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INVESTIGATION OF HCl-EXTRACTION OF HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

Diluted HCl at pH values of lower than 3 extracts about 45% of the proteins of the erythrocyte membrane. An analysis was made to give insight into which proteins are split off by this procedure and which remain in the insoluble membrane. The results indicate that the majority of the available proteins are released from the membrane. Proteins I and II (molecular weight approx. 250000 and 230000, respectively) are dissociable into subunits.

INTRODUCTION

Together with lipids and carbohydrates, proteins are the main constituents of biological membranes. Their arrangement in the membrane is determined primarily by the type and strength of the bonds which they can form among themselves and with the molecules of the other two classes. Three types of bonding are possible: covalent bonding forces (specifically –S–S– bridges), ionic bonding forces, and intermolecular forces such as hydrogen and hydrophobic bonding.

An experimental means of acquiring knowledge concerning the types of bonds in membranes is the use of solutions which will specifically split certain of these bonds. Until now experiments have been performed mainly on erythrocyte membranes using pure hydrophilic media[1–4] and media with a more or less hydrophobic character[5–8], which have set various parts of the membrane or individual molecules free. The same occurs with the use of chelating agents[9] or with high salt concentration[10]. The present work involves an experiment, hitherto not performed, using a medium of low ionic strength and pH to acquire knowledge concerning the participation of pure ionic and hydrogen bonds. To this end, initial clarification was necessary concerning the amount of protein split from the membrane at pH values below 4, the type of proteins which were split off, and the type of proteins which remain in the remaining insoluble membrane. Finally an attempt is made, based on the experimental findings, to draw conclusions concerning the membrane structure.

MATERIALS AND METHODS

Human erythrocyte membranes, isolated according to the method of Steck *et al.*[11], served as the source of material. The concentrated membrane suspension

acquired by 4–5 times washing with a 5 mM sodium phosphate buffer (pH 8) was used in the experiments.

The treatment of the membranes with HCl was carried out at a temperature between 0 and 4 °C. Thereafter, centrifugation at $4000 \times g$ was performed at low temperature for 30 min. The part of the membranes that under these conditions is present as a sediment shall be designated as membrane residue, the non-sedimented part as the HCl-supernatant.

To obtain the membrane residues free from the HCl-supernatant, 1–2 ml of the membrane concentrate was diluted to 10 ml with a 10 mM NaCl solution and then brought to a pH of 2.0 or 2.5, with 0.1 M HCl. 20 min after pH adjustment $4000 \times g$ centrifugation for 30 min was carried out. The sedimented membrane residue was then washed once with 10 mM NaCl solution of pH 2 or 2.5 and then prepared for disc electrophoresis.

To acquire the HCl-supernatant at the concentration necessary for disc electrophoresis, 1–2 ml of the membrane concentrate was adjusted to a pH of 2 or 2.5, with 0.1 M HCl. After 20 min, centrifugation was performed at low temperature ($4000 \times g$ for 30 min). The HCl-supernatant was then prepared for disc electrophoresis.

To measure the percent of protein remaining unsedimented after treatment of the membranes with HCl under the given conditions: the membrane concentrate was diluted with 10 mM NaCl solution and the protein content of this suspension was measured according to the Lowry-method with the addition of 1% sodium dodecyl sulfate. Five 10-ml portions of this solution were then brought to pH values of 4, 3.5, 3, 2.5 and 2 using 0.1 M HCl. After centrifugation at $4000 \times g$ at low temperature for 30 min, the protein content of the residual membranes was determined. Using the difference between the protein content of the initial membrane suspension and that of the membrane residue one can calculate the percent protein remaining.

The quantitative determination of protein in the membrane residue was carried out according to Lowry *et al.*[12]. To this end the sediment acquired from centrifugation was dissolved in 4 ml of a 10 mM NaCl solution +0.5 ml of 10% dodecyl sulphate dissolved in 0.2 M NaOH and then brought to exactly 5 ml with several drops of 0.2 M NaOH. 0.5 ml of this solution, the pH of which was approx. 10, was then added to 1.8 ml of the basic copper solution and 0.2 ml Folin solution (1:1 water dilution). After 30 min the absorbance was measured at 750 nm. Bovine serum albumin was used for standardization.

The determination of the molecular weight of the proteins with the aid of disc electrophoresis in dodecyl sulphate-containing solutions was performed according to Fairbanks *et al.*[13]. A 5.6% gel was used. The HCl-supernatant, as well as the membrane residue, was prepared as follows. The test solution was brought to a pH between 7 and 9 using 0.1 M NaOH, then mixed with dodecyl sulphate (final concentration 1%) to dissolve the residue and insoluble proteins and with 2-mercaptoethanol (final concentration 0.04 M) for the reduction of any –S–S– bonds present. It was then incubated at 37 °C for 20 min to complete the reaction and thereafter mixed with a small amount of bromphenol blue (as front-marker for the electrophoresis) and some sucrose. Separation was performed at a constant current of 6 mA/gel; the accompanying potential reached a value of 60 V. Under these conditions the bromphenol blue migrated 70–75 mm in 2 h. The proteins were stained according to the procedure

given in ref. 13 with coomassie-brilliant blue R250 for 24 h and the background then destained with 10% acetic acid. Since a densitometer was not available at the time, measurement of the individual gel cylinders was performed with a microspectrophotometer at 570 nm in order to represent the position and hight of the protein bands graphically. Based on my experience, this method is as efficient as the profile-representation acquired with a densitometer. The determination of the M_r of the protein bands was possible by comparing the electrophoretic migrations with those of proteins of known molecular weight.

The periodic acid–Schiff reaction was carried out on the gels according to Zacharius *et al.*[14].

For the electron microscopic investigation of the membrane residue, prefixation was performed with a 5% glutaraldehyde solution dissolved in a phosphate buffer (pH 7.2). After 24 h washing in Michaelis buffer (pH 7.2), it was post-fixated for 2 h with a 1% OsO_4 solution according to Palade[15]. Then followed 24 h of washing out the OsO_4, dehydration and embedding in Epon. The prepared thin sections were then contrasted with uranyl acetate and lead citrate and observed with a Siemens Elmiskop I.

Spectrin was isolated according to the method given by Marchesi *et al.*[16]. The $(NH_4)_2SO_4$ precipitate was centrifugated and then dialysed against a 5 mM Tris–acetate buffer (pH 7.5). The pH was then adjusted to 2.5 with 0.1 M HCl and, after standing for 1 h the solution was prepared for disc electrophoresis. All steps were performed at a temperature of 0–4 °C.

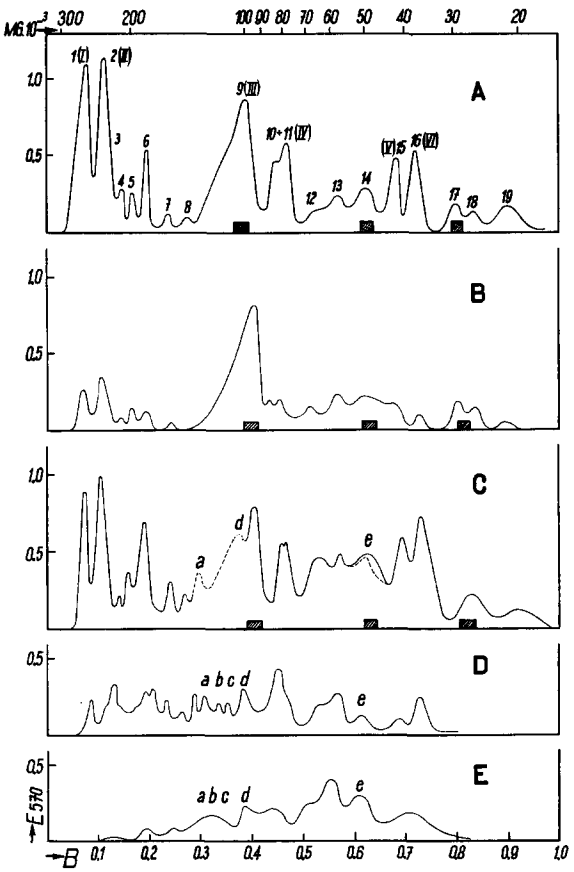
RESULTS

Amount of protein in HCl-supernatant

The change in the concentration of the protein which was split off the membranes by HCl at pH values between 2 and 4, is shown in Fig. 2. The *y*-axis gives the percent of protein, which can be found in the supernatant at any given pH value. Each point represents the mean value of 6 measurements. It can be seen that the amount of protein split off does not increase continuously with the decrease in the pH value. Above a pH value of 3 the amount of protein is small, whereas at pH 2.5 the maximum value of 45% is almost reached. The duration of HCl treatment does not affect the results. From these results it can be inferred that the protein remaining in the supernatant was set free by the breaking of ionic bonds, in which the $-COO^-$ group was involved. Carboxyl groups lose their charge in proteins at pH's between 3–2.7 and become undissociated –COOH groups.

