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REACTIONS OF THE ALKYLATING AGENT TRIS(2-CHLOROETHYL)-AMINE WITH THE ERYTHROCYTE MEMBRANE

EFFECTS ON SHAPE CHANGES OF HUMAN ERYTHROCYTES AND GHOSTS

DIETER B. WILDENAUER, HERBERT REUTHER and JÖRG REMIEN

Pharmakologisches Institut der Universität München, D-8000 München 2 (F.R.G.)

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Summary

The influence of tris(2-chloroethyl)amine on shape changes of human erythrocytes and ghosts was studied in vitro and correlated with alterations in the molecular structure of the membrane.

(1) Reaction with 1—2 mM tris(2-chloroethyl)amine, a concentration which caused polymerisation of spectrin as detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis, protected intact erythrocytes against metabolically induced shape changes.

(2) When induced by Mg^{2+}-ATP, ghosts prepared from alkylated erythrocytes underwent normal changes in shape. However, when ghosts were treated directly with tris(2-chloroethyl)amine, no Mg^{2+}-ATP-induced shape changes occurred. This fixation in shape appeared to be due to a higher degree of reaction with the alkylating agent.

(3) The amount of chlorpromazine necessary for transformation of erythrocytes into stomatocytes was increased for tris(2-chloroethyl)amine-pretreated cells and was dependent on the degree of reaction with tris(2-chloroethyl)-amine.

(4) Deformability of red cells after reaction with tris(2-chloroethyl)amine was estimated by measuring their rheological behaviour in glass capillary arrays. A slight reduction of the flow rate was observed for cells alkylated with 1—2 mM tris(2-chloroethyl)amine.

(5) Extractability of spectrin was diminished and corresponded to the degree of spectrin polymerisation.

(6) No difference in the incorporation of ^{32}P by alkylated and untreated cells was found. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and autoradiography of the membrane proteins from ^{32}P-treated cells showed that the spectrin component 2 and the polymerisation products generated by the reaction with tris(2-chloroethyl)amine were labelled.

It is suggested that the observed conservation of cell shape is preferentially caused by the reaction of tris(2-chloroethyl)amine with spectrin.

Introduction

Human erythrocytes undergo shape changes dependent on their metabolic state [1—3]. Intracellular ATP depletion causes crenation and thus formation of echinocytes, a process which is reversible upon replenishing the cells with ATP. Analogous effects have been observed in experiments with erythrocyte membranes [4,5].

Shape changes are also generated by amphipathic drugs [6,7]. Chlorpromazine for instance, causes formation of stomatocytes.

The membrane protein, spectrin, seems to be involved in these shape alterations [5,8—10]. Spectrin is a peripheral protein located on the inside of the membrane [11—13]. It is thought to be a major component of the cytoskeleton and to be responsible for regulation and maintenance of the cell shape [14]. It has been reported that phosphorylation and dephosphorylation of the spectrin component 2 are important [5,8,15]. Modification of the cytoskeleton should have an effect on shape changes.

Palek et al. [10] were able to show that the irreversible spheroechinocytic shape of erythrocytes induced by Ca^{2+} was due to an alteration of the spectrin assembly. This spheroechinocytic transformation could not be reversed by cationic amphipathic agents like chlorpromazine or procaine [16].

While studying the effect of the alkylating antitumor agent tris(2-chloroethyl)amine, a trifunctional nitrogen mustard derivative, on erythrocyte membrane proteins, we observed extensive reaction with spectrin [17] which resulted in the formation of polymers.

The present paper describes the effect of this reaction on shape changes in intact human erythrocytes and isolated erythrocyte membranes. Our findings show that the spectrin polymerisation caused by tris(2-chloroethyl)amine can be correlated with a fixation of the cell shape. In addition, we investigated the impact of the reaction with tris(2-chloroethyl)amine on erythrocyte deformability, spectrin extractability and spectrin phosphorylation.

Materials and Methods

Materials. Tris(2-chloroethyl)amine was obtained from EGA Chemie. Sodium dodecyl sulphate (SDS) and polyacrylamide were purchased from Bio-Rad. All other chemicals used were reagent grade from E. Merck, Darmstadt. [U-^{14}C]Tris(2-chloroethyl)amine hydrochloride (spec. act. 5 mCi/mmol) was synthesized by Behringwerke AG. Radiochemical Laboratory, Frankfurt/Main, F.R.G. $^{32}P_i$, as orthophosphoric acid, carrier-free, was from New England Nuclear.

Reaction of erythrocytes and ghosts with tris(2-chloroethyl)amine. Human blood was freed from plasma and the buffy coat by sedimentation and the remaining erythrocytes washed three times with phosphate-buffered saline containing 150 mM NaCl and 5 mM phosphate, pH 8.0. The packed cells were diluted with 9 vol. of 100 mM Tris buffer, pH 7.0, containing 0.37% NaCl and tris(2-chloroethyl)amine at various concentrations was added. The mixture was incubated at 37°C for 60 min with gentle shaking. To remove non-reacted reagent, the cells were washed two or three times by centrifugation. For reaction with ATP-depleted erythrocytes, the cells were preincubated at 50% haematocrit in 146 mM NaCl, 20 mM Tris, pH 7.4, containing 100 μg/ml penicillin and 0.1 mg/ml streptomycin for 20 h at 37°C. Erythrocytes used for deformability experiments were alkylated in phosphate-buffered saline. Reaction with ghosts was achieved after dilution of packed ghosts with 9 vol. of 50 mM Tris, pH 7.0, and incubation with tris(2-chloroethyl)-amine as in the case of intact cells.

Shape changes. ATP-dependent shape changes in intact cells were carried out using the method of Sheetz et al. [4]. ATP depletion was achieved by incubation of washed erythrocytes as described above. To replenish ATP, the cells were incubated for 2 h at 37°C in 5 mM adenosine. For scanning electron microscopy the erythrocytes were fixed as described by Sheetz and Singer [7].

Shape changes of ghosts were carried out according to the method of Sheetz and Singer [5]. Washed erythrocytes at about 50% haematocrit, untreated or alkylated with tris(2-chloroethyl)amine as described above, were lysed in 10 vol. of 10 mM Tris buffer, pH 7.4, at 0°C and the haemolysate removed by centrifugation at $22\,000 \times g_{max}$ for 15 min. The pellet was suspended in 9 vol. of a solution of 2 mM Mg^{2+}-ATP in distilled water at 0°C. The suspension was made isotonic by the addition of 1 vol. of 1.4 M KCl, 0.2 M NaCl and 10 mM Tris, pH 7.4. The mixture was incubated at 37°C for 30 min.

