THE REVERSIBILITY OF N-ETHYLMALEIMIDE (NEM) ALKYLATION OF RED CELL GLUTATHIONE

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N-ethylmaleimide (NEM) rapidly alkylates both protein and nonprotein sulfhydryl groups. The red cell membrane appears to be freely permeable to NEM, and it has therefore been used by a number of investigators to measure red cell sulfhydryl groups[1], or to determine the function of reduced glutathione (GSH)[2].

Since the binding of red cell GSH by NEM was believed to be irreversible, and because it had been shown that NEM-treated red cells survived normally in the circulation even when nearly all of the GSH had been alkylated[2], we thought that labeled NEM might be an excellent tag for the study of red cell survival. However, when rabbit red cells were tagged with C^{14} NEM it was found that the label quickly disappeared from the blood, even though it could be shown that the tagged cells still circulated. Further investigations showed that even on *in vitro* incubation a labeled derivative of NEM left the red cells over a period of several hours and that GSH reappeared in the erythrocytes. The dissociation of the GS-NEM (the alkylated product of GSH and NEM) occurs nonenzymatically, and the instability of this alkylation product must be taken into account in interpreting the results of long term experiments in which NEM is employed to alkylate sulfhydryl groups.

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MATERIALS AND METHODS

In vivo studies were carried out on the red cells of normal New Zealand rabbits; *in vitro* investigations on normal human blood drawn into acid-citrate-dextrose (U.S. Pharmacopeia formula B).

C^{14} NEM was obtained from New England Nuclear Corporation. Phosphate-NaCl solution, pH 7.4 was prepared by mixing 9 parts of 0.154 M NaCl solution with 1 part of 0.150 M potassium phosphate buffer, pH 7.4. Phosphate-NaCl solution, pH 6.6 was prepared by mixing 8 parts of 0.154 M NaCl solution with 2 parts of 0.150 M potassium phosphate buffer, pH 6.6. Tris-NaCl solution, pH 8.2, was prepared by mixing 8 parts of 0.154 M NaCl solution with 2 parts of 0.200 M Tris-chloride, pH 8.2. Reduced glutathione determinations were carried out using 5,5' dithiobis-(2-nitrobenzoic acid) (DTNB) [3]. Oxidized glutathione (GSSG) was estimated enzymatically after alkylation of GSH with NEM [4]. Radioactivity was counted using a Packard tricarb scintillation spectrometer for C^{14} and a Packard autogamma counter for Cr^{51}.

Rabbit red cells were prepared for labeling with NEM by washing 3 ml of blood 2 or 3 times in 0.15 M NaCl solution and then preparing a 50% suspension of red cells in a phosphate-NaCl solution, pH 6.6. A 200 μM solution of NEM containing 0.25 μcuries of C^{14} NEM/ml was prepared in phosphate-NaCl, and 0.2 ml of this solution added to each ml of 50% rabbit red cell suspension. After standing at 25° for 10-20 minutes, the red cells were washed 2 times in 0.15 M NaCl solution, an aliquot was saved for counting and the remainder injected intravenously. To determine C^{14} activity of the red cells 0.2 ml of packed cells were pipetted into 0.3 ml of water and 0.5 ml of 20% trichloroacetic acid (TCA) were added. The supernatant after centrifugation was removed and the precipitate was washed with 0.5 ml of 10% TCA. The pooled supernatants were brought to

a volume of 2 ml with water and extracted twice with 5 ml of ether. The radioactivity of 1 ml of the extracted solution was determined in 10 ml of Bray's solution.

Human red cells were washed 2 times in 0.154 M NaCl solution. The packed cells were resuspended in various volumes of 2 mM NEM containing 0.025 μcuries C^{14} per ml in phosphate-NaCl, pH 7.4. Sufficient phosphate-NaCl solution was then added to make a 50% suspension, which was allowed to stand for 10-20 minutes at 25°. The cells were washed 5 times in phosphate-NaCl solution, pH 7.4. Incubation experiments were carried out using a 25% suspension of cells in phosphate-NaCl solution, pH 7.4, containing substrate as indicated. At appropriate intervals the suspension was centrifuged, the supernatant removed for counting, and the cells lysed in water for GSH determination. To avoid the possible effect of swelling or shrinkage of the cells, the GSH measurements were based on the hemoglobin content of the lysed cells, and converted to μmoles of GSH per ml of red cells based on the initial mean corpuscular hemoglobin concentration of the cells.

RESULTS

Rabbit red cells were labeled with radioactive NEM and reinfused into the donor rabbit. The quantity of carrier NEM in the system is sufficient only to alkylate approximately 4% of the red cell GSH. In the case of one rabbit (Rabbit #3) the red cells were labeled with Cr^{51} as well. Blood was drawn from the ear opposite to the one used for injection 10 minutes and 24 hours after injection of the labeled red cells. The results are summarized in Table I. It is apparent that in all 3 instances virtually all of the radioactivity had disappeared within 24 hours. However, the persistence of the Cr^{51} label in the red cells of Rabbit 3 demonstrated that treatment with NEM had not damaged the rabbit erythrocytes

Table I

C^{14} Activity of Rabbit Red Cells Labeled with C^{14} NEM and Cr^{51} and Reinfused into the Donor Rabbit

Rabbit No.	Percent of Injected Counts per ml of RBC			
	10 Minutes		24 Hours	
	C^{14}	Cr^{51}	C^{14}	Cr^{51}
1	1.56	--	.010	--
2	1.26	--	0.00	--
3	.76	1.07	0.00	.86

sufficiently to impair markedly their viability.