Molecular weight determination of the proteins in the membrane residue

Disc-electrophoresis in the presence of 1% dodecyl sulphate was introduced by Shapiro *et al.*[17] as a method for the determination of protein molecular weights. By this means, with additional aid from a standard curve drawn from known proteins the molecular weight and the approximate size of a protein can be determined. The analysis of erythrocyte membrane proteins by this method results in a pattern of protein bands, with M_r from 250000 to 20000 (Fig. 1A)[13,18]. For the purpose of comparison the individual peaks in Fig. 1A are numbered 1–19. The designation of the larger peaks normally found in the literature[13] are given next to these in



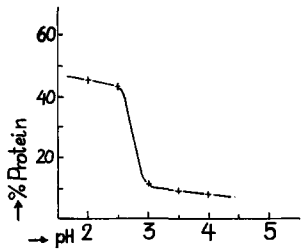


Fig. 1. Profiles of the disc-electrophoresis. A, untreated erythrocyte membranes; B, membrane residue after HCl treatment at pH 2; C, erythrocyte membrane after HCl treatment at pH 2; D, HCl-supernatant after 30 min HCl action at pH 2; E, HCl-supernatant after 24 h HCl action at pH 2. The newly appeared protein peaks are drawn with dotted lines. The horizontal bars in A and B and C designate the peaks, which are periodate–Schiff-positive.

Fig. 2. The alteration of the protein concentration (as percents of the total protein content of the erythrocyte membranes) in HCl-supernatants of various pH values. Each point represent the mean value of 6 measurements.

Roman numerals. Parallel periodate–Schiff staining shows that Peaks 9, 14 and 17 are periodate–Schiff positive. In addition, the glycolipids found on the front[18,19] appear colored by this reaction. The proteins of the membrane residue acquired by HCl treatment at pH 2 (a treatment at pH 2.5 gives the same result, only Peak 2 varies somewhat) are shown in the protein pattern of Fig. 1B. Comparison with Fig. 1A shows that many peaks have greatly decreased in height. For analysis of the change in each protein peak, the area under each was calculated. Apart from the cases of extreme high and low protein concentrations the area is proportional to the protein content. The area under Peak 9 was used as a basis of comparison and the areas under all other peaks expressed as fractions of that under Peak 9. Each protein band can therefore be given a comparison number, which remains constant over a

TABLE I

MOLECULAR WEIGHT AND COMPARISON NUMBERS OF THE INDIVIDUAL PROTEIN BANDS (NUMBERED AS 1–19) OF THE ERYTHROCYTE MEMBRANES, AS WELL AS OF THE MEMBRANE RESIDUE REMAINING AFTER HCl TREATMENT AT pH 2 AND 2.5, RESPECTIVELY

Peak No.	M_r	*Comparison number*		
		Untreated membrane	*Membrane residue*	
			pH 2	*pH 2.5*
1	above 250 000	52±2	9±0.17	9±0.6
2	220 000–240 000	52±1.4	16±2	26±0.15
3	about 230 000	+	+	+
4	210 000	9±1.9	3±0.25	2±0
5	200 000	8±1.6	4±0.25	5±1
6	180 000	19±1.9	4±0.25	5±1
7	150 000–160 000	+	+	+
8	130 000	+	+	+
9	92 000– 95 000	100	100	100
10	84 000	} 38±2.4	} 8±0.22	} 8±0.15
11	78 000– 80 000			
12	68 000– 70 000	6±1.6	14±0.4	12±1.5
13	57 000	18±1.6	12±0.12	10±1
14	50 000	27±2.1	25±0.1	24±2
15	41 000– 42 000	25±1.8	10±0	11±1
16	37 000	33±1.8	6±0.25	6±1.6
17	30 000	10±1.2	13±0.12	12±0.2
18	27 000	10±1.5	9±0.12	7±2.3
19	21 000	5±0.25	4±2	4±1

wide range of concentrations. Peak 9 lends itself easily for use as a comparison peak because it was not altered by HCl treatments. The evidence leading to this assumption is the negative periodate–Schiff reaction of the HCl-supernatant and the consistency of the molecular weights of Peak 9 in the membrane residues. Table I shows the comparison numbers for untreated membranes and for the membrane residue after HCl treatment at pH 2 and 2.5, respectively. The values given are mean values of 8 separate measurements. The standard deviation of the larger peaks is ±7.5% on the average. One can see that the Proteins 1 and 2 as well as 11+12, 15 and 16 in the membrane residue are reduced to slight traces. Protein 2 proved stabler than the rest, since it appears in higher concentrations at pH 2.5 than at pH 2. Analysis of some of the smaller peaks was not possible, since they overlap with other peaks or are very diffuse. Their standard deviation from the mean value is therefore relatively large. As well as Peak 9, the proteins of Peaks 14 and 17 showed no change. It may be noted that all three of the non-extractable bands are periodate–Schiff-positive.

