REACTION OF ACETALDEHYDE WITH HUMAN ERYTHROCYTE MEMBRANE PROTEINS

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1. Introduction

Erythrocyte abnormalities associated with chronic alcoholism include alterations in cell morphology, increased osmotic fragility and decreased erythrocyte filterability [1–4]. Proposed mechanisms for these abnormalities include an altered membrane cholesterol to phospholipid ratio, folate deficiency and direct bone-marrow toxicity by ethanol. We suggest that acetaldehyde, the first product of ethanol oxidation, may account for some of the erythrocytic alterations by reacting with erythrocyte spectrin and actin. It is known that free blood acetaldehyde accumulates to about 0.05 mM in chronic alcoholics [5]. Furthermore, spectrin and actin are thought to be involved in the maintenance of erythrocyte shape and flexibility [6–13].

Although there is no direct evidence that acetaldehyde binds to proteins of the human erythrocyte, it has been known that this compound can cross-link proteins [14] and can alter certain physical properties of erythrocytes [15–17]. Unfortunately, these studies were performed mainly at unusually high acetaldehyde concentrations (2% or greater) and at temperatures at or beyond the boiling point of acetaldehyde (20°C), making interpretation difficult. Steck [18] has presented evidence that related compounds (e.g., formaldehyde and glutaraldehyde) may cross-link spectrin at low aldehyde concentrations. However, evidence for cross-linking of spectrin and actin is lacking.

In this communication, we present evidence which suggests that acetaldehyde reacts with proteins of the human erythrocyte membrane in a manner analogous to the formaldehyde and glutaraldehyde reactions reported by Steck [18]. However, unlike these aldyhydes, acetaldehyde appears to react with both spectrin and actin. We also present evidence that acetaldehyde binds to erythrocyte membrane proteins in the intact cell and produces characteristic changes in cell morphology (stomatocytes). Evidence indicating that acetaldehyde binds to intracellular hemoglobin is also discussed. These results suggest that acetaldehyde can bind irreversibly to proteins of the human erythrocyte and they may offer a possible molecular basis for the transient stomatocytosis seen in chronic alcoholism. Some of the results communicated in this report were presented at the 1976 annual Meeting of the American Society of Hematology [19].

Definitions of cell morphology used: Stomatocyte an erythrocyte with a linear-appearing central depression as opposed to the usual circular-appearing central depression. Triangulocyte a type of stomatocyte with a triangular-shaped central depression. Poikilocytosis the presence of varying shapes within an erythrocyte population.

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2. Materials and methods

Human erythrocyte ghosts were prepared from freshly outdated bank blood in 5 mM sodium phosphate, pH 8, 5P(8), according to Fairbanks et al. [20]. Erythrocyte spectrin and actin were isolated by incubating intact Hb-free ghosts in 0.5 mM sodium phosphate, pH 8, plus 0.1 mM EDTA at 37°C for 15 min, after a 1:40 dilution [21]. The membranes were pelleted and the supernatant collected and volume depleted by vacuum in a 10 S and S Collodion bag

No. 100 (Scheicher and Schuell, Inc., Keene, New Hampshire). Hemoglobin was isolated according to Salhany et al. [22].

Due to the 20°C boiling point of acetaldehyde, reactions were carried out in sealed containers in a cold room (2–4°C). Acetaldehyde was from Eastman Kodak (Rochester, NY) and was greater than 98% pure as determined in this laboratory using the gas-chromatographic method of Cederbaum et al. [23]. The only detectable impurity was acetic acid, which does not produce any of the results reported below.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS–PAGE) was performed according to Fairbanks et al. [20] and Steck [18]. The gels contained 3.9% acrylamide (w/v) and 0.2% SDS. A typical run consisted of solubilization of the membranes (usually 3 mg protein/ml) with final concentrations, 1% SDS, 5% sucrose, 10 mM Tris–HCl, 1 mM EDTA and 32 mM dithiothreitol, pH 7.4. The sample was heated (40°C), mixed and 40 μl applied to the top of the gel. Electrophoresis and staining were performed as described elsewhere [18, 20]. In some experiments, membranes or intact cells were treated with [^{14}C]acetaldehyde (4.2 mCi/mM, New England Nuclear, Boston, MA). Tandem gels were either stained or sliced after electrophoresis, with the unstained slices solubilized in Aquasol (New England Nuclear) and counted in a Beckman Liquid Scintillation Counter. Efficiencies were determined by external standardization.

