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Chemo-mechanical leak formation in human erythrocytes upon exposure to a water-soluble carbodiimide followed by very mild shear stress. I. Basic characteristics of the process

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Human erythrocytes treated with low concentrations (1–5 mM) of the carboxyl group-modifying reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) lose their native deformability in parallel with extensive cross-linking of the membrane skeleton. After treatment with higher (5–40 mM) concentrations of the reagent the cells develop a hitherto undescribed property: when subjected to even very low shear stresses (resuspension after packing by centrifugation or viscometric shearing at up to 4 s^{-1}) they become highly leaky to ions, lose their K^+ with a half-time of about 5 min and subsequently undergo hemolysis. Lysis is not accompanied by cell fragmentation as occurs with mechanical hemolysis, but is colloid-osmotic, due to the formation of aqueous membrane leaks with an apparent radius of about 3 nm. Leakiness and lysis affect an increasing fraction of the cell population, in relation to (a) the concentration of EDC applied, (b) the shearing intensity, and (c) particularly, the hematocrit during shearing. The physical parameter determining the mechanical component of this 'chemo-mechanical' leak formation is not predominantly the shear stress. Rather, cell–cell interactions of as yet undefined nature seem to be involved. The analysis of chemo-mechanical leak formation may provide interesting insights into the influence of mechanical forces on membranes.

Introduction

The barrier function of the human erythrocyte membrane can be disrupted [1,2] by mild oxidative modification [3] of SH groups with diamide, by stronger oxidants such as periodate [4] or *t*-butylhydroperoxide [5,6], by photo-oxidation [7], and by electric breakdown [8]. Moreover, application of high shear stresses at low apparent viscosity of the suspension [9] produces hemolysis and morphological changes although no such effects occur in native blood at shear stresses below 2000 dynes/cm^2.

In the studies presented here, we have investigated the effects, on the barrier properties of the erythrocyte, of the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC).

Carbodiimides have long been known as protein-modifying reagents with a predominant selectivity for carboxyl groups. They are widely used tools in the modification of enzymes, transport and channel proteins [10–12]. EDC and other

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Mes, 4-morpholineethanesulfonic acid.

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water-soluble carbodiimides have already been applied to human erythrocyte membranes by investigators of anion transport [13–15]. Besides irreversible inhibition of band 3-mediated anion exchange, cross-linking of membrane proteins was observed as a side effect [15].]

The reaction of a carbodiimide with a protein carboxyl group is known to produce an unstable *O*-acylisourea intermediate, which is reactive with exogenous or adjacent endogenous nucleophiles, in particular amino groups. These reactions lead to the formation of a carboxamide group or to protein cross-linking by an amide bond [16].

In this report it will be shown that carbodiimide-induced leak formation in the erythrocyte membrane requires an interplay between chemical modification of carboxyl groups and shear forces acting simultaneously on the modified cells. Since this leak formation involves chemical modification and frictional forces, the EDC-modified erythrocyte membrane may be a further interesting model for studying features which provide for the reliable barrier properties of plasma membranes.

Materials and Methods

Materials

Fresh human blood from healthy donors was obtained from the local blood bank. EDC and Mes were from Merck, Darmstadt. Dextrans were from Serva, Heidelberg (D 4 (M_r 4000–6000), D 8 (M_r 8000–12000)) and from Sigma, Munich (D 18 (M_r 15000–20000)). D 1 (M_r 900–1200, Promit®) was from Schiwa, Glandorf, F.R.G.

Methods

Preparation of cells

Freshly taken human blood anticoagulated with citrate was stored at 4°C in a conventional storage medium containing glucose (20 mM) and adenine (25 μM) and used for experiments within the following 7 days. Erythrocytes were isolated by centrifugation (5750 × *g*, 5 min), plasma and buffy coat removed, the erythrocytes washed three times in an excess of isotonic NaCl and packed at 5750 × *g* for 5 min.

Treatment of the cells with EDC and application of shear stress by subsequent centrifugal packing and resuspension of the cells ('ejection technique')

1 vol. of packed cells was suspended in 7 vol. of isotonic NaCl at room temperature. The pH was adjusted to 6.0 by stepwise addition of 1 vol. 100 mM Mes (free acid, pH 3.6) in isotonic NaCl and, if necessary, by small amounts of 1 M HCl. A Mes-buffered system was chosen because phosphate buffer (12.4 mM) led to a disappearance of EDC with a half-time of 45 min, in line with earlier observations of George and Borders [17]. EDC, freshly dissolved in 1 vol. of isotonic NaCl, was added after warming the suspension to 37°C. The resulting suspension (hematocrit 10%) was then routinely incubated for 20 min under gentle agitation in an incubator (SWR 5/1, Julabo, Heidenheim), using a shaking frequency of 100/min at an amplitude of 1.5 cm. Subsequently, the EDC-pretreated cells were packed to various densities (55–95% hematocrit) by centrifugation in small cylindrical centrifuge tubes (2 ml, Sarstedt, Nümbrecht, F.R.G.). The supernatant was removed by suction. A tightly fitting syringe plunger with two holes was then inserted into the tube (Fig. 1) closely above the cell pellet. The hematocrit of pellets obtained under these packing conditions was derived indirectly from the K^+ and Na^+ contents of the pellets after washing a known amount of the cells in isotonic choline chloride.

