Biochimica et Biophysica Acta 861 (1986) 277–286
Elsevier

BBA 73310

Transcellular cross bonding of the red blood cell membrane

Thomas M. Fischer

Abteilung Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, D-5100 Aachen (F.R.G.)

(Received 28 April 1986)

Key words: Erythrocyte membrane; Membrane cross bonding; Membrane bridge; Sulfhydryl reagent; Heat denaturation; Osmotic lysis

When red blood cells are osmotically shrunk, opposing regions of the inner membrane surface touch each other in the dimple area. In normal red cells such a mechanical contact is undone by reswelling the cells. When the cells are treated with the SH reagents diamide or *N*-ethylmaleimide, or simply heated to temperatures between 42 and 48°C such a mechanical contact can be made permanent by a process termed 'membrane cross bonding'. Cross bonding also occurred when the cells were treated before mechanical contact was established. The bridge between the two cross-bonded membrane regions may be assumed to be formed by membrane skeletal material. Membrane bridges become visible microscopically when the cells are swollen. These bridges are strong enough to resist the membrane tensions occurring at osmotic lysis. Bridged red cells can be a useful tool in rheology, since they are deformable but cannot adapt to shear flows by membrane tank treading.

Introduction

During the study of the influence of chemical modification of membrane proteins on the mechanical properties of red cell membranes [1] a small percentage of odd-looking cells was often observed. Opposing regions of the membrane appeared to be laminated together in the dimple region. The generation of these shapes was explained as follows: the slightly hypertonic medium (330 mosM) which was used for incubation produced internal membrane contact in a small percentage of cells. This contact, normally purely mechanical and reversible in nature, became obviously irreversible due to the chemical treatment. The present paper defines the experimental conditions required to produce such 'membrane bridges' and reports on studies of their stability under mechanical stress. A preliminary report of part of this work has been previously presented [2].

Correspondence address: Abteilung Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, D-5100 Aachen, F.R.G.

Materials and Methods

Suspending media. Suspending media are designated by M carrying as a subscript their osmolarity in mosM. M_{300} contains (mM): KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (10). Hypotonic media were prepared by diluting M_{300} with H_2O. Hypertonic media were produced by adding appropriate amounts of sucrose to M_{200}. The pH of all solutions was adjusted to 7.4. Bovine albumin (fraction V, Sigma) was added to all media. For hypotonic media, the concentration was 0.3 mg/ml. For hypertonic media, concentrations up to 1.3 mg/ml were used.

Incubation procedures. Erythrocytes from freshly collected heparinized human blood were washed three times with M_{300}. Internal membrane contact

media. For no contact, the cells were swollen in M_{200}.

Incubations with chemical reagents were performed at 37°C and a haematocrit of 10%. After washing, the cells were treated with iodoacetate (10 mM) in M_{200} for 15 min to block intracellular GSH. The cells were then washed and treated with *N*-ethylmaleimide (Merck) or diamide (diazene dicarboxylic acid bis-(*N*,*N*'-dimethylamide), Calbiochem) in either hypotonic or hypertonic medium. Heat treatment was performed at a haematocrit of 10%.

In the incubations following the modifying treatments, the haematocrit was 10%. For a quick change of temperature and/or osmolarity it dropped to 5%.

Counting of bridged red cells. For counting of bridged cells, a drop of the cell suspension was put into 1 ml M_{200}. Swelling in this medium had two effects: first, it stopped further bridge formation; second, it made the membrane bridges clearly visible under interference contrast (cf. Fig. 1). Between 100 and 300 cells were counted directly under the microscope to determine the percentage of bridged red cells. When the counted percentage was zero but outside the counting grid a bridged cell was seen, the percentage was given as $< 1\%$. In order to select a random sample, the positioning of the field to be counted was done according to a fixed scheme without looking through the microscope.

Preparation of haemoglobin-rich resealed ghosts. In some experiments the cells were haemolysed in order to put the membrane under maximal isotropic tension. At 0°C 1.3 ml of 4 mM $MgSO_4$ were added to 0.6 ml packed red cells. After 2 min isotonicity was restored by adding 70 μl of a stock solution containing (mM): KCl (940), NaCl (460). After another 2 min the temperature was elevated to 37°C and kept there for 15 min. A drop of the lysate was then added directly to 1 ml M_{200} for counting as described above. These ghosts had enough microscopical contrast to allow recognition of bridges.

Results and Interpretation

Influence of hypotonic swelling on the shape of bridged cells

The shape of bridged red cells did not depend on the kind of treatment. For example, membrane bridges were induced by incubation of cells in M_{500} at 46°C for 15 min. In this medium membrane bridges could not be seen (Fig. 1a). After the treatment the cells were resuspended in solutions with different osmolarities (Fig. 1b–f). Upon swelling some cells changed shape into inflated disks. In the other cells craters appeared on both sides. These craters decreased in diameter and increased in depth upon progressive swelling. The geometry of the craters indicates enormous tensions in the membrane [3] which can only exist when opposing regions of the membrane are firmly connected. In the following the process of connection will be called membrane cross bonding and the structure mediating the connection will be termed a membrane bridge. These bridges could be counted best at osmolarities between 180 and 240 mosM.

