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N-ETHYLMALEIMIDE CAUSES MECHANICAL FRAGILITY AND ACCUMULATION OF SPECTRIN DIMERS IN THE RAT ERYTHROCYTE MEMBRANE

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SUMMARY: Treatment of rat erythrocytes with N-ethylmaleimide is found to render them mechanically fragile. Membranes of the lysed cells show degradation of band 3 and, to a lesser extent, of spectrin; as well as considerable accumulation of dimeric spectrin. The predominant action of N-ethylmaleimide on isolated membranes, however, is the conversion of spectrin to its dimeric form.

Sulfhydryl groups play an important role in the stability of the human erythrocyte membrane (1-3). Reaction of isolated ghosts with organic mercurials or dithiobisnitrobenzoic acid leads to fragmentation of the membrane with concomitant release of skeletal proteins. The mercurials have also been reported to cause the breakdown of Triton-skeletons (4). Other sulfhydryl reagents, NEM and iodoacetic acid, neither fragment the membrane nor protect it from the action of the mercurials or DTNB, denoting selectivity of the SH groups involved in membrane stability. The disruptive action of the agents, moreover, is species specific, causing only marginal breakdown of the erythrocyte membrane of species other than the human (3).

We report that NEM and IAA, which penetrate the red cell (5), destabilize the membrane of rat erythrocytes, so that mild mechanical perturbation leads to lysis of the cells.

ABBREVIATIONS: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); IAA, iodoacetic acid; NEM, N-ethylmaleimide; PBS, 0.15 M NaCl in 0.01 M phosphate, pH 7.4.

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MATERIALS AND METHODS

NEM, IAA, DTNB and dithiothreitol were products of Sigma. Sepharose 4B was obtained from Pharmacia.

Four times washed rat (Wistar strain) erythrocytes (0.3% hematocrit) were incubated in PBS containing various concentrations of the sulfhydryl reagent. After incubation, either undisturbed or with shaking at 100 reciprocal strokes per minute, at 37° C, the intact cells were sedimented and absorbance of the supernatant was measured at 450 nm. Percent hemolysis was calculated by comparison with the absorbance of completely lysed equivalent number of cells.

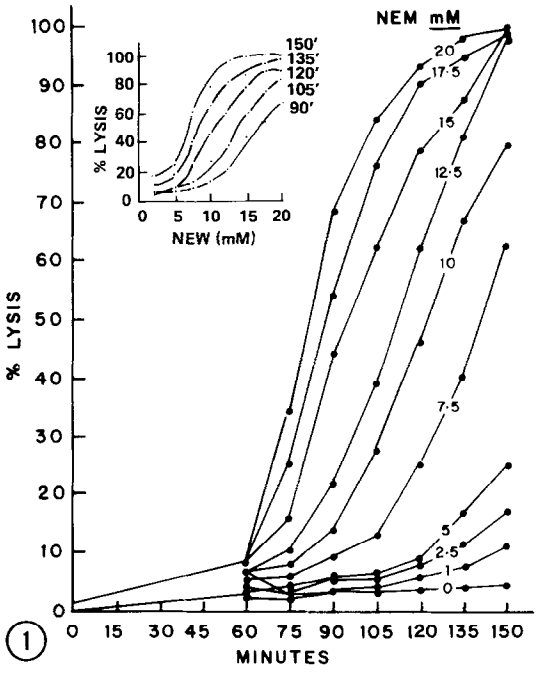
For isolation of membranes from NEM-lysed cells (10% hematocrit), the lysate was chilled and centrifuged at 1,500 x g for 10 min. Hereafter the temperature was maintained below 4° C. The supernatant was centrifuged at 20,000 x g for 15 min and the membranes were washed at least three times in 0.01 M Tris-HCl, pH 7.4. Ghosts from normal erythrocytes were prepared by lysis of washed cells in 0.01 M Tris-HCl, pH 7.4, and the membranes were pelleted and washed as above. The ghosts were incubated in 10 vol of PBS or 20 mM NEM in PBS for 120 min with shaking.