The cell pellet (0.2 ml) was washed out of the tube by a flow of isotonic NaCl passing through the cylinder from an inlet to an outlet (opening diameters 2.0 mm) in the plunger. Usually 5 ml saline were driven through this system within 1 s

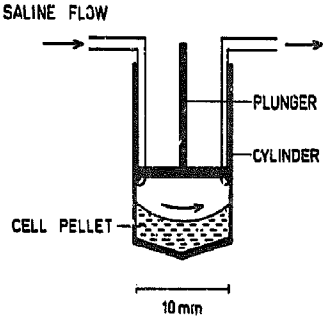

Fig. 1. Resuspension device for applying shear stresses by the ejection technique. For details see text.

by an automatic pipette (Diluette, Brand). The resuspended cells were ejected directly into a vessel containing 15 ml isotonic NaCl. This suspension was incubated at room temperature and pH 6. K^+-release from the cells was measured by taking 1 ml samples after appropriate intervals which were centrifuged and analysed for K^+ content in the supernatant by flame photometry (Instrumentation Laboratory Type 543) using an internal lithium standard. Hemolysis was estimated by measuring the hemoglobin content (as cyanmethemoglobin) in the supernatant by spectrophotometry (Hitachi model 100-20). All values were normalized to the total content of K^+ and hemoglobin in the suspension, measured after solubilisation of the cells by addition of a small amount of Triton X-100.

The procedure reported here of application of low shear stresses to erythrocytes by resuspension after centrifugation is called 'ejection' in the following. The resuspension at a hematocrit of 1% also served to lower the concentration of EDC trapped in the pellet to an ineffective level.

Treatment with EDC and application of shear stress in a rotational viscometer (viscometric shearing)

5 vol. of packed erythrocytes were mixed with 3 vol. isotonic NaCl. Stepwise addition of 1 vol. 300 mM Mes (pH 3.3) in isotonic NaCl and of small amounts 1 M HCl lowered the pH to 6.0. This suspension was filled into a syringe cylinder from the open end and cooled to 0 °C. 1 vol. of a 10-fold-concentrated ice-cold EDC stock solution was then added under careful mixing. The low temperature prevented the onset of the reaction of EDC since at 0 °C none of the EDC effects described below occurs, even at 80 mM EDC (data not shown). The plunger was then set into the syringe and 3 ml of the ice-cold erythrocyte suspension (50% hematocrit) filled into a viscometer with Mooney (a combination of cone-plate and Couette) configuration (Brookfield LVT 0.5). The reaction with EDC was then started by rapid warming of the viscometer chamber to 37 °C. Shearing began after the 5 min required for reaching this temperature. Shear rates were varied between 0.4 and 78.0 s^{-1}. After shearing the cells for periods between 20 and 60 min, the suspension was carefully removed from the viscometer by a pipette at minimal suction. It was added to 49 vol. isotonic NaCl to give a suspension of 1% hematocrit, which was incubated at room temperature for 60 min and then centrifuged. K^+-release into the supernatant and hemolysis were then measured in the same way as described for the ejection method.

Cell morphology and measurement of membrane rigidification

Changes of erythrocyte shape were observed by interference contrast microscopy. Membrane rigidification by EDC was evaluated semiquantitatively by the suppression of the echinocyte formation usually occurring upon addition of the echinocytogenic reagent, sodium salicylate, to erythrocyte suspensions [18].

Furthermore, the mean elongation of erythrocytes subjected to viscometric shearing was quantified by laser ectacytometry, using the prototype of a simple apparatus (Rheodyn, Tecel; Kreuzlingen, Switzerland). Measurements were carried out in phosphate-buffered saline solutions containing Dextran T 40 (Pharmacia) (viscosity = 23 cP at 23 °C) at shear stresses between 12.5 and 105.0 $dyn \cdot cm^{-2}$ and a plate–plate distance of 0.5 mm. Elongations (%) were calculated from $(A - B)/(A + B) \times 100$, where A and B are values of light intensities taken from the ectacytometric diffraction patterns. A is related to the length and B to the width of the cells [19].

Estimation of the leak size

The apparent size of membrane leaks induced by the combination of EDC and shear stress was determined using nonelectrolytes of varying molecular size as potential protectants against colloid-osmotic lysis as described by Heller et al. [20].

