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SELECTIVE ALTERATION OF ERYTHROCYTE DEFORMABILITY BY SH-REAGENTS

EVIDENCE FOR AN INVOLVEMENT OF SPECTRIN IN MEMBRANE SHEAR ELASTICITY

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Summary

In order to elucidate the molecular basis of membrane shear elasticity, the effect of membrane protein modification by SH-reagents on the deformability of human erythrocytes was studied. Deformability was quantified by measuring the elongation of erythrocytes subjected to viscometric flow in a transparent cone-plate viscometer. Impermeable SH-reagents proved to have no mechanical effect. Many, but not all, permeable SH-reagents markedly decreased the elongation. Among these, bifunctional SH-reagents (e.g. diamide, tetrathionate and *N,N'-p*-phenylenedimaleimide) able to cross-link membrane SH-groups were more effective than monofunctional SH-reagents (e.g. *N*-ethylmaleimide and ethacrynic acid). The bifunctional SH-reagents produced a 50% decrease of elongation after modification of less than 5% of the membrane SH-groups. In contrast, for a comparable effect, more than 20% of the SH-groups had to be modified by the monofunctional reagents. The effect of SH-oxidizing agents was fully reversible after treatment with disulfide-reducing agents. All bifunctional SH-reagents induced a dimerization of a small fraction of spectrin. Analysis of the distribution of the diamide-induced disulfide bonds among the various membrane protein fractions showed that this agent preferentially acts on the spectrin polypeptides.

The results provide direct experimental evidence that the native arrangement of spectrin is essential for the shear resistance of the erythrocyte membrane and that introduction of small numbers of intermolecular cross-links as well as modification within the molecule lead to a rapid loss of this function.

Introduction

The plasma membrane provides considerable mechanical stability to the erythrocyte but allows also deformations of the cell when it is subjected to hydrodynamic forces [1], e.g. in the circulation. This deformability is required for the passage of the erythrocyte through capillaries narrower than the cell diameter [2]. Moreover, a high deformability of the erythrocyte reduces the apparent viscosity of the blood [3] and thereby facilitates its flow through districts of the circulation with high shear rates. A simple model to study this adaptation to the forces of flow is a diluted suspension of erythrocytes in a viscous medium subjected to shear in a viscometer [4]; the low hematocrit of the suspension is necessary to avoid cell-cell interactions.

Under the influence of shear forces erythrocytes become elongated to prolate ellipsoids stationarily oriented in the shear field. These elongated cells can be inspected by microscopy under suitable conditions. Upon stopping the shear flow the cells immediately return to their original shape (for details of this approach cf. ref. 5). The length of the major axis of the ellipsoids increases with increasing shear rate (velocity gradient of the shear field) and can reach more than twice the diameter of the resting cell without mechanical lysis [6].

According to present concepts [7] membrane proteins must be responsible for this remarkable property of the erythrocyte. Experiments involving chemical modification of membrane proteins have rendered support to this concept [8,9].

The aim of the present study was to collect more precise biochemical evidence concerning the relevance of proteins for the elastic properties of the membrane. To this end we studied the influence of protein modification by SH-reagents on the elongation of erythrocytes subjected to shear flow.

Materials and Methods

Incubation procedures. Erythrocytes from freshly collected heparinized human blood were washed three times with 154 mM NaCl. 1 vol. of the washed cells was then suspended in 10 vols. of a medium containing 90 mM KCl, 45 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4 and 44 mM sucrose (medium A). After adjustment of pH to 8.0, 10 mM iodoacetate was added in order to block intracellular GSH, and the cells incubated for 15 min at 37°C. Subsequently, the cells were washed three times with 10 vols. of medium A and incubated for 15 or 30 min at pH 8.0 and 37°C in 10 vols. of medium A containing one of the following SH-reagents: *p*-chloromercuriphenylsulfonic acid (Sigma), 5,5'-dithio-bis-(2-nitrobenzoic acid), *N*-ethylmaleimide, $HgCl_2$ and iodoacetamide (Merck), 4,4'-dithiodipyridine and sodium tetrathionate (Fluka), *N,N'-p*-phenylenedimaleimide (Aldridge), diamide (diazinedicarboxylic acid bis-[dimethylamide], Calbiochem) or 4-chlor-7-nitrobenzo-2-oxa-1,3 diazole (NBD-Cl, Serva), ethacrynic acid (a gift from Merck, Sharpe and Dohme, Rahway). The concentrations of the agents are indicated in the text.

