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Comparison of the Effects of *N*-Ethylmaleimide and Iodoacetamide on Red Blood Cell Membranes[1] (39387)

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(Introduced by M. E. Kaplan)

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The importance of free sulfhydryl groups in maintaining red blood cell (RBC) membrane structure and function has been extensively studied, employing *N*-ethylmaleimide (NEM) and other agents (1-3). NEM produces a rapid reduction in RBC filterability (4) and a loss in RBC viability (5). RBC membrane polypeptides reacting with [^{14}C]NEM have been compared with those reacting with [^{14}C]iodoacetamide (IA) by polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (PAGE SDS) (6, 7). IA at low concentrations produces specific labeling of glyceraldehyde-3-phosphate dehydrogenase (G3PD), whereas NEM at similar levels or IA at higher concentrations binds with less specificity to many membrane components (7). Here we compare the effects of NEM with those of IA on RBC membrane polypeptides, G3PD, ATP levels, and filterability.

Methods. Using aseptic technique, heparinized whole blood was freshly obtained from normal healthy donors, centrifuged (1200 *g*, 10 min), and the plasma, buffy coat, and top one-fifth of the RBC removed. Similar efforts to remove white cells were made after three subsequent washes of the RBC with Dulbecco's phosphate buffered saline (PBS) (8). Cell counts were performed on 50% RBC suspensions with a Coulter model S cell counter and the RBC were observed under phase microscopy. Aliquots of RBC were measured in this standard freshly washed state, so that volume changes induced by incubation would not be a factor in measurements reported. Washed RBC were incubated with two volumes of 10 m*M* IA or 10 m*M* NEM in PBS, for 2 hr at 37° to obtain maximal effect with less than 1% hemolysis (5). For labeling experiments [^{14}C]NEM and IA 10 mCi/mmole were used (New England Nuclear). RBC filtration employed the method of Teitel *et al.* (4) employing 9-cm Schleicher and Schuell No. 589 white band filter paper at 25° using twice-washed cells, at 50% hematocrit in PBS. RBC membranes were prepared by the method of Dodge *et al.* (9) as modified by Fairbanks *et al.* (10). To measure total membrane protein, RBC were hemolyzed with exactly 60 volumes of 0.005 *M* phosphate buffer, pH 8, centrifuged for 30 min at 80,000 *g*, and the pellet collected and brought to standard volume. Total protein was measured by the Lowry procedure (11), hemoglobin by the benzidine method (12), and nonhemoglobin protein reported as the difference. Prior to electrophoresis the protein was dissolved in 1% SDS, containing 1% mercaptoethanol, and heated to 100° for 2 min to destroy proteases. SDS PAGE of from 10-300 μg of protein was performed on 0.5 × 10-cm gels. The gels, containing 5% acrylamide in 0.1% SDS and 0.1 *M* phosphate buffer, pH 7, were run at 25° (10 mA/tube), stained for proteins with Coomassie blue (10), and the relative amounts of protein components were determined on a Canalco gel scanner. Autoradiography of [^{14}C]NEM- and [^{14}C]IA-labeled polypeptides was performed on longitudinally sliced gels, which were dried on filter paper (13), and wrapped with Kodak no-screen film for 28 days. The autoradiograms were scanned and compared with the PAGE SDS protein scan. G3PD was assayed on isolated membranes, one unit of actively reducing 1 μmole/min of nicotinamide-adenine dinucleotide at 25° (14), and ATP concentrations were determined enzymatically (15) using a Gilford spectrophotometer.

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TABLE I. EFFECTS OF RBC INCUBATION IN PBS, NEM, AND IA.[a]

	G3PD[b] (units/mg membrane protein)	ATP[b] (μmole/dl RBC)	Nonhemoglobin protein[c] (g × 10^{-13}/cell)	1/2 Filtration time[b] (min)
PBS (0 time)	1.8 ± 0.3	96.25 ± 3.5	9.9 ± 0.3	4.5 ± 1
PBS (2 hr control)	1.4 ± 0.1	94.0 ± 1.0	10.0 ± 0.8	4.3 ± 1
N-Ethylmaleimide	0	56.8 ± 2.2	13.1 ± 0.7	∞
Iodoacetamide	0	55.8 ± 3.5	9.2 ± 0.4	5.0 ± 1

[a] Two volumes of 10 m*M* inhibitor, 37°, pH 7.4, 2 h).
[b] Mean from four individual determinations ± SD.
[c] Mean from six individual determination ± SD.