Results and Discussion

EDC treatment induces membrane rigidification

Treatment of erythrocytes with low concentrations (1–5 mM) of EDC at pH 6 and 37 °C under gentle shaking leads to a marked decrease of cell deformability. This loss of deformability is indicated by the observation that cells no longer turn

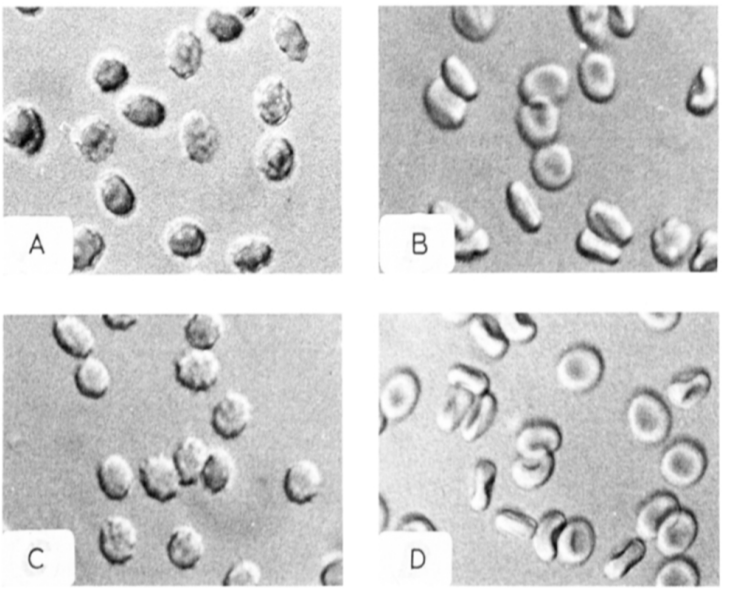

Fig. 2. Shape of human erythrocytes in the presence of the echinocytogenic agent sodium salicylate, before and after treatment with EDC. The cells were incubated with or without EDC at the indicated concentrations for 20 min (pH 6.0, 37°C), then added to an equal volume of isotonic saline medium containing 50 mM or 150 mM sodium salicylate. These suspensions were inspected, between two plastic cover slips, by interference contrast microscopy and photographed. (A) Control cells in 25 mM salicylate. (B) Cells treated with 1 mM EDC, then suspended in 25 mM salicylate. (C) Cells treated with 1 mM EDC, then suspended in 75 mM salicylate. (D) Cells treated with 5 mM EDC, then suspended in 75 mM salicylate.

into echinocytes when they are transferred into an isoosmotic medium containing high concentrations of the echinocytogenic agent sodium salicylate (25 or 75 mM), after exposure to EDC at various concentrations for 20 min (Fig. 2). The effect occurs within less than 1 min.

The loss of deformability was further substantiated by ectacytometric measurements of shear-induced cell elongations. While normal erythrocytes progressively elongate up to 30% when subjected to increasing shear stresses between 0 and 90 $dyn \cdot cm^{-2}$, cells pretreated with EDC are much less deformable or even undeformable (Fig. 3). This effect, which reaches its maximum at 3 mM EDC, is irreversible, since removal of the reagent by washing the cells and subsequent incubation for 1–2 h will not restore deformability. The loss of deformability is due to selective membrane rigidification and not the consequence of a hardening of the whole cell contents as, for example, in the case of extensive glutaraldehyde treatment [21]. Ghosts can be prepared from EDC-

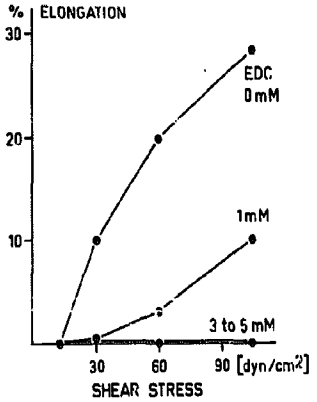

Fig. 3. Decrease of the shear-induced elongation of erythrocytes after treatment with EDC. Cells were treated with various concentrations of EDC for 20 min (pH 6.0, 37°C). Elongation under viscometric shearing was then measured in a laser ectacytometer in saline solutions (pH 7.4) containing Dextran T 40 (Pharmacia) at a viscosity of 23 cP at 23°C and increasing shear stresses as given on the abscissa.

treated cells (20 mM, 20 min, pH 6) by osmotic lysis, which contain only very little membrane-attached hemoglobin.

The cell interior obviously remains in a liquid state during EDC treatment. On the other hand, SDS-polyacrylamide gel electrophoresis revealed extensive cross-linking (aggregation) of all membrane proteins (data not shown), as already observed by Craik and Reithmeier [15]. This cross-linking was very pronounced even at the lowest concentrations of EDC, leading to a loss of deformability, rendering impossible resolution of the cross-linked bands on the gels. Hemoglobin, on the other hand, was not converted into dimers or oligomers (data not shown). Rigidification is thus likely to derive from cross-linking of the membrane skeleton by formation of endogenous amide bonds.

