Modifications of the Erythrocyte Membrane by Glutaraldehyde: Effect on Acetylcholinesterase[1] (36539)

FRITZ HERZ AND EUGENE KAPLAN

Departments of Pediatrics, Sinai Hospital, Baltimore, Maryland 21215 and The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Passive hemagglutination is widely used for the assay of small amounts of antigen and antibody. Recent studies have indicated that a variety of antigens can be covalently coupled to the erythrocyte surface with the aid of glutaraldehyde (GA), a bifunctional cross-linking reagent (1–3). Although GA-treated erythrocytes have been employed in the study of macrophage adherence (4), cell permeability (5), cell structure (6) and hemorrheological properties (7), the effect of GA on specific components of the red cell membrane has not been investigated. In this report it will be shown that GA renders erythrocytes resistant to hemolytic agents and to mechanical stresses, and that these alterations are accompanied by irreversible modifications of acetylcholinesterase (ACHE), an integral part of the human red cell membrane (8).

Materials and Methods. Preparation of red cells. Blood from normal adult individuals and from newborn infants was collected with EDTA. The erythrocytes were washed thrice with 20 vol of ice-cold 0.1 *M* sodium–potassium phosphate buffer (pH 8.0). The supernatant and buffy coat were removed by suction after each centrifugation. After the last spinning an approximate 50% (v/v) red cell suspension was prepared and used immediately. When the influence of pH was investigated, the cells were washed with and suspended in phosphate buffer adjusted to the appropriate pH. Hemoglobin-free membranes were prepared by osmotically induced hemolysis (9).

GA treatment. A 50% (w/w) GA solution (Biological grade, Fisher Scientific Co.) was diluted with phosphate buffer and used at once. Unless otherwise indicated, 20 vol of an appropriately diluted GA solution were added to 1 vol of a 50% cell suspension at 4° and incubated for 20 min at 25°. Cells treated with buffer alone served as controls. At the end of the incubation period, erythrocytes were washed five times with 100 vol of phosphate buffer and after the last centrifugation adjusted to a 50% suspension.

Enzyme assay. ACHE activity was measured at 412 nm on duplicate 0.1% cell suspensions in 0.1 *M* phosphate buffer (pH 8.0) using acetylthiocholine as substrate and 5:5′-dithiobis-(2-nitrobenzoic acid) as color reagent (10). Enzyme activity was related to packed cell volume, which was determined after centrifugation of triplicate cell aliquots in microhematocrit capillary tubes at 15,500*g* for 5 min. No correction was made for trapped fluid. Percent of ACHE activity remaining after GA treatment was computed from the activity of controls.

Stability of ACHE. For heat stability studies, GA-treated membranes were diluted 1:100 in 0.01 *M* phosphate buffer (pH 8.0) and triplicate 1.0-ml aliquots were incubated at 56° for various time intervals. The controls were kept at 4°. After incubation, 10 ml of ice-cold 0.1 *M* phosphate buffer (pH 8.0) were added and ACHE activity was measured subsequently. The effect of urea on ACHE activity was investigated by adding 10 ml of various concentrations of urea in 0.1 *M* phosphate buffer (pH 8.0) to duplicate 1.0-ml aliquots of 1% (v/v) membrane sus-

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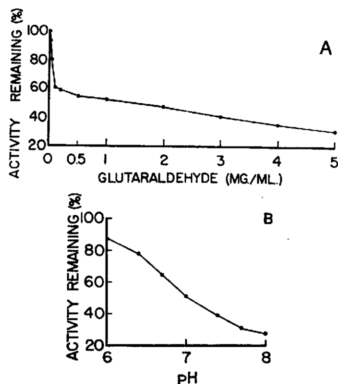

FIG. 1. Effect of concentration and pH on ACHE inactivation by GA. (A, top) At 4°, to duplicate 0.2 ml of a 50% (v/v) red cell suspension in 0.1 M phosphate buffer (pH 8.0), 4 ml of GA in the same buffer were added. After 20 min at 25°, the cells were washed five times with 10 ml of buffer. A 50% suspension was prepared after the last centrifugation. ACHE activity was determined colorimetrically and related to packed cell volume. The percent activity remaining was calculated from the activity of cells incubated without GA. (B, bottom) Same as above, except that washing, suspension, GA treatment and subsequent washing of erythrocytes were carried out with phosphate buffers adjusted to pH indicated. The concentration of GA was 5 mg/ml.

pensions in the same buffer. The residual enzyme activity was determined after 15 min at 25° and related to the respective controls treated with buffer alone.