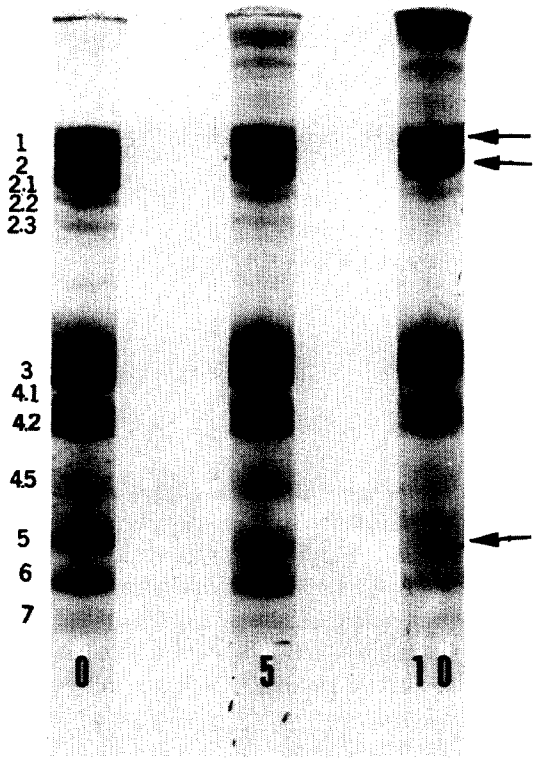

Fig.1. Effect of acetaldehyde on human erythrocyte membrane proteins in Hb-free ghosts. Ghosts (3 mg protein/ml) and acetaldehyde were incubated in 5P(8) for 30 min at 4°C. Samples were solubilized and electrophoresed as described in the text. Final concentrations of acetaldehyde were 0 mM, 5 mM and 10 mM and are indicated at the bottom of each gel. Bands are classified according to Steck [18]. Arrows indicate positions of bands 1, 2 and 5. The gels were stained with Coomassie Blue.

3. Results and discussion

The major gel bands from the erythrocyte are numbered according to Steck [18]. The effect of increasing acetaldehyde concentration on ghost proteins is illustrated in fig.1. The intensities of bands 1, 2 and 5 strongly decreased with new, slowly migrating bands appearing at the top of the gel. Like Steck's [18] formaldehyde and glutaraldehyde reactions, the amount of new high molecular weight protein (HMWP) increased with aldehyde concentration, incubation time, pH 6–8, temperature and ionic strength. However, unlike the formaldehyde or glutaraldehyde treatments, we could see the involvement of bands 1, 2 and 5 for the first time. The minor bands 2.1, 2.2, 2.3, 4.2 and 6 may also be involved.

Ghosts were reacted with [^{14}C] acetaldehyde such that the final concentration of acetaldehyde was 1 mM containing 5 μCi of ^{14}C. Figure 2 shows a plot of the counts along a sliced, solubilized tandem gel versus a Coomassie Blue stained gel. Most of the counts occurred in the HMWP region of the gel. Some counts were found further along the gel, but the intensity was at least an order of magnitude less. This result suggests that the new HMWP does contain bound acetaldehyde. We do not rule out some small amount of binding to other membrane proteins.

When isolated spectrin and actin, containing some band 2.1 protein, were reacted with acetaldehyde, we observed the formation of apparently very high

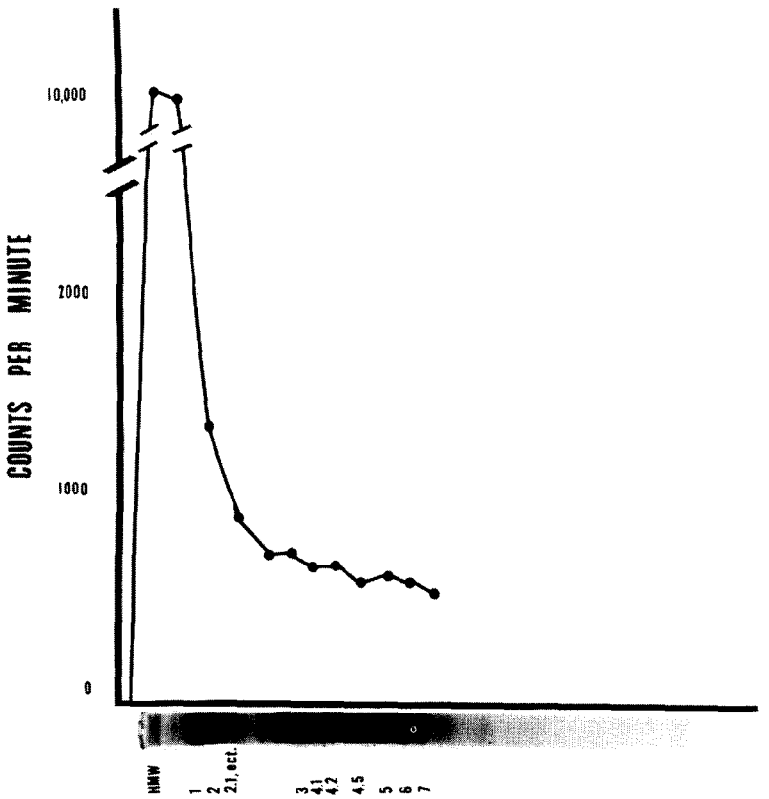

Fig.2. Binding of [^{14}C]acetaldehyde to membrane proteins of Hb-free ghosts. Ghosts were reacted with stock buffer solutions such that the final concentration of acetaldehyde was 1 mM containing 5 μCi of ^{14}C, at 4°C for 10 min. Some of the gels were stained as usual, while other unstained gels from the same run were sliced, solubilized and counted as described in the text.

molecular weight material (fig.3). These results suggest that the component proteins form a complex (either stable or transient) which may be cross-linkable by acetaldehyde. There is other evidence to suggest that spectrin and actin can form a definite structure even in the absence of the overlying membrane [7].

We have studied the reaction of these low levels of acetaldehyde with washed, intact erythrocytes and whole blood under isosmolar conditions. Washed erythrocytes were reacted for 30 min at 4°C with [^{14}C]acetaldehyde (final concentration 5 mM acetaldehyde containing 10 μCi of ^{14}C). Free acetaldehyde was vaporized into a semicarbizide trap and was found to comprise 16% of the initial amount of label added, with 84% remaining in the suspension. The cells were pelleted and the clear supernatant measured for ^{14}C. None was detected. The cells were lysed in 5P(8) (1:40), the ghosts pelleted and the initial hemolysate collected. The ghosts were washed three times in 5P(8) to remove hemoglobin, solubilized and counted. Protein concentration was determined according to Lowry et al. [24]. We found both the Hb-free membranes and the initial hemolysate to contain about the same amount of label (hemolysate 5.2 cpm/μg protein, membrane 4 cpm/μg protein). SDS–PAGE of the ghosts and counting of tandem gels showed results similar to fig.2. This appears to indicate that acetaldehyde can react with erythrocyte membrane proteins in both the intact cell and the isolated membrane. SDS–PAGE of the initial hemolysate showed a pattern suggesting hemoglobin cross-linking. This was confirmed by reacting isolated hemoglobin (3.27 mg/ml) in 5P(8) with 5 mM acetaldehyde (final concentration) and performing SDS–PAGE. The two new bands

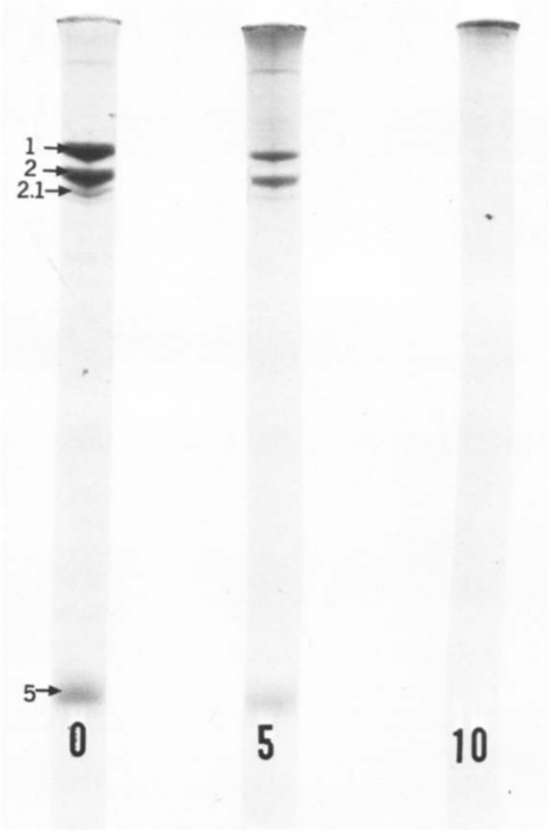

Fig.3. Reaction of acetaldehyde with isolated spectrin and actin. Spectrin and actin were eluted from erythrocyte ghosts and volume-depleted as described in the text, then incubated for 30 min at 4°C with 0 mM, 5 mM and 10 mM acetaldehyde (final concentrations). The numbers at the bottom of each gel indicate the concentration of acetaldehyde used.

seen on the gel were at positions consistent with the molecular weights of dimers and tetramers of hemoglobin.

In order to check for possible acetaldehyde-induced changes in cell morphology, we withdrew fresh venous blood in heparin from a normal donor and then added isosmolar acetaldehyde to the sealed container so as to give a final concentration of 1 mM. The container was allowed to stand for 120 min at 4°C. A portion of the blood was withdrawn and examined by light microscopy after staining a smear with Wright's Stain. Fig.4 shows a photomicrograph from a typical experiment. We repeatedly observed marked poikilocytosis, stomatocytosis and what have been previously referred to as 'triangulocytes' [25]. Thus, there appears to be a correlation between cross-linking the spectrin–actin complex and a change in erythrocyte morphology

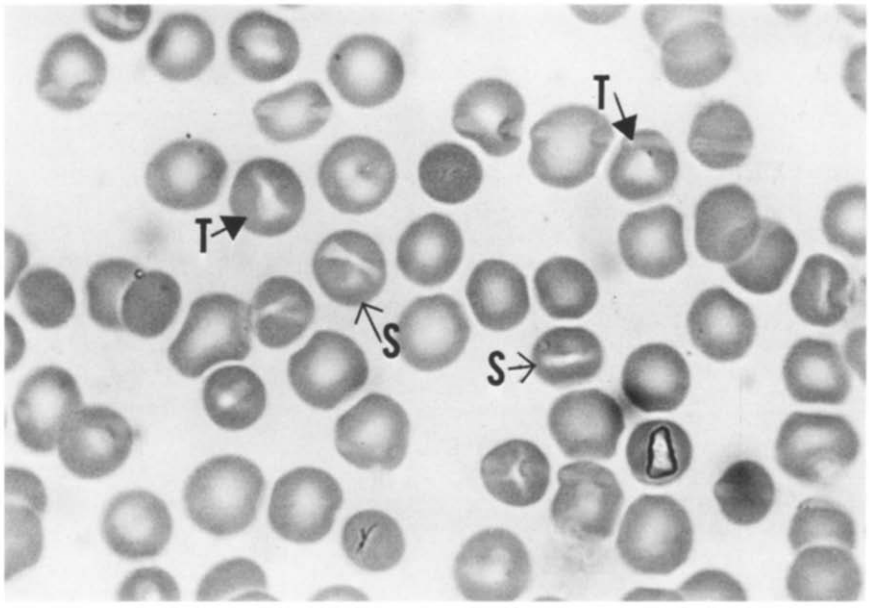

Fig.4

which appears consistent with the proposed function of this complex.

