Surface Alteration and Acetylcholinesterase Activity of Human Red Cells[1] (34808)

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Treatment with formaldehyde renders erythrocytes capable of adsorbing antigens and antibodies from solution and this manipulation is widely used in passive hemagglutination and in hemagglutination–inhibition studies (1). Although the mechanisms by which this substance exerts its effects on the erythrocