Comparison of Hemoglobin Köln Erythrocyte Membranes With Malondialdehyde-Reacted Normal Erythrocyte Membranes

By David W. Allen, Claude F. Burgoyne, Jerome D. Groat, C.M. Smith II, and James G. White

Splenectomized patients with hemoglobin (Hb) Köln have rigid RBCs with membrane polypeptide aggregates that are not dissociable with disulfide-reducing agents. Malondialdehyde (MDA) action on normal RBCs produced rigid RBCs with similar nondissociable aggregates. To test the hypothesis that Hb Köln RBC aggregates contained unsaturated MDA-type bonds, we reduced normal control RBC membranes, Hb Köln RBC membranes, and MDA-reacted membranes with \(^{3}H\)NaBH\(_{4}\). Hb Köln RBC membranes and MDA-reacted membranes both had significantly more \(^{3}H\) incorporation than control membranes. Furthermore, \(^{3}H\) incorporation in both Hb Köln and MDA-treated membranes was located in the membrane polypeptide aggregates, presumably saturating the crosslinking bonds. After reaction of RBCs with \([^{14}C]\)MDA, the MDA label was similarly concentrated in the membrane polypeptide aggregates. Normal RBC membranes incubated with MDA were analyzed with and without reduction by NaBH\(_{4}\) prior to amino acid determination by high-performance liquid chromatography (HPLC). Reduction with NaBH\(_{4}\) after MDA treatment decreased the lysyl residues by 33% and the serine by 7% and increased by 10% the methionyl residues, but did not affect 12 other amino acids. Similar changes could be detected in NaBH\(_{4}\)-reduced Hb Köln aggregates in methionine and serine content. MDA may also alter protein configuration, as evidenced by an increase in the protease susceptibility of membrane proteins from MDA-treated and Hb Köln RBCs. We conclude that Hb Köln RBC membranes, like MDA-treated membranes, have similar high molecular weight aggregates conferring decreased membrane deformability. \(^{3}H\)NaBH\(_{4}\)-reducible unsaturated bonds, changes in amino acid composition upon reduction, and protease-sensitive configurational changes.

Red Blood Cells from patients with hemoglobin (Hb) Köln generated increased malondialdehyde (MDA) in the hydrogen peroxide stress test.\(^1,2\) Lipid extracts of fresh RBCs from patients with Hb Köln also showed a fluorescence spectrum characteristic of MDA-produced 1-amin-3-imino propenes.\(^2\) Thus, the RBC membrane polypeptide aggregates in splenectomized patients with Hb Köln disease that are not dissociated by mercaptethanol and were associated with decreased RBC deformability were thought to be crosslinked by similar MDA-produced bonds shown to produce membrane polypeptide aggregates,\(^3\) decreased deformability,\(^4,5\) and RBC survival.\(^5\) However, MDA is not the only possible mechanism with nondissociable crosslinks.\(^6,8\) and thus, additional proof of MDA involvement in Hb Köln disease should be sought.

For experimental purposes, MDA is generally produced just before use by acid hydrolysis of its methyl acetal, 1,1,3,3-tetramethoxypropane.\(^9\) This reaction is contaminated with the methanol released, the methanol oxidation product formaldehyde, reactive partial hydrolysis products (3,3-dialkoxypromionaldehyde, and \(\beta\)-alkoxyacrolein), and straight-chained and branched-chained self-condensation products of MDA.\(^10\) Almost all of these side products may react with proteins and may perhaps be more effective at denaturing enzymes and polymerizing proteins than MDA itself.\(^11\) Thus, most investigators have employed a variety of experimental conditions and controls to decrease the possibility of confusing the effects of contaminants with those of MDA.\(^3,5,9\)

