). The phospholipid/protein ratio decreased with increasing pH (Table II).

TABLE II

RELATIVE AMOUNTS OF PROTEIN AND PHOSPHOLIPID SOLUBILIZED FROM THE HUMAN ERYTHROCYTE MEMBRANE RESIDUE WITH 1 % TWEEN 20 AT DIFFERENT pH VALUES

The solubilization was done as described in Materials and Methods in 0.010 M buffers. The amount of protein and of phospholipid solubilized are given as percentages of the total amounts in the suspensions. The concentration of protein in the suspensions corresponded to $A^{1\,cm}_{280\,nm} - A^{1\,cm}_{310\,nm} =$ 2.0–2.5 and the phosphate concentration was 1.8–2.4 mM. Phospholipid was determined as phosphate. Number of observations are given in parenthesis.

1 % Tween 20		Protein solubilized (%)	Phospholipid solubilized (%)	Phospholipid/protein (mM phosphate per $A^{1\,cm}_{280\,nm} - A^{1\,cm}_{310\,nm}$)
pH	Buffer			
4.5	Acetate–NaOH	2** (3)	7* (4)	2.8**
6.7	Imidazole–acetic acid	10** (4)	15** (4)	1.5**
8.5	Tris–acetic acid	18** (4)	18** (4)	1.0**
9.8	Glycine–NaOH	34** (25)	24** (17)	0.7**

*,** The standard errors (expressed as percent of the tabulated values) were in the ranges: (*) 0–10, (**) 10–20.

TABLE III

RELATIVE AMOUNTS OF PROTEIN AND PHOSPHOLIPID SOLUBILIZED FROM THE HUMAN ERYTHROCYTE MEMBRANE RESIDUE AT DIFFERENT CONCENTRATIONS OF TWEEN 20 AND AT DIFFERENT IONIC STRENGTHS

The solubilization was done as described in Materials and Methods and in the legend to Fig. 2. The buffer was glycine–NaOH (pH 9.8). The total amounts and the amounts of protein and phospholipid solubilized are as given in Table II. The calculations were with the assumption that the volume of the supernatant was equal to the total volume, since the pellets were small. Phospholipid was determined as phosphate. Phospholipid/protein ratio is expressed as mM phosphate per $A^{1\,cm}_{280\,nm} - A^{1\,cm}_{310\,nm}$. Number of observations is given in parenthesis.

Concn of Tween 20 (%)	0.2	1	5	1
Concn of buffer (M)	0.010	0.010	0.010	0.10
First supernatant				
Protein (%)	13** (10)	34** (25)	42** (11)	14** (14)
Phospholipid (%)	8** (9)	24** (17)	49** (6)	18** (11)
Phospholipid/protein ratio	0.6**	0.7**	1.2**	1.3**
Second-fourth supernatant (sum)				
Protein (%)	36** (4)	33* (3)	23*** (3)	15* (4)
Phospholipid (%)	27*** (3)	26** (2)	34** (2)	25** (3)
Phospholipid/protein ratio	0.7***	0.7**	1.2**	1.6***
Pellet after four extractions				
Protein (%)	54** (4)	38*** (3)	36*** (3)	70* (4)
Phospholipid (%)	68* (3)	52* (2)	41* (2)	61* (3)
Phospholipid/protein ratio	1.3***	1.5***	1.2***	0.9*

*,**,*** The standard errors (expressed as percent of the given values) were in the ranges: (*) 0–10, (**) 10–20, (***) 20–30.

Dependence on ionic strength. The amount of protein solubilized by 1 % Tween 20 in 0.10 M glycine–NaOH buffer (pH 9.8) was less than half of the amount of protein solubilized in 0.010 M buffer (Table III, Columns 3 and 5, Fig. 2, Sections 3 and 5). At both ionic strengths the supernatant contained about 20 % of the phospholipids.

Dependence on concentration of detergent. With 5 % Tween 20 in 0.010 M glycine–NaOH (pH 9.8) about 40 % of the membrane residue protein was solubilized in the first extraction, compared to only 13 % with 0.2 % Tween 20 (Table III) and 2 % without detergent. The phospholipid/protein ratio in the supernatant was higher at 5 % than at 0.2 % of the detergent.