Influence of the sequence of treatments

Cross bonding could be induced either by membrane modification while there was internal membrane contact, i.e., in hypertonic medium (procedure A), or in the absence of internal membrane contact, i.e., in hypotonic medium (procedure B). In procedure B membrane contact was established by transferring the cells into hypertonic media after the modifying treatment.

In the case of treatment with diamide or *N*-ethylmaleimide, procedures A and B produced basically the same percentage of bridged cells (Table I), indicating that the chemcial modification was equally effective in both media and was irreversible. This suggests that membrane cross bonding is a process consisting of two sequential steps: (1) chemical modification rendering the inner membrane surface 'sticky' (2) membrane cross bonding which occurs upon membrane contact.

In the case of heat treatment, procedure A produced about twice as many bridges as procedure B. There are two possibilities to explain this finding. First, heat treatment is more effective in the hypertonic medium than in the hypotonic medium or, second, a partial reversion of the heat effect takes place during the washing procedure (lasting 10–15 min) between the two incubations.

Omitting the washing (procedure B*) did not change the percentages of bridged red cells, thus

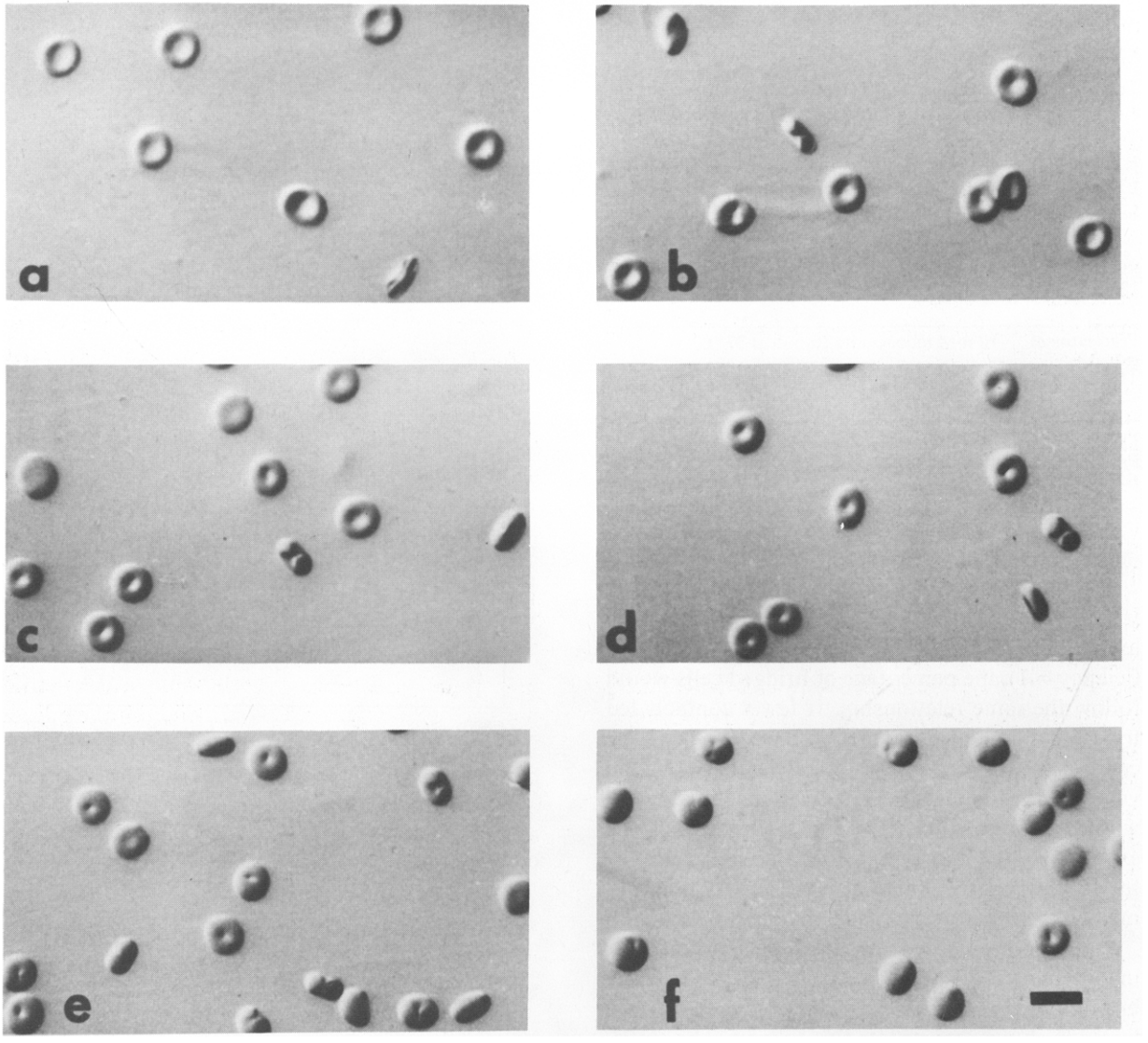

Fig. 1. Morphology of bridged red cells swollen in solutions of different osmolarity. Red cells were incubated in M_{500} for 15 min at 46 °C. They were then suspended at room temperature in media of different osmolarities (mosM): (a) 500, (b) 300, (c) 260, (d) 220, (e) 180, (f) 140 and photographed. Scale bar = 10 μm.

arguing against the second possibility. The first possibility seems more likely when another effect of heat treatment is considered: increase of the membrane shear modulus [4]. This increase was found to be much smaller when the cells were treated at 46 °C in M_{200} than in M_{400} (Fischer, T.M., unpublished data).