Alkylation of ghosts was carried out before addition of the nucleotide solution as described above. The ghosts were fixed by the addition of 1 vol. of ghost suspension to 0.5 vol. of 2% glutaraldehyde in 105 mM phosphate buffer, pH 7.4. After 15 min at 0°C, another 0.5 vol. of glutaraldehyde was added and after 1 h at 0°C, the suspension was washed with distilled water. The ghost preparation was post-fixed with 1% OsO_4 in 105 mM phosphate buffer, pH 7.4, and after washing with water, dehydrated in absolute alcohol.

Drug-induced shape changes were carried out by addition of an isotonic chlorpromazine solution (100 mM) to an erythrocyte suspension at 3% heamatocrit. The cells were incubated for 5 min at room temperature and viewed in the light microscope.

Microscopy. Erythrocytes and ghosts were observed under a light microscope with Nomarski interference optics before and after fixing with glutaraldehyde and OsO_4. The fixed preparation in absolute ethanol was dried in a Critical Point Dryer SPC 900 and shadowed in a Polaron Scanning Electron Microscope Coating Unit E 5000. The cells or ghosts were viewed in a Stereoscan SEM Type 96113 (Cambridge).

SDS-polyacrylamide gel electrophoresis. Ghosts from tris(2-chloroethyl)-amine-reacted erythrocytes prepared according to the method of Steck and

Kant [18] and tris(2-chloroethyl)amine-reacted ghosts were solubilized in a buffer containing 0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol. The solution was boiled for 2 min and subjected to SDS-polyacrylamide gel electrophoresis on a slab gel. The separating gel was prepared by mixing two acrylamide solutions (5 and 12% prepared according to the method of Laemmli [19]) in a gradient mixer to give a linear gradient from 5 to 12% acrylamide. A 5% stacking gel was used as described by Laemmli [19]. The gels were stained by Coomassie blue. ^{32}P-containing bands were detected by autoradiography using Kodak medical X-Ray film RP/R54.

Deformability. The deformability of red cells and red cell membranes (haemoglobin-free ghosts), prepared according to the method of Steck and Kant [18], was estimated by measuring the rheological behaviour of the suspensions at 30% haematocrit using glass capillary arrays (Galilea Optics, Sturbridge, MA) as described earlier [22]. The capillary length was 2 mm, the capillary diameter was 5 μm. At a driving pressure of 40 mmHg, the suspension medium (phosphate-buffered saline) and the suspension were alternatively perfused through the array. The relative flow rate was calculated by dividing the flow rate of the suspension medium by the flow rate of the suspension. Each measurement was made in triplicate using three different arrays.

Spectrin extractability. Ghosts from tris(2-chloroethyl)amine-reacted, freshly prepared erythrocytes as described above were suspended in 40 vol. of 0.1 mM EDTA, pH 8.0, and incubated at 37°C for 15 min [21]. The mixture was centrifuged for 45 min at 39 000 $\times g_{max}$. The supernatant was lyophilized and the protein content was estimated in the lyophilate and in the pellet by using the procedure of Lowry et al. [20]. Aliquots were solubilized and subjected to SDS-polyacrylamide gel electrophoresis.

Incorporation of $^{32}P_i$ into red blood cells. Fresh erythrocytes or erythrocytes after ATP depletion were incubated with tris(2-chloroethyl)amine using the standard conditions. 2.5 mCi $^{32}P_i$ per ml were added to a suspension of erythrocytes at about 50% haematocrit in 146 mM NaCl, 20 mM Tris-HCl, pH 7.4, containing 5 mM adenosine and 0.2% glucose. The mixture was incubated for 2 h at 37°C and washed three times with 146 mM NaCl, 20 mM Tris-HCl, pH 7.4, by centrifugation at 2500 $\times g_{max}$. Ghosts were prepared by hypotonic lysis according to the method of Steck and Kant [18]. Incorporated ^{32}P was detected by Cerenkov counting. Dephosphorylation was detected by incubation of the phosphorylated cells in 146 mM NaCl, 20 mM Tris-HCl, pH 7.4, for an additional 20 h at 37°C followed by the three washes as described above, and further treatment as in the case of phosphorylation.

Results

Shape changes of intact erythrocytes are affected by tris(2-chloroethyl)amine

To study the effect of tris(2-chloroethyl)amine on metabolically induced shape changes, discocytes which had been preincubated for 1 h with different concentrations of tris(2-chloroethyl)amine were incubated at 37°C for 20 h. Cells alkylated with a tris(2-chloroethyl)amine concentration of 2 mM maintained their shape, whereas untreated erythrocytes became crenated and formed echinocytes. As shown in Fig. 1, treatment with 1.0 mM tris(2-chloro-