Selectivity of Tween 20 solubilization of the membrane residue

Tween 20 at pH 9.8 released most of Components 1, 2 and 4.1 from the membrane residue in one extraction, and four extractions released these components completely (Fig. 2). Only small amounts of components other than 1, 2 and 4.1 were solubilized in 0.010 M buffer containing 0.2 % Tween 20 and in 0.10 M buffer

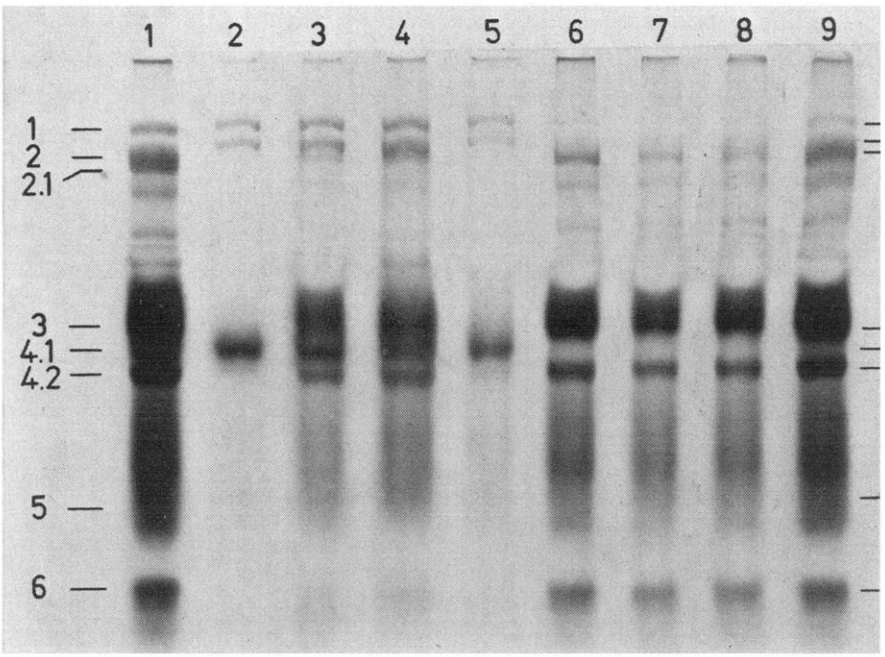

Fig. 2. Solubilization of the membrane residue with Tween 20. Dependence on detergent concentration and ionic strength. Polyacrylamide-gel electrophoresis in dodecylsulfate. Section 1, membrane residue; Sections 2–4, supernatants after solubilization with 0.2, 1, and 5 % of Tween 20, respectively, in 0.010 M glycine–NaOH buffer (pH 9.8); Section 5, supernatant after solubilization with 1 % Tween 20 in 0.10 M glycine–NaOH (pH 9.8); Sections 6–9, pellets obtained after four washes with the buffers used for Sections 2–5, respectively, and solubilization with dodecylsulfate.

The sample composition and the electrophoresis conditions were as described in Fig. 1. The initial solubilization was done as described in Materials and Methods. Each pellet obtained after centrifugation was suspended in buffer containing Tween 20 to the volume of the initial suspension. The suspension was kept at 0 °C for 22 h and then centrifuged. This was done 3 times. The pellet obtained after four centrifugations was suspended in 0.04 M Tris–acetic acid (pH 8.5) containing 0.1 M dodecylsulfate to the same volume as before and centrifuged. A very tiny pellet was obtained. Two faint zones (not shown in the figures) were seen well ahead of Zone 6 in gel section 1.

containing 1 % Tween 20 (Fig. 2, Sections 2 and 5, respectively). With 1–5 % Tween 20 at low ionic strength the solubilization was less selective (see Fig. 2, Sections 3 and 4), as was also the case at pH values higher than 9.8.

Polyacrylamide-gel electrophoresis in Tween 20 of solubilized components

Probably because the erythrocyte membrane proteins form complexes or aggregates, they have low electrophoretic migration velocities in gels containing non-ionic detergents. The membrane residue proteins, solubilized with Tween 20 migrated in a polyacrylamide gel $T = 4\,\%$, $C = 2\,\%$ and did not precipitate at the gel surface. When 2 % or more of sucrose was included in the samples, the zones became very distorted.