The difference between procedures A and B was less strong when the temperature was 46 °C (Fig. 4). This may be due to saturation, since bridge percentages were close to 100%.

Influence of the osmolarity of the shrinking medium for producing membrane contact

Extent of bridging. Due to the statistical distribution of cell porperties, the percentage of red cells developing internal mechanical contact can be expected to rise sigmoidally from 0 to 100%

TABLE I

PERCENTAGES OF BRIDGED RED CELLS

Treatment and shrinking done simultaneously (procedure A) and successively (procedure B and B*). Pretreatment in M_{200}. Treatment in M_{600} (procedure A) or M_{200} (procedure B and B*). Successive incubation: 30 min in M_{600} (procedure B and B*). Procedure B* is like procedure B except that the washing before the successive treatment was replaced by diluting the suspension two-fold so that the suspending medium changed quickly from M_{200} to M_{600}. The haematocrit dropped from 10% to 5%. NEM, *N*-ethylmaleimide.

Pretreatment:	10 mM iodoacetate 15 min at 37°C		
Treatments:	0.2 mM diamide 30 min at 37°C	0.2 mM NEM 30 min at 37°C	44.4°C 30 min
Procedure A	51±14	28±15	74±8
Procedure B	49±12	26±9	35±6
Procedure B *			31±4

with the osmolarity of their suspending medium. If, upon membrane modification, every contact led to membrane cross bonding (bridging efficiency = 1), the percentage of bridged cells would follow the same relationship. If fewer contacts led to cross bonding (bridging efficiency < 1), the curve would also rise sigmoidally but its plateau value (corresponding to its bridging efficiency) would be lower. In order to test these assumptions experimentally, the cells were modified in M_{200} for 15 min and incubated afterwards for 30 min in media of osmolarities between 300 and 900 mosM.

The curve obtained for diamide (Fig. 2a) rises in a sigmoidal fashion as expected. This curve can be compared to a theoretical curve (Fig. 2a broken line) which was constructed as follows. The statistical distribution of the minimal cell thicknesses at isotonicity in a population of normal red cells was assumed to be normal. The mean value and standard deviation were taken from Evans and Fung [5]. For the average red cell [5], the reduction of minimum cell thickness upon lowering the cell volume was calculated using theoretical models [6]. Minimum cell thickness proved to be a linear function of cell volume and was assumed to be the same for all cells of the population. Using this functional dependence, the volume corresponding to the first membrane contact was obtained for all cells of the population. The osmolarity corresponding to this volume was obtained using data