Mechanical measurements. After the incubation the cells were separated by centrifugation and suspended in 50 vols. of a medium containing 75 mM KCl, 37.5 mM NaCl, 8.3 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4) and enough Dextran 60

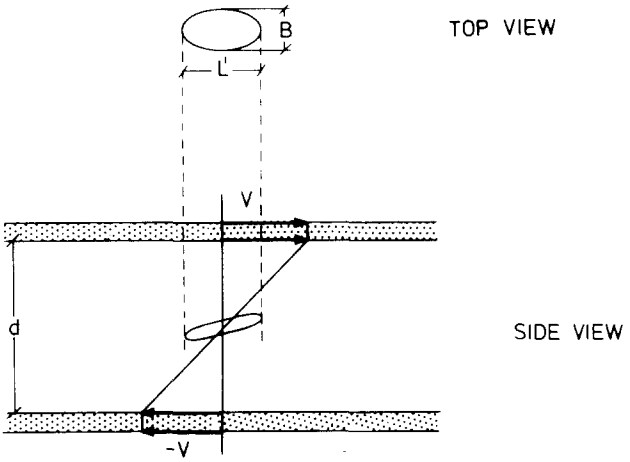

Fig. 1. Schematic drawing of the orientation and elongation of an erythrocyte in the shear field. Side view: cross-section through cone and plate of the rheoscope and an elongated and stationarily-oriented erythrocyte. v, peripheral speed of the cone; $-v$, peripheral speed of the plate; d, distance between cone and plate. $\frac{2v}{d} = \dot{\gamma}$ = shear rate. Top view: ellipsoidal outline of the projection of the erythrocyte onto the plate. L', length of the ellipsoid; B, width of the ellipsoid. $(L' - B)/(L' + B)$ = elongation.

(Knoll AG, Ludwigshafen) to obtain a viscosity of 16 cP, as measured in a capillary viscometer. The suspension was transferred to a transparent counter-rotating cone-plate viscometer [10], sheared and photographed through an interference contrast microscope. In this "rheoscope" the shear rate equals the difference in peripheral speed of the cone and the plate, divided by their distance (Fig. 1). On the photographic copies, the projected lengths (L') and widths (B) of about 20 erythrocytes were determined for each data point. The elongation (E') was calculated according to the formula, modified from Taylor [11]:

$$E' = (L' - B)/(L' + B)$$

Determination of membrane SH-groups. 0.1 ml of packed erythrocyte membranes, prepared according to Dodge et al. [12], were solubilized with 0.1 ml sodium dodecyl sulfate (SDS) (10%, w/v) and the SH-groups determined according to a modification [13] of the procedure of Habeeb [14].

Polyacrylamide gel electrophoresis. Electrophoresis was performed on gels containing 5.0% acrylamide, 0.1% *N,N'*-methylene-bis-acrylamide and 1.0% SDS, following the procedure of Fairbanks et al. [15]. No disulfide-reducing agents were added to the solubilized ghost preparation, to the gel solution and to the electrophoresis buffer.

Localization of membrane disulfide bonds. 1 vol. of erythrocytes was pre-treated with iodoacetate, washed, incubated with or without a disulfide-forming agent as described above, and washed three times with medium A. Ghosts [12], prepared from the cells, were incubated in 10 vols. of a 10 mM solution of *N*-ethylmaleimide in 10 mM phosphate buffer (60 min, pH 8.0, 37°C) in order to block all reactive SH-groups as well as *N*-ethylmaleimide-reactive amino groups. After washing the ghosts three times, native and diamide-induced disulfide bonds were reduced [16] by a 30 min treatment with 10 vols. of a 10 mM solution of dithioerythritol in 10 mM phosphate buffer (pH 8.0, 37°C).

Subsequently, the ghosts were washed three times and the released SH-groups labeled with 0.2 mM *N*-[*ethyl*-^{3}H]ethylmaleimide (specific radioactivity 6.25 Ci/mol; NEN) for 15 min (37°C, pH 8.0). The ghosts were washed another three times, solubilized with SDS and subjected to gel electrophoresis.

Results

Human erythrocytes incubated for 15 min with the rather impermeable [17,18] SH-reagents, *p*-chloromercuriphenylsulfonate (0.4 mM) or 5,5′-dithiobis(2-nitrobenzoate) (5 mM) could be elongated to the same extent as untreated cells when sheared at rates between 14 and 700 s^{-1} in the rheoscope. Likewise, a 15 min exposure to iodoacetate, a rapidly penetrating SH-reagent [19] did not affect the elongation, although the reagent alkylates all of the intracellular GSH and reacts with 10% of the membrane SH-groups (data not shown). In further experiments with permeable SH-reagents, all incubations could therefore be preceded by a treatment with iodoacetate which prevented