Membrane residue material solubilized with Tween 20 at low ionic strength, pH 9.8, showed six major zones, A–F, in polyacrylamide-gel electrophoresis, after

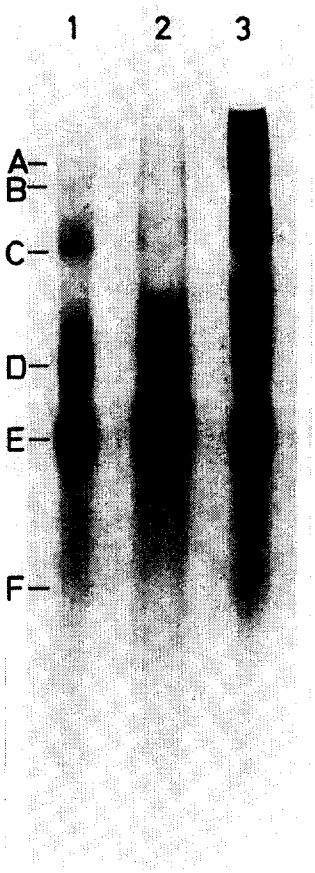

Fig. 3. Polyacrylamide-gel electrophoresis in 0.5 % of Tween 20 of the Tween-20 soluble material. Section 1, supernatant after solubilization with 0.2 % Tween 20 in 0.010 M glycine-NaOH buffer (pH 9.8). Sections 2 and 3, supernatants after solubilization with 1 % Tween 20 in 0.010 and 0.10 M glycine–NaOH buffer (pH 9.8), respectively. Before application, all samples were made 2 % in Tween 20 to simplify layering in the well. The gel was $T = 4\,\%$, $C = 2\,\%$ and the buffer (included in the gel) was 0.020 M glycine–NaOH (pH 9.8) containing 0.5 % of Tween 20. Voltage, 80 V; current, 0.03 A; time, 5 h.

The sample in Section 2 contained 0.1 M glycine–NaOH buffer and therefore gave broader and more diffuse zones.

staining with Coomassie Brilliant Blue (Figs 3 and 4). Behind Zone D (the shape of which varied) a zone with a sharp curved front could be seen, which did not stain, but became opalescent in the staining procedure. This zone contained 30 % of the phospholipids extracted from the gel.

Material solubilized from the membrane residue and stored for 10 weeks at 5 °C gave the same staining pattern after polyacrylamide-gel electrophoresis as fresh material.

