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Chemo-mechanical leak formation in human erythrocytes upon exposure to a water-soluble carbodiimide followed by very mild shear stress. II. Chemical modifications involved

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The chemical reactions underlying the chemo-mechanical leak formation in human erythrocytes upon treatment with the carboxyl-modifying reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and subsequent minimal shearing, described in the preceding paper (Thelen, P. and Deuticke, B. (1988) Biochim. Biophys. Acta 944, 285–296), are here characterized in more detail. The capacity to form leaks under minimal shearing results from the transformation of certain membrane carboxyl groups into an activated state, i.e., the *O*-acylisourea derivative of the original COO^- group. This activated state re-disappears, i.e., the cells become shear-resistant again, when the *O*-acylisourea derivative under goes spontaneous hydrolysis upon removal of excess EDC in the suspension by addition of a cation-exchange resin. The activated state can be stabilized by addition of *N*-hydroxysuccinimide or *N*-hydroxysulfosuccinimide, which both form activated esters. The additional presence of nucleophilic amines, e.g., glycine methyl ester or aminomethane sulfonate, during the pretreatment with EDC strongly suppresses leak formation during subsequent shearing, which substantiates the involvement of COO^- groups. EDC reactive side groups other than COO^- (e.g., tyrosyl-OH or sulfhydryls) can be discarded as candidates for the underlying chemical reaction. The formation of the chemo-mechanical leaks most likely results from the cross-linking between a subpopulation of activated carboxyl groups and endogenous amino groups. This cross-linking, however, seems only to occur when the two reacting groups are brought into contact by the shear-induced cell–cell interactions required for leak formation. Besides information on chemo-mechanical leak formation the study provides new data helpful for future work with carbodiimides.

Introduction

'Chemo-mechanical' leak formation in erythrocytes was introduced in the preceding paper [1] as a joint effect of chemical modification of membrane carboxyl groups by the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and very low shear stresses applied by cell–cell interactions. Chemo-

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; GME, glycine methyl ester; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSI, *N*-hydroxysuccinimide; HSSI, *N*-hydroxysulfosuccinimide; IRC-50, Amberlite® ion-exchange resin IRC-50.

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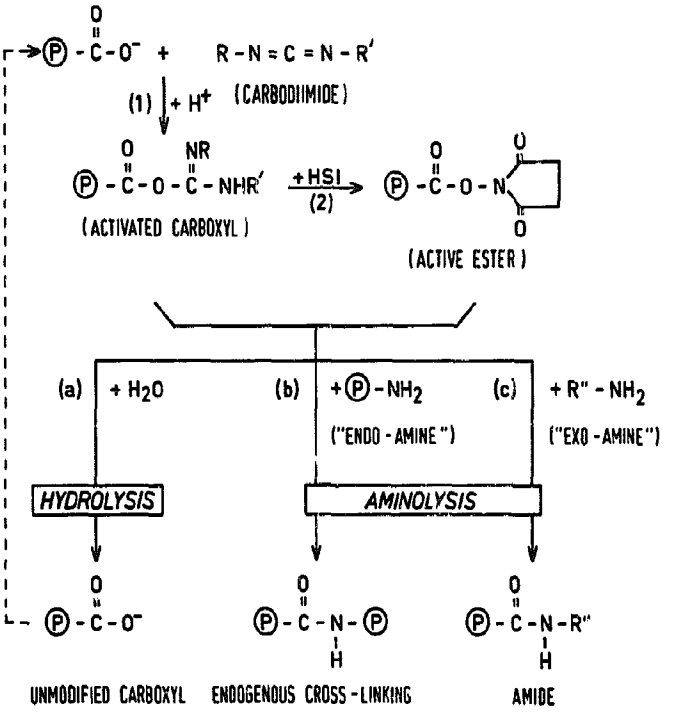

Fig. 1. Reaction scheme for carbodiimides.

mechanical leak was shown in that report to be an all-or-none phenomenon affecting more and more cells of a population with increasing intensity of the chemical or mechanical component. The preceding report [1] dealt with the characteristics of the shear stresses which lead to membrane leaks of defined diameter in erythrocytes treated with EDC. In this second paper the chemical reactions involved in the effect of the carbodiimide will be elucidated.

According to presently available information [2–4] and new data to be reported below, the reaction between a carbodiimide and a protein carboxyl group may take the pathways shown in Fig. 1. An unstable intermediate is formed primarily (reaction 1). In this *O*-acylisourea intermediate (an 'activated carboxyl group'), the carboxyl-carbon is strongly electrophilic and thus a target for various nucleophiles including water.

