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# AGGREGATION OF INTRAMEMBRANE PARTICLES IN ERYTHROCYTE MEMBRANES TREATED WITH DIAMIDE

JOSEPH KURANTSIN-MILLS [b] and LAWRENCE S. LESSIN [a]

[a] *Division of Hematology and Oncology, Department of Medicine and* [b] *Department of Physiology, The George Washington University Medical Center, Ross Hall, Room 513—514, 2300 Eye Street, Washington, DC 20037 (U.S.A.)*

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## Summary

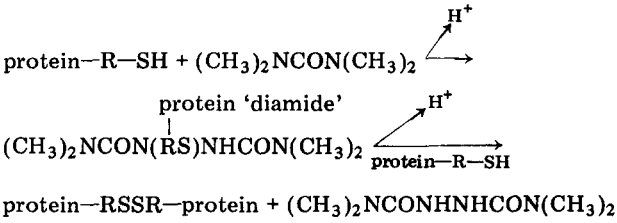
Treatment of erythrocytes with diamide (diazene dicarboxylic acid bis-(*N*,*N*-dimethylamide)) results in oxidation of sulfhydryl groups of the membrane, and cross-linking of membrane proteins into high molecular weight complexes. Concomitant freeze-etching studies show aggregation of intramembrane particles on the protoplasmic fracture face of erythrocyte ghost membranes treated with the oxidant. Furthermore, after a 3 h incubation of erythrocytes with 10 mM diamide at 37°C, cellular energy levels declined to about 70% of control values. The data suggest that disulfide cross-linking of the major membrane proteins releases the apparent physical occlusion of the band 3 proteins within the interstices of the cytoskeletal shell. This results in the translational mobility of band 3 proteins which is reflected ultra-structurally in the freeze-etch images.

## Introduction

Diamide (diazene dicarboxylic acid bis(*N*,*N*-dimethylamide)) was first introduced by Kosower and associates [1] as a specific oxidant for gluthathione in human erythrocytes. However, subsequent studies have shown that the com-

Abbreviations: Temed, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; SDS, sodium dodecyl sulfate.

pound is active in general reactions such as the following [2,3]:




where R is the cysteinyl residue in a peptide. Such intermolecular disulfide linkages result in altered cellular reactions. Thus, it has been reported that diamide impairs oxidative reactions in rat liver mithochondria [4]; inhibits amino acid and sugar transport in rat kidney cortex [5]; and non-specifically inhibits AMP-dependent and -independent auto-phosphorylation of erythrocyte membranes [6]. Diamide also inhibits ATP-dependent echinocyte or spheroechinocyte-discocyte transformation [7] and cross-links the polypeptides of erythrocyte membranes into high molecular weight complexes [8—10]. In order to obtain further information on the perturbation of membrane structure, we have examined erythrocyte membranes treated with diamide by freeze-etching and transmission electron microscopy, as well as by polyacrylamide gel electrophoresis. Our results show that in the presence of diamide there is aggregation of intramembrane particles.

## Materials and Methods

### *Chemicals*

Chemical reagents used for the preparation of buffers were all of analytical grade. Diamide (diazene dicarboxylic acid bis(*N*,*N*-dimethylamine)) was obtained from Sigma Chemical Co. St. Louis, MO, U.S.A. Dithiothreitol and other reagents for electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.

### *Freeze-etching and transmission electron microscopy*

Freeze-etching of erythrocyte membranes was performed in a Balzers 300 Ultrahigh vacuum device by the method of Moor and Muhlethaler [11]. Ghost membranes treated with and without 5 or 10 mM diamide for 60 min were cryo-protected by incubation in 25% glycerol solution in 5 mM phosphate-buffered saline, pH 7.4, at 37°C for 2 h with three changes of the buffered glycerol medium. Droplets of thick ghost suspension were then pipetted onto gold disks and frozen in liquid dichlorodifluoromethane (—150°C) and stored in liquid nitrogen. The frozen specimens were transferred onto the freeze-etching microtome stage and the chamber evacuated to $10^{-6}$ torr. The specimen was fractured by a single stroke using a cold (—100°C) microtome knife, and then sublimated (etched) for 2 min. A replica of the fractured surface was obtained by evaporating platinum and carbon onto it. The specimen was removed from the device and the replica floated in buffered 30% glycerol or water. The replica was then cleaned of cellular debris in 70% sulfuric acid

(1—2 h), in dilute sodium hypochlorite (20—30 min), and distilled water (30 min). Replicas were placed on copper grids and examined in a JEOL JEM 100B electron microscope. Images were studied and photographed at original magnification of 10 000 and higher magnification obtained by photographic enlargement.