A likely, but unproven, site of MDA addition to proteins is the epsilon amino group of lysine. Normally, the major reducible components of RBC membranes are hexosyllysines.\(^12\) \(^{3}H\)NaBH\(_{4}\) labels all the membrane proteins of normal or diabetic RBCs in like proportion to Coomassie blue staining, indicating similar glycosylation of RBC membrane proteins.\(^13\) However, if MDA is added to proteins, additional lysine-aldehyde adducts might form, and unsaturated bonds might result.\(^10\) Thus, we reacted control, Hb Köln, and MDA-treated RBC membranes with \([^{14}C]\)MDA, to label, saturate, and stabilize any MDA-produced 1-amin-3-imino propene bonds. We compared the incorporation of this label and its distribution in membrane polypeptides separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and gel filtration. To demonstrate the propene carbons of MDA in such aggregates, we also reacted normal RBCs with \([1,3,^{14}C]\)MDA.

Genetic abnormalities of RBC membrane proteins alter their normal interaction required for membrane function and RBC survival.\(^14\) In addition to mutational changes, denaturation of spectrin with urea, SDS,\(^15\) or N-ethylmaleimide\(^6\) has been shown to increase its susceptibility to tryptic digestion. Thus, we compared

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the sensitivity to trypsin and chymotryptic digestion of Hb Köln RBC membranes with control membranes and with RBC membranes that had been treated with MDA.

Chio and Tappel\(^9\) reacted ribonuclease with MDA and compared the amino acid content with that of the original protein. They noted 40% destruction of tyrosine and 25% destruction of methionine and lysine. These authors did not reduce with NaBH\(_4\) prior to acid hydrolysis, although any imino propene linkages formed would be acid labile.\(^{17}\) Here, we extend Chio and Tappel’s observations to normal RBC membranes reacted with MDA and to Hb Köln membrane polypeptide aggregates, both with and without NaBH\(_4\) reduction. Using these various techniques, we define further the chemical nature of the Hb Köln membrane changes, finding additional similarities to the effects produced by MDA on normal RBC membranes.

MATERIALS AND METHODS

Subjects

Members of the previously described family with Hb Köln\(^2\) and normal control laboratory personnel were studied. Each gave informed written consent in accordance with a protocol approved by the Human Studies Subcommittee of the Research and Development Committee of the Minneapolis Veterans Administration Medical Center.

Preparation of MDA

MDA stock (1.2 mol/L) was routinely prepared just prior to use by hydrolysis of 1,1,3,3-tetramethoxypropane (Eastman Kodak, Rochester, NY) in 1 to 10 mmol/L HCl at 25 °C for 0.5 to 128 minutes.\(^{14}\) After neutralization, subsequent dilutions were made in either pH 7.4 phosphate-buffered isotonic saline (PBS) (RBCs) or 5 mmol/L phosphate (membranes), and the incubation started immediately. 1,1,3,3-Tetramethoxy [1,3,4\(^\text{C}\)]propane (33 mCi/mmol) was obtained from Amersham Searle (Arlington Heights, Ill).

Studies on Intact RBCs

Blood samples were drawn into tubes containing heparin or disodium EDTA. The blood was centrifuged, white cells and platelets were removed, and the RBCs washed and suspended in PBS. RBCs were then incubated with 2 m mol/L to 200 mmol/L MDA (PBS, 37 °C, 15 to 120 minutes). Following the incubation, the MDA was removed by washing.

Studies on RBC Deformability

Membrane deformability was studied in normal control and MDA-treated RBCs. A small portion of membrane at the central biconcavity of the RBCs was drawn into micropipettes with internal radii (R) between 0.3 and 0.5 μm at pressures under 800 dyne/cm\(^2\).\(^{18,20}\) Several pressure-extension length pairs were determined for each cell. The shear extensional elastic modulus (μ) of the membrane was obtained by linear regression analysis of the pressure-length pairs, utilizing the mathematical relationship described by Chien.\(^{18}\) The RBCs were studied in PBS (0.2 g/dL albumin, glucose 0.1 g/dL) while inside a 1-mm high glass cave, similar to that reported by Linderkamp.\(^{20}\) The same pipette was used for paired control and MDA-treated RBCs because of the large error introduced into the analysis by the measurement of pipette radius. The data are presented as an elastic index which was defined as the ratio of the elastic modulus of the MDA RBCs (μ\(_\text{MDA}\)) to the control elastic modulus (μ\(_\text{C}\)) obtained for that particular pipette.