Skeletal proteins were extracted and analyzed by fractionation on Sepharose 4B as described by Gratzer (6). SDS-polyacrylamide electrophoresis on 5-15% gradient gels was according to Laemmli (7), and staining was done with coomassie blue. Protein was estimated by the method of Lowry et al. (8) using bovine serum albumin as the standard.

Ghosts and skeletons (prepared by extracting the ghosts in 1% Triton X-100 in 0.01 M Tris-HCl, pH 7.4 in the cold) were viewed by darkfield microscopy.

RESULTS

Incubation of rat erythrocytes with NEM caused lysis of the cells after a lag of one hour. The lysis then increased with time and concentration of the reagent (Fig. 1), but only if the cells were moderately shaken. If left stationary, they did not lyse for several hours. It was additionally observed (data not shown) that (i) IAA, but not DTNB, produced lysis similar to NEM; (ii) inclusion of dithiothreitol together with NEM or IAA at twice the molar concentration of the sulfhydryl reagent prevented lysis completely; (iii) the slight increase in osmolarity of 20 mM NEM in PBS was not the cause of lysis as NEM at the same concentration in 0.14 M NaCl was equally lytic; (iv) the NEM-treated ghosts or those derived from the NEM-lysed cells were intact; (v)



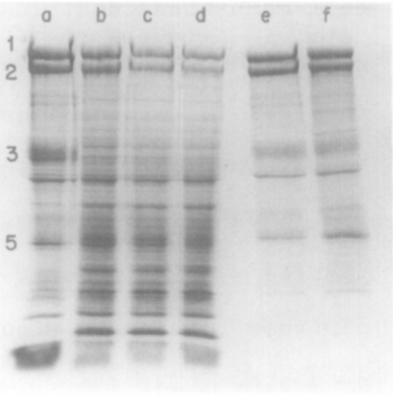


Figure 1. NEM-induced lysis of rat erythrocytes. Experimental conditions are described under Materials and Methods. Cells were incubated with shaking. The data points between 0 and 60 min are not shown. The data are replotted as function of NEM concentrations in the inset.

Figure 2. SDS-polyacrylamide gel electrophoresis of membrane proteins. Ghosts from the cells incubated in PBS for 120 min (a); membranes isolated from the lysate produced with: 10 mM NEM, 120 min (b); 20 mM NEM, 90 min (c); 20 mM NEM, 120 min (d). Ghosts from normal cells incubated in PBS (e) and 20 mM NEM (f) for 120 min. All incubations were with shaking. 100 µg protein for lanes (a) to (d), and 80 µg for lanes (e) and (f) each.

the Triton-skeletons of normal and NEM-treated membranes appeared indistinguishable; and (vi) human erythrocytes incubated in 20 mM NEM with shaking for 2 hr did not show any lysis.

SDS-PAGE (Fig. 2) of the membranes isolated from NEM-lysed cells showed loss of band 3 at 10 mM NEM, and an apparent reduction in the amount of spectrin at 20 mM NEM. Appearance of new polypeptides, probably degradation products, in these cases was notable. However, when isolated ghosts were incubated for 120 min in 20 mM NEM, band 3 and spectrin degradation were minimal, with only small amounts of degradation products evident.

There was a small amount of protein in the supernatant of NEM-treated ghosts, which was comparable to that released by buffer alone. In both cases, this consisted of small molecular weight peptides as revealed by SDS-PAGE (not shown).

The skeletal proteins from NEM-treated ghosts (or lysed cell-membranes) contained reduced amounts of the spectrin-band 4.1-actin complex and tetrameric spectrin (I and II, respectively in Fig. 3), with an increased amount of material in the peak corresponding to dimeric spectrin (peak III, Fig. 3). Following peak III, a few smaller peaks can be seen in the NEM-treated sample, which were pooled and on SDS-PAGE showed smaller peptides (not shown), possibly representing spectrin degradation products.