If the nucleophile is an endogenous amino group ('endo-amine'), e.g., a lysine residue of the same or another protein, an amide bond is formed (reaction b), producing an intra- or intermolecular cross-link. If the nucleophile is an exogenous amine ('exo-amine'), e.g., glycine methyl ester (GME), a stable terminal amide is formed (reaction c). When no amino group is available, hydrolysis rapidly re-establishes the native carboxyl group (reaction a), which can, however, again be re-activated by carbodiimide. Hydrolysis can be delayed considerably when the activated carboxyl group is converted into an active ester by addition of *N*-hydroxysuccinimide (HSI) (reaction 2). Although much more stable against hydrolysis, the active ester is as susceptible to a nucleophilic attack by an amine as the activated carboxyl group.

As a further reaction of the activated carboxyl group an intramolecular rearrangement of the activated carboxyl group into a stable *N*-acylurea may occur. As will be shown below, no evidence for a participation of this reaction in 'chemo-mechanical' leak formation could be found. The same is true for the following two reactions of carbodiimides, which are therefore also omitted

from the scheme: carbodiimides may react with sulfhydryl groups to produce a stable adduct [5]; moreover, tyrosine residues react with carbodiimides to produce an adduct, which is stable to acid hydrolysis, but can be decomposed upon extensive treatment with the strong nucleophile, hydroxylamine [6]. In the following it will be shown that all features of the chemistry of chemo-mechanical leak formation can be explained by reactions between carboxyl groups and nucleophiles.

Material and Methods

Materials

Fresh human blood from healthy donors was obtained from the local blood bank. EDC, Mes, Hepes, neuraminidase, taurine, ethylenediamine and mercaptoethanol were from Sigma, Munich. GME, semicarbazide, HSI and Triton X-100 were from Merck, Darmstadt; glycine ethyl ester, aminomethanesulfonic acid and proline methyl ester from Aldrich, Steinheim (F.R.G.); and *N*-hydroxysulfosuccinimide (HSSI) and choline chloride from Fluka, Buchs (Switzerland). Amberlite® IRC-50, a weakly acidic cation-exchange resin with carboxylate groups, was from Serva, Heidelberg.

Methods

Combined treatment of erythrocytes with EDC and further additives (nucleophiles, HSI or HSSI)

Treatment of erythrocytes with EDC was carried out in the same manner as described in the companion report [1] but in the presence of additives specified in the results. The additives were dissolved at 10-times the desired final concentration in 1 vol. isotonic NaCl, adjusted to pH 6 and added to 8 vol. Mes-buffered (pH 6) cell suspension. EDC was added freshly dissolved in a further 1 vol. isotonic NaCl. The final hematocrit was 10%. After the chemical modification the cells were subjected to shear stress by ejection or by viscometric shearing as described in the companion paper [1].

Removal of excess EDC after pretreatment prior to shearing

Since cells treated with higher concentrations of EDC become leaky upon shearing [1] they could not be washed to remove excess carbodiimide by the usual procedures. EDC could, however, be removed by a cation-exchange resin (Amberlite IRC 50, mesh size 20–50 μm, converted into the Na-form and washed several times) in a batch procedure. To remove EDC, 0.1 g resin was added to 2 ml cell suspension for 20 min at 37 °C under gentle agitation. No pH changes ensued from the addition of the cation-exchange resin. The cell suspension could be separated from the resin without centrifugation due to the spontaneous sedimentation of the resin grains.

Determination of EDC concentrations

To monitor EDC consumption during the treatment of cells and to control the effectiveness of the EDC removal procedure it was necessary to determine residual EDC concentrations in suspensions after cell modification. This could be achieved by quantifying the proton uptake occurring when EDC reacts with mercaptoethanol to form a more basic, stable adduct [5]. Most probably the imide nitrogen in a carbodiimide reacting with a sulfhydryl group becomes more basic in the *S*-amidino group formed and binds a H^+ according to the following reaction scheme.

$$\underset{\text{carbodiimide}}{R\text{-}SH + RN = C = NR'} \xrightarrow{+H^+} \underset{S\text{-amidine}}{R\text{-}S\text{-}C(NHR') = N^+HR}$$

This H^+ uptake is stoichiometric due to the high pK' value of the *S*-amidino group.