Molecular weight determination of HCl-supernatant proteins

By analysis of an HCl-supernatant up to 3 h old, one acquires the protein band pattern as shown in Fig. 1D. A supernatant brought to pH 6 immediately upon

isolation will give the same result after several days. The proteins found in the HCl-supernatant eventually become insoluble as the pH is increased. A comparison of the molecular weight of individual proteins with those in Fig. 1A gives the following picture: (a) Proteins 1 and 2 are present in much smaller amounts compared to their proportion in the untreated membrane. Their peaks become smaller with increased HCl treatment. (b) The majority of the proteins found in the HCl-supernatant have molecular weights identical to those of proteins not present in the membrane residue. These are Proteins 10+11, 12 and 13, as well as 15 and 16, along with several smaller peaks with molecular weights from 210000 to 130000. (c) The periodate–Schiff-positive band Nos 9, 14 and 17 are totally missing (the reaction gels are negative). (d) Several new protein bands are present compared to Fig. 1A. To these new proteins belong three smaller peaks in the molecular weight region of 125000–110000 (Figs 1D, a, b, c), a peak with a molecular weight of 100000 (Figs 1D, d) and a peak whose molecular weight is identical with that of Protein 14 (Fig. 1D, e). The last new peak is not periodate–Schiff-positive and is therefore not identical with Protein 14 in this respect. The concentrations of the individual proteins vary somewhat depending on the preparations used, HCl-action time, and pH values.

The clear protein bands in Fig. 1C are obtained by the following procedure: The pH of an erythrocyte membrane suspension was brought to 2 using small amounts of HCl. By this means, a mixture of membrane residue and HCl-supernatant proteins, in the same relative amounts as found in untreated membranes, was acquired. Using this mixture, disc electrophoresis to determine the molecular weight of the proteins present was performed. The lines (solid) in Fig. 1C indicate sharp peaks with the same molecular weight as the peaks in Fig. 1A. This means that the proteins in the HCl supernatant complement exactly the missing peaks of the membrane residue, and thus give a profile as would be found in an untreated membrane. The areas of Peaks 1 and 2 are reduced markedly. The newly added peaks (dotted line) agree well with the molecular weight of Peaks a–e in Fig. 1D.

A somewhat different picture is given by the molecular weight determination of the HCl-supernatant proteins when one allows the HCl to act longer than 3 h (Fig. 1E). The results are the same whether the HCl-supernatant proteins are under the long HCl action together with membrane residue, or if one separates them immediately after pH adjustment, from the residue and allows them to remain at pH 2–2.5 for a longer period of time. Proteins 1 and 2 are totally missing from Fig. 1E, whereas Peaks d and e are relatively strongly represented. Many of the remaining peaks are poorly resolved and very diffuse and indicate, dependent on the duration of the HCl action, a variable, reciprocal height relationship. By and large, though, molecular weights agree with those in Fig. 1D.

Ultrastructure of the membrane residues

An example of what the membrane residues look like under the electron microscope is seen in Fig. 3. On the whole, one sees extended membrane structures, which are more or less closely packed. Amorphous material is not present. The presence of many small vesicles of approximately the same size is also conspicuous.