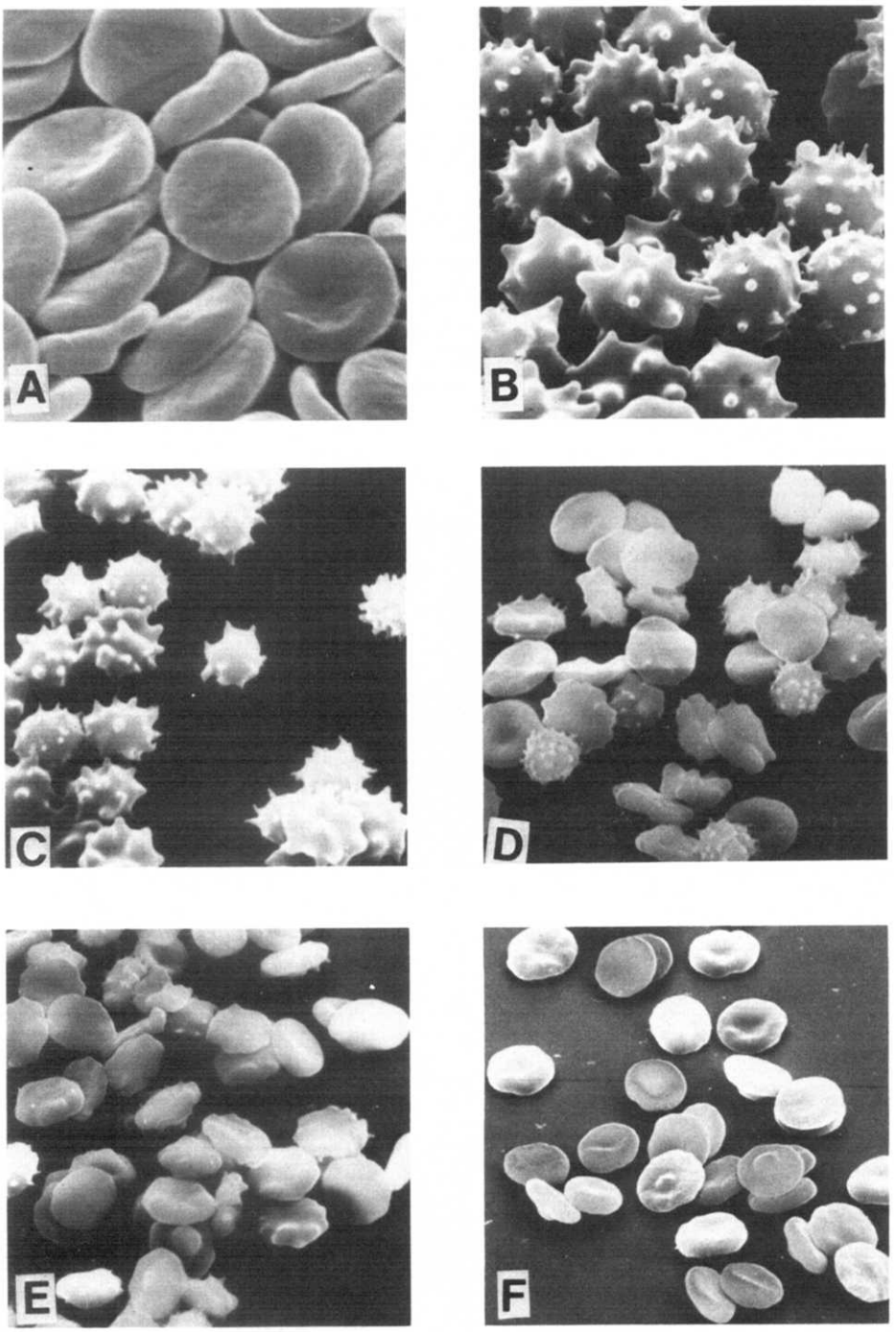
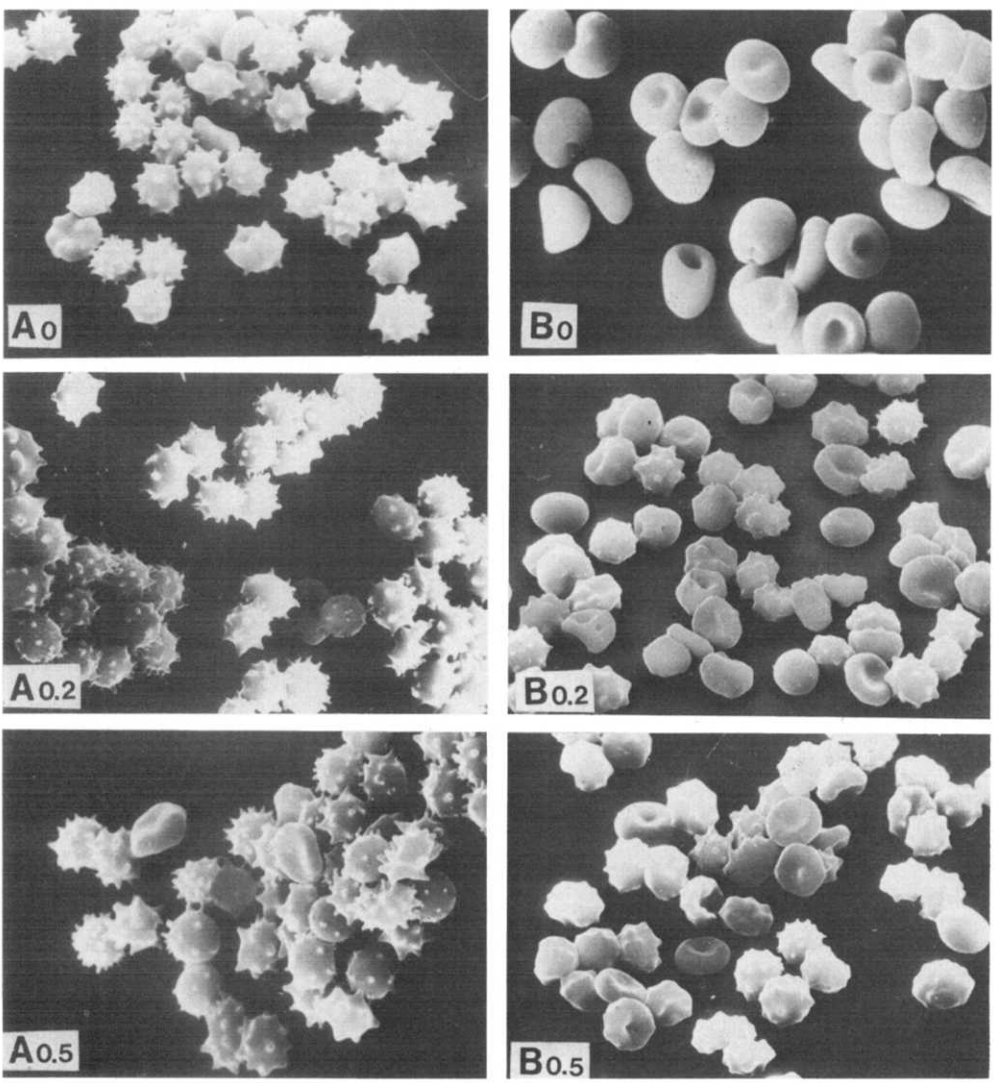


Fig. 1. Shape changes of erythrocytes after depletion of intracellular ATP. Erythrocytes (A) were alkylated with 0.2, 0.5, 1.0 and 2.0 mM tris(2-chloroethyl)amine as described in Materials and Methods and incubated for 20 h at 37°C in 146 mM NaCl, 20 mM Tris buffer, pH 7.4. A, cells before incubation; B—F, cells after incubation. B, untreated; C, 0.2 mM; D, 0.5 mM; E, 1.0 mM; F, 2.0 mM tris(2-chloroethyl)amine. The cells were fixed by glutaraldehyde and post-fixed by OsO_4 as described in Materials and Methods and examined in the scanning electron microscope.

ethyl)amine was not sufficient to arrest the cell shape entirely. In another experiment, discocytes were first incubated for 20 h at 37°C. After this period the cells had turned into echinocytes. The cells were then incubated for 1 h at 37°C with different tris(2-chloroethyl)amine concentrations (0, 0.2, 0.5, 1.0, 2.0 mM). The non-reacted reagent was washed out, 5 mM adenosine was added and after incubation for 2 h at 37°C, the cells were viewed in the microscope. As expected, the control erythrocytes had altered in shape to form stomatocytes (Fig. 2 B_0). The shape change of the erythrocytes which had been treated with tris(2-chloroethyl)amine, however, was inhibited depending on the degree of reaction with the alkylating agent. When the cells were pre-treated with 0.2—0.5 mM tris(2-chloroethyl)amine, transitions between the two forms could still be seen (Fig. 2 B), while erythrocytes which had been treated with 1—2 mM tris(2-chloroethyl)amine did not change in shape signif-



icantly. It should be noted, however, that the modifying agent itself has the tendency to transform echinocytes into discocytes (Fig. 2 A_1 and A_2).