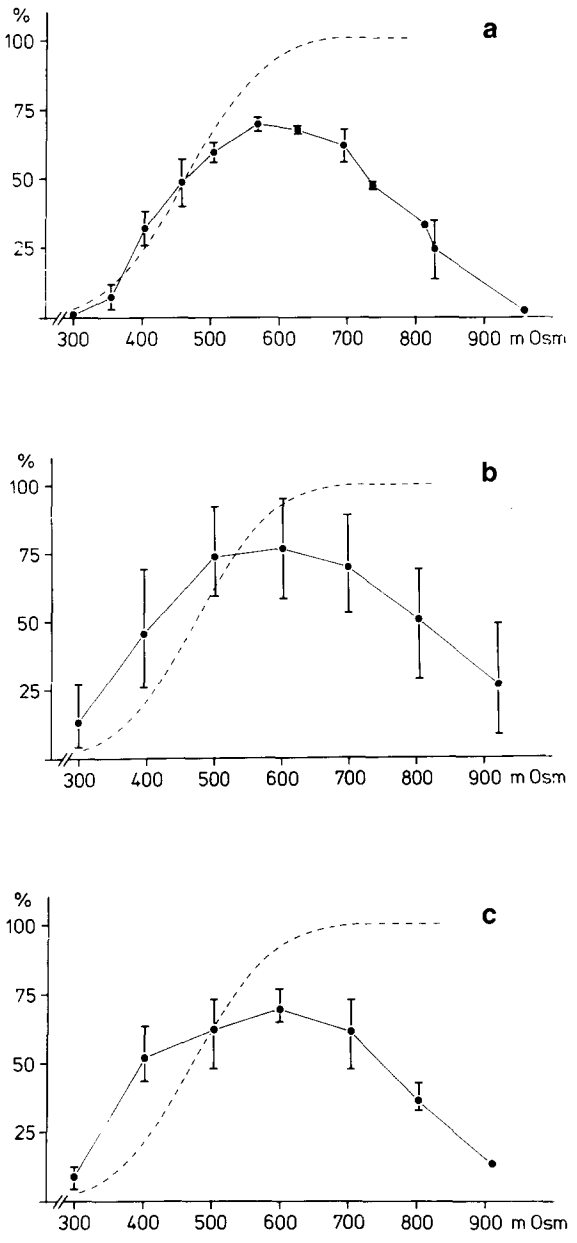

Fig. 2. Percentage of bridged red cells vs. osmolarity of the shrinking medium. Continuous line: red cells were modified for 15 min in M_{200} and subsequently incubated for 30 min at 37°C in the shrinking media. Mean values and ranges (bar) are given unless the bar would be smaller than the symbol. Broken line: theoretical calculation taking into account the shrinking behaviour of red cells and the statistical distribution of the minimum cell thickness, and assuming a bridging efficiency of 1. For details see text. (a) Treatment with 1 mM diamide at 37°C ($n = 4$, each point represents the mean of two experiments). (b) Treatment with 1 mM *N*-ethylmaleimide at 37°C ($n = 3$). (c) Incubation at 47°C ($n = 3$).

of Solomon and coworkers [7,8]. A bridging efficiency of unity was assumed.

The agreement between theory and experiment (Fig. 2a) is excellent up to an osmolarity of 470 mosM, validating the assumption of a bridging efficiency of unity. Above 470 mosM the curve deviates from the expected sigmoidal rise in that it goes through a maximum and declines.

The curves for bridging by *N*-ethylmaleimide (Fig. 2b) and heat (Fig. 2c) also go through a maximum. The concentration of *N*-ethylmaleimide as well as the temperature were chosen so that the maximum percentage was about the same as in the diamide treatment. The rising part of the curves in Fig. 2b and 2c is shifted to the left, relative to the theoretical prediction, leading to 50% bridged red cells approximately at 400 mosM. At this osmolarity the theoretical calculations predict a membrane distance, in the dimple, of 0.2 μm. One might speculate that this distance is overcome by the thermal thickness variations of the red cell which are observed as membrane flickering [9]. This speculation is supported by direct observations of the amplitude of thermal thickness variations on a chemically unmodified cell at a location halfway between the dimple and the rim ranging from 0 to more than 1 μm [10]. These thickness varations might produce membrane contact for short periods of time even when the membranes are separated enough on average to avoid contact. Membrane flickering has been shown to be damped strongly after increase of the membrane shear modulus with diamide [10]. Therefore, the absence of flickering in the case of diamide treatment is not surprising. On the other hand, treatment with *N*-ethylmaleimide [1] or heating in M_{200} (Fischer, T.M., unpublished data) increases the membrane shear modulus much less than treatment with diamide.

Instead of reaching a plateau at 100% bridging as expected, the curves in Fig. 2a, b, c decline at osmolarities exceeding 600 mosM with no significant difference among the three treatments. Two explanations may be offered. First, the cytoplasm may become so viscous due to cell shrinkage that the membrane does not reach its equilibrium shape. It rather encloses an almost rigid pancake of haemoglobin which prevents membrane contact. Second, if membrane cross bonding were due to electrostatic interactions, the bonds could become weakened by the increase in intracellular ionic strength following cell shrinkage.

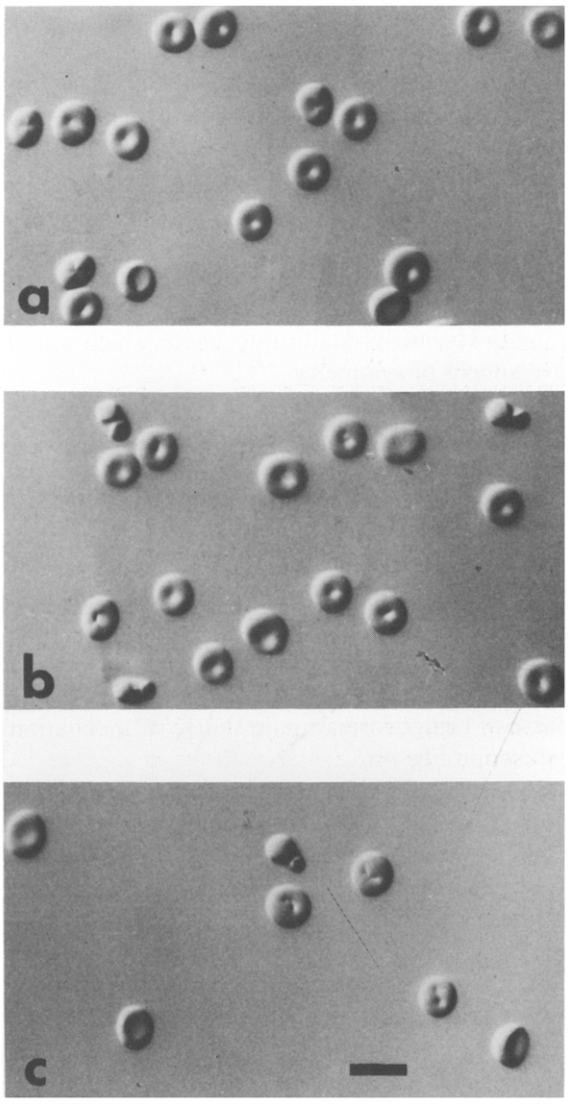

Fig. 3. Shape of membrane bridges produced by membrane cross bonding in different shrinking media. Cells were treated for 15 min with 1 mM *N*-ethylmaleimide at 37°C in M_{200} and subsequently incubated in hypertonic media of different osmolarity (mosM): (a) 400, (b) 600, (c) 800 for 30 min at 37°C. Then they were resuspended in M_{200} and photographed. Scale bar = 10 μm.