Disc-electrophoresis of HCl-treated spectrin

In comparison to untreated spectrin (composed from Proteins 1 and 2) after

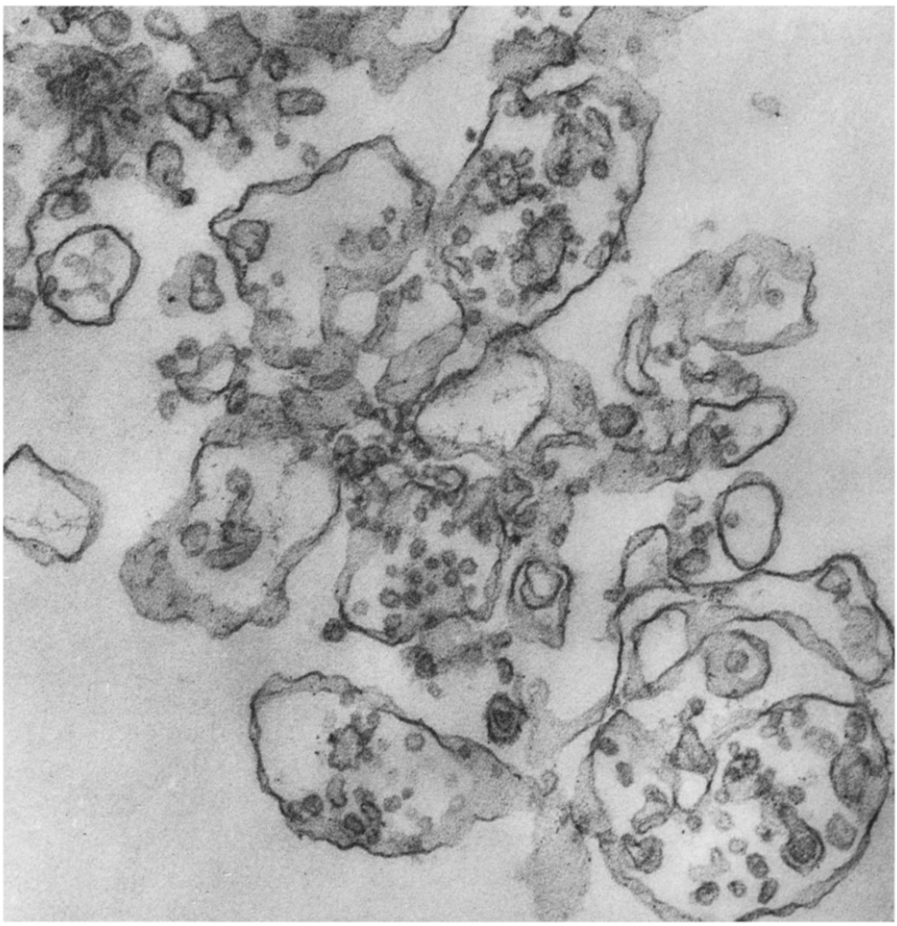


Fig. 3. Electron micrograph of the membrane residue after HCl treatment at pH 2. Magnification: 55000×.

HCl treatment of this protein new bands emerge, namely, 2 bands with electrophoretic mobilities of approx. 0.34 and 0.40, and several bands with electrophoretic mobilities lower than 0.16. The spectrin of this preparation has a mobility of 0.2–0.25 under the same conditions.

DISCUSSION

The experiments have shown that the erythrocyte membrane has 45% of its proteins split immediately from it when treated with HCl below a pH of 3. Under the given centrifugation conditions these remain as a supernatant and are thus separable from the sedimented residue. Use of very high membrane concentrations cause the thus acquired supernatants to be somewhat cloudy rather than clear. This would seem to indicate that the proteins are present as larger aggregated complexes.

Preliminary electron microscopic analysis indicates that they are present in the form of vesicles which contain also some phospholipids.

Protein analysis using disc electrophoresis indicates which proteins are split off and whether or not these have subsequently undergone molecular weight changes. All of the proteins which are missing from the residue are found in the HCl-supernatant without alterations in molecular weight. Exceptions to this are Peaks 1 and 2. These 2 proteins seem to be initially split from the erythrocyte membranes in the form of units with molecular weights of 250000 and 230000, since an analysis of the HCl-supernatant immediately after pH adjustment showed the presence in large quantities of proteins with just these molecular weights, whereas the newly appearing proteins are present in only small amounts. The disappearance of Peak 1 and 2 and the corresponding increase in the concentration of the newly appearing peaks with increased HCl action, seems to indicate that Proteins 1 and 2 break up into subunits with time at pH values below 2.6. Nonetheless they still represent dissociable protein complexes, as opposed to a single polypeptide chain[13]. Further information concerning the number and the size of the subunits cannot be deduced, since the applied molecular weight determination method can give no sure indication of the actual molecular weight[20,21]. In addition, secondary aggregates built from the subunits can falsify their actual molecular weights.

Other recently published results[22,22] support the view that the erythrocyte membranes possess protein complexes, which are held together by especially strong binding forces between the various components and are, therefore, only decomposable by drastic means.

The results obtained from the HCl-treated spectrin allows a very similar interpretation.

The sedimented membrane residue contains, along with the proteins of Peaks 9, 14 and 17 all of the carbohydrates of the erythrocyte membranes.

Preliminary electron microscopic findings, which shall be published in a later work in more detail, indicate together with Fig. 3 that the membrane residue has a distinctly visible membrane character. Also, other morphological characteristics of the erythrocyte membranes were found to be unaltered in the membrane residue. An evaluation of all these findings concerning the residue allows the conclusion that it does not just represent an unordered precipitate of all the membrane parts which are insoluble in HCl.

In general, therefore, it can be said that dilute HCl splits from the erythrocyte membranes vesicular structures, which must be attached to the membrane by pure hydrophilic binding forces. Electron microscopic investigations, which shall be undertaken in the near future, will elucidate the exact morphology of the split-off membrane parts as well as the membrane residue.

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