Inhibition of drug-induced shape changes was studied by the addition of chlorpromazine to discocytes which had been pretreated with different concentrations of tris(2-chloroethyl)amine (Table I). To exclude the possibility of competition between free tris(2-chloroethyl)amine or its hydrolyzed product and chlorpromazine the cells had been washed three times with the incubation buffer. Higher concentrations of chlorpromazine were necessary to induce the transformation of alkylated dicocytes into stomatocytes. Alkylation with 2 mM tris(2-chloroethyl)amine rendered shape transformation difficult (Table I). This is consistent with the results obtained in the experiments with metabolically depleted erythrocytes.

In order to correlate the inhibition of shape alterations caused by the alkylating agent with changes in the molecular structure of the membrane, membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The gel pattern in Fig. 3 shows the disappearance of bands 1 and 2 (spectrin) with increasing tris(2-chloroethyl)amine concentrations. This is accompanied by the appearance of bands with higher molecular weight on top of the gel.

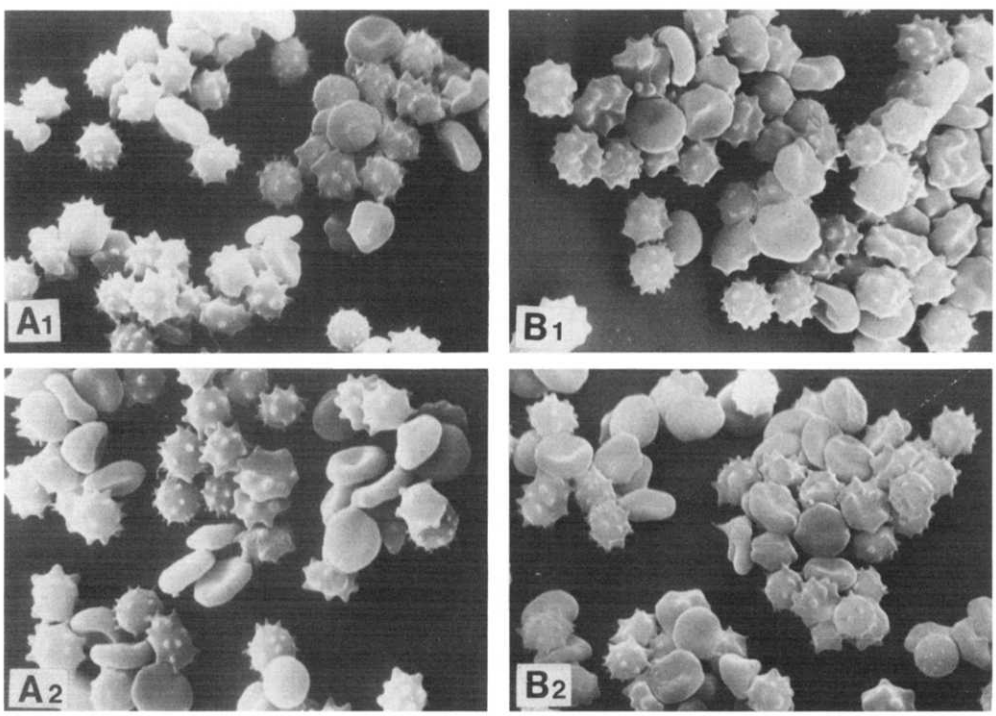


Fig. 2. Shape change of erythrocytes after treatment with adenosine. Erythrocytes were incubated in 146 mM NaCl, 20 mM Tris, pH 7.4, for 20 h and after centrifugation, alkylated with the indicated concentrations of tris(2-chloroethyl)amine by incubation for 1 h at 37°C in 0.37% NaCl, 100 mM Tris buffer, pH 7.0. Aliquots were taken and fixed for scanning electron microscopy as described in the legend to Fig. 1. The cells were then treated with 5 mM adenosine and incubated in 146 mM NaCl, 20 mM Tris buffer, pH 7.4, for 2 h. A, erythrocytes after incubation for 20 h followed by alkylation with tris(2-chloroethyl)amine. The numbers indicate the tris(2-chloroethyl)amine concentrations (mM). B, the same cells after addition of 5 mM adenosine and incubation for 2 h.

TABLE I

CHLORPROMAZINE-INDUCED SHAPE CHANGES ON TRIS(2-CHLOROETHYL)AMINE-PRETREATED ERYTHROCYTES

Erythrocytes in the disc form were incubated with the indicated concentrations of tris(2-chloroethyl)-amine for 1 h at 37°C in 0.37% NaCl, 100 mM Tris buffer, pH 7.0. The cells were washed tree times with the same buffer in order to remove non-covalently bound tris(2-chloroethyl)amine or its hydrolysis products and suspended in the incubation buffer to make up a haematocrit of 10%. A solution of chlor-promazine was added and after 5 min the cells were examined in the light microscope.

Pretreatment with tris(2-chloroethyl)amine (mM)	0	0.2	0.5	1.0	2.0
Chlorpromazine (mM) *	0.04	0.05	0.1	0.2	— **

* Concentration of chlorpromazine (mM) necessary to transform discocytes to 80% (±10%) stomato-cytes.

** 2 mM tris(2-chloroethyl)amine-pretreated erythrocytes showed a partial change (30—50%) into stomatocytes at a concentration of 0.2 mM, but lysed at higher concentrations.