Morphology of bridges. The morphology of membrane bridges did not depend on the kind of treatment. For example, cells were treated with

N-ethylmaleimide (1 mM) in media of three different hypertonicities and then suspended in M_{200} and photographed. Treatment at 400 mosM produced only membrane bridges with a point-like contact area (Fig. 3a). At 600 mosM the average cross-bonded area was increased as expected (Fig. 3b). At 800 mosM multiple point-like or single line-shaped membrane bridges could be seen (Fig. 3c). This corroborates the interpretation that the almost solid cytoplasm does not allow the membrane to assume its equilibrium shape which would have an axis of symmetry.

Influence of incubation time

The effect of incubation time was determined for the three modifications (diamide, *N*-ethylmaleimide and heat). As a typical example, the results for heating are presented. In procedure A heat treatment and bridge formation were performed in M_{600} (Fig. 4a). In procedure B the cells were heated for 30 min in M_{200}, then transferred into M_{600}, and incubated there at 37°C. The time course of bridge formation in the 37°C incubation is shown in Fig. 4b.

The rate of bridge formation increases with temperature. The corresponding enthalpy of activation calculated from initial slopes in Fig. 4a is about 420 kJ/mol which is a typical value for protein denaturation [11].

Stability of membrane cross bonding

It is conceivable that cross-bonded membrane

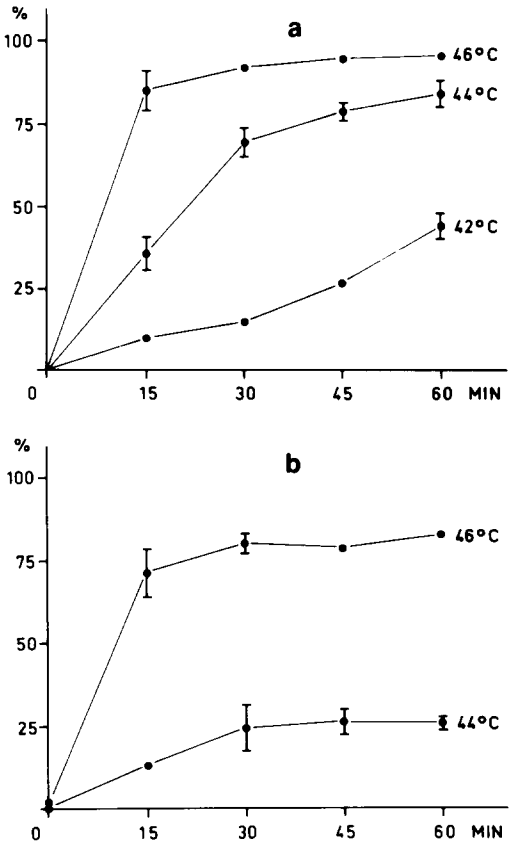

Fig. 4. Percentage of bridged red cells vs. incubation time in M_{600} at three temperatures. Values are means of two experiments. Bars show range of measurements unless the bar would be smaller than the symbol. (a) Cells incubated up to 1 h in M_{600} at the temperatures indicated (procedure A). (b) Cells preincubated in M_{200} for 30 min at the indicated temperatures and subsequently suspended at 37°C in M_{600} up to 1 h (procedure B).

TABLE II

PERCENTAGES OF MEMBRANE BRIDGES BEFORE AND AFTER OSMOTIC HAEMOLYSIS

Red cells were treated in M_{600} for 15 min as indicated, then washed, haemolysed, reconstituted, and resealed (for details see Methods). Values are means (and range). NEM, *N*-ethylmaleimide.

Iodoacetate 10 mM						
Treatment:	Diamide (mM)		NEM (mM)		Temperature (°C)	
	1	5	1	5	46	48
Before haemolysis	58 (43–65)	84 (82–88)	58 (56–60)	88 (83–93)	81 (75–88)	95 (91–88)
After haemolysis	28 (27–30)	64 (50–70)	0	54 (46–63)	6 (2–12)	85 (77–92)
Number of samples	3	3	3	3	4	4

regions might be torn apart when bridged red cells are swollen (see above). 'Dose'-response curves were obtained according to procedure A (Fig. 5a, b, c upper curve). As a test for the mechanical stability of membrane bridges the cells were subsequently suspended in M_{200} and incubated for another 15 min at 37°C (Fig. 5a, b, c lower curve). The relative number of membrane bridges that opened up decreased with increasing 'dose'. When the incubation in M_{200} was prolonged beyond 15 min (up to 1 h) no significant further decrease in percentage of bridged red cells was observed.