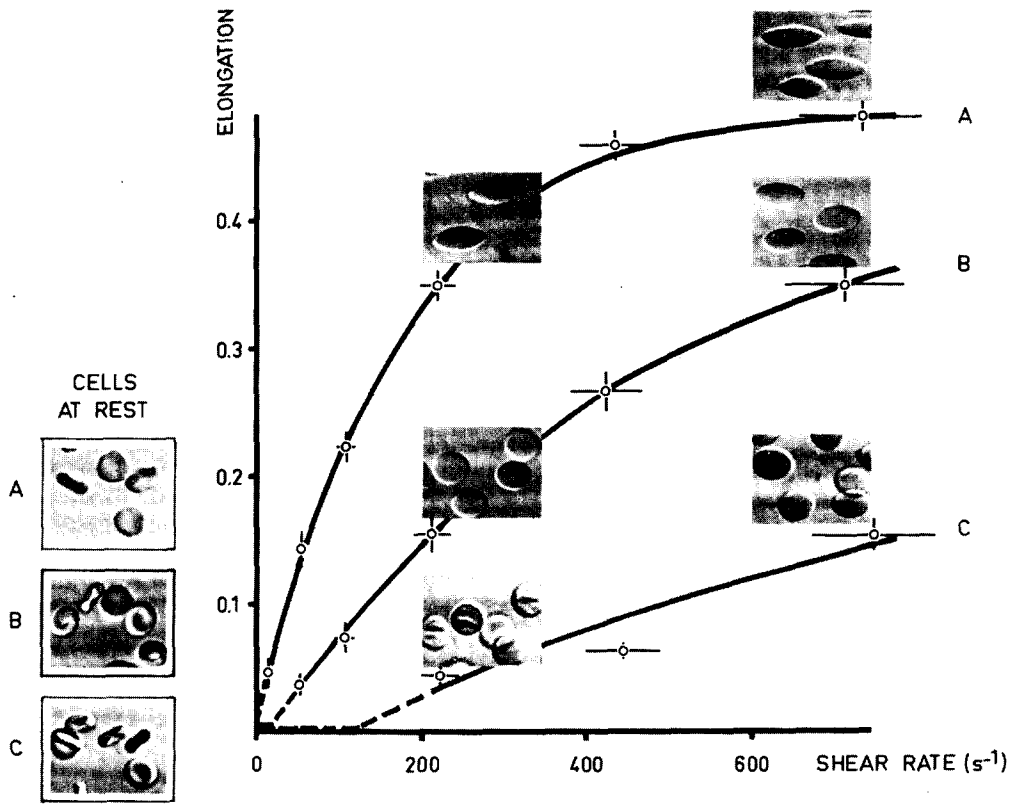

Fig. 2. Elongation of control and diamide-treated erythrocytes at different shear rates ($\dot{\gamma}$). Erythrocytes were pretreated with iodoacetate (10 mM, 15 min, pH 8.0, 37°C), washed three times and incubated (A) without any additives; (B) with 0.05 mM diamide; (C) with 0.1 mM diamide for 15 min (37°C, pH 8.0). The elongation, at different shear rates, was determined as described in Fig. 1. Values are means ± S.E. Left column, cells at rest; middle column, cells at $\dot{\gamma} \approx 200\ s^{-1}$; right column, cells at $\dot{\gamma} \approx 700\ s^{-1}$.