The erythrocytes were visualized directly on the micropipette with a Reichert-Austria (Vienna) model NR 257310 Wright microscope equipped with Leitz 100 x water immersion lens. (Leitz, Rockleigh, NJ). The mean cell volumes (MCVs) of the RBC suspensions were obtained by the quotient of the spun microcentrifuge hematocrit and Coulter S electronic red cell count (Coulter Electronics, Hialeah, Fla). The distribution of cell density was evaluated by visualization of a ten layer Dextran 40 discontinuous gradient with specific gravities varying from 1.078 to 1.125.\(^{21}\)

Studies on RBC Membranes

RBC membranes, prepared by the method of Dodge et al.\(^{22}\) were incubated with various concentrations of MDA in 5 mmol/L phosphate, pH 8, at 25 °C for 20 hours. Polypeptide aggregates were detected on SDS-PAGE (20 μg protein)\(^{23,24}\) and prepared by gel filtration of 10 to 20 mg protein\(^2\) with Biogel A50M (BioRad, Richmond, Calif). [\(^3\)H]NaBH\(_4\) (21 mCi/Mmol/L) (New England Nuclear, Boston) was reacted (16 mmol/L, 25 °C, 45 minutes, 5 mmol/L phosphate, pH 7.4) in 200 molar excess to the calculated lysyl content of the RBC membranes. Before determination of the incorporated radioactivity, the aliquots were precipitated and washed three times with trichloroacetic acid at 0 °C, and before SDS-PAGE, the membranes were washed with 5 mmol/L PO\(_4\) pH 8, at 4 °C by repeated ultracentrifugation.

Proteinase Sensitivity of Membranes and Spectrin Extracts

Crude spectrin extracts containing actin were obtained by extraction of RBC membranes with 0.1 mmol/L phosphate, EDTA, mercaptoethanol, and phenyl-methyl-sulfonyl fluoride (PMSF) (pH 8, 37 °C, 30 minutes), modified from Speicher et al.\(^{25}\) After centrifugation at 50,000 rpm for 50 minutes, aliquots of the supernatant were reacted with 20 mmol/L MDA. Control, Hb Köln, and MDA-treated membranes and spectrin were digested with trypsin or chymotrypsin equal to 1:100 weight of substrate (pH 8, 0 °C, 20 hours), and the peptide composition compared by SDS-PAGE of 40 μg protein.

Amino Acid Analysis

For amino acid analysis, NaBH\(_4\)-reduced and unreduced samples were hydrolyzed with 6 N HCl at 110 °C for 24 to 96 hours, then dried and resuspended in water. The samples were derivatized with orthophthalaldehyde (Pierce Chemical Co, Rockford III) before analysis on a Bondapak C-18 reverse phase column (Waters Associates, Milford, Mass). We used sequential linear gradients adding acetonitrile to a triethylamine-acetic acid buffer (0.016 mmol/L, pH 7.4)—first 0% to 40% (40 minutes), then 40% to 70% (10 minutes)—modifying the method of Larsen and West.\(^{26}\) Standard amino acid mixtures (60 and 480 pmol, Pierce Chemical Co) were run daily to calibrate the fluorescence for each amino acid.