*Assay of adenosine triphosphate*

Fresh red cells were washed three times with 5 mM phosphate-buffered saline, pH 7.4, and resuspended to a packed cell volume of 30%. 2-ml aliquots of the cell suspension were then incubated with 10 mM diamide for various intervals at 37°C. After incubation the suspensions were centrifuged at 3000 × *g* for 10 min, the supernatant discarded and the cells re-suspended to the same packed cell volume as the preincubation values. The ATP content of the cell preparations was determined by the method of Kornberg [12] as modified by Lamprecht and Trautschold [13] using the enzymatic assay on neutralized perchlorate extracts.

*Incubation of erythrocytes with diamide*

Fresh erythrocytes were washed three times with 5 mM phosphate-buffered saline, pH 7.4. Appropriate aliquots of the cell suspension were then pipetted into the buffer solution containing 5 or 10 mM diamide in a final volume of 10 ml. A control cell suspension was set up without diamide. The cells were incubated at 37°C for 30 or 60 min. At the end of the incubation, the cells were centrifuged, washed twice in suspension, and centrifuged again. Packed cells were then assayed for adenosine triphosphate content. Another tube of packed cells was processed for freeze-etching. In the latter procedure, ghosts prepared by hypotonic lysis followed by two washings were incubated with 5 or 10 mM diamide at 37°C for 60 min. The ghosts were then glycerinated for freeze-etching and transmission electron microscopy. Ghost membranes for protein electrophoresis were prepared by the method of Dodge et al. [14], and their protein content determined by the method of Lowry et al. [15].

*Electrophoretic analysis of membrane proteins*

Because of the supramolecular complexes of cross-linked membrane proteins, agarose-acrylamide composite gels were used for the electrophoresis according to the formulations of Steck [16]. The gels were cast of 3% acrylamide and 0.4% agarose according to the method of Peacock and Dingman [17]. The gels and the electrophoresis buffer both contained 0.2% SDS. It was found that temperature control during the casting of the gel was essential in relation to the polymerization and the flatness of the stained protein bands. Best results were achieved by warming all stock solution to 45°C in a water bath. The stock solutions were then mixed, and the hot agarose (95°C) was added to the mixture maintained at 60°C. After adding the ammonium persulfate and Temed, the gel solution was immediately pipetted into glass tubes which had been warmed to 60°C. The gels were overlaid with warm solution of 0.1% SDS, 0.15% ammonium persulfate and 0.05% Temed or 0.2% SDS. The warm gels were immediately solidified by immersion in an ice-water bath for 5 min, and left at room temperature for 24—48 h before the electrophore-

sis. Electrophoresis was completed in about 2 h. The gels were stained for proteins with 0.5% Coomassie Blue in a 25% isopropanol/10% acetic acid mixture and destained as previously described [16].

## Results

*Adenosine triphosphate concentration in diamide-treated erythrocytes*

The concentration of ATP in erythrocytes incubated with 10 mM diamide declined to about 70% of the values determined on control samples during the 1 h incubation at 37°C (Table I). Extension of the incubation time to 3 h resulted in a further decrease in cellular ATP levels to about 30% of the control samples.

*Freeze-etching erythrocyte membranes treated with cross-linking agents*

Replicas of freeze-etched red cell ghosts treated with 5 mM diamide revealed the protoplasmic and exoplasmic fracture faces. Electron micrographs of the protoplasmic fracture face revealed intramembrane particles manifesting varying degrees of particle aggregation (Fig. 1). The pattern of aggregation included: (i) clumping; (ii) linear arrays; (iii) clumping and arrays together; and (iv) reticular arrangement of particles in the plane of the fracture face. Scrutiny of several micrographs disclosed that intramembrane particle aggregation was exclusive to the protoplasmic fracture face. Aggregates of intramembrane particles varied widely in the number of particles. Estimates of number of intramembrane particles per aggregate ranged from 4 to 20 (Fig. 1). Because of the low density of intramembrane particles on the exoplasmic fracture face, any effect of diamide on particle organization was not apparent. The degree and variability of particle aggregation is illustrated in Fig. 1; no differences could be discerned with the use of 5 mM or 10 mM diamide in a final 15% cell suspension. When excess diamide of the treated cells was washed off with buffer, and the cells reincubated with 25 mM dithiothreitol for 30 min at 37°C no intramembrane particle aggregation was observed, indicating a reversal of the intramembrane particle aggregation (data not shown).