Results. Stability of GA-treated erythrocytes. Human erythrocytes treated at 25° with 0.5 mg/ml (5×10^{-3} M) of GA were not lysed by water, nor could hemolysis be induced by ultrasonication for 30 min with a Raytheon 10-kc oscillator or by freezing and thawing ten times. The treated cells retained normal morphological appearance. Although vigorous agitation was required to resuspend the modified cells after centrifugation, no spontaneous autoagglutination was observed.

Inactivation of ACHE. The increased stability of treated red cells to hemolytic agents was accompanied by irreversible modifications in ACHE activity. Incubation of erythrocytes with GA for 20 min at 25° and pH 8.0 caused a concentration-dependent loss of enzyme activity (Fig. 1A). Approximately 40% of the activity was lost at a GA concentration of 0.1 mg/ml and 70% at a concentration of 5 mg/ml. Repeated washing with buffer or with water of GA-treated erythrocytes did not restore or augment enzyme activity and none was detected in the supernatant liquid after incubation with the aldehyde. No differences in inactivation were seen with erythrocytes from individuals of different blood groups and the enzyme of ACHE-deficient red cells from newborn infants with ABO hemolytic disease (10) was affected to the same extent as the ACHE of normal red cells. The exposure of whole blood to GA also caused ACHE inactivation. Red cell integrity was not essential for the effect of GA, because the enzyme of hemoglobin-free membrane preparations behaved like that of intact erythrocytes.

The reduction in ACHE activity was dependent on the pH of the incubation mixture. Whereas at pH 8.0, 70% of the activity was lost after treatment with 5 mg/ml of GA for

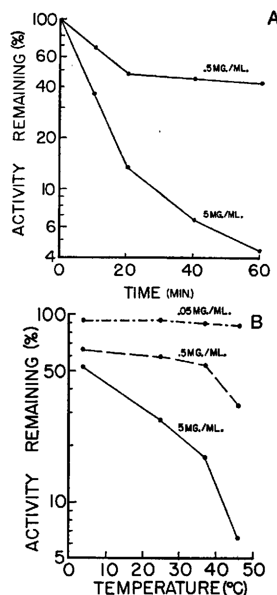

FIG. 2. Effect of time and temperature on ACHE inactivation. (A, top) To duplicate 0.2 ml of a 50% cell suspension, 4 ml of GA in 0.1 M phosphate buffer (pH 8.0) were added. After incubation at 37° for time indicated, cells were washed with buffer and the percent of ACHE activity remaining was determined as indicated in text. (B, bottom) Same as above, except that GT treatment was carried out for 20 min at the temperature indicated.

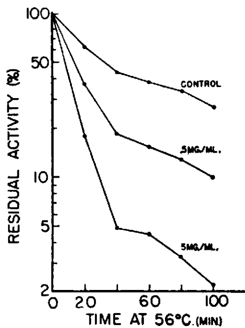

FIG. 3. Effect of time on thermostability of ACHE. Erythrocyte membranes obtained by osmotic lysis were treated for 20 min with GA at pH 8.0 and 25°. GA was omitted from controls. After five washings with 0.01 *M* phosphate buffer (pH 8.0) a 1% (v/v) suspension was prepared with the same buffer. Triplicate 1.0-ml aliquots were incubated at 56° for time indicated, immediately diluted with 10 ml of ice-cold 0.1 *M* phosphate buffer (pH 8.0) and stored at 4°. Enzyme activity was measured simultaneously as indicated in text and related to aliquots kept at 4°.

20 min at 25°; at pH 7.0 and 6.0, the loss was 50% and 10%, respectively (Fig. 1B). Prolonging the time of incubation resulted in increased enzyme inactivation, but the relatively rapid initial loss of activity was followed by a slower rate (Fig. 2A). The action of GA on ACHE was temperature dependent. A concentration of 0.5 mg/ml at pH 8.0 caused 35% inactivation in 20 min at 4° and 70% at 45°. With 5 mg/ml the values were 50% and 94%, respectively (Fig. 2B).

Changes in stability of ACHE. The reduction in ACHE activity was not associated with alterations in substrate specificity, K_m and pH profile. However, it was accompanied by changes in the stability of the enzyme. Thus, the residual ACHE activity after exposure to GA was more thermolabile than the normal enzyme (Fig. 3). The decrease in stability was dependent on the concentration of reagent used. Similar results were obtained when membrane preparations with partially heat-inactivated ACHE (11) were exposed to GA, washed with buffer and then incubated at 56°. The latter findings suggested that the reagent did not preferentially affect a more stable ACHE species. The decreased stability of the residual enzyme activity was also evident by exposing GA-treated membranes to urea. Thus, after 15 min in 3 *M* urea at pH 8.0 and 25°, 40% of the activity was lost in control preparations as compared to 70% and 55% in membranes pretreated with 5 mg/ml and 0.5 mg/ml of GA, respectively.

Discussion. The foregoing results demonstrate that GA-induced modifications of the erythrocyte membrane were accompanied by ACHE inactivation. These observations extend earlier studies in which it was shown that formaldehyde-mediated surface alterations of the red cell were associated with reduction in ACHE activity (12). The failure to restore enzyme activity by repeated washing of GA-treated cells indicated that the effect was irreversible. This is in keeping with the action of GA on other systems, reflecting the characteristic reactivity of this agent with proteins and its tendency to form stable cross-linked derivatives (13). Although the mechanism by which GA interacts with proteins is not fully understood, it has been shown that it reacts preferentially with lysine (13) and that α-β-unsaturated polymeric forms may be involved in the formation of stable cross-linked derivatives (13). The resistance of GA-treated erythrocytes to hemolytic agents and to mechanical stress is probably related to the production of such cross-linked structures.

In experiments to determine the factors which influence the coupling of bovine serum albumin to red cells in the presence of GA, it was found that concentration, volume, time and pH were of importance (2). ACHE inactivation was also dependent on these factors as well as on the temperature of incubation. Of particular interest is the finding that the residual activity after GA treatment was strikingly less resistant to the action of heat and urea than the normal enzyme. These observations can be contrasted with the increased stability of GA-modified glycogen phosphorylase b (14) and of the residual ACHE activity of membranes treated with 1,5-difluoro-2,4-dinitrobenzene (15). Although the present investigation was restricted to a specific protein located at or near the cell surface (8, 16), the possibility exists that GA may also affect other surface-related

characteristics of the red cell such as electrophoretic mobility and agglutinability.

Summary. When erythrocytes are treated with glutaraldehyde they become resistant to hemolytic agents (water) and to mechanical stresses (sonication, freeze–thawing). These alterations are accompanied by the irreversible inactivation of acetylcholinesterase, an integral part of the erythrocyte membrane. Enzyme inactivation is dependent on concentration, pH, time and temperature. The reduction in activity is not associated with changes in substrate specificity, K_m and pH profile. However, the residual acetylcholinesterase activity is more labile to denaturation by heat and urea than the normal enzyme.

1. Avrameas, S., Taudou, B., and Chuilon, S., Immunochemistry **6**, 67 (1969).

2. Onkelinx, E., Meuldermans, W., Joniau, M., and Lontie, R., Immunology **16**, 35 (1969).

3. Bing, D. H., Weyand, J. G. M., and Stavitsky, A. B., Proc. Soc. Exp. Biol. Med. **124**, 1166 (1967).

4. Rabinovitch, M., Proc. Soc. Exp. Biol. Med. **124**, 396 (1967).

5. Morel, F. M. M., Baker, R. F., and Wayland, H., J. Cell Biol. **48**, 91 (1971).

6. Lewis, S. M., Osborn, J. S., and Stuart, P. R., Nature (London) **220**, 614 (1968).

7. Ham, T. H., Dunn, R. F., Sayre, R. W., and Murphy, J. R., Blood **32**, 847 (1968).

8. Herz, F., Kaplan, E., and Stevenson, J. H., Jr., Nature (London) **200**, 901 (1963).

9. Dodge, J. T., Mitchell, C., and Hanahan, D. J., Arch. Biochem. Biophys. **100**, 119 (1963).

10. Kaplan, E., Herz, F., and Hsu, K. S., Pediatrics **33**, 205 (1964).

11. Coleman, M. H., and Eley, D. D., Biochim. Biophys. Acta **67**, 646 (1963).

12. Herz, F., and Kaplan, E., Proc. Soc. Exp. Biol. Med. **134**, 437 (1970).

13. Richards, F. M., and Knowles, J. R., J. Mol. Biol. **37**, 231 (1968).

14. Wang, J. H., and Tu, J.-I., Biochemistry **8**, 4403 (1969).

15. Herz, F., Kaplan, E., and Gleiman, E. J., Experientia **24**, 215 (1968).

16. Bender, W. W., Garan, H., and Berg, H. C., J. Mol. Biol. **58**, 783 (1971).

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