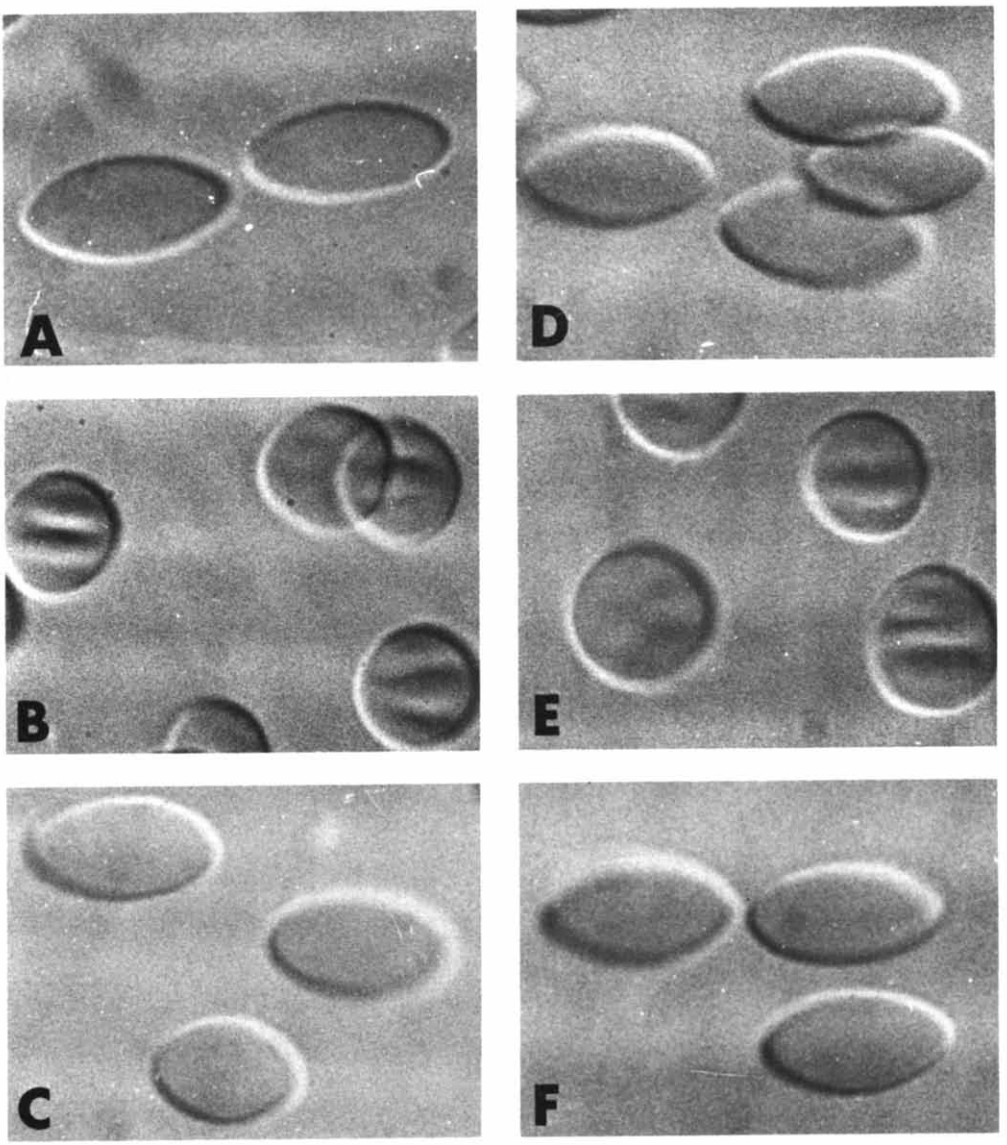

Fig. 3. Reversibility of the effect of SH-oxidizing agents on erythrocyte elongation in shear fields ($\dot{\gamma}$ = 200 s^{-1}). Erythrocytes were treated (pH 8.0, 37°C) for 15 min with 10 mM iodoacetate (A,D), washed and incubated for 15 min with 0.1 mM diamide (B) or 0.1 mM 4,4′-dithiodipyridine (E) and washed. The diamide-treated cells were then incubated for 120 min with 10 mM sulfite (C) and the dithioerythritol (F). After each incubation period the elongation was measured (see Fig. 1).

interference by GSH and reduced the concentrations of SH-reagent required for modification. Iodoacetate-treated erythrocytes also served as controls.

In contrast to the results with the impermeable SH-reagents and with iodoacetate, all other reagents tested produced a marked, concentration-dependent decrease of the elongation at all shear rates studied. A typical result of an experiment with diamide is shown in Fig. 2. While at rest, cells treated with the reagent do not exhibit any major alteration of the normal biconcave shape

TABLE I

PERCENTAGE OF MEMBRANE SH-GROUPS MODIFIED BY SH-REAGENTS AT CONCENTRATIONS THAT DIMINISH THE ELONGATION BY 50% AT $\dot{\gamma} = 150\ s^{-1}$

Erythrocytes were incubated for 15 min (pH 8.0, 37°C) with 10 mM iodoacetate, washed three times and incubated for15 min with one of the SH-reagents listed. The deformability of the cells was measured (4—6 experiments) and membrane SH-groups determined (2—3 measurements). See Methods for further details. NBD-chloride, 4-chlor-7-nitrobenzo-2-oxa-1,3 diazole.