Cross-linking and rigidification, which are most pronounced at pH 6, could not be prevented by added nucleophiles such as glycine methyl ester (data not shown). This lack of effect is not due to an impermeability of the nucleophile (pK' about 7) which rapidly enters the erythrocyte by nonionic diffusion of the deprotonated form of the base. This could be demonstrated using a technique previously applied to study nonionic diffusion of organic acids in erythrocytes [22]. In spite of being permeable, the exogenous nucleophiles can thus obviously not compete with the endogenous amino groups involved in the cross-linking reactions.

Shear stress applied to EDC-treated cells induces membrane leaks and hemolysis

Cells treated with EDC (10–40 mM) in dilute suspension (10% hematocrit) under very gentle agitation (100 strokes per min) neither hemolyse nor lose intracellular K^+ during the 20 min EDC treatment. When such pretreated cells are, however, packed by centrifugation and resuspended by a flow of saline into a small vessel ('ejection' procedure as described in Methods), a rapid and massive loss of potassium and of hemoglobin occurs. A typical experiment is shown in Fig. 4.

As becomes evident from the graph, untreated control cells subjected to shear by ejection neither lose K^+ nor hemolyse, while cells treated with

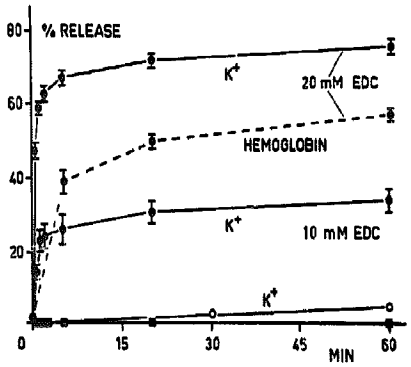

Fig. 4. Time course of K^+-release (———) and hemolysis (— — —) of control cells (■) and of cells treated with various concentrations of EDC at pH 6.0 and 37°C. After 20 min EDC treatment the cells were either sheared by ejection as described in Methods (●) or just diluted under minimal shearing (○) and incubated at room temperature. Samples were taken from the suspension after appropriate time intervals and analysed for K^+ and hemoglobin. Mean values ± S.D. from 4–6 experiments.

EDC and sheared by ejection lose considerable fractions of the total intracellular K^+ within the first minute after ejection in relation to the concentration of EDC. This rapid phase, the duration of which depends on the concentration of EDC, is followed by a second, much slower phase of K^+ loss. Subsequent to K^+-release hemolysis occurs with a somewhat longer half-time, which is also biphasic, a rapid phase followed by a much slower phase. The extent of induced leakiness is well reproducible as indicated by the rather low standard deviations.

Cells transferred from the EDC-containing incubation medium into an excess of isotonic NaCl solution, avoiding shear stresses as far as possible, lose K^+ only very slowly (Fig. 4) without detectable hemolysis during the time-period studied. This shear-independent leakage has about the same rate as the second, slower phase of K^+-leakage after EDC and shearing. This slower phase will not be considered in the following. To quantify the extent of leakiness deriving from the joint effect of chemical treatment and low shear stress, the K^+-release from the cells after 60 min was used as a standard parameter.

It might be questioned whether the observed leakiness is induced by centrifugation of the

EDC-treated cells or by the shear resulting from resuspension by ejection. We could demonstrate that no K^+ appears in the supernatant after mere centrifugation even after prolonged incubation of the centrifuged sample to allow for diffusion of K^+ out of the pellet (data not shown). Therefore it seems safe to assume that centrifugation is not the mechanical stress responsible for leak formation.

The nature of the K^+-release and lysis induced by EDC treatment and subsequent shearing

Shear-induced hemolysis of EDC-pretreated cells is colloid-osmotic

Leakiness resulting from EDC treatment and shear stress did not lead to complete loss of K^+ and hemoglobin. This raised the question of the mechanism underlying the loss of cellular constituents and cell lysis. The possibility of cell fragmentation as observed in the case of mechanical hemolysis of normal erythrocytes at high shear rates [23] can be dismissed, since cells were morphologically intact after the treatment and no fragments or vesicles could be detected by microscopic inspection.

The most common mechanism of red cell lysis in isotonic salt media is the colloid-osmotic type [24], due to leaks permeable to small ions such as Na^+, K^+ and Cl^- but not to large intracellular solutes like hemoglobin. A colloid-osmotic type of lysis may be assumed if lysis is suppressed by large molecules added to the extracellular medium, which do not permeate the induced leaks and thus counterbalance the osmotic drag of intracellular impermeant solutes, which otherwise would lead to swelling and lysis. Such protectants can also be used to size the induced leaks [20].