RESULTS

Since Hb Köln RBCs had previously been shown to generate MDA, with high molecular weight membrane polypeptide aggregates and decreased deformability,\(^7\) we examined the effect of MDA on normal RBC membranes. High molecular weight aggregate
formation and decreased deformability became evident when the MDA concentration to which the RBCs were exposed reached 20 mmol/L (Table 1). In this experiment, intact RBCs were incubated in PBS for 30 minutes at pH 7.4 and 37 °C with various concentrations of either MDA or the theoretical amount of methanol released by hydrolysis of the tetramethoxypropane. The RBCs were then washed with PBS, aliquots were studied by micropipette, and the remaining RBCs were used for preparation of membranes and analysis by 4% SDS-PAGE. The gels were scanned, and the amounts of components determined by planimetry. The content of aggregates formed was measured as the percent of band 3, an intrinsic membrane component. The extensional elastic modulus of the membrane was not altered by exposure to MDA until the MDA concentration reached a level that caused easily discernible high molecular weight aggregates. The presence of aggregates and the altered material property of the membrane was not associated with recognizable changes in cell shape, size, or density. The MCV and density gradient distribution of all MDA-treated samples, including the 20 mmol/L exposure, were not significantly different than the control suspensions. Exposure of control RBCs to 80 mmol/L methanol (Table 1) and, in other experiments, up to 800 mmol/L methanol, produced no aggregates or change in elastic index. The methanol was incubated under conditions identical to the MDA to help rule out effects from any formaldehyde produced by oxidation of methanol released by hydrolysis. Susceptibility to trypsin was also measured (see below).

Figure 1 shows an evaluation of the time course of hydrolysis of the tetramethoxypropane in 10 mmol/L HCl at 25 °C. Such an experiment was needed, since various hydrolysis times have previously been used.3 The extent of hydrolysis was evaluated by comparing aggregate formation from RBC membranes exposed to the products formed immediately after completion of hydrolysis. Note that the reaction was incomplete in 0.5 minutes, although this time interval has been used previously.4 Aggregate formation was nearly maximal at 30 minutes of hydrolysis, which was the interval employed in the experiments shown in Table 1. This achieved nearly maximal reaction, but avoided effects from MDA modification.11

Progressively more incorporation of [3H]NaBH₄ from [3H]NaBH₄ into control RBC membranes, Hb Köln RBC membranes, and MDA-reacted RBC membranes and MDA-reacted polylysine is shown in Fig 2. The aggregates of the MDA-treated membranes considerably exceed those of the Hb Köln RBC membranes, hence, the increased specific activity of the former is not unreasonable. The increased activity of the MDA-treated polylysine is likely due to the increased lysyl sites available for reaction with MDA. In three additional experiments, the specific activity of RBC membranes from Hb Köln patients after [3H]NaBH₄ reduction was also greater than controls.

To determine the polypeptide sites of this tritiation by [3H]NaBH₄, the labeled RBC membrane polypeptides were separated by SDS-PAGE, stained with Coomassie blue, dried, and photofluorographed (Fig 3). Note the increased radioactivity of the polypeptide aggregates at the origin of the MDA-reacted and of the Hb Köln RBC membranes compared to the normal control. The drying has decreased the apparent separation of the bands, and there is quenching of the photofluorography by the Coomassie blue, resulting in apparently less radioactivity with more stained aggregates in the MDA-treated membranes. Band 2.1 (ankyrin), band 4.2, and band 6 (glyceraldehyde-3-phosphate dehydrogenase) are labeled in all three

### Table 1. Effect of MDA Concentration on RBC Membranes

<table>
<thead>
<tr>
<th>MDA (mmol/L)</th>
<th>Methanol (mmol/L)</th>
<th>Aggregates (% Band 3)</th>
<th>Elastic Index* (μMDA/μC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>0.4 ± 0.3</td>
<td>1.01 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.7 ± 0.8</td>
<td>1.08 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.3 ± 0.5</td>
<td>1.01 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>16.7 ± 1.1†</td>
<td>1.47 ± 0.26†</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>80</td>
<td>0.3 ± 0.5</td>
<td>1.09 ± 0.16</td>
</tr>
</tbody>
</table>

*μMDA/μC is the elastic modulus of MDA-treated RBCs divided by the elastic modulus obtained for control RBC with the same micropipette. The 0.02 mmol/L and 20 mmol/L results were based on the aspiration of the 60 MDA-treated cells, and the 0.2 mmol/L and 2 mmol/L data were derived from the analysis of 30 cells.