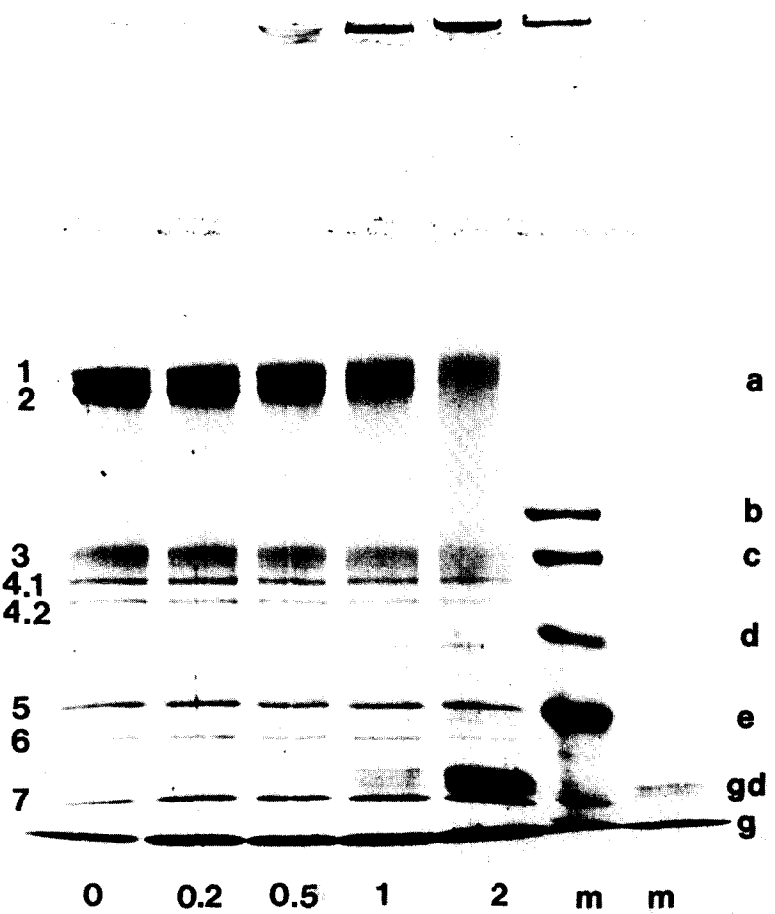


Fig. 3. SDS-polyacrylamide gel electrophoresis of membranes from alkylated erythrocytes. Ghosts were prepared from cells which had been treated with different concentrations of tris(2-chloroethyl)amine as in Figs. 1 and 2, and solubilized as described in Materials and Methods. Samples corresponding to about 20 μg protein, determined according to the method of Lowry et al. [20], were applied to a 5—12% gradient gel [19]. The numbers at the bottom indicate the concentration of tris(2-chloroethyl)amine in mM. m, marker proteins (a, myosin, M_r 200 000; b, β-galactosidase, M_r 130 000; c, phosphorylase B, M_r 94 000; d, bovine serum albumin, M_r 68 000; e, ovalbumin, M_r 43 000). 1 and 2, spectrin; 5, actin; 6, glyceraldehyde-3-phosphate dehydrogenase; g, globin; gd, globin dimer. (The nomenclature is adapted from that of Steck [12]).

A concentration of 2 mM tris(2-chloroethyl)amine causes the total disappearance of the spectrin bands and also a complete arresting of the cell shape. When the Coomassie blue-stained gels were viewed, no other membrane protein seemed to be affected. A new band appeared in the gel pattern representing a peptide with a molecular weight of 32 000 (Fig. 3 gd). Since it appeared at the same position as a globin dimer [17,23], it is thought to be a cross-linking product of haemoglobin which was non-covalently bound to the membrane and therefore isolated together with the ghosts.

Shape changes of ghosts

When ghosts were loaded with ATP and resealed in an isotonic medium, they exhibited a crenated shape. After incubation at 37°C for 30 min, they were transformed into cup-form structures (Fig. 4). Alkylation of erythrocytes did not influence this effect, even at a concentration of 2.0 mM tris(2-chloroethyl)amine, which inhibits the shape change of intact erythrocytes completely. Reaction of isolated ghosts with tris(2-chloroethyl)amine, however, arrested the crenated form at any tris(2-chloroethyl)amine concentration used. An untreated control preparation which had been incubated under the same conditions was able to undergo the expected shape change. With identical tris(2-chloroethyl)amine concentrations, ghosts incorporated much more tris(2-chloroethyl)amine than intact cells as detected by the uptake of radioactivity. The large amount of covalently bound tris(2-chloroethyl)-amine molecules (Fig. 5) suggests reaction with other proteins. The SDS-polyacrylamide gel electrophoresis pattern showed, in addition, the disappearance of several membrane proteins (Fig. 6).

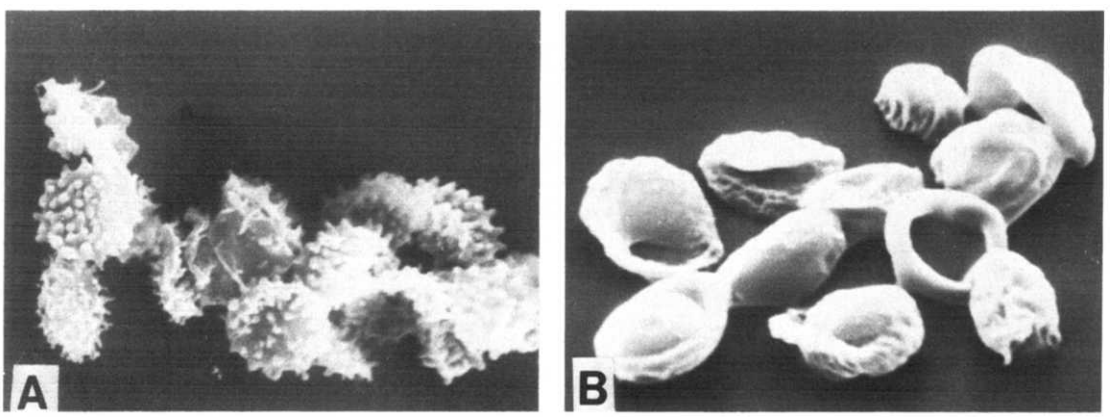


Fig. 4. Shape changes of ghosts. Ghosts were prepared from alkylated (0.2, 0.5, 1.0, 2.0 mM tris(2-chloroethyl)amine) or untreated erythrocytes by hypotonic haemolysis, suspended in a 2 mM Mg^{2+}-ATP solution and made isotonic by the addition of 1 vol. of 1.4 M KCl, 0.2 M NaCl, and 10 mM Tris, pH 7.4. The mixture was then incubated at 37°C for 30 min. Incubation of ghosts with tris(2-chloroethyl)amine (at the same concentrations as for erythrocytes) was carried out in 50 mM Tris buffer, pH 7.0, for 1 h before addition of the ATP solution. A, tris(2-chloroethyl)amine-treated ghosts after 30 min incubation. Before incubation, the same shape was assumed by untreated control ghosts and by ghosts from alkylated erythrocytes. B, ghosts from tris(2-chloroethyl)amine-treated erythrocytes after 30 min incubation at 37°C.

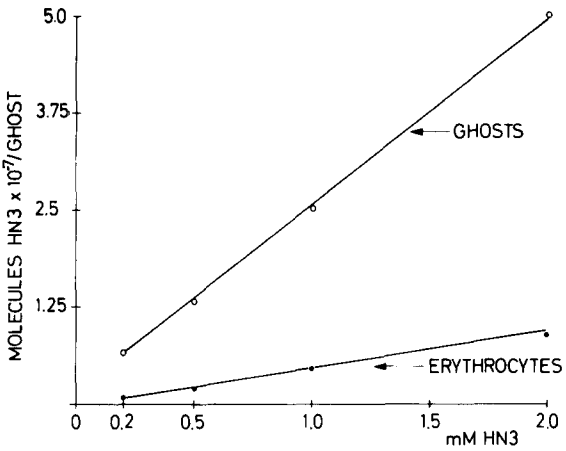


Fig. 5. Incorporation of radioactivity in the membrane of erythrocytes (●———●) and in ghosts (○———○). Washed human erythrocytes at 10% haematocrit were incubated at 37°C with the indicated tris(2-chloroethyl)amine concentrations (^{14}C-labelled) in 100 mM Tris, pH 7.0, containing 0.37% NaCl for 1 h. Ghosts were prepared by hypotonic haemolysis. For incorporation of radioactivity in ghosts, erythrocytes were lysed and, without further washing, suspended in 9 vol. of 50 mM Tris, pH 7.0. The mixture was incubated for 1 h at 37°C with the indicated tris(2-chloroethyl)amine (HN3) concentrations. The membranes were then washed with 5 mM phosphate buffer, pH 8.0. The amount of incorporated radioactivity and the protein content [20] were determined in both preparations. The value for the number of molecules of tris(2-chloroethyl)amine/ghosts were calculated from the specific activity of tris(2-chloroethyl)amine (5 mCi/mmol) and the protein content, assuming $5.7 \cdot 10^{-10}$ mg of protein/-ghost [25].