Nonelectrolytes of increasing molecular weight were added at a concentration of 40 mM to the saline medium used for resuspending and shearing of packed, EDC-treated cells by the ejection method. Hemolysis was determined after 24 h incubation at room temperature. As becomes evident from Fig. 5, sucrose and raffinose provided only minor protection. Increasing but incomplete protection against hemolysis was obtained with dextrans D 1, D 4 and D 8, indicating a heterogeneous distribution of leaks over the cell population. Only dextrans larger than D 8 fully protected

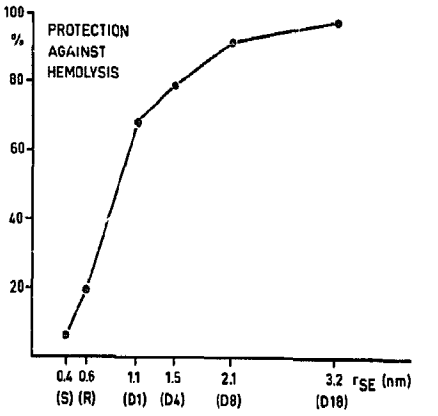

Fig. 5. Estimation of apparent pore radii in cells subjected to EDC treatment and shear stress, based on protection against colloid-osmotic hemolysis by nonelectrolytes of increasing molecular size. The cells were treated with 20 mM EDC as usual, sheared by ejection after centrifugation at 9600 × *g* for 20 s, and incubated for 24 h at room temperature in the presence of 40 mM of protectant. Subsequently the supernatants were analyzed for K^+-release and hemolysis. S = sucrose, R = raffinose, D 1 = dextran 1000, D 4 = dextran 4000, D 8 = dextran 8000, D 18 = dextran 18000. r_{SE} = Stokes-Einstein radii, adopted from Ref. 25

the cells against hemolysis. K^+-release was not affected by the additives. Thus, lysis induced by EDC treatment and subsequent shearing is colloid-osmotic in nature and based on the formation of defects with radii up to 3.2 nm, the Stokes-Einstein radius of D 18 [25].

The induced leaks are not spontaneously reversible

Dextran 18 was also used as a protectant against hemolysis in order to determine whether the induced leaks are permanent or can reseal. To this end cells treated with 20 mM EDC were ejected into an isotonic KCl medium containing 40 mM D 18. In such a medium net K^+-release and hemolysis will not occur since there are no appropriate driving forces. After incubation of the cells in that medium for 2 h at 37 °C the cells were packed by centrifugation and sheared by ejection into isotonic NaCl without protectant. The extent of K^+-release and hemolysis after this interposed incubation period was not different from that of cells ejected into isotonic NaCl immediately after treatment with EDC (data not shown). This find-

ing indicates that no resealing had taken place during the 2 h incubation.

Chemical parameters affecting the extent of fractional leak formation

Under the conditions of the experiment shown in Fig. 4 the release of intracellular K^+ is not complete. The extent of the release depends on the experimental conditions, such as the concentration of EDC or the extent of shear stress. As already evident from Fig. 4 and shown in more detail in Fig. 6A, the extent of the initial rapid K^+-release after shearing EDC-treated erythrocytes by ejection is a function of the concentration of EDC. Leakage becomes detectable at about 5 mM. At this level of EDC the cells are already completely undeformable under our experimental conditions. Rigidification may thus be a prerequisite for leak formation, but other modifying effects of EDC are certainly also necessary for this event. In order to obtain 100% rapid K^+-release, concentrations of EDC exceeding 40 mM are required. A similar concentration dependence was obtained when the shear stress was applied to the EDC-treated cells by cone-plate viscometry as described in the Methods (Fig. 6B). Besides the concentration of EDC, other conditions of the chemical modification prior to application of shear stress influence the extent of induced leakiness.

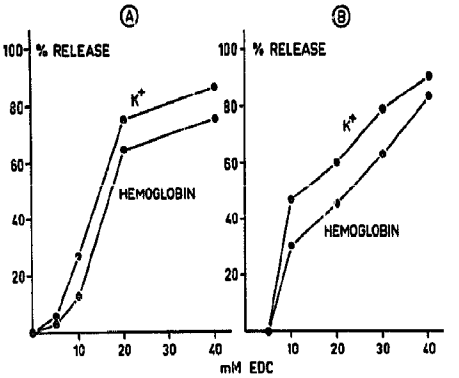

Fig. 6. (A) Influence of the concentration of EDC on the extent of K^+-release. K^+-release from cells treated with various concentrations of EDC and sheared by ejection after packing by centrifugation 9600× g for 20 s was measured 1 h after the ejection. (B) Influence of the concentration of EDC on the extent of leakiness for cells subjected to viscometric shearing. K^+-release and hemolysis are shown for cells sheared in the viscometer for 1 h at a shear rate of 78 s^{-1} and a hematocrit of 50% at the indicated EDC concentrations and subsequently incubated further for 1 h at room temperature after diluting the suspension to a hematocrit of 1% with isotonic NaCl.