In the light of these results it is likely that by merely suspending cells in M_{200} for the counting procedure, some membrane bridges are already torn open. Hence, only bridges above a certain threshold of stability would be counted.

Resistance of membrane bridges against osmotic haemolysis

High 'doses' in the modification produce bridges able to sustain even the extreme membrane tensions occurring during osmotic haemolysis. This is documented by cinematography. After treatment with diamide, red cells were swollen at a controlled rate [12] by suspension in isotonic NH_4Cl/HCO_3 (Fig. 6). During the swelling process the craters became deeper and thinner until they disappeared when the cells looked perfectly spherical (12.5 s). A second later the craters suddenly reappeared, indicating that the bridges were not broken but too thin to be resolved by light microscopy.

The spherical shape at 12.5 s indicates that the membrane shape was dominated by isotropic tension. The release of this tension during the sudden transition can be best explained by formation of leaks allowing exit of small solutes and water. After a lag of about 7 s, the contrast of the cells began to fade which means that the cells lost haemoglobin. During the leaking process the cell did not change shape, indicating that there was practically no pressure difference between cell interior and exterior. Hence, considerable plastic shear flow of the membrane had occurred during the swelling phase, since the red cell did not return to its biconcave shape after release of the isotropic tension.

Quantitative data are shown in Table II. Red

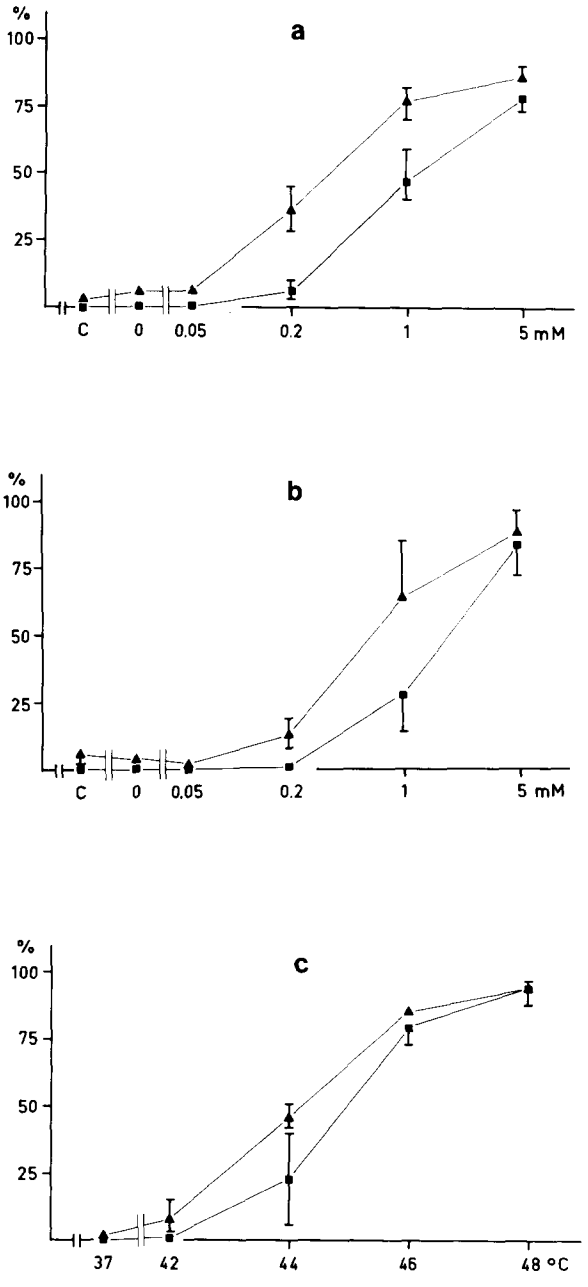

Fig. 5. Percentages of bridged red cells vs. concentration of SH reagents or temperature, respectively. Values are means. Bars show range of measurements unless the bar would be smaller than the symbol. C denotes control treatment at 37°C without modification. (a) diamide ($n = 3$), (b) *N*-ethylmaleimide ($n = 4$), (c) heat ($n = 3$). ▲, cells were treated at 37°C for 15 min in M_{600} (procedure A). ■, after treatment the cells were resuspended in M_{200} and incubated at 37°C for 15 min.