SH-reagent	Mean value of concentration (μM) required for a 50% decrease of elongation at $\dot{\gamma} = 150\ s^{-1}$ (range in brackets)	Decrease of membrane SH-groups (%)
Diamide	55 (35—75)	<5
Tetrathionate	220 (120—320)	<5
4,4′-Dithiodipyridine	18 (12—24)	<5
N,N′-p-Phenylenedimaleimide	16 (8—24)	<5
$HgCl_2$	20 (12—28)	—
N-Ethylmaleimide	450 (250—650)	23
NBD-chloride	230 (150—310)	24
Ethacrynic acid	1600 (800—2400)	22
Iodoacetamide	>10.000	24

(photos in the left column). Cells treated with 0.1 mM diamide and sheared at $200\ s^{-1}$ show characteristic buckling of their membranes (curve C, middle column). This buckling was also observed in cells modified by the other reagents tested (see Fig. 3, 4,4′-dithiodipyridine), but not in untreated cells. It appears at shear rates which induce the first measurable elongation of the erythrocytes. At higher shear rates the buckling disappears. As is further evident from Fig. 2, control and diamide-treated cells become elongated progressively with increasing shear rates, although with decreasing increments. Diamide markedly reduces the elongation at any given shear rate. It should be noted that the S.E. values for the elongation at a given shear rate are of similar magnitude for control and diamide-treated cells. These findings indicate that the decrease of elongation develops gradually and simultaneously in all cells of the population and does not involve an all-or-none phenomenon, which is also evident from the photographs.

Even at high concentrations of diamide the cells still elongate progressively upon increase of the shear rate. It was not determined whether the limiting elongation at which mechanical lysis occurs [6] is the same in untreated and treated cells or whether the treated cells rupture at lower elongations.

To facilitate a comparison of the effects of the various SH-reagents, elongations were measured at a standard shear rate of $150\ s^{-1}$ and concentrations required for a 50% diminution of the elongation determined. The data are compiled in Table I. In general, reagents able to produce disulfide bonds from two SH-groups or to form cross-links between them, are more effective than reagents only reacting with one SH-group. In line with this difference in effectivity, the "bifunctional" reagents, including tetrathionate, diamide, 4,4′-dithiodipyridine and *N,N′-p*-phenylenedimaleimide, reacted with less than 5% of the membrane SH-groups at the concentrations required for a 50% decrease of elongation, whereas the "monofunctional" reagents, *N*-ethylmaleimide, ethacrynic acid, iodoacetamide and 4-chlor-7-nitrobenzo-2-oxa-1,3-diazole [20]

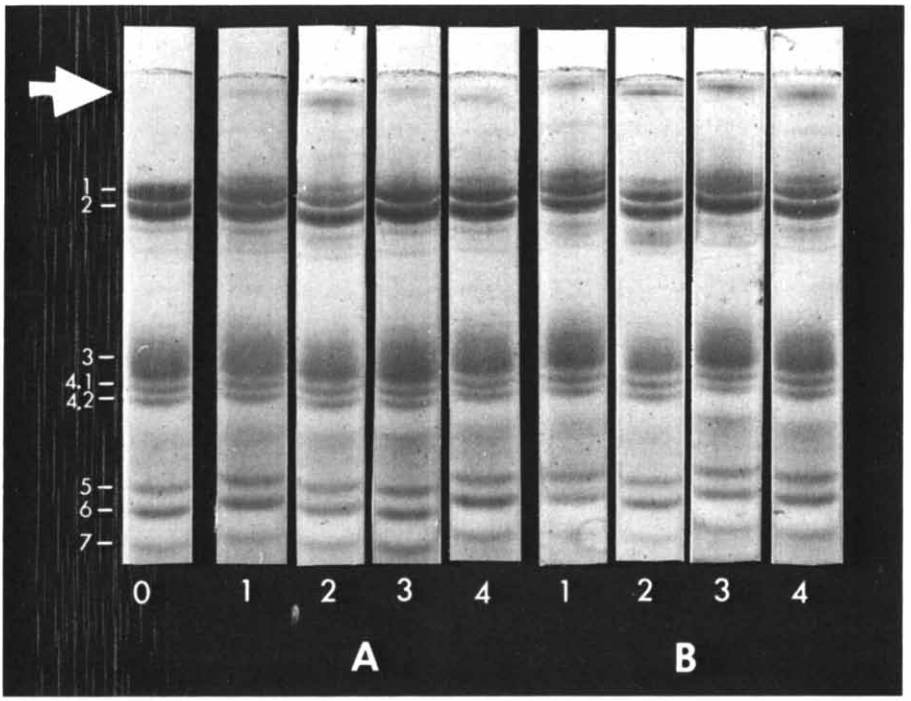

Fig. 4. Gel electrophoretic profiles of membrane proteins from human erythrocytes treated with bifunctional SH-reagents. Erythrocytes were incubated (pH 8.0, 37°C) with 10 mM iodoacetate for 15 min, washed three times and incubated (pH 8.0, 37°C) for 15 min with no additives (0) or one of the following SH-reagents: tetrathionate (1), diamide (2), *N,N'-p*-phenylenedimaleimide (3) 4,4'-dithiodipyridine (4) at the concentrations listed in Table I which induce a 50% decrease of elongation (A) or at twice these concentrations (B).

modified more than 20% of the SH-groups before producing the same decrease of elongation.