To determine the EDC content of a cell suspension, 1 ml supernatant after centrifugation was added to 1 ml isotonic saline containing 10 mM Mes (pH 6.0) at room temperature. After addition of 0.025 ml pure mercaptoethanol the pH increased quickly and reached a stable maximum within a few seconds. This increase of pH was then titrated back to pH 6.0 with 1 M HCl. HCl consumption was proportional to the EDC concentration in the supernatant. Absolute values could be calculated on the basis of a standard value (HCl consumption per 1 ml freshly prepared solution of 20 mM EDC) or a calibration curve (Fig. 2). All values were corrected for the small pH change arising from the addition of 0.025 ml mercaptoethanol to an EDC-free solution.

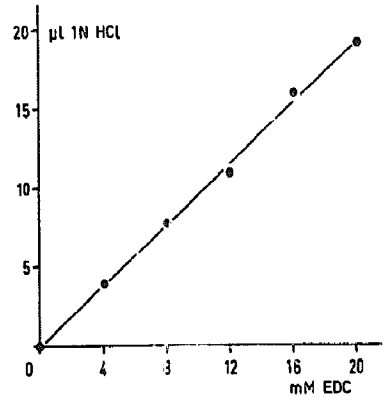

Fig. 2. Calibration curve for the determination of EDC concentrations. 0.025 ml pure mercaptoethanol were added to a mixture of 1 ml Mes-buffered isotonic NaCl and 1 ml Mes-buffered (pH 6.0) EDC solution at the concentrations indicated. The indicated volumes of 1 M HCl were required to restitute pH 6.0 after proton uptake by the basic product of the reaction between EDC and mercaptoethanol.

Determination of thiols

GSH was determined according to Beutler et al. [7]. Membrane SH groups were quantified in membranes isolated from modified erythrocytes as described earlier [8].

Results and Discussion

The presence of activated carboxyl groups is required for leak formation

As shown in the companion report [1], erythrocytes become rigidified upon treatment with low (1–5 mM) concentrations of EDC, while appreciable leak formation only occurs at higher (10–40 mM) concentrations. In view of the high reactivity of EDC with the erythrocyte membrane indicated by the complete rigidification at 5 mM EDC, it was somewhat surprising that such high concentrations of the agent are required to produce leakiness. To clarify this problem it is important to know which groups of the membrane have to be modified to make the red cells leaky upon shearing. Is it the stable modification of a cysteine or a tyrosine residue, or is a carboxyl group involved? In the latter case, is it the intermediate, labile activated carboxyl group (the *O*-acylisourea compound), its stable *N*-acylurea iso-

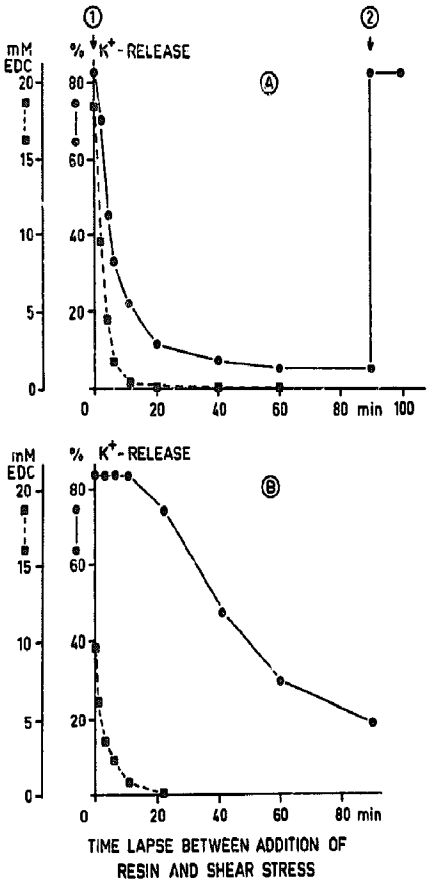

Fig. 3. (A) Time course of the decrease of EDC concentration (■) and of the concomitant loss of shear sensitivity (●) after addition of Amberlite IRC 50 to erythrocytes pre-exposed to EDC. Erythrocytes were treated with 20 mM EDC at pH 6 in a Mes-buffered saline solution hematocrit 10%, 37°C) for 20 min as described in Methods and the preceding paper [1]. Subsequently, they were sheared by ejection immediately before and at the time intervals given on the abscissa after addition of Amberlite IRC-50 which removed unbound carbodiimide. Residual free EDC in the medium was determined after these time intervals as described in Methods. Fractional release of K^+ served as a measure of the fraction of cells still shear-sensitive at various times after removal of free EDC. Arrow (1), addition of IRC-50; arrow (2), removal of IRC-50 and re-addition of 20 mM EDC. (B) Time course of the decrease of EDC concentration (■), and of the concomitant decrease of shear sensitivity (●) after addition of Amberlite IRC-50 to erythrocytes pre-exposed to EDC and HSI. Same experiment as described in (A) except that 2.5 mM HSI were added and the EDC concentration was only 10 mM.

mer, or the stable amide bond within one or between two proteins?