Deformability

The rheological behaviour of the red cells was not changed significantly by the pretreatment with 0.5 mM tris(2-chloroethyl)amine (Table II). At 1 and 2 mM tris(2-chloroethyl)amine, there was a slight reduction of the relative flow rate of the cell suspension indicating a decrease in deformability of the cells.

Spectrin extractability

Shape change, erythrocyte deformability and spectrin extractability are thought to be interrelated and dependent on the state of the spectrin. Extract-

TABLE II

DEFORMABILITY OF RED CELLS AFTER REACTION WITH TRIS(2-CHLOROETHYL)AMINE

Human erythrocytes were incubated in phosphate-buffered saline at 37°C for 1 h with the indicated tris-(2-chloroethyl)amine (HN3) concentrations. The rheological behaviour was determined in glass capillary arrays (capillary length 2 mm, diameter 5 μm, driving pressure 40 mm H_2O).

Control	HN3 (mM)		
	0.5	1.0	2.0
0.62 ± 0.08 *	+5.5 ± 10.6% **	−13.8 + 9% **	−34 ± 36% **

* Relative flow rate of a suspension at 30% haematocrit.

** Per cent deviation of the control value ± S.D. (n = 4).

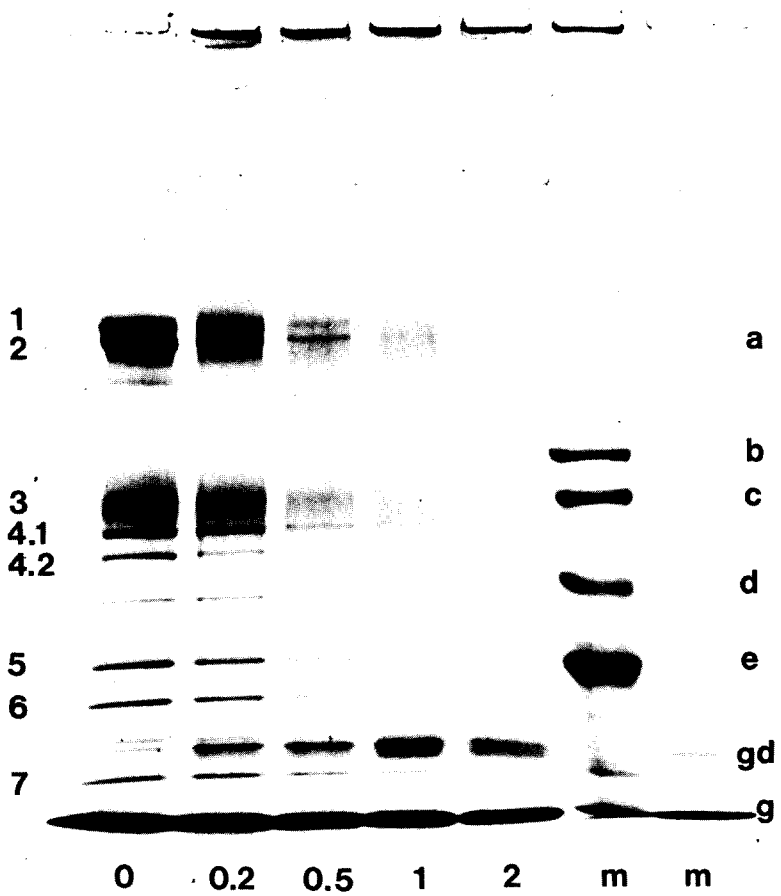


Fig. 6. SDS-polyacrylamide gel electrophoresis of alkylated ghosts. Ghosts prepared by hypotonic lysis without further washing were treated with the indicated tris(2-chloroethyl)amine concentrations as described in the legend to Fig. 6, solubilized and subjected to SDS-polyacrylamide gel electrophoresis (5—12% gel) as described in Materials and Methods. The nomenclature of the protein bands corresponds to that in Fig. 4.

ability of spectrin was decreased in alkylated erythrocytes, dependent on the concentration of tris(2-chloroethyl)amine used for alkylation (Fig. 7A). Examination of the EDTA-treated ghosts in the light microscope revealed small fragments from untreated erythrocytes and large connecting structures from alkylated cells (not shown). SDS-polyacrylamide gel electrophoresis of the EDTA-treated ghosts showed, as expected, retention of polymerized spectrin in the membrane (Fig. 7B). The protein represented by band 5 (actin) was also retained in the membrane, although it did apparently not react with the alkylating agent. Non-covalent bound haemoglobin was extracted by EDTA treatment in all preparations (Fig. 7B).

Phosphorylation of spectrin

In order to exclude the possibility that tris(2-chloroethyl)amine might be inhibiting phosphorylation of the spectrin subunit 2, ATP-depleted alkylated