Disulfide bond formation by SH-oxidizing agents (tetrathionate, diamide and 4,4'-dithiodipyridine) has been shown to be fully reversible by treatment with sulfite or dithioerythritol [16]. As is evident from Fig. 3, a treatment with these reducing agents also fully restores the decreased deformability of cells modified by diamide or 4,4'-dithiodipyridine. It can be concluded from this observation that only a reversible formation of disulfide bridges and not a subsequent irreversible denaturation of membrane proteins is responsible for the effects of the oxidants.

In order to clarify whether the bifunctional reagents induce intermolecular or only intramolecular cross-linking of membrane proteins under our experimental conditions, membranes of modified erythrocytes were subjected to SDS gel electrophoresis. At reagent concentrations which induce a 50% decrease of elongation, only minor changes in the peptide patterns were observed (Fig. 4, gels A1—A4). These changes became more pronounced at higher concentrations of the agents (gels B1—B4). All bifunctional modifiers produce a new band of about 500 000 daltons (arrow), presumably a dimer of spectrin [21,

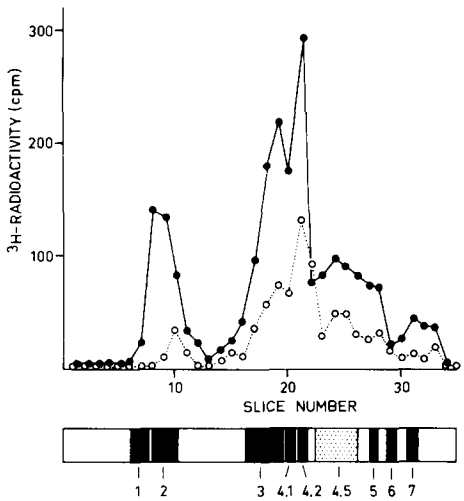

Fig. 5. Distribution among the membrane proteins of native and diamide-induced disulfide bonds in human erythrocytes. Erythrocytes were pretreated with iodoacetate (10 mM, 15 min, pH 8.0, 37°C), washed three times, incubated with or without 0.04 mM diamide and washed three times (0°C). SH-groups released from native and diamide-induced disulfide bonds by reductive cleavage were labeled with *N*-[*ethyl*-^{3}H]ethylmaleimide as described in Methods. Ghosts were solubilized in SDS and subjected to electrophoresis. After staining and destaining, the gels were cut into 2-mm slices, the protein material solubilized with soluene 350 (Packard Instruments) and the radioactivities determined by liquid scintillation counting. ○- - - - -○, without diamide; ●———●, with diamide.

22] *. High concentrations of diamide in addition produce a band of 180 000 daltons (gel B2), most likely a dimer of the major intrinsic protein (band 3 according to Steck [21]).

While these gel electrophoretic studies may indicate an involvement of spectrin and band 3 in the effects of SH-oxidants on cell elongation, they do not exclude a possible role of other proteins. Therefore, the distribution, among the various membrane protein fractions, of the diamide-induced disulfide bonds was studied by a labeling technique. Cells were first treated with diamide in the usual manner. In a second step, the SH-groups not oxidized by diamide were blocked irreversibly by extensive treatment of the cells with *N*-ethylmaleimide. In a third step, all (native and diamide-induced) disulfide bonds were reduced by dithioerythritol and the released SH-groups labeled with *N*-[*ethyl*-^{3}H]ethylmaleimide. The native disulfide bonds in control cells were labeled by an analogous procedure omitting the treatment with diamide.

Fig. 5 shows the distribution of the radioactivity among the various membrane protein fractions after separation by gel electrophoresis. After subtracting the labeling due to the native disulfide bonds from the labeling in diamide-treated cells, the number of SH-groups affected could be determined more precisely to be 3%. The induced disulfide bonds are predominantly distributed betweeen bands 1 + 2, 3 and the band 4 area. It is therefore likely that proteins in these fractions are responsible for the altered deformability. In order to

* This assumption rests on the finding that reductive cleavage of the 500 000 dalton oligomer leads to an exclusive reappearance of the two spectrin bands (data not shown).

TABLE II

NUMBERS OF DIAMIDE-INDUCED DISULFIDE BONDS PER MOLECULE OF THE VARIOUS MEMBRANE PROTEIN FRACTIONS

The experimental conditions were exactly the same as described in Fig. 5 with the exception that the radioactivity of separate bonds was determined by slicing of the gel according to the Coomassie Blue staining profile. Specific activity of *N*-[*ethyl*-^{3}H]ethylmaleimide: 6.25 Ci/mol. Band enumerations according to Steck [23].