TABLE I

ATP CONCENTRATION OF RED CELLS INCUBATED WITH 10 mM DIAMIDE FOR VARIOUS TIME PERIODS AT 37°C

The control was incubated for 3 h at 37°C without diamide. ATP values are the mean of three separate experiments, with each ATP assay done in duplicate.

| Time of incubation (h) | ATP concentration (mM/l red blood cells) |
|---|---|
| Control | 1.28 |
| 0.5 | 0.96 |
| 1.0 | 0.90 |
| 2.0 | 0.44 |
| 2.5 | 0.58 |
| 3.0 | 0.39 |

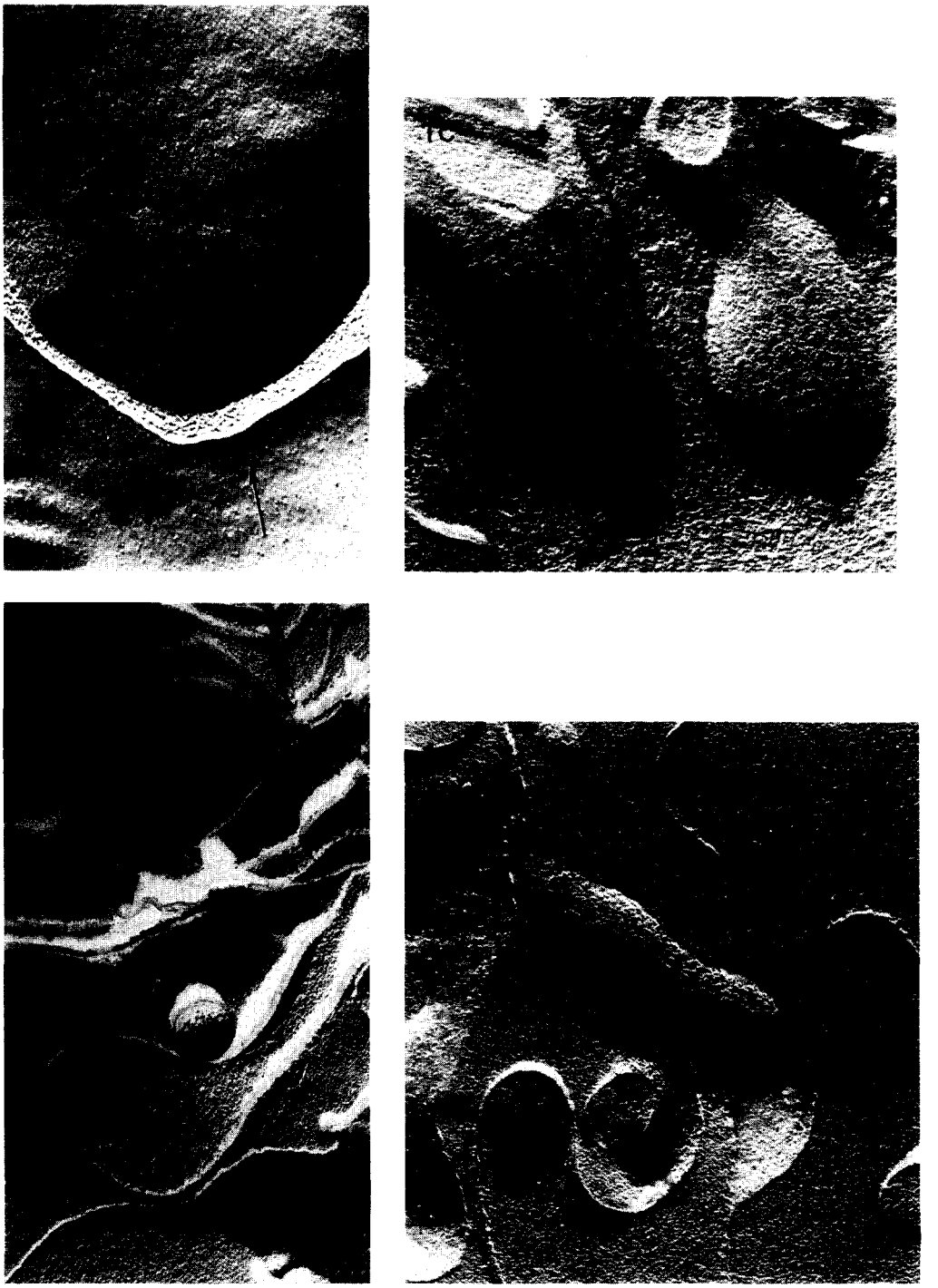


Fig. 1. Freeze-etched micrographs of membrane of erythrocytes untreated (a) and treated (b, c, d) with 5 mM diamide in phosphate-buffered saline, pH 7.4, for 60 min at 37° C prior to glycerination and freeze-etching. The micrographs illustrate the varying types of aggregation of intramembrane particles observed in this study. Note that intramembrane particle aggregation appears only at the protoplasmic fracture face (arrows). The rather low density of intramembrane particles on the exoplasmic fracture (EF) face make it difficult to discern any rearrangement of the intramembrane particles. Magnification: a, control 27 200X, untreated with diamide; b, 32 670X; c, 34 670X; d, 36 960X.