(i) At a given concentration of EDC, the extent of K^+-release induced by subsequent shearing increases during the first 10 min of EDC treatment, but then reaches a saturating value (data not shown). Obviously, the chemical reaction sensitizing the cells to mechanical forces is completed after that time.

(ii) The extent of leakiness produced by subsequent shearing decreases with increasing pH of the suspension during EDC treatment above pH 6, which is optimal for this reaction. No leak formation occurs above pH 7.0, although the cells still become rigidified at that pH.

(iii) The extent of leakiness produced by subsequent shearing increases with increasing temperature during the EDC treatment between 0° and 37° C. No measurable effects of EDC (1–80 mM) became evident at 0° C, even after 60 min treatment (data not shown).

Rapid release of cell contents after exposure to EDC and shearing is a population phenomenon

As is evident from Figs. 4 and 6, the extent of rapid K^+- and hemoglobin release from cells exposed to EDC and shearing varies considerably depending on the conditions of the treatment. Since leak formation is irreversible, as shown above, this fractional nature of the leakiness is most likely the result of a population phenomenon in the sense that parts of the cell population lyse completely while a residual fraction remains intact. This assumption could be proven by the following experiments. Samples taken from a suspension of cells treated with EDC and sheared by ejection were centrifuged very gently (290 × g for 2 min) to separate the remaining intact cells from the supernatant containing the majority of the ghosts. These remaining cells were either resuspended in a defined volume of isotonic saline and counted in a cell counter (ELT 800, Ortho Diagnostics) or lysed to determine cellular K^+ and hemoglobin content as described.

As shown in Table I, the increase of the fractional lysis with increasing concentration of EDC results in a corresponding loss of cells, while the

TABLE I

CELLULAR CONTENTS OF HEMOGLOBIN (MCH) AND OF K^+ IN RESIDUAL UNLYSED CELLS AFTER LYSIS INDUCED BY 20 MIN EXPOSURE OF ERYTHROCYTES TO EDC AND SUBSEQUENT SHEARING BY EJECTION

For experimental details see Methods and text. Mean values from two experiments.

EDC (mM)	K^+ released (%)	Hemoglobin released (%)	Cells disappeared (%)	MCH (pg/cell)	K^+ (fmol/cell)
0	0	0	0	30.2	8.0
5	5.9	2.7	(0)	30.3	8.0
10	29.7	17.8	17.7	30.9	7.1
20	77.7	66.0	70.3	33.1	7.8
40	83.9	73.8	80.7	36.8	6.6

hemoglobin and K^+-contents of the residual unlysed cells are not very different from those of the original cells. Due to the very high extent of leakiness induced by EDC and shearing the resulting lysis is thus essentially an all-or-none phenomenon within the time regime of our study.

Mechanical parameters affecting the extent of fractional leak formation

Since the observed leaks seem to result from a joint effect of chemical and mechanical modification, shear-related parameters should also play a role in the occurrence of these leaks. If this is the case, the extent of leakiness should also be a function of the shear stress applied and of the hematocrit of the suspension. This problem was investigated in four sets of experiments.

Very low shear stresses suffice to evoke leak formation in EDC-treated cells

When cells treated with EDC and packed by centrifugation are sheared by ejection, the shear stress applied can be varied by varying the flow rate of saline between 0.5 and 5.0 ml/s (at a constant packing density). This is easily achieved by varying the pressure under which the saline is driven through the device. The fraction of leaky cells decreased only slightly when the cells were ejected at a flow rate of only 0.5 ml/s as compared to the usual ejection at 5 ml/s (data not shown). Even this minor decrease is probably merely due to an incomplete resuspension of the pellet. Thus, either the shear rate plays only a minor role in leak formation or the shear stresses applied in the ejection procedure are already in a saturating range.

In order to characterize in more quantitative terms the role of the shear rate, cells were sheared at a hematocrit of 50% in a rotational viscometer in the presence of EDC for 60 min. The fraction of leaky cells was determined 60 min after shearing as described in Methods. The shear rate in the viscometer used could be varied between 0.4 s^{-1} and 160 s^{-1}. For cells suspended at an EDC concentration of 20 mM and a hematocrit of 50%, corresponding to an apparent suspension viscosity of 5.0–6.0 cP, a saturation in leak formation is already reached at a shear rate of only 4 s^{-1} (Fig. 7). Very low shear rates thus obviously suffice to induce leak formation.