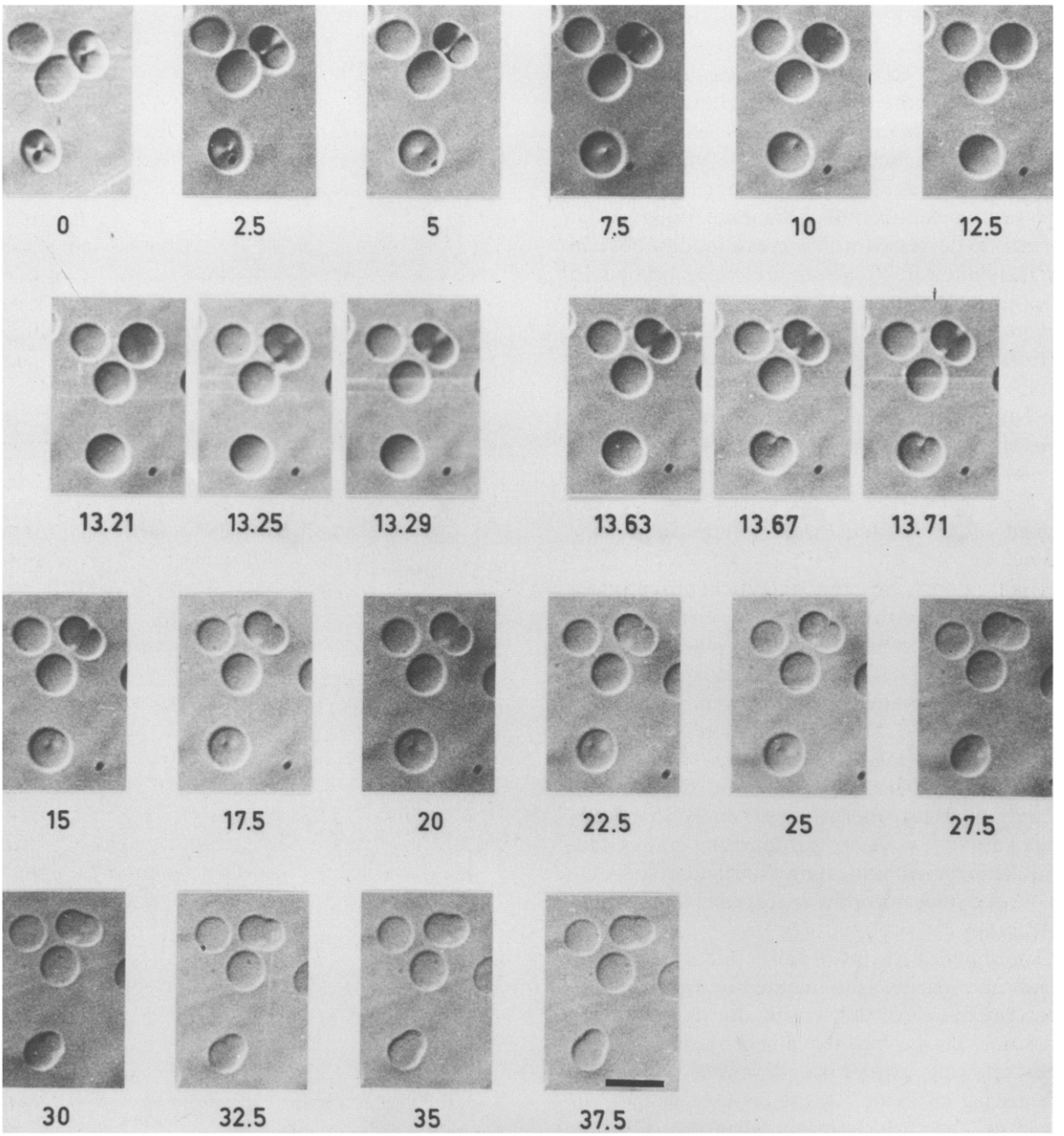

Fig. 6. NH_4Cl/HCO_3-induced swelling of bridged red cells at 23°C. Photomontage from a motion picture (24 frames/s). Numbers denote elapsed time in seconds. Zero time is for technical reasons about 10 s after suspending the cells in 150 mM NH_4Cl + 10 mM $NaHCO_3$. Cells were treated with 1 mM diamide in M_{400}. Scale bar = 10 μm.

cells were treated in M_{600} for 15 min and then haemolysed. The threshold 'dose' for resistance against osmotic haemolysis is between 1 and 5 mM for *N*-ethylmaleimide, between 46 and 48°C for heat, and below 1 mM for diamide treatment. Osmotic fragility curves were not influenced by the percentage of bridged red cells (data not shown), indicating that the membrane surface area of the craters and the cross-bonded region in the moment of lysis is small compared to the total surface area of the membrane.

Discussion

According to the results presented above, treatment of red cells with permeable SH reagents modifies the membrane in such a way that cross bonding occurs when two areas of the cytoplasmic face come into contact. It is interesting to note that the monofunctional SH reagent *N*-ethylmaleimide is about as effective as the disulphide-producing agent diamide (Tables I and II, and Fig. 5a, b). This trend is in contrast to the action of these reagents in increasing the shear modulus of the membrane. With *N*-ethylmaleimide, ten times as high a concentration was required to produce the same effect as with diamide [1]. Therefore, disulphide formation or covalent substitution are probably not the direct causes for the 'stickiness' of the membrane. More likely, some secondary rearrangement of membrane constituents is involved. The fact that heating of red cells between 42 and 48°C produces cross bonding suggests that spectrin [13] is involved in bridge formation. It can be hypothetized that bridging by SH reagents is also mediated by spectrin and that the secondary rearrangement (see above) is similar to heat denaturation of spectrin.