†Significantly greater than control: P < .01.
preparations. Labeling of aggregates and bands 2.1, 4.2, and 6 was confirmed upon slicing and counting 10% slab24 and 4% disk gels. Further, gel filtration of 3H-Hb Köln RBC membranes on Biogel A50M2 demonstrated the increased specific activity of the aggregates, largely polymerized spectrin2 (1,980 cpm/μg protein), v nonaggregated spectrin (350 cpm/μg protein). Reaction of RBCs with 20 mmol/L [14C]MDA with subsequent membrane isolation and SDS-PAGE showed similar membrane polypeptide aggregates labeling and demonstrated participation of carbons 1 and 3 of the MDA in this crosslinking.

Since spectrin is especially sensitive to MDA action,1 susceptibility of spectrin to protease action as a measure of the effect of MDA on this protein is evaluated in Figs 4 and 5. Figure 4 also shows the effect of MDA on spectrin after four hours (Fig 4C) and 16 hours (Fig 4D). With time, MDA resulted in a progressive increase of high molecular weight aggregates. Comparison of the digests of MDA-treated spectrin with control spectrin after trypsin (Fig 4, F through I) and chymotrypsin (Fig 4, K through N) digestion demonstrates that in both cases, MDA pre-treatment resulted in increased proteolysis with increased background and loss of the distinct peptide pattern of the controls (control trypsin, Fig 4, F and G; control chymotrypsin, K and L; MDA, then trypsin, H and I; then chymotrypsin, M and N). Evidently, MDA produced a change in the secondary and tertiary structure that was responsible for the limited digestion of unmodified spectrin at 0 °C. For intact RBCs, 20 mmol/L MDA was required to produce these changes, as limited trypptic digestion of the samples in Table 1 showed.

Because MDA decreases spectrin extractability, it was preferable to compare the protease susceptibility of total membrane proteins of Hb Köln and MDA-reacted RBCs rather than extractable spectrin. Therefore, as seen in Fig 5, we compared the susceptibility to proteolytic digestion of membranes by trypsin equal to 1:100 weight of membrane protein (pH 8, 0 °C, 20 hour). Compared to the control digests (Fig 5, C and I-I), note the increase in low molecular weight peptides (< 43 x 10^3 mol wt) in the Hb Köln membranes (Fig 5, D and E) and MDA-treated control and MDA-reacted high reticulocyte RBCs (Fig 5, F and G). Notice also that while the control membranes (Fig 5, C and H), note the increase in low molecular weight peptides (< 43 x 10^3 mol wt) in the Hb Köln membranes (Fig 5, D and E) and MDA-treated control and MDA-reacted high reticulocyte RBCs (Fig 5, F and G). Notice also that while the control membranes (Fig 5, C and H) had relatively more of the 80 x 10^3 mol wt peptides than the 43 x 10^3 mol wt peptides, there were relatively more of the latter in Hb Köln (Fig 5, D and E) and MDA-reacted membrane peptides (Fig 5, F and G). Some aggregates were still present in the Hb Köln and MDA-reacted membranes.

To attempt to detect the amino acid residues combined with MDA, we analyzed RBC membranes with HPLC using precolumn derivatization with orthophthalaldehyde, a reverse-phase C18 column, and a sensitive fluorescence detector. Since the polypeptide
HEMOGLOBIN KÖLN RBC MEMBRANES

Fig 4. Protease susceptibility of MDA-spectrin aggregates. SDS-PAGE as in Fig 3, but the gel, stained with Coomassie blue, was not dried prior to photography. Controls: (A) molecular weight markers (Worthington) galactosidase 130 x 10^3, phosphorylase a 100 x 10^3, albumin (human serum) 66 x 10^3, pyruvate kinase 57 x 10^3, ovalbumin 43 x 10^3, and deoxyribonuclease 1 31 x 10^3; (B) incubated control spectrin extract (16 hours) (4 μg protein); (C) MDA-reacted spectrin extract (4 hours) (4 μg protein); (D) MDA-reacted spectrin extract (16 hours) (4 μg protein); (E) control membranes (20 μg protein). Trypsin digest (0.01 wt/wt, 20 hours, 0 °C, 40 μg protein); (F) spectrin extract (0 hours); (G) control incubated spectrin extract (16 hours, 37 °C); (H) 20 mmol/L MDA-reacted spectrin extract (16 hours, 37 °C); (I) control membranes (20 μg protein, 0 °C, 20 μg protein).