Results. Table I compares the G3PD activity, ATP levels, and RBC membrane protein content with RBC filtration of control RBC, RBC incubated for 2 hr at 37° in PBS, or in two volumes of 10 m*M* NEM or IA. Both NEM and IA completely inactivated the G3PD, and produced a similar 40% reduction in ATP levels. While this decrease in ATP was identical, NEM markedly inhibited RBC filtration whereas IA did not (Table I). Thus, the decrease in filtration rate with NEM cannot be ascribed to effects on ATP. The modification by NEM of the RBC membrane was confirmed by the increased amounts of nonhemoglobin protein per RBC membrane isolated ($P < 0.005$, by the Student's t test). Membranes from NEM-treated RBC were noted to pack better than those from control cells, perhaps related to the cell shrinkage observed with NEM (5), and thus are contained in a smaller volume in the one-step hemolysis procedure, and require fewer centrifugations to yield white ghosts.

The sequential changes in membrane polypeptides induced by NEM treatment are shown in Fig. 1. The polypeptide bands are numbered as in Fairbanks *et al.* (10). The lowest curve represents the PAGE SDS of membranes from unincubated control RBC, and the remaining curves the effects of incubation with 20 μmole NEM/ml RBC for 15, 30, and 120 min at 37°. Marked changes are clearly present at 15–30 min and included the apparent increase in bands already detected in control membranes, such as band 6 previously identified as G3PD (16) and band 4.2, the appearance of new bands such as those between polypeptides 2 and 3, and a complex increase in bands in the region of 4.5.

The relation of the protein changes induced by [^{14}C]NEM at 2 hr to binding of this inhibitor is shown in Fig. 2. It is apparent that many of the NEM-produced polypeptides are heavily labeled with [^{14}C]NEM.

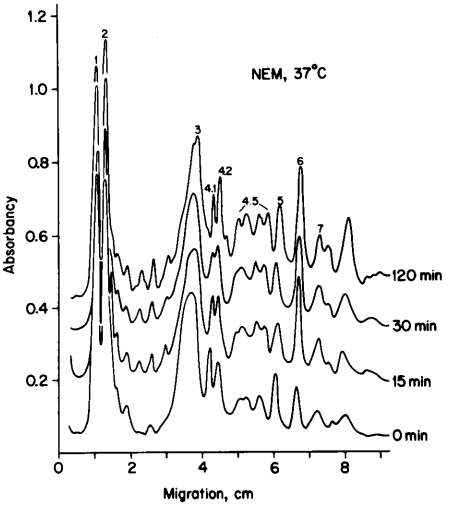

FIG. 1. Effect of NEM (20 μmole/ml RBC, 37°) for 0, 15, 30, and 120 min on PAGE SDS. 120 μg of protein was applied in each case. Note the progressive appearance of many new polypeptide bands produced by NEM. The 15-, 30-, and 120-min scans have been displaced upwards on the ordinate to aid visualization.

The NEM-induced accumulation of new bands occurred only in intact cells. Treatment of isolated membranes with NEM either with or without prior exposure to 1% mercaptoethanol (100°, 2 min) yielded no additional bands, as shown in Fig. 3. These findings are consistent with the cytoplasmic origin of the additional nonhemoglobin protein recovered in the membrane fraction after NEM treatment of intact RBC. Since deliberately increasing the amount of white blood cell contamination from 400/μl to 40,000/μl failed to increase the rate or extent of NEM-induced effects, these changes

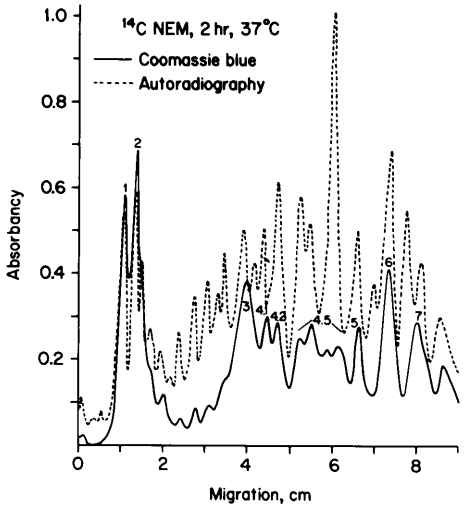

FIG. 2. [^{14}C]NEM-labeled membranes: comparison of polypeptide stain and autoradiograph. RBC were labeled with [^{14}C]NEM (20 μmole/ml RBC, 2 hr, 37°), and 300 μg of solubilized membrane protein was applied to PAGE SDS gels.