Band number	cpm per band (percent of total radioactivity)	Number of labeled SH-groups per protein molecule	Approximate number of protein molecules per disulfide bond
1 + 2	445 (31.6)	0.618	3
3	470 (33.3)	0.302	7
4.1	20 (1.4)	0.059	33
4.2	140 (9.9)	0.321	6
4.5	145 (10.3)	0.223	9
5	70 (5.0)	0.139	14
6	65 (4.6)	0.097	20
7	55 (3.0)	0.061	33

quantitate the alterations on a stoichiometric basis, the numbers of induced disulfide bonds per molecule of each of the proteins was calculated from: (a) the percent activity in the single fractions, (b) the specific activity of the labeled *N*-ethylmaleimide, (c) the percentage of total proteins in the single fractions and (d) the apparent molecular weight of these fractions [23,24]. As can be seen from Table II, 1 disulfide bond per 3 copies of spectrin, 1 bond per 6 copies of band 4.2, 1 bond per 7 copies of band 3 and less than 1 disulfide bond per 9 molecules of each of the other membrane proteins is formed during an incubation with 0.04 mM diamide, which decreases the elongation by about 50% (at a shear rate of 150 s^{-1}).

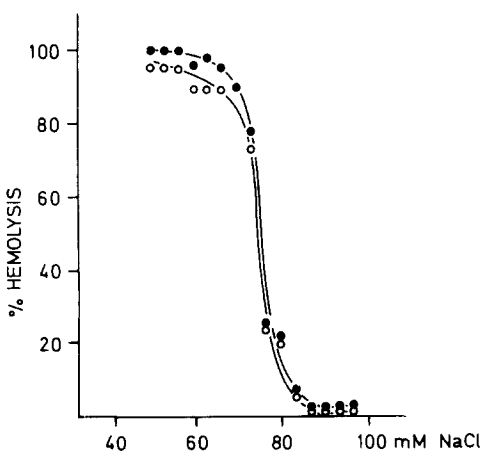

Fig. 6. Lack of influence of diamide on the osmotic fragility of erythrocytes. Erythrocytes were pretreated with iodoacetate (10 mM) or iodoacetate and diamide (0.1 mM, 15 min) as described in Methods. Cells were diluted 1 : 1 with medium A. 50 μl of this suspension were pipetted into 5 ml of NaCl solutions of decreasing osmolarities. After 15 min, concentrated NaCl was added to reconstitute isotonicity, the samples were centrifuged and the hemoglobin content of the supernatants determined by photometry at 546 nm. ○———○, iodoacetate; ●———●, iodoacetate, then diamide.

TABLE III

HALF TIMES OF HEMOLYSIS OF ERYTHROCYTES SUSPENDED IN ISOTONIC SOLUTIONS OF 1,3-PROPANEDIOL

A, freshly drawn erythrocytes; B, erythrocytes pretreated with 10 mM iodoacetate (see Methods); C, erythrocytes pretreated with iodoacetate, followed by 0.1 mM diamide (see Methods). After washing, 1-ml cells were suspended in 10 ml of medium A. 10 μl of this suspension were mixed into 2.5 ml of a 310 mosM solution of 1,3-propanediol in a photometric cuvette at room temperature and the decrease of absorbance at 700 nm registered.

	Half-time of hemolysis (s)
A	5.6
B	5.4
C	5.4

The changes of elongation produced by the blockade of SH-groups indicate alterations of mechanical membrane properties provided that the cell volume does not change in the presence of the reagents. Swollen and shrunken cells are known [25] to have a reduced deformability. In order to exclude this possibility, the water content of cells treated with a number of the effective SH-reagents was determined, but found to be essentially unaltered (data not shown). An unchanged cell volume is also indicated by a lack of differences in the osmotic fragility curves of control and diamide-treated cells (Fig. 6). Measurements of the half-times of osmotic lysis furthermore indicate that the cells treated with diamide do not withstand the biaxial stress in the membrane which precedes the process of osmotic lysis [26] for a longer period of time than untreated cells (Table III).

Discussion

Proteins of the erythrocyte membrane are involved in transport phenomena which control the volume of the cells, contribute to the transfer of CO_2 (by means of the chloride-bicarbonate exchange), and maintain the substrate supply for the glycolytic metabolism of the cell. Moreover, they constitute the immunological properties of the cell. Recently, proteins have also been invoked to explain the remarkable mechanical properties of the erythrocyte [7]. The findings presented above can be regarded as substantial evidence for this latter role originally based on theoretical considerations. In the following, the new data shall be discussed with respect to present physical descriptions of erythrocyte membrane mechanics, but particularly with regard to the biochemical basis of these mechanical properties.