In the following, three sets of data (Fig. 3 and Table I) are presented which clearly suggest that the presence of the labile activated state of a carboxyl group during shear stress is required for leak formation. As evident from Fig. 3A, free EDC can be removed from a cell suspension originally containing 20 mM EDC by addition of Amberlite IRC 50 as described in Methods. The half-time of this process is 2.5 min. As a consequence, the shear sensitivity of the cells disappears with a half-time of about 5 min. 60 min after addition of IRC-50 only 5% of the cells become leaky upon shear stress by ejection as compared to more than 80% before addition of the resin, which removes almost all the EDC within 20 min.

This finding indicates that leak formation does not derive from a primary, stable modification of a class of membrane carboxyl groups (or cysteine or tyrosine residues). Rather, leak formation seems to depend on the presence of the labile activated carboxyl group. Excess EDC has to be present until the cells are sheared, to compensate for the continuous hydrolysis of the labile activated carboxyl groups (cf. Fig. 1). Removal of EDC leads to a rapid decrease of shear sensitivity resulting from the restitution of the free carboxyl group by hydrolytic cleavage of the *O*-acylisourea derivate. This interpretation is strongly supported by the observation that the shear sensitivity lost after addition of IRC 50 can be restored upon removal of IRC-50 and re-addition of EDC prior to shear stress 90 min after addition of IRC-50 (cf. Fig. 3A). The reversibility of shear sensitivity is in contrast to the irreversibility of membrane rigidification induced by EDC already at lower concentrations [1]. Thus, on top of the stable modification of an unknown number of membrane carboxyl groups responsible for irreversible membrane rigidification, carboxyl groups in membrane proteins and maybe in other membrane constituents such as sialic acid or phosphatidylserine are modified reversibly by EDC in a continuous cycle of adduct formation and hydrolysis. This adds to the corresponding cyclic modification of the cytoplasmic cell constituents. Altogether, the rate of this reversible modification as derived from the consumption of EDC amounts to 1.5 μmol · ml cells^{-1} · min^{-1} at a level of EDC of 20 mM.

Conversion of the activated carboxyl group into a more stable active ester prolongs the state of shear sensitivity after removal of EDC

The requirement of an activated carboxyl group for leak formation could be further substantiated in experiments in which the activated carboxyl group was converted into an active ester by addition of HSI [4]. Active esters (cf. Fig. 1) are more resistant towards hydrolysis than the activated carboxyl groups [9] but are still capable of forming amide bonds [4]. The addition of HSI together with EDC has two effects (Fig. 3B).

(i) The same extent of leakiness (82%) can now be reached with lower concentrations of EDC (10 mM instead of 20 mM). This indicates that the EDC-consuming cycle of activation and hydrolysis has been slowed down.

(ii) The disappearance of shear sensitivity after removal of EDC by IRC-50 is much slower in the presence of HSI. Its half-time increases from 5 to 47 min. The half-time even increases to 210 min when the cell suspension is cooled to 0°C after complete removal of EDC (20 min after addition of IRC-50) and only warmed up to 37°C again immediately prior to shear (data not shown).

In a preliminary communication, Grabarek and Gergely [10] working on actomyosin recently reported similar results for the hydrolysis of the active ester. They obtained an apparent first-order rate constant of 0.015 min^{-1} at 22°C, equivalent to a half-time of 46 min.

These experiments demonstrate that leak formation can still occur when EDC is completely removed from the suspension. An 'activated state' either as an EDC-activated carboxyl group or as an active ester seems to suffice to induce leak formation upon shearing.

HSI could be replaced by its sulfonated congener, HSSI [9] (data not shown). Since the active ester with HSSI carries a negative charge like the original carboxyl group, this finding demonstrates that the simple removal of an anionic group is not the cause of shear sensitivity.