composition of Hb Köln membranes differs from normal membranes because of the attached precipitated globin (Heinz bodies), direct comparison of the amino acid content of control and Hb Köln membranes proved not to be helpful for this purpose. However, it was reasonable to compare Hb Köln RBC membrane polypeptide aggregates that had been hydrolyzed with 6 N HCl, both with and without prior reduction with NaBH₄, since such reduction is required to stabilize MDA amino adducts. A decrease in the amino acid content of reduced samples would indicate MDA adducts. As can be seen in Table 2, there was a decrease in serine, but no decrease in lysine content in reduced Hb Köln RBC membrane proteins. There was, however, an increase in methionine, as in MDA-treated membranes (see below). The amino acid composition of the aggregates determined by HPLC was similar to that obtained previously by another method.

Given the 5% to 10% standard deviation of the HPLC amino acid determinations, to see any effect of MDA on lysine, we needed to increase the proportion of amino acids reacted with MDA beyond that apparently present in Hb Köln RBC membranes. Furthermore, we had to avoid the change in membrane polypeptide composition that occurs when intact RBCs are incubated with MDA and cytoplasmic proteins become linked to the membrane. Thus, we treated isolated, but not lipid-extracted, membranes with 20 mmol/L MDA and compared the amino acid composition to incubated control membranes. Half of each was reduced with NaBH₄, then both portions were hydrolyzed with 6N HCl and analyzed on HPLC. The results are shown in Table 2. When the MDA-reacted membranes were reduced with NaBH₄, the lysine content was decreased by 33%. As in the Hb Köln aggregates, methionine increased in the NaBH₄, MDA-treated membranes. Interestingly, it increased back to levels present in controls, suggesting that MDA may have reversibly oxidized methionine. Methionine has been reported to be reversibly oxidized in glycophorin. Serine was increased in the nonreduced MDA-treated membranes, possibly crosslinked.
from phosphatidyl serine of the inner membrane lipids by an acid-labile MDA bond. The content of 12 other amino acids was the same in each preparation.

Presumably, reduction of the MDA lysine adducts was required to stabilize it prior to acid hydrolysis, since, unless reduced, the lysine content was unchanged in the MDA-treated membranes. The acid lability of MDA-lysyl adducts was confirmed using MDA-treated polylysine and N-α-acetyl lysine. Both yielded only lysine on hydrolysis unless there had been prior reduction with NaBH₄.

**DISCUSSION**

We found that 20 mmol/L MDA produced membrane polypeptide aggregates, altered protein configuration, and decreased RBC membrane deformability. Earlier reports that 2 to 20 μmol/L MDA was effective in producing aggregates have since been corrected by the senior author to be more consistent with our findings.

The use of sufficiently high concentrations of MDA to produce readily measurable abnormalities is a reasonable first step in the clarification of the role of lipid peroxidation in the pathophysiology of Hb Köln and related anemias. By this means, the content of the membrane polypeptide aggregates and MDA adducts can be increased. Eventually, proof of the importance of these relationships in vivo will demand decreasing the amount of MDA in the model system until RBCs are produced with lesions similar to those of RBCs from Hb Köln, and increasing the sensitivity and specificity of MDA detection so that the role of MDA is unequivocal. To detect such effects, however, will require the refinement of techniques for the recognition of MDA-lysine adducts. Reduction and stabilization of these adducts with [³H]NaBH₄ may be the key to this isolation and identification. Figure 6 illustrates the labeling and stabilization by [³H]NaBH₄ of hypothetical lysine adducts formed by MDA-derived 1-amino 3-imino propene crosslinks. Note that unsaturated nitrogen-carbon bonds are acid labile and are stabilized by reduction prior to acid hydrolysis. It is unlikely that the high concentrations of MDA used here are attained in vivo in the entire RBC cytosol. However, MDA might be formed locally in high concentrations at the inner membrane surface where polyunsaturated fatty acids, reactive oxygen intermediates formed from oxygen, and catalytic iron from degraded unstable hemoglobin are all present. MDA