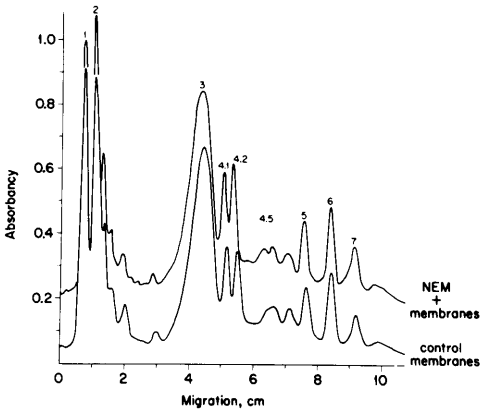

FIG. 3. Lack of effect on isolated membranes of NEM (20 μmole/ml packed membranes, 60 min, 37°) (upper scan), compared to control membranes, incubated in buffer alone, 60 min, 37° (lower scan).

almost certainly do not arise from leukocyte proteinase action. Moreover, no release of a proteinase by NEM was demonstrable using the detector Azocoll (Calbiochem); however, activation of membrane-fixed proteinases could not be ruled out (17, 18). Omitting calcium from the PBS had no effect on the NEM-induced changes, unlike sterile incubation of glucose-deprived RBC *in vitro* (19).

Figure 4 compares the protein scan of the stained [^{14}C]IA-labeled polypeptides on PAGE SDS with the scan of the autoradiograph. The relative concentration of polypeptides on the scan is similar to that of fresh RBC seen in the lowest curve in Fig. 1, except an increase in band 6 and a possible increase in band 4.2. The autoradiograph shows that these bands are also particularly heavily labeled with [^{14}C]IA and that the labeling of other bands is generally proportional to their protein content, as reported by others with these concentrations of [^{14}C]IA (7). When RBC were treated with IA for 1 hr, the changes shown in Fig. 4 appeared. When the IA-treated cells were then incubated with NEM for an additional hour, the membranes isolated, and electrophoresed, the multiple new membrane bands in NEM-treated RBC membranes became apparent.

Discussion. The correlation of the filtration rate with changes in the protein content and composition of the RBC membranes provides some evidence that changes in the isolated membranes may apply to the intact cell. Thus, a decrease in filterability of RBC occurs with NEM which alters the PAGE SDS and protein content of the membrane significantly, and not with IA which does not. With NEM the rate of the decrease in

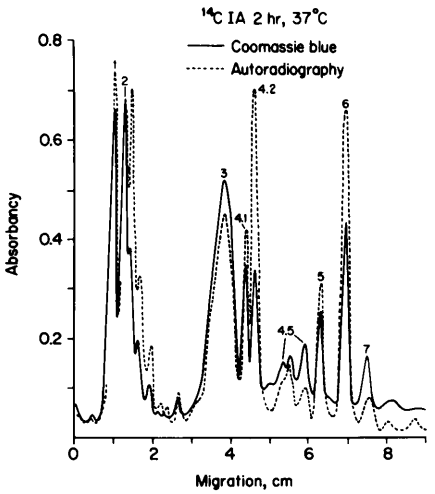

FIG. 4. [^{14}C]IA-labeled membranes: comparison of polypeptide stain and autoradiograph. RBC were labeled with [^{14}C]IA (20 μmole/ml RBC, 2 hr, 37°) and 300 g of solubilized membrane protein applied to PAGE SDS.

filterability (4) and appearance in new bands (Fig. 1) is similar. Teitel *et al.* (4) observed that the amount of NEM required to alter filterability had to exceed, mole for mole, RBC glutathione content (2 μmole/ml). Similarly, more than 2 μmole NEM/ml RBC were needed before there were any changes in the polypeptides on PAGE SDS. The lack of significant modification of the PAGE SDS polypeptide scan makes IA labeling of reactive sulfhydryl groups more readily interpretable than [^{14}C]NEM labeling. Since pretreatment with IA did not block subsequent NEM-induced changes, it is presumed that NEM combined at additional protein sites.

The mechanisms causing the polypeptide alterations in the red cell membrane have been only partially clarified. The increase in band 6 (G3PD) caused by its specific combination with IA or NEM may be explained by stabilization of the membrane-bound state of the enzyme by the inhibitor, with prevention of substrate-induced dissociation (20). How generally this type of mechanism might apply to other membrane components (such as band 4.2) is not known. The correspondence of the [^{14}C]NEM reactivity of a polypeptide and its appearance in the membrane fraction suggests a direct chemical relation between these events. For example, NEM may convert cytoplasmic components to a denser or more hydrophobic configuration by its direct addition to a side group or by disruption of tertiary protein structure. Such changes might result in the decreased solubility of the proteins and their subsequent recovery in the membrane fraction. Additional effects, such as increased susceptibility to an endogenous protease following such configurational changes, have not been entirely ruled out.

Summary. Although both NEM and IA completely inhibited G3PD activity and decreased RBC ATP levels 40%, IA produced little effect on RBC filterability whereas NEM greatly reduced the filtration rate. IA caused only an isolated increase of band 6 (G3PD) and no change in membrane protein content. However, the decreased filterability of NEM-treated RBC was associated with significantly increased membrane-associated nonhemoglobin protein which appeared as a complex array of polypeptide bands on PAGE SDS.

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