Two-dimensional gel electrophoresis

Material solubilized by 0.2 % of Tween 20 was separated by two-dimensional

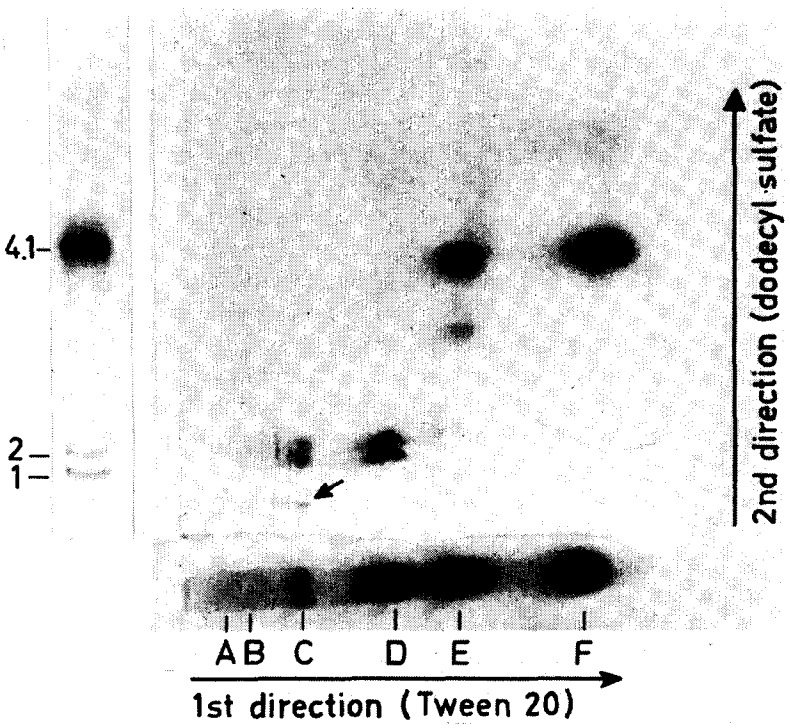

Fig. 4. Two-dimensional polyacrylamide-gel electrophoresis using Tween 20 in the first direction and dodecylsulfate in the second (cf. [17]). Sample: supernatant after solubilization of human erythrocyte membrane residue with 0.2 % Tween 20 in 0.010 M glycine–NaOH buffer (pH 9.8). After electrophoresis as described in Fig. 3 one section was cut out and immersed for 1 h in 0.040 M Tris–acetic acid buffer (pH 8.5), containing 0.1 M dodecylsulfate and 0.05 M 2-mercaptoethanol. The section was then placed at the bottom of the gel chamber and the monomer solution containing dodecylsulfate was poured into the chamber. After polymerization the electrophoresis was started with the anode in the upper vessel. The composition of the second gel was the same as in Fig. 1. Voltage, 70 V; current, 0.05 A; time, 4.5 h. Two identical samples were run in the first direction. One of the corresponding gel sections was used in the two-dimensional electrophoresis. The comparison gel section is included in the figure to illustrate the separation in the first direction. A photograph of a dodecylsulfate electrophoresis pattern from a sample solubilized under the same conditions as the sample used for the two-dimensional electrophoresis has also been inserted to facilitate the interpretation of the two-dimensional electrophoresis.

polyacrylamide-gel electrophoresis, using Tween 20 in the first and dodecylsulfate in the second direction (Fig. 4). Zone E splits into two zones with nearly the same migration velocity in dodecylsulfate as the zone corresponding to Zone F, and one minor, slower zone. Zones C and D divide into two major zones with similar migration velocities. In addition, Zone C shows one faint zone of low migration velocity (see arrow).

DISCUSSION

Repeated extraction of human erythrocyte membranes with detergent-free solutions of low ionic strength solubilizes about one third of the protein, in agreement

with most results reported for similar procedures [3, 4, 22, 23]. The solubilization is highly selective and the components released are 1, 2 and 5 [3, 4, 9, 24, our results].

To release the components remaining in the membrane residue we have treated it with the non-ionic detergent Tween 20. At a low ionic strength (approx. 0.005) more protein was solubilized than at a moderately high ionic strength (approx. 0.05) in agreement with the result of others [8, 25].

The solubilization by non-ionic detergents is also enhanced by a high pH (cf. [8, 25]), at which proteins are negatively charged. Electrostatic attractions are thus eliminated, or counteracted by repulsion between negative charges. The intramolecular repulsion at high pH should also facilitate the solubilization by distorting hydrophobic regions at the protein surfaces. The solubilizing effect of high pH is most pronounced at low ionic strength, where electrostatic interactions are most favored.

Tween 20 can accomplish highly selective solubilization. Component 4.1 was extensively released by 0.2 % Tween 20 at pH 9.8 in the first extraction, together with the small amounts of Components 1 and 2 present in the membrane residue, while only traces of the other components were extracted (Fig. 2, Section 2). The selective solubilization of Component 4.1 greatly facilitates the purification of this component.

Yu et al. [8] have selectively solubilized Components 3, 4.2 and 6 from the erythrocyte membranes with Triton X-100 at low ionic strength (approx. 0.008), pH 8.0, and mainly Component 3 at the ionic strength approx. 0.04. In both cases also the major glycoprotein was solubilized. A comparison of these results with ours indicates that non-ionic detergents can differ in selectivity.

Solubilization of membrane proteins with non-ionic detergents can be done under conditions where complexes of proteins are preserved or formed [26]. Some of the zones obtained in electrophoresis in the presence of Tween 20 seem to contain complexes (native or not) that are split by dodecylsulfate (Fig. 4, Zones C and E, for example). Zone C separates in dodecylsulfate into two zones (probably corresponding to Components 1 and 2) and a third slowly migrating zone (arrow) (Fig. 4) which we have never seen in one-dimensional dodecylsulfate electrophoresis of membrane residue proteins. Since Zones C and D seem to show the same main components (1 and 2) in dodecylsulfate but differ in migration velocities in Tween 20 electrophoresis, Zone C might represent a complex (or complexes) of components in Zone D. (Evidence that Components 1 and 2 might be associated in the membrane has been obtained by cross-linking [27, 28].) Also Zone E of the Tween 20 electrophoresis shows more than one zone in dodecylsulfate electrophoresis. The relationships between the dodecylsulfate components of the Tween 20 extracts and the constituents of the Tween 20 Zones A–F (Figs 3 and 4) cannot be deduced solely from experiments such as those illustrated in Fig. 4.

ACKNOWLEDGEMENTS

We thank Mrs Barbro Ahlström for skilful technical assistance. We are grateful to our colleagues in the Membrane Group at our institute and to Dr Ole Bjerrum for stimulating discussions. This work has been supported by the Foundation of Ollie and Elof Ericsson, the von Kantzou Foundation, the Lennander Foundation, the Nathorst Foundation, the Nobel Foundation, the Swedish Natural Science Research Council and the Wallenberg Foundation.

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