DISCUSSION

Contrary to the rapid disruptive action of organic mercurials and DTNB on human erythrocyte ghosts, NEM and IAA lyse the rat

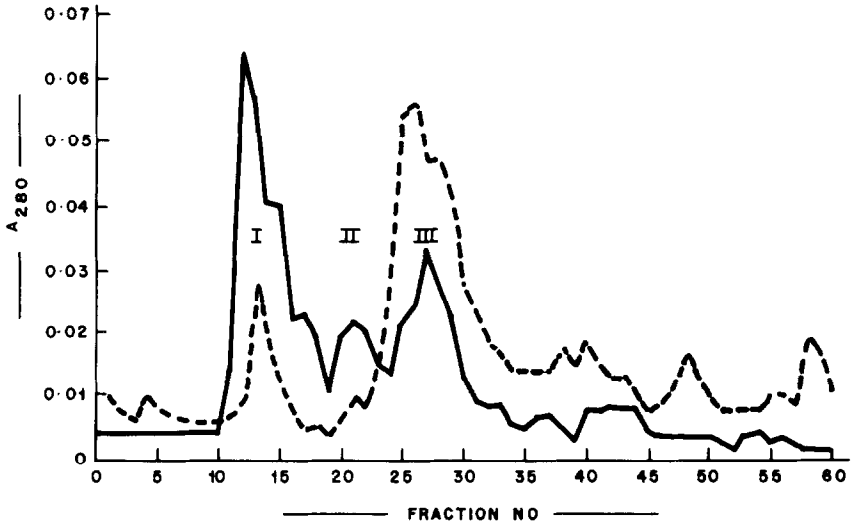


Figure 3. Elution patterns of membrane skeletal extracts. The proteins were extracted from ghosts incubated in PBS (——) and 20 mM NEM for 120 min (- - -). One ml extract ($A_{280\ nm}$ = 0.33) was loaded on 38 x 1 cm Sepharose 4B column. One ml fractions were collected at the rate of 5 ml/hr.

cells after a long lag, and without fragmentation of the membrane or elution of skeletal proteins. The fact that NEM and IAA (both able to penetrate the cell) cause lysis, but not DTNB (which does not penetrate (5)), suggests that the target(s) of the sulfhydryl reagents lies on the cytoplasmic face of the membrane.

The degradation of band 3 and to some extent of spectrin is observed only in the membranes of NEM-lysed cells. While band 3 breakdown could sever the ankyrin-mediated linkage between the membrane proper and the skeleton (9), and spectrin degradation could also destabilize the membrane, their significance in the cell lysis questionable: In these cases fragmentation of the membrane should be expected, as occurs when the skeleton is eluted (10) or spectrin is degraded (11). Since fragmentation does not occur, and the two proteins remain nearly intact in NEM-incubated ghosts (suggesting that their degradation in the cells must be due to cytosolic proteases), the accumulation of dimeric spectrin assumes significance in regards to mechanical fragility of the cells. High concentration of experimentally-induced dimeric spectrin is known to cause mechanical fragility in the membrane (12), and some hemolytic anemias are known where the membrane contains spectrin dimers in a large proportion (13,14). NEM-treated cells and isolated membranes both show a high degree of conversion of spectrin to its dimeric form.

The components of the membrane skeleton dissociate and reassociate constantly at a slow rate (15). It is possible that sulfhydryl groups play an important role in these phenomena. The accumulation of spectrin dimers in the membrane following NEM treatment suggests that sulfhydryl groups may play a role in spectrin tetramer-dimer equilibrium. Sulfhydryl groups have also been reported to participate in band 4.1 and spectrin interaction (16), and this may be another site of NEM action. Obviously many

interactions would have to be affected before the membrane loses its ability to resist mechanical stress, possibly accounting for the long lag observed before the onset of lysis. Since the membranes and Triton-skeletons of NEM-lysed cells remain unfragmented, mechanical stress appears to produce localized damage permitting the leakage of cytoplasmic material.

It is of interest to note that the spectrin-band 4.1-actin complex isolated from the human erythrocyte membrane undergoes dissociation on incubation with p-hydroxymercuribenzoate for several hours (17). Thus in both human and rat erythrocytes, SH groups play an important role in skeletal component interactions. However, the crucial sulfhydryl groups in the two species appear to be located in different chemical environments as indicated by their differing sensitivity to different SH-reagents.

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