| Table 2. Effect of MDA Treatment and NaBH₄ Reduction on Amino Acid Composition (mol/100 mL Amino Acid) |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Hb Köln Aggregates (n = 5) | Control Membranes (n = 3) | MDA Membranes (n = 3) |
| Aspartic acid | + NaBH₄ | Aspartic acid | + NaBH₄ | Aspartic acid | + NaBH₄ |
| Glutamic acid | + NaBH₄ | Glutamic acid | + NaBH₄ | Glutamic acid | + NaBH₄ |
| Serine | + NaBH₄ | Serine | + NaBH₄ | Serine | + NaBH₄ |
| Histidine | + NaBH₄ | Histidine | + NaBH₄ | Histidine | + NaBH₄ |
| Threonine | + NaBH₄ | Threonine | + NaBH₄ | Threonine | + NaBH₄ |
| Glycine | + NaBH₄ | Glycine | + NaBH₄ | Glycine | + NaBH₄ |
| Arginine | + NaBH₄ | Arginine | + NaBH₄ | Arginine | + NaBH₄ |
| Methionine | + NaBH₄ | Methionine | + NaBH₄ | Methionine | + NaBH₄ |
| Isoleucine | + NaBH₄ | Isoleucine | + NaBH₄ | Isoleucine | + NaBH₄ |
| Leucine | + NaBH₄ | Leucine | + NaBH₄ | Leucine | + NaBH₄ |
| Phenylalanine | + NaBH₄ | Phenylalanine | + NaBH₄ | Phenylalanine | + NaBH₄ |
| Lysine | + NaBH₄ | Lysine | + NaBH₄ | Lysine | + NaBH₄ |

*Significantly more than the unreduced samples (P < .001).
†Significantly less than the unreduced samples (P < .001).
appears to reversibly oxidize methionine to methionine sulfoxide. Methionine is then restored to its original content by NaBH₄ reduction. A similar effect is seen with Hb Köln membrane polypeptide aggregates, which is further evidence that oxidation resulted in their formation. In part, the ability to detect serine and methionine, but no lysine, changes in Hb Köln membrane aggregates may relate to the decreased reproducibility of lysine derivatization and the evident decreased precision in its determination. The high concentrations of MDA used without a significant effect on other amino acids helps rule out other residues as sites of MDA action. Thus, tyrosine, said by Chio and Tappel to be a principal site of MDA addition and implicated in crosslinking, does not seem to be affected in MDA-treated RBC membranes. Another amino acid affected by MDA according to these authors, histidine, is poorly measured by our present HPLC techniques and may be involved. As noted, we found that NaBH₄ treatment prior to hydrolysis was required to stabilize serine and lysine adducts, and thus to decrease their content upon analysis after hydrolysis.

Remaining to be determined is the relative importance in the decreased RBC survival in Hb Köln disease of membrane protein changes due to crosslinking, as evidenced by aggregates or configurational changes shown by increased protease sensitivity. While the increased tritium incorporation and changes in amino acid composition upon reduction are inconsistent with transglutamination or bityroine formation as exclusive crosslinking mechanisms, they do not rule out other oxidative processes or amino group crosslinking.

REFERENCES

15. Knowles W, Speicher D, Morrow J, Marchesi V: Renatural-
Comparison of hemoglobin KoIn erythrocyte membranes with malondialdehyde-reacted normal erythrocyte membranes

DW Allen, CF Burgoyne, JD Groat, CM 2d Smith and JG White