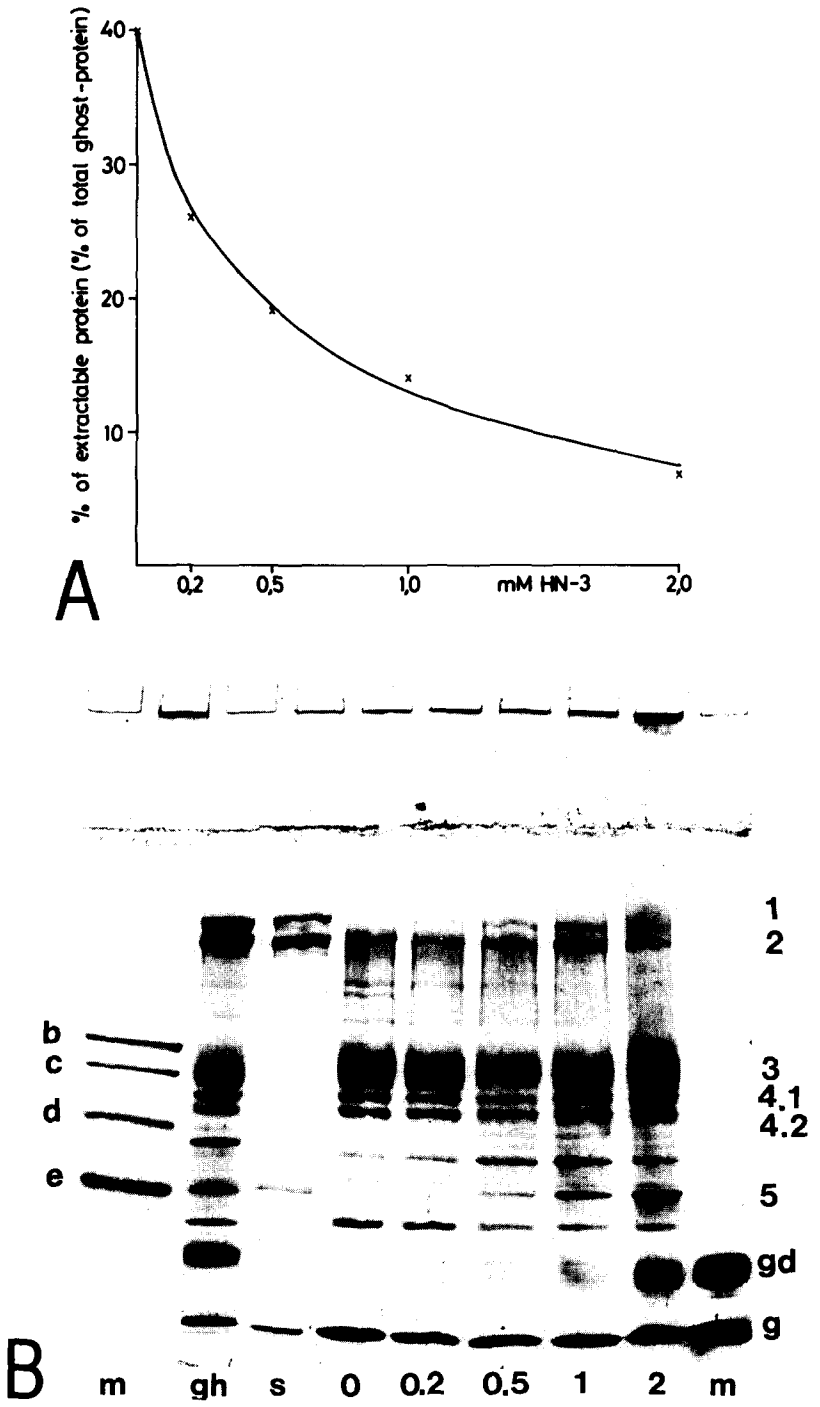


Fig. 7. Spectrin extractability of alkylated erythrocytes. Ghosts from tris(2-chloroethyl)amine-treated, freshly prepared erythrocytes were extracted by incubation with 40 vol. of 0.1 mM EDTA, pH 8.0, at 37°C for 15 min. After centrifugation at 39 000 × g_{max} for 45 min. the protein content was estimated according to the method of Lowry et al. [20] in the supernatant and in the pellet (A). For SDS-polyacrylamide gel electrophoresis (B), aliquots were taken from the pellet and solubilized as described in Materials and Methods. m, marker proteins as described in the legend to Fig. 4; gh, untreated ghosts. The numbers at the bottom indicate different tris(2-chloroethyl)amine concentrations in mM. s, EDTA extract of untreated cells. HN-3, tris(2-chloroethyl)amine.

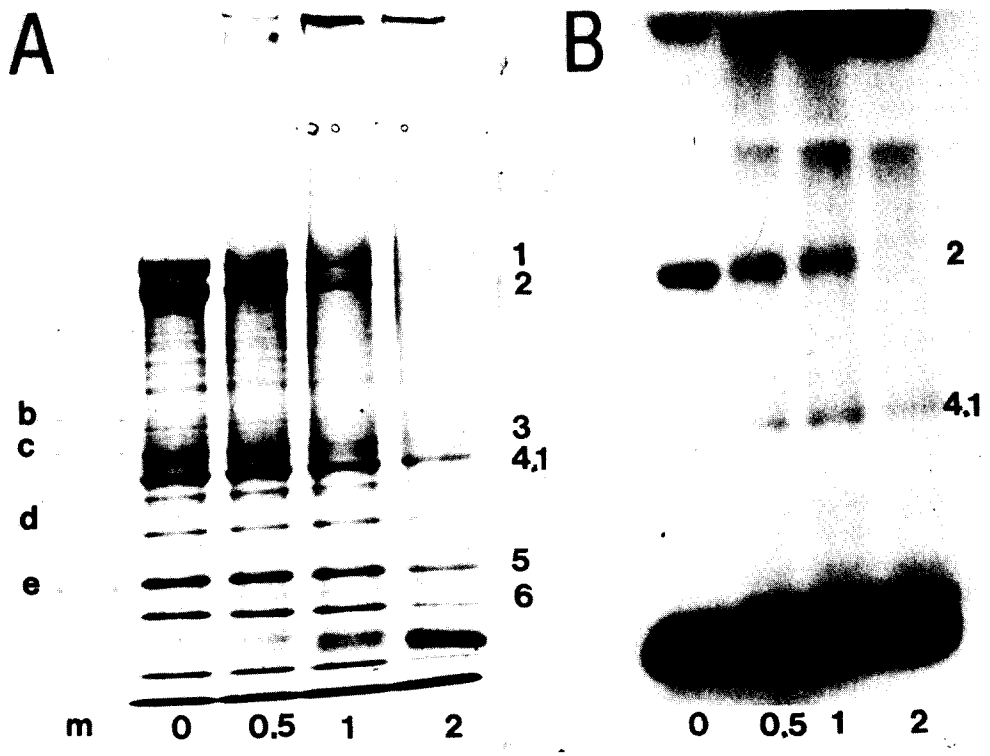


Fig. 8. SDS-polyacrylamide gel electrophoresis (5—12% acrylamide) of membrane proteins after incubation of erythrocytes with $^{32}P_i$. Tris(2-chloroethyl)amine-treated erythrocytes were incubated for 2 h with 2.5 mCi $^{32}P_i$/ml, 5 mM adenosine and 0.2% glucose in isotonic buffer as described in Materials and Methods. Ghosts were prepared, solubilized and subjected to polyacrylamide gel electrophoresis. A, gel stained with Coomassie blue; B, autoradiography of the dried gel. The numbers at the bottom indicate the different tris(2-chloroethyl)amine concentrations used for alkylation. m, marker proteins as outlined in the legend to Fig. 4.

cells were incubated with adenosine and $^{32}P_i$ for 2 h at 37°C. The uptake of $^{32}P_i$ was the same in cells which had been exposed to 0.5, 1.0 and 2.0 mM tris(2-chloroethyl)amine as the uptake in untreated control cells (not shown). Phosphorylation of spectrin component 2 was detected by SDS-polyacrylamide gel electrophoresis of the solubilized membranes and autoradiography of the gel (Fig. 8). In the case of alkylated cells, radioactivity was found in the polymerisation products of spectrin. 20 h of incubation at 37°C of cells pretreated with $^{32}P_i$ resulted in dephosphorylation and was in the same range for alkylated and for non-alkylated erythrocytes (data not shown).