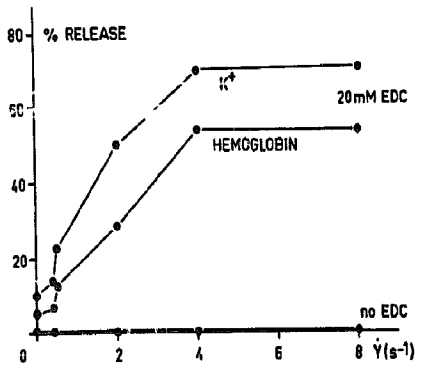

Fig. 7. Influence of the viscometric shear rate on the extent of leakiness in EDC-treated cells. Cells were sheared in the presence of 20 mM EDC at a hematocrit of 50% for 1 h in the viscometer at the indicated shear rates. K^+ released and extent of hemolysis were measured 1 h after subsequent dilution of the suspension to a hematocrit of 1%.

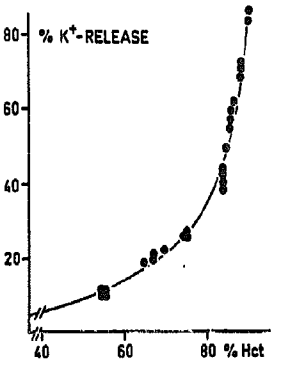

Fig. 8. Influence of the packing density of EDC-modified cells on the extent of K^+-release. Cells treated with 20 mM EDC for 20 min were packed, by centrifugation between 750 × g for 20 s and 10500 × g for 120 s, to hematocrit values indicated on the abscissa prior to application of shear stress by ejection. K^+-release into the supernatant was measured 1 h after resuspension.

The fraction of leaky cells increases with increasing density of packing prior to shearing

The density of cell packing prior to shearing proved to be of high importance for the extent of induced K^+-release. The packing density was varied by varying the duration (between 20 s and 120 s) and intensity (between 750 × g and 10500 × g) of the centrifugation of the cells prior to ejection by saline. As shown in Fig. 8, the fractional loss of K^+ upon shearing by ejection at a fixed flow rate increased hyperbolically with increasing hematocrit of the packed cells.

This extraordinary dependence of the induced membrane damage on the packing density indicated an important role in leak formation either of the suspension viscosity, which is essentially a function of the hematocrit in saline suspension, or of as yet undefined cell–cell interactions operative during shearing. These alternatives were tested in the following.

Suspension viscosity is not the parameter responsible for the effect of packing density

Cells were treated with EDC and subjected to viscometric shearing at low hematocrit in media set at the same or even higher viscosities than those of the usual 50% hematocrit suspensions in saline medium. This was achieved by addition of dextrans. When these suspensions were subjected to a saturating shear rate of 78 s^{-1} for 20 min at 40 mM EDC, the resulting K^+-release was considerably lower in the low-hematocrit suspensions in spite of the same or even higher viscosities (Table II). These findings would seem to indicate that the effect of packing density described in Fig. 8 reflects cell–cell interactions.

The fraction of leaky cells is a function of cell density at constant shear stress

In order to substantiate the role of direct cell–cell interactions, cell suspensions were sheared in the viscometer under conditions of constant shear stress (τ) of 0.52 dyn · cm^{-2} ($\tau = \dot{\gamma} \cdot \eta$), but at various hematocrit values.

Since an increasing hematocrit increases the apparent viscosity (η) of the suspension [26], and thus influences the effective shear stress (τ), the shear rates ($\dot{\gamma}$) were decreased when suspensions of higher hematocrit (higher viscosity) were sheared, in order to keep the shear stress constant. If shear stress were the relevant parameter, the extent of leakiness after viscometric shearing should remain constant in this set-up. This is, however, not the case. As is evident from Fig. 9, the extent of leakiness increased considerably when the hematocrit was raised from 10 to 50% at constant shear stress.

Taken together, these results indicate that leaks in cells modified with EDC already occur at minimal shear stresses when these shear stresses derive from cell–cell interactions at high hema-

TABLE II

DEPENDENCE OF THE EXTENT OF LEAKINESS (% K^+-RELEASE) ON THE HEMATOCRIT AND THE VISCOSITY OF THE SUSPENSION

Leakiness was induced in erythrocytes by shearing at 78 s^{-1} for 20 min at 40 mM EDC, pH 6. K^+-release was determined as described in Methods.

	Suspension medium	Suspension viscosity (cP)	Hematocrit (%)	K^+-release (%)
A.	Saline	5.5	50	92.7
B.	Saline + D 18, 160 g/l	5.5	10	41.2
C.	Saline + D 40, 160 g/l	10.0	10	47.1

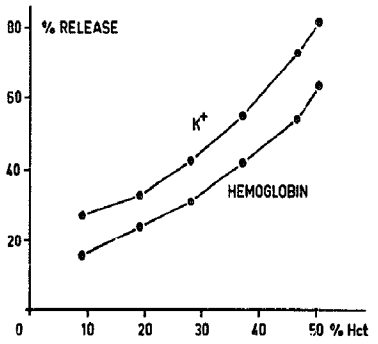

Fig. 9. Hematocrit dependence of the extent of leakiness in EDC-treated cells sheared at a constant mean shear stress. Cells were sheared for 20 min at an EDC concentration of 40 mM and a constant shear stress of 0.52 dyn·cm^{-2} at various values of hematocrit as indicated. The shear stress was kept constant by raising the shear rate compensatory to the decrease of viscosity at decreasing hematocrit. K^+-release and hemolysis were measured 1 h after dilution of the suspension to a hematocrit of 1%.

tocrit, while even high shear stresses cannot compensate for a low hematocrit in more dilute suspensions in which the cells are at greater distance from each other.