Chemically, cross bonding may be due to precipitation of altered spectrin from its stable two-dimensional suspension at the membrane interface and binding of spectrin aggregates of opposing membranes upon mechanical contact. Support for this hypothesis comes from gel electrophoresis in which spectrin aggregation was demonstrated after treatment with 2 mM *N*-ethylmaleimide or heating to 50°C [14] and after 2–5 mM diamide treatment [15,16]. Further support comes from the observation, by freeze etching and electron microscopy, of aggregation of membrane skeletal elements after 0.5–5 mM diamide treatment [17].

Physically, membrane cross bonding is an example of an adhesive contact which does not spread. Spreading means that a contact initially small in area propagates spontaneously until equilibrium between adhesive forces and elastic recoil of the membrane due to deformation is reached. In contrast, Fig. 1a shows that the membrane contour of all cells is smooth, indicating that cross bonding does not induce membrane deformation. According to Evans [18], non-spreading is due to a sparse distribution of cross links (the term cross link does not necessarily imply a covalent chemical bond), their lateral distance in the plane of the membrane being greater than their range of attraction (normal to the membrane).

When cross-bonded cells are swollen in a hypotonic medium there is mechanical equilibrium between the tension in the membrane and the force the cross links bear at the circumference of the cross-bonded region. The cross-bonded region decreases and apparently a new equilibrium is attained when the osmolarity of the swelling medium decreases (Fig. 1b–f). There are two possible contributions to this effect. First, separation of the cross-bonded membranes from each other at the rim of the cross-bonded region releases membrane tension because the surface area that encloses the red cell volume increases. Second, the cross links may not be broken during the process of separation of opposite membranes but may move inward laterally and become concentrated [19] at the rim of the cross-bonded region, thus reducing the force per cross link.

Rheologically, membrane cross bonding has an interesting application. Membrane bridges abolish the unique capacity of the normal human red cell to adapt to adverse flow conditions by membrane tank treading [20]. The bridged red cell is somewhat similar to all other cells which are stabilized by a cytoskeleton. Since the cytoplasm is still liquid and bridged red cells have normal volumes, they can undergo deformations such as squeezing or folding which do not require membrane tank treading. Therefore, bridged red cells open the possibility to quantify the relative contributions of membrane tank treading and red cell deformation to rheological effects under various flow conditions. However, care has to be taken to use as controls non-cross-bonded red cells with the same membrane shear modulus as the cross-bonded sample because all treatments presented in this paper increase the membrane shear modulus [1,4].

Acknowledgements

I thank Dr. C.W.M. Haest and Professor B. Deuticke, RWTH Aachen, for stimulating discussions, H. Heidtmann, H. Scheidt and P. Steffens for technical help, F.J. Kaiser for preparing the

figures, and Professor R. Skalak, Columbia University New York, for allowing the use of his finite element programme. Support came from DFG (Fi 258/3-1) for materials and from NIH (HL-16851) for computer time.

References

1 Fischer, T.M., Haest, C.W.M. Stöhr, M., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 510, 270–282
2 Fischer, T.M. (1983) Biorheology 20, 425 (Abstr.)
3 Evans, E.A. and Skalak, R. (1979) CRC Crit. Rev. Bioeng. 3, 181–418
4 Nash, G.B. and Meiselman, H.J. (1985) Biorheology 22, 73–84
5 Evans, E. and Fung, Y.C. (1972) Microvasc. Res. 4, 335–347
6 Zarda, P.R., Chien, S. and Skalak, R. (1977) J. Biomech. 10, 211–221
7 Savitz, D., Sidel, V.W. and Solomon, A.K. (1964) J. Gen. Physiol. 48, 79–94
8 Gary-Bobo, C.M. and Solomon, A.K. (1968) J. Gen. Physiol. 52, 825–853
9 Parpart, A.K. and Hoffman, J.F. (1956) J. Cell comp. Physiol. 47, 295–303
10 Fricke, K. (1985) Doctorial Dissertation TU Munich, F.R.G.
11 Stearn, A.E. (1949) in Advances in Enzymology (Nord, F.F., ed.), pp. 25–74, Interscience Publishers, New York
12 Jausel-Hüsken, S. and Deuticke, B. (1981) J. Membr. Biol. 63, 61–70
13 Brandts, J.F., Erickson, L., Lysko, K., Schwartz, A.T. and Taverna, D. (1977) Biochemistry 16, 3450–3454
14 Smith, D.K. and Palek, J. (1983) Blood 62, 1190–1196
15 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
16 Smith, D.K. and Palek, J. (1982) Nature 297, 424–425
17 Mohandas, N., Wyatt, J., Mel, S.F., Rossi, M.E. and Shohet, S.B. (1982) J. Biol. Chem. 257, 6537–6543
18 Evans, E.A. (1985) Biophys. J. 48, 185–192
19 Evans, E. and Leung, A. (1984) J. Cell Biol. 98, 1201–1208
20 Fischer, T.M., Stöhr-Liesen, M. and Schmid-Schönbein, H. (1978) Science 202, 894–896