Chemo-mechanical leak formation can be prevented by addition of an exogenous nucleophile

The shear-induced leak formation in EDC-treated cells was markedly suppressed, when a small amine compound such as GME was added to the reaction mixture together with the EDC (Table I). Under standard conditions (cells exposed to 20 mM EDC, 20 min at 10% hematocrit, then sedimented and sheared by ejection), about 75% of the intracellular K^+ was released. This value is normalized here as 1.0 (line A). The additional presence of 50 mM GME in the suspension suppresses K^+ release to 0.1 (line C), equivalent to a protection of 90% of the cells, probably due to the formation of an amide. Half-maximal protection is already reached at 5 mM GME (line B) and no further protection becomes evident at concentrations of GME exceeding 50 mM. Glycine ethyl ester and aminomethanesulfonic acid (50 mM) protect to the same extent as GME, while other nucleophiles (proline methyl ester, semicarbazide, taurine (i.e., aminoethanesulfonic acid) and ethylenediamine) are somewhat less effective. Nucleophiles do not react directly with EDC, thus quenching the reagent, since the concentration of EDC is not affected by the presence of nucleophiles (data not shown). The differences in the protective efficiencies between the various agents are not likely to arise from differences in permeability since, on the one hand, GME, glycine ethyl ester, ethylenediamine and probably semicarbazide are highly permeable by nonionic diffusion, while taurine [11] (and probably its structural homologue, aminomethanesulfonic acid) are essentially impermeable. The protective effect of impermeable nucleophiles provides evidence that the activated carboxyl groups involved in leak formation are probably located outside the permeability barrier for anions.

90% protection against leak formation is also achieved when GME (or aminomethanesulfonic acid, data not shown) is added immediately prior to shear after 20 min exposure of the cells to EDC alone (line D). This observation substantiates the conclusion, already drawn from the findings shown in Fig. 3, that the carboxyl groups crucial for leak formation remain in the activated state up to the shearing procedure.

From the experiment described in line C and D, and in analogy to the shear dependence of leak formation by EDC, one might presume that protection by GME against leak formation also requires shearing. This is, however, not the case, as becomes evident from the experiment shown in Table I, line E. In this experiment, cells were treated with EDC and GME for 20 min, and then freed of EDC by addition of IRC-50, and of GME by three careful washings. Subsequently they were treated again with EDC alone and sheared by ejection. Under these conditions 60% of the cells proved to have become protected during the exposure to EDC plus GME (normalized K^+-release 0.4). Thus, considerable, although not complete, protection can be achieved without shearing.

The reaction pattern outlined in Table I thus indicates that the largely shear-independent reaction of an activated carboxyl group with an exogenous amine (GME) prevents most of its shear-dependent reaction with an endogenous amino group, the latter reaction being required for leak formation.

The protective influence of exogenous amines demonstrated in Table I suggests that chemo-mechanical leak formation involves the formation

TABLE I

EFFECT OF ADDED EXOGENOUS NUCLEOPHILES ON LEAKINESS PRODUCED BY EDC AND SUBSEQUENT SHEAR BY EJECTION

Cells were exposed to EDC and GME (37°C, pH 6) as indicated in lines A–E. In the experiment shown in line E, EDC and GME were removed after the first treatment by addition of Amberlite IRC-50 and three washings.

Treatment	K^+-release (normalized) after treatment and shearing by ejection
A. 20 mM EDC, 20 min	1.0
B. 20 mM EDC + 5 mM GME, 20 min	0.5
C. 20 mM EDC + 50 mM GME, 20 min	0.1
D. 20 mM EDC, 20 min, then 50 mM GME added immediately before shearing	0.1
E. Pretreatment with 20 mM EDC + 50 mM GME, 20 min, then removal of EDC and GME by IRC-50 and 3 washing steps, then second exposure to 20 mM EDC, 20 min	0.4

TABLE II

DECREASE OF GSH AND MEMBRANE SH GROUPS AFTER TREATMENT OF ERYTHROCYTES WITH EDC

Erythrocytes were treated for 30 min with EDC at the concentrations indicated (pH 6.0, 37°C, 10% hematocrit).