Physical analyses indicate [7] that the elongation of an erythrocyte in a shear field is linked to a two-dimensional elastic shear deformation of the membrane. Two-dimensional shear applied to the membrane deforms a small square piece of membrane into a rhomb of the same surface area. The elastic resistance of the membrane against this deformation can be quantified by the shear modulus [7].

The reduction in the elongation E' (Fig. 1) occurring after modification of membrane proteins by SH-reagents can be interpreted as an increase of this

modulus. This view is confirmed by the observation of buckling of the treated cells (Figs. 2 and 3). Buckling is to be expected, on theoretical grounds, when the shear modulus of the membrane increases at a given geometry of the cell while the resistance of the membrane against bending remains constant [27]. Our data provide experimental evidence for this concept.

Consideration of the quantitative differences between the various SH-reagents, with respect to their mechanical effects and the amounts of modified membrane SH-groups, leads to a number of conclusions concerning the structural basis of the shear elasticity of the erythrocyte membrane, discussed in the following sections.

Impermeable SH-reagents (*p*-chloromercuriphenylsulfonate and 5,5′-dithiobis-(2-dinitrobenzoate)) are ineffective. This may indicate that the proteins responsible for the shear elasticity of the membrane are not located on the external surface of the membrane. The permeable SH-reagents fall into two classes with respect to their effectivity to increase the shear modulus. One class comprises monofunctional reagents. With these compounds, more than 20% of the membrane SH-groups have to be modified in order to diminish elongation by 50%. In contrast, a comparable mechanical effect can be obtained with the second class of reagents after blockade of less than 5% of the membrane SH-groups. This class comprises compounds able to cross-link SH-groups. The marked differences in effectivity between cross-linking and monofunctional agents could be interpreted by assuming that monofunctional reagents have a different pattern of reactivity and react with a large number of SH-groups not involved in membrane deformability before reaching the SH-groups essential for deformability. Alternatively, deformability could be postulated to be much more sensitive to the formation of cross-links than to the simple blockade of SH-groups.

According to the labeling experiments on diamide-treated cells, cross-links formed by this agent are predominantly localized in spectrin, band 3 and band 4.2 (Table II). Spectrin, which is supposed to form a network on the cytoplasmic surface of the erythrocyte membrane [7], has frequently been claimed, due to its topology and molecular configuration, to be responsible for the shear elastic properties of the erythrocyte membrane (for a recent review see ref. 28). Membranes depleted of spectrin have been shown to exhibit a decreased elasticity [9]. Regardless of the particular arrangement of the spectrin network, with [29] or without [30] fixed junctions between the filaments, the generation of additional attachment sites will strongly increase its shear modulus [7]. Intermolecular cross-linking of spectrin, but also of a number of other membrane proteins, has very recently been reported in erythrocytes with a lowered filtrability induced by photooxidation [8].

From our gel electrophoretic studies it follows that of the three proteins sensitive to diamide only spectrin forms intermolecular cross-links in the presence of all the bifunctional SH-reagents studied. Assuming that intermolecular cross-links constitute the above-mentioned additional attachment sites, our labeling and cross-linking data provide further and more precise evidence for a predominant relevance of spectrin for the elastic deformability of the erythrocyte membrane. A rough estimate indicates that the formation of only one intermolecular disulfide bond per 30 copies of spectrin is sufficient to decrease

elongation by 50% *. These quantitative data support the above concept that the shear elasticity of the spectrin network should be very sensitive to the generation of a small number of additional junctions.

Although the involvement of intermolecular disulfide bridges is a very attractive hypothesis, the formation of intermolecular disulfide bonds cannot be discarded as a reason for the decreased deformability: besides one intermolecular cross-link per 30 copies of spectrin, diamide and probably the other bifunctional SH-reagents too, produce a considerable amount of intramolecular cross-links (about 1 per 3 copies of spectrin). These intramolecular cross-links could increase the shear modulus of the membrane due to an increased resistance to changes of angles in a kinked spectrin filament.

Moreover, even a simple blockade of SH-groups by alkylating and other monofunctional reagents affects deformability. Since preferential binding of *N*-ethylmaleimide to spectrin has recently been observed in our laboratory [13] it seems conceivable that the monofunctional agents also act via spectrin, for instance by impeding changes of angles in the protein chain. It is not unlikely that a higher number of SH-groups has to be blocked by monofunctional agents in order to obtain changes comparable to those produced by the introduction of a small number of intra- or intermolecular disulfide bonds.

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* This number can be derived from the staining intensity of the spectrin dimer as compared to that of the spectrin monomer fraction (Fig. 4), and the labeling data in Table II.

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