Concluding remarks

In this work we present a new kind of experimentally induced leak formation in the erythrocyte membrane differing from established leaks involving purely chemical [1–7] or purely mechanical types [9] of membrane damage. The leak formation reported here requires a chemical modification by EDC in parallel with very low shear stresses resulting from cell–cell interactions. This combination induces stable membrane leaks of defined but heterogeneous diameters. We propose the term 'chemo-mechanical' for this mode of leak formation. To what extent the membrane rigidification also produced by the treatment with EDC contributes to chemo-mechanical leak formation is as yet unknown. The different ranges of EDC concentrations and exposure times required for membrane rigidification and for leak formation at least indicate that leak formation involves additional effects of EDC. Membrane rigidification induced by other cross-linking reagents (diamide, glutaraldehyde) does not render erythrocytes more susceptible to the low mechanical stresses applied here [27]. Pretreatment of cells with the SH-alkylating agent *N*-ethylmaleimide induces increased shear sensitivity of erythrocytes [27,28], but the resulting lysis is accompanied by cell fragmentation, characteristic for mechanical lysis. Chemo-mechanical leak formation occurs without any fragmentation or shedding of membrane vesicles.

As an additional peculiarity, this new type of leak formation exhibits a somewhat unusual dependence on the intensity of both the chemical modification (by variation of EDC concentration) and the frictional cell–cell interactions (e.g., by variation of the centrifugational packing density, of the hematocrit during viscometric shear, and of shear stress). Both components at least predominantly affect the fraction of cells that become leaky, while there is not much variation in the extent of leak permeability of the leaky fraction of the cells, as characterized by the rate of K^+-release (see Fig. 4). This feature suggests that chemo-mechanical leak formation is an all-or-none phenomenon for the individual cell, comparable to osmotic lysis, but quite different from leak formation due to mere chemical membrane modifications by oxidants or group-specific reagents [1–5], or to electric breakdown [8]. Both types of damage induce leaks in all the cells of a population, the major variable being the extent of leak permeability of the individual cell. Noncovalent types of leak formation, e.g., by membrane-perturbing toxins [29,30] have an intermediate position in that both the fraction of leaky cells and the extent of leakiness are variable.

Membrane leaks responsible for osmotic lysis are of course much larger than the leaks described here, but they also exhibit sieving properties [31]. The size of leaks responsible for purely mechanical lysis has not been elucidated, but these leaks also discriminate among solutes of varying size (Barth, P. and Deuticke, B., unpublished results).

In former studies on experimentally induced leaks in human erythrocytes we have determined not only the sizes of leaks but also apparent numbers of 'holes' per cell. These can be calculated from the measured leak permeabilities on the basis of simplifying assumptions [1,2,4–6]. A rough estimate is also possible for the chemo-mechanical leaks although it can only be based on approxima-

tions of the induced leak permeabilities as derived from rates of K^+-loss. According the data given in Fig. 4 and additional measurements, the cells in the leaky fraction lose all their K^+ (90 mmol/l cells on average) within the first minute after shearing. If this is equated, to a first approximation, to a net flux of 90 μmol/ml cells per min, we can calculate, disregarding the unknown but certainly low membrane potential, a leak permeability of 10^{-6} cm $\cdot$ s^{-1}. This value can then be used to derive the average total area of the holes per cell (see Refs. 4–6 and 8 for details) assuming an aqueous pathway. From this, and the apparent radius of the single hole (3 nm as reported above), one can calculate the apparent number of holes per cell. We obtain the low number of 0.1 hole per cell. Similar low numbers, below 1 per cell, were previously obtained for other types of membrane leakiness and interpreted in terms of one or more dynamic defects in each cell fluctuating between an open and a closed state [4–6,8].

In summary, chemo-mechanical leak formation is thus not comparable to any other known type of leak formation or lysis, although it has features in common with many of them. It is a fractional all-or-none phenomenon like osmotic lysis, although on the basis of much smaller leaks comparable to those induced by chemical or noncovalent membrane modification. With the latter modification it also shares the fractional character. It requires mechanical forces but does not involve cell fragmentation.

The subsequent paper deals with the chemical reactions involved in the phenomenon reported here and also attempts to answer the question whether the mechanical stress is already required for the crucial chemical membrane modification or only for the leak formation in the modified membrane.

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