EDC (mM)	μmol GSH/ ml cells	Membrane thiols (nmol/mg protein)
0	2.47	88.2
0.8	1.33	-
5	0.21	-
20	0.00	77.8

of an endogenous amide bond. This endogenous amide bond, however, is not formed prior to the exposure to shear stress, since removal of EDC by IRC-50 abolishes shear sensitivity even after the usual 20 min exposure to EDC (Fig. 3A). The formation of the activated carboxyl group and its conversion into an endogenous carboxamide cross-link are thus temporally distinct processes. The observation that a labile, easily reversible product of the reaction between EDC and the erythrocyte membrane is the cause for shear sensitivity also excludes the involvement of any primarily stable reaction product of EDC, e.g., with sulfhydryl or tyrosine-hydroxyl groups. A causal role of membrane SH groups could further be excluded by the observation (data not shown) that pretreatment of cells with *N*-ethylmaleimide (10 mM, pH 8.0, 60 min), which blocks about 75% of the membrane SH groups [12], did not suppress the effects of EDC. On the other hand, EDC itself does react with a few membrane SH groups and all of the cellular GSH (Table II). For the reason outlined above, these reactions are, however, not likely to contribute to the effect of EDC.

While a carboxyl group is thus almost certainly involved in leak formation it might be questioned whether this carboxyl group is located in a protein. Carboxyl groups in phosphatidylserine or in sialic acids can indeed not be ignored a priori. A contribution of phosphatidylserine is unlikely, however, since we could not detect phosphatidylserine carboxamides by thin layer chromatography in erythrocytes pretreated with EDC plus GME (Thelen, P. and Haest, C.W.M. unpublished data). A causal role of carboxyl groups in sialic acids also seems unlikely since removal of most of the sialic acids by pretreatment of cells with neuraminidase (500 mU per 10 ml (hematocrit 10%), 45 min, pH 6.5) did not abolish chemo-mechanical leak formation (data not shown).

In order to substantiate our concept of the chemical reactions underlying chemo-mechanical leak formation we finally had to exclude an alternative interpretation: leak formation might require the presence of a carboxyl group in any modified state but does not involve the formation of an endogenous amide bond. This alternative is rather unlikely for two reasons:

(i) Although the activated states of carboxyl groups presented here (*O*-acylisourea, active esters with HSI and HSSI) are chemically very heterogeneous, shear sensitivity derives from all of them. On the other hand, a stable amide formed by the addition of, for example, GME, which also represents a modification of a carboxyl group, does not produce shear sensitivity.

(ii) Modification of carboxyl groups without formation of an endogenous amide would not account for the particular pH-dependence of the leak formation by EDC treatment and subsequent shearing, described in the following section.

pH-dependence is different for carboxyl group activation and ensuing reactions

If our assumption that leak formation derives from the formation of an endogenous amide bond is correct, the pH-dependence of the effect might be a further clue. The reactions of carbodiimides with carboxyl groups are favoured at acidic pH [3] but ensuing reactions might have their own pH optimum.

In our standard experiments cells were treated with EDC at pH 6. As evident from Fig. 4A, this is an optimum for the treatment with EDC or with EDC plus HSI. Treatment at pH values below or above pH 6 induces leakiness to a lesser extent. A slightly acidic pH is obviously appropriate for the formation of an activated carboxyl group. However, since the subsequent amide formation, supposed to cause leak formation during shearing, involves an amino group in a nucleophilic reaction, a more alkaline pH might enhance that reaction, even if the primary reaction step (the formation of the activated carboxyl group) has taken

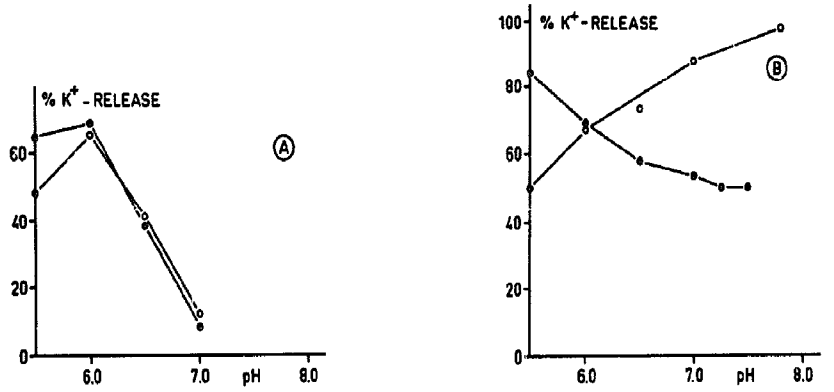

Fig. 4. (A) pH-dependence of the reaction of EDC inducing shear sensitivity. The cells were treated with 20 mM EDC (●) or 10 mM EDC and 2.5 mM HSI (○) for 20 min at the indicated pH values. Subsequently, they were sheared by ejection into buffered isotonic saline at pH 6. K^+ released was measured 60 min later. (B) pH-dependence of the reaction occurring in EDC-treated erythrocytes during the application of shear stress. The cells were exposed to 20 mM EDC (●) or 10 mM EDC and 2.5 HSI (○) at pH 6.0 for 20 min. The pH was then rapidly shifted to lower or higher values by adding small amounts of isotonic Mes or Hepes solution and the cells sheared immediately by ejection at the new pH into isotonic saline buffered at pH 6. K^+ released was measured 60 min later.

place at its pH optimum, pH 6.