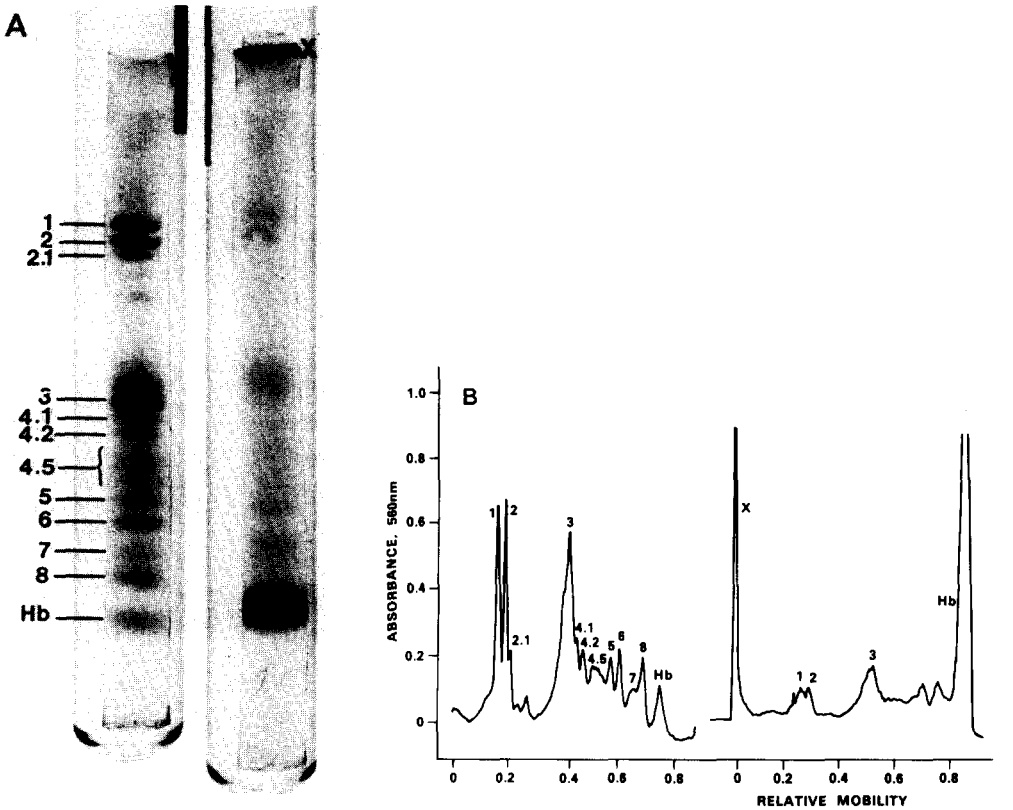



Fig. 2. (a) Electrophoretograms of red blood cell ghost proteins in SDS-agarose polyacrylamide gels showing the effect of diamide on the ghost proteins. Control ghost without diamide (left). Red blood cells were incubated with 5 mM diamide in phosphate-buffered saline at 22—24°C for 30 min prior to preparation of ghosts. Ghosts were dissolved in 10 mM Tris-acetate buffer, pH 8.0/1% SDS, but without dithiothreitol in the diamide-treated cells, incubated at 37°C for 39 min and then electrophoresed as described in Materials and Methods. 30 μg protein was loaded onto each gel. Gels were stained with Coomassie blue as described in Materials and Methods. Note the presence of high molecular weight protein aggregates on top of the gels of the diamide-treated samples, and the depletion of all major protein bands (right). (b) Densitometric scans of the Coomassie-blue-stained gels of the control sample and the diamide-treated cells shown at the left. Hb, hemoglobin.

*Electrophoretic analysis of membranes of erythrocytes and ghosts treated with diamide*

Electrophoretic analysis of membranes of ghosts from erythrocytes treated with 5 or 10 mM diamide revealed the presence of very high molecular weight complexes when the protein preparation was electrophoresed without dithiothreitol (Fig. 2). Estimation of the molecular weights of these complexes indicated that they ranged from 540 000 to over $10^6$. The latter complexes failed to enter the 3% acrylamide/0.4% agarose composite gels or 2.5% acrylamide/0.3% agarose composite gels. Concomitantly, most of the major polypeptides showed substantial reduction in their staining intensity (bands 1, 2, 3, 4.1—4.5). The only exception was globin subunits which showed increased staining due to greater hemoglobin retention of diamide-treated ghosts (Fig. 2).