Discussion

Using the trifunctional alkylating agent, tris(2-chloroethyl)amine, which penetrates the membrane of intact erythrocytes and reacts preferentially with spectrin [17], we were able to detect a correlation of this reaction with the inhibition of erythrocyte shape changes. Alkylation in 1—2 mM tris(2-chloro-

ethyl)amine was necessary to arrest the cell shape. These concentrations also caused complete polymerisation of spectrin, but did not alter significantly other membrane proteins as detected by SDS-polyacrylamide gel electrophoresis (Fig. 3). This is in contrast to the action of glutaraldehyde, which reacts with most of the proteins in membrane and cytoplasm and therefore serves as fixative for the cell shape. The defined distance of 7.5Å between two reactive groups in tris(2-chloroethyl)amine and the high reactivity of the compound seem to be the reason for the predominant reaction with the spectrin components 1 and 2. The number of molecules of tris(2-chloroethyl)-amine, however, which are bound to the membrane (Fig. 5) is too high in order to assume an exclusive reaction with spectrin. Therefore, we cannot exclude that cross-linking of minor amounts of membrane or cytoplasmic proteins may contribute to the observed effect. Reaction with phospholipids could not be detected in earlier experiments [17] using similar reaction conditions.

When echinocytes which had been obtained by ATP depletion were treated with 1 or 2 mM tris(2-chloroethyl)amine, the cells were partially transformed into biconcave discs even without the addition of adenosine (Fig. 2 A_1 and A_2). This effect might be due to stomatocytogenic properties of tris(2-chloroethyl)amine. It is possible that the polymerisation of spectrin by tris(2-chloroethyl)amine causes this effect. Sheetz and Singer [7] suggested that polymerisation of spectrin by phosphorylation is the mechanism for transformation of ghosts into biconcave discs.

The results obtained in the case of chlorpromazine-induced shape changes add further evidence to the idea that the shape of red cells is influenced by the state of spectrin. The concentration of chlorpromazine required for an 80—90% transformation of discocytes into stomatocytes increases with the degree of spectrin polymerisation generated by the reaction with tris(2-chloroethyl)-amine.

There is no simple explanation for the finding that ghosts prepared from alkylated erythrocytes, in contrast to the parent cells, undergo ATP-induced transformation into discs. It is also possible that the tris(2-chloroethyl)amine-induced spectrin polymerisation favours the biconcave shape. This would be similar to the effect we observed when echinocytes were treated with tris(2-chloroethyl)amine and the shape changed partially to the disc form (Fig. 2A). Another possibility could be that the cytoplasm with its large amount of haemoglobin is an important factor in determination of the cell shape. Without cytoplasm, the tris(2-chloroethyl)amine-modified spectrin might be flexible enough to allow the shape changes of the membrane. The shape fixation of ghosts which had been alkylated can be explained by the reaction of the membrane proteins with tris(2-chloroethyl)amine which occurs to a higher degree than in the case of intact red cells. This results in an enhanced polymerisation of proteins and thus in loss of flexibility of the membrane. The properties of the membrane might be also influenced by reaction of tris(2-chloroethyl)amine with haemoglobin, which is present in the ghosts used for these experiments (Fig. 6).

Since deformability of red cells is supposed to be determined by the state of spectrin [9,21,24], we measured the rheological properties of tris(2-chloroethyl)amine-reacted red cells. Physical forces were applied in the same range

as those which occur in capillaries in vivo under physiological conditions (wall shear stress 12 dyne/cm^2). Even under the condition of complete cross-linking of spectrin, the cells retained their ability to deform and pass through the 5-μm glass capillaries. The slight decrease in the relative flow rate in the case of 2 mM tris(2-chloroethyl)amine-treated cells indicates a decrease in membrane deformability. This is assumed to be due to the fact that at higher tris(2-chloroethyl)amine concentrations, other protein molecules are involved in the cross-linking reaction. The experiments show that fixation of the membrane against shape changes does not necessarily cause changes in the rheological behaviour of the cells.

Spectrin extractability has been found to be diminished in cases where the protein was assumed to be aggregated or polymerized; for instance, when the intracellular pH fell below 5.5 [9] or when erythrocytes were heated above 48°C [9,21]. Lux et al. [9] observed that the amount of inextractable spectrin increased during ATP depletion of the cells. Thus, it is not surprising that the tris(2-chloroethyl)amine-polymerized spectrin in our experiments becomes inextractable (Fig. 7). Intramolecular cross-linking between the spectrin units or intermolecular cross-linking between spectrin and other components of the membrane renders the fragmentation of the membrane in small vesicles difficult. The degree of extractability was followed by measuring the protein content in the extract and in the EDTA-extracted ghosts. It was not possible to determine the spectrin content directly because of the difficulty in identifying the polymerized products of spectrin. Although not cross-linked, actin (band 5 in the SDS-polyacrylamide gel electrophoresis) showed the same behaviour as the modified spectrin, i.e., it was retained in the membrane after EDTA treatment. Actin seems to form a complex with spectrin which resists the EDTA treatment. Removal of globin, however, was possible in all experiments.

A mechanism for the observed inhibition of shape changes by tris(2-chloroethyl)amine could be its interference with the phosphorylation-dephosphorylation cycle of the spectrin unit 2. Greenquist et al. [15] suggested that diamide, which also inhibits the adenosine-induced transformation of echinocytes into discocytes, influences the rate of spectrin phosphorylation and dephosphorylation. Our experiments showed that incorporation of $^{32}P_i$ into the polymerized spectrin occurred. Although only a detailed analysis of the polymerisation products might be conclusive, it is assumed that spectrin phosphorylation is not impaired by tris(2-chloroethyl)amine. Another argument against a possible interference with phosphorylation is the inhibition of drug-induced shape changes by alkylation with tris(2-chloroethyl)amine. It is unlikely that phosphorylation or dephosphorylation plays a role in drug-induced shape changes.

Using the reaction of the trifunctional mustard derivative, tris(2-chloroethyl)amine, we have investigated the fixation of the cell shape by this reagent against transformation induced metabolically or by the drug, chlorpromazine. We were able to correlate cross-linking of spectrin as detected by SDS-polyacrylamide gel electrophoresis with this shape fixation. This suggests that spectrin is an important component of the membrane for regulation and maintenance of the cell shape. However, we cannot excluding the possibility

that reactions with other proteins which are not detectable on SDS-polyacrylamide gel electrophoresis are contributing to the observed effects.

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