Indeed, as shown in Fig. 4B, cells pretreated with EDC and HSI at pH 6 develop increasing leakiness when the pH of the suspension has been shifted to increasingly alkaline pH values prior to shearing. This would seem to reflect the higher reactivity of an amino group upon deprotonation at alkaline pH. In contrast, cells pretreated at pH 6 with EDC, but without HSI, do not show this enhanced leakiness upon shearing at alkaline pH. They become less leaky at high than at low pH. This observation, however, does not invalidate our hypothesis. In the case of treatment with EDC alone a stimulating effect of alkalinization cannot establish itself since an alkaline pH will intensify the spontaneous hydrolysis of the activated carboxyl group (see Fig. 1). This will cancel carboxyl group activation immediately. Only the stable active ester survives alkaline pH to react with an amine. The difference in shear sensitivity between cells treated with EDC alone and cells treated with EDC and HSI also excludes the possibility that increased leak formation derives from pH changes per se. The experiment shown in Fig. 4B furthermore provides preliminary insight into the localization of the pH effect. In this experiment the time interval between the addition of the alkaline buffers and the shearing procedure could be shown to be too short to allow for changes of the intracellular pH. This was probably due in part to the fact that EDC treatment induces blockage of the anion exchanger (band 3 protein) [13], which mediates transmembrane pH-equilibration. Since the pH effect in Fig. 4B is thus an extracellular one, the pH-dependence of the leak formation in cells premodified by EDC and HSI probably reflects the involvement of an activated carboxyl group located outside the permeability barrier of the membrane.

Concluding remarks

In the first paper of this series it was shown that very low shear stresses applied to EDC-treated erythrocytes by cell–cell interactions induce membrane leakiness. This second paper has presented evidence that leak formation requires the presence of an activated state of a certain subpopulation of membrane carboxyl groups, which undergo cross-linking with membrane amino groups during the subsequent exposure of the cells to shear stresses.

In the course of this study we have also made a number of discoveries and elaborated procedures which may be helpful to other investigators using water-soluble carbodiimides. These results can be summarized as follows:

(1) The mere activation of carboxyl groups by carbodiimides produces a labile intermediate, which can only be maintained in the presence of high concentrations of the carbodiimide and is lost rapidly but not immediately due to hydrolytic cleavage when the carbodiimide is removed.

(2) Carbodiimides can be removed from systems, in which mere washing away of the agent by centrifugation and resuspension is not feasible, by addition of a cation-exchange resin. The mechanism of action of the resin is not fully clear at present. On the one hand, EDC as a tertiary amine will be mainly in the positively charged state at the pH of our experiments and thus bind to the anionic resin by coulombic interactions. On the other hand, a covalent reaction of the carbodiimide with the carboxylate groups of the cation exchanger cannot be excluded. This new quenching procedure is probably superior to procedures used hitherto, such as dialysis, which is time-consuming, or addition of an excess of a compound reacting with EDC, like acetate [2]. This latter quenching procedure creates a freely mobile reactive species, which might modify protein amino groups or other nucleophilic membrane sites and thereby create artifacts.

(3) Conversion of a carbodiimide-activated membrane carboxyl group into an active ester by HSI or HSSI prolongs the lifetime of the activated, reactive state. This is helpful for elucidating mechanisms of modification, but can also be used to enhance subsequent amide formations by pH changes, as demonstrated in Fig. 4A.

(4) Concentrations and consumptions of carbodiimides can be quantified by a simple method based on their irreversible reaction with thiols at pH 6.

(5) Using this quantitative assay the interference of certain constituents of an incubation medium with the carbodiimide reaction may be quantified. Confirming earlier observations [14], it could be demonstrated [1] that inorganic phosphate buffers should be avoided in experiments involving water-soluble carbodiimides since these buffers quench the agent.