The present findings may be important in understanding the molecular basis for the transient stomatocytosis seen in chronic alcoholism [26]. The demonstration that acetaldehyde can react irreversibly with proteins of the human erythrocyte may suggest that the free blood acetaldehyde levels measured in vivo underestimate the amount released by the liver in the chronic alcoholic. In vivo measurements of both free and bound blood acetaldehyde seem warranted.

Acknowledgements

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References

[1] Eichner, E. R. and Hillman, R. S. (1971) Amer. J. Med. 50, 218–232.
[2] Eichner, E. R. (1973) Amer. J. Med. 54, 621–630.
[3] Jacob, H. S. and Amsden, T. (1971) New Engl. J. Med. 285, 1446–1450.
[4] Territo, M. C. and Tanaka, V. R. (1974) Arch. Intern. Med. 134, 445–447.
[5] Korsten, M. A., Matsuzaki, S., Feinman, L. and Lieber, C. S. (1975) New Engl. J. Med. 292, 386–389.
[6] Sheetz, M. P., Painter, R. G. and Singer, S. J. (1976) Biochemistry 15, 4486–4492.
[7] Steck, T. L. (1974) J. Cell. Biol. 62, 1–19.
[8] Guidotti, G. (1972) Arch. Inter. Med. 129, 194–201.
[9] Marchesi, V., Furthmayr, H. and Tomita, M. (1976) Ann. Rev. Biochem. 45, 667–698.
[10] Painter, R. G., Sheetz, M. and Singer, S. J. (1975) Proc. Natl. Acad. Sci. USA 72, 1359–1363.
[11] Tilney, L. G. and Detmers, P. (1975) J. Cell. Biol. 66, 508–520.
[12] Kirkpatrick, F. H. (1976) Life Sci. 19, 1–18.
[13] Lux, S. E., John, K. M. and Karnovsky, M. F. (1976) J. Clin. Invest. 58, 955–963.
[14] Mohammed, A., Olcott, H. S. and Fraenkel-Conrat, H. (1949) Arch. Biochem. 24, 270–280.
[15] Heard, D. H. and Seaman, G. V. F. (1961) Biochim. Biophys. Acta 53, 366–374.
[16] Vassar, P. S., Hards, J. M., Brooks, D. E., Hagenberger, B. and Seaman, G. V. F. (1972) J. Cell. Biol. 53, 809–818.
[17] Palmer, A. A. and Betts, W. H. (1975) Biorheology 12, 283–292.
[18] Steck, T. L. (1972) J. Mol. Biol. 66, 295–305.
[19] Salhany, J. M., Gaines, K. C., Tuma, D. J. and Sorrell, M. F. (1976) Blood 48, 966.
[20] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[21] Steck, T. L. (1974) in: Methods in Membrane Biology (Korn, E. D. ed) Vol. 2, pp. 245–281, Plenum Press, New York.
[22] Salhany, J. M., Ogawa, S. and Shulman, R. G. (1975) Biochemistry 14, 2180–2190.
[23] Cederbaum, A. I., Lieber, C. S. and Rubin, E. (1974) Arch. Biochem. Biophys. 161, 26–39.
[24] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[25] Schumacher, H. R., Khanna, S. and Moyer, B. (1976) 235, 2285–2286.
[26] Douglass, C. C. and Twomey, J. J. (1970) Ann. Inter. Med. 72, 159–164.

Fig.4. Photomicrograph of normal, freshly drawn venous blood treated as described in the text with 1 mM acetaldehyde (final concentration). S stomatocyte and T 'triangulocyte' (see ref. [25]). Magnification × 970. There was no abnormal morphology observed in the same blood in the absence of acetaldehyde.