Incubation of diamide-treated ghosts with 42 mM dithiothreitol for 30 min at 37 or 24°C prior to electrophoresis restored the electrophoretograms to a pattern similar to the control untreated ghost membranes. The rapid reversibility to a normal protein gel profile in the presence of the reducing agent suggested that sulfhydryl groups are involved in the formation of protein complexes. Even in the presence of dithiothreitol, the staining intensities of the diamide-treated samples was slightly diminished. Furthermore, some of the $10^6$ dalton complexes still persisted on top of the gel (data not shown).

## Discussion

Diamide has been shown to react extensively with glutathione (γ-Glu-Cys-Gly; GSH), and perhaps exposed reactive thiols in red cells [18]. Most of these previous studies have emphasized the thiol-oxidizing properties of diamide without consideration of the effect of the agent on red cell membrane ultrastructure. Greenquist et al. [7] showed that diamide inhibited echinocyte-discocyte transformation and suggested that thiol groups of membrane proteins which may be involved in the cell shape changes have been oxidized. The present study has demonstrated that diamide induces reversible aggregation of intramembrane particles on the protoplasmic fracture face of the erythrocyte membrane. Reversible clumping of intramembrane particles has been observed in red cell ghosts exposed to a pH 4.5—5.0 buffer [19]. Elgsaeter and Branton [20] have also demonstrated that a substantial proportion of bands 1, 2 and 5 need to be extracted from ghosts during washing with a pH 5.0 buffer before particle aggregation could be observed. In the present studies the cells were incubated in buffered saline at pH 7.4 prior to freeze-etching and the pH of the incubated cell suspensions did not change. Hence, pH-induced aggregation of particles can be discounted.

Glycophorin and band 3 protein are currently believed to be the major constituents of the intramembrane particles revealed by freeze-etching and electron microscopy of red cell membranes [21,22]. Elgsaeter and Branton [20] demonstrated that the translational mobility of protoplasmic fracture face intramembrane particles is restricted by spectrin, supporting an earlier hypothesis that both glycophorin and band 3 are physically linked to spectrin [21]. On the contrary, Cherry et al. [23] have demonstrated that band 3 rotates relatively freely about an axis normal to the plane of the membrane, and that spectrin does not restrict this rotational diffusion (relaxation time, 1 ms). The lateral mobility of membrane glycoproteins has also been demonstrated by the technique of fluorescence redistribution after fusion [24]. In the latter study, it was found that lateral mobility of erythrocyte membrane glycoproteins can be increased by the addition of ATP and 2,3-diphosphoglycerate, and decreased by neomycin and spermine. This control was effective only when these molecules were in contact with the cytoplasmic side of the membrane. Our results are consistent with the notion [23,24] that glycoprotein lateral mobility is influenced by protein-protein interaction. Therefore, the degree of interaction or cross-linking of cytoskeletal shell proteins (spectrins, actin and band 4.1) would conceivably control the translational mobility or the state of aggregation of the glycoproteins (band 3 and

glycophorin). It is interesting to note that bands 4.1 and 2.1 (ankyrin) have been shown to form a stable complex with spectrin and band 3 in aqueous medium [25—27], although the exact interaction of these proteins within the membrane bilayer is at present unknown.

Liu and Palek [28] have also demonstrated specific association between spectrin band 1 and band 3 in erythrocyte ghosts by disulfide coupling (using $CuSO_4$/*o*-phenanthroline mixture) under physiologic pH and isotonic conditions producing a complex of 330 000 daltons. Although the disulfide coupling of membrane proteins in our study was accomplished under different conditions, it is reasonable to consider that the 540 000—$10^6$ dalton complexes include proteins of the cytoskeleton and band 3. It is then possible that disulfide cross-linking of the cytoskeletal proteins releases their apparent restriction on the translational mobility of the band 3 proteins. This viewpoint supports the hypothesis that the actual physical state of the cytoskeleton is labile [29,30]. Based on our results, and other data [23,24,28,30], we conclude that diamide perturbs membrane ultrastructure of erythrocytes by disulfide cross-linking of cytoskeletal proteins (spectrin, actin, component 4.1). This perturbation results in the translational mobility and aggregation of membrane glycoproteins which is reflected ultrastructurally in the freeze-etch images of the protoplasmic fracture face.

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