Concerning the phenomenon of chemo-mechanical leak formation, our present results can be summarized and evaluated as follows:

The activation of the crucial carboxyl groups which 'sensitizes' the cells derives from their covalent modification by compounds (EDC, HSI, HSSI) very different in charge or steric properties. The common denominator of these modifications is the catalysis of cross-linking reactions. The particular cross-linking reactions involved in leak formation, however, do not occur as spontaneously as in other cases of carbodiimide treatment [15]. As evident from the data compiled in Fig. 3 and Table I, simultaneous or subsequent shear stresses are required which may be very low but have to result from frictional interactions between cells.

While endogenous amino groups involved in leak formation seem to react with the activated crucial carboxyl groups only when shearing is applied, the reaction of exogenous nucleophiles with these groups is not dependent on the application of shear forces for the formation of the amide bonds that protect against leak formation. This difference may indicate that the endogenous amino groups are normally too far away from the activated carboxyl groups to react in the normal state. They probably come into closer contact under the conditions of the shear stress. Some speculations about this process will be presented below.

Cross-linking deriving from a reaction between activated carboxyl groups and endogenous amino groups is also likely to be the cause for carbodiimide-induced membrane rigidification. The situation concerning nearest neighbours and reactivities is, however, quite different. The rigidifying cross-links form spontaneously and cannot be prevented by exogenous amines. This indicates that the carboxyl and amino groups involved are normally in close contact and not accessible to external amines. In view of this high reactivity, of the lack of interference of amines, and of the lack of a marked pH-dependence of EDC-induced rigidification [1], reactions of EDC with side groups other than carboxyl groups might be involved in rigidification. The observation that blocking a highly reactive class of membrane SH-groups with iodoacetamide (10 mM, pH 7.4, 60 min) does not inhibit rigidification (Thelen, P., unpublished results) is difficult to reconcile with this possibility. Moreover, endogenous, enzymatically induced carboxamide cross-links have already been shown to induce rigidification in erythrocytes upon

activation of transglutaminase by Ca^{2+} [16].

The sequence of events leading from cross-linking to leak formation is as yet unclear. It may be speculated that conformational changes in the proteins involved produce defects at a protein/lipid interface disturbing the membrane barrier function. On the other hand, the conspicuous size of the induced leaks, which have radii up to 3.2 nm [1], would also be compatible with proteinaceous structures, comparable to the leaks induced by bacterial toxins [17]. Since EDC is a highly permeable and reactive carbodiimide the proteins involved will be hard to localize. Labeled nucleophiles and nucleophiles of different permeability and polarity may become helpful for this purpose.

The role of shear stresses in the process of leak formation is also a highly intriguing problem. As a rather simplistic interpretation one might presume that shearing has two sequential effects. First, it brings into contact activated carboxyl groups on the surface of one cell with superficial amino groups on another cell, thus providing intercellular cross-linking. Subsequently it tears apart the cross-linked cells, thereby destroying their membrane integrity. This interpretation does not seem tenable for two reasons: First, intercellular covalent bonding between erythrocyte surfaces would require contacts too close to be possible for electrostatic reasons. Intercellular coulombic repulsion between EDC-treated cells is still operative since no spontaneous aggregation was observed. Second, no irreversible cell doublets or multiplets, indicative of intercellular cross-bonding, could be detected in EDC-treated cell suspensions after shearing.

The possibility of a shear-induced macroscopic deformation of the whole cell and its membrane can also be dismissed, since the shear stresses applied are extremely low and the membrane is rigidified. In view of the probable significance of cell–cell interactions it might, however, be hypothesized that it is not so much 'bulk' shear stress applied to the cell that causes leak formation but some sort of locally inflicted distortion of membrane elements, especially the glycocalyx. One may speculate that some of the integral membrane glycoproteins by means of their long-chain oligosaccharides acting like antennae [18] might behave as force receptors that transmit local deformation to other membrane elements and thereby provide new nearest-neighbour relationships among them.

To what extent chemical modifications of surface structures are involved is as yet unknown. To clarify this question investigations are under way using an impermeant carbodiimide. Studies with such a compound should also help to further elucidate the question whether the functional groups involved in the formation of the crucial amide bonds are indeed located external to the permeability barrier of the membrane as suggested by the studies with impermeant protective nucleophiles and with variation of extracellular pH. It is expected that further studies on chemomechanical leak formation will reveal interesting new information about erythrocyte membrane structure.

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