To determine whether the labeled NEM had become attached to an acid insoluble or ether extractable component of the red cell, the TCA insoluble material was dissolved in Soluene (Packard), counted in Bray's solution, and the ether extracts were also counted. No C^{14} activity was found.

Human red cells were treated with 0.75 volumes or with 1.25 volumes of 2 mM labeled NEM in phosphate-NaCl solution, pH 7.4, or with phosphate-NaCl solution, pH 7.4. The five times washed cells were suspended in phosphate-NaCl solution, pH 7.4, containing 5.56 mM glucose. Samples were removed at 2 hour intervals, and the radioactivity of the supernatant and glutathione content of the red cells were determined. As shown in Figure 1, GSH reappeared in the red cells during the course of incubation, and C^{14} activity appeared in the incubation medium.

The properties of the C^{14} labeled material differed in several respects from NEM. The C^{14} activity in the supernatant was only slightly ether extractable, while unaltered NEM is totally ether extractable. The material in the supernatant no longer had the capacity to combine with GSH to form a compound inactive with DTNB.

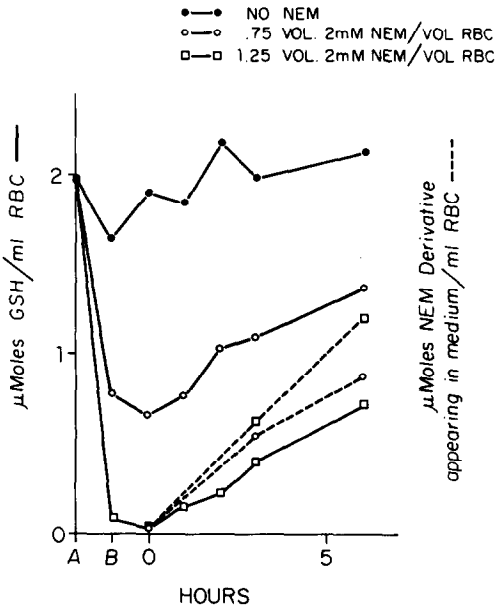


Figure 1: The effect of NEM treatment and subsequent incubation in phosphate-NaCl solution, pH 7.4 on red cell GSH and on appearance of C^{14} in the medium. A: pretreatment; B: after treatment with NEM and washing.

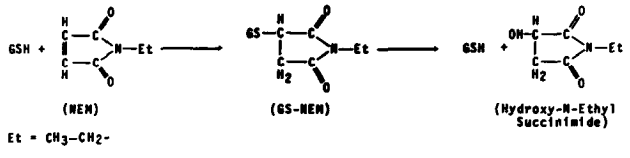
It did not diffuse into the red cell when mixed with normal human erythrocytes.

In order to clarify the mechanism of GSH release from GS-NEM further studies were carried out. Red cells from fresh heparinized blood were washed two times in 0.15 M NaCl solution, frozen and thawed and centrifuged at 20,000 g for 30 minutes. The stroma-free hemolysate was dialyzed for at least 48 hours against phosphate-NaCl solution, pH 7.4 or pH 6.6 or tris-NaCl solution, pH 8.2. GS-NEM substrate was prepared by making 0.1 M NEM and 0.1 M GSH solutions by weight, mixing equal volumes, and neutralizing with NaOH. The mixture was found to contain a 5% excess of GSH, assuring that no free NEM remained in the mixture. Dialyzed hemolysate representing approximately 5 ml packed red cells per 10 ml incubation mixture was incubated with 10 mM $MgCl_2$, 0.1 mM TPN, 2 mM ATP, 5.6 mM glu-

cose and 10 mM GS-NEM substrate for 6 hours. Hydrogen ion concentrations were controlled at pH 6.6, 7.4 and 8.2, by the buffer against which the hemolysate had been dialyzed. An increase in the quantity of GSH in the mixture occurred, so that at pH 7.4, 0.1 to 0.2 µmoles of GSH had been released from the NEM-GSH complex per ml of reaction mixture. Dissociation of the complex occurred more rapidly at pH 8.2 and more slowly at pH 6.6 than at pH 7.4. When the TPNH generating system was omitted from the reaction mixture, a part of the glutathione appeared in the oxidized form. It was found, however, that the rate of release of GSH or GSSG from the GS-NEM was similar if the hemolysate was boiled prior to addition to the mixture. When no red cell extract was added to the system some GSSG also appeared, but this usually amounted to less than 0.05 µmoles per ml of mixture. Although GSSG was found as a product, it seems likely that the primary product is GSH: if an excess of NEM was added to the system no GSSG or GSH were found.

DISCUSSION

The investigations lead us to conclude that the GSH-NEM complex formed in the red cells dissociates at physiologic pH levels resulting in the formation of GSH and a NEM derivative, possibly in the following reaction:



If, indeed, it is the first product of hydrolysis of GS-NEM, hydroxy-N-ethyl succinimide very likely undergoes further degradation to N-ethyl succinimide.

The fact that the complex of NEM with GSH is not stable clarifies previously unexplained observations regarding the role of GSH in the red cell. Allen and Jandl[2] had demonstrated that virtually all of the GSH in red cells could be alkylated with NEM with-

out impairing survival of the reinfused erythrocytes. Yet, GSH-synthetase deficiency, a genetic defect which results in GSH deficiency was found to impair markedly the survival of erythrocytes[5]. This discrepancy can now be explained. The alkylation of GSH by NEM is a gradually reversible process. Therefore, only short-term experiments using this agent have validity. Alkylation of protein-SH groups by NEM is widely employed in structural studies, and the stability of the protein-S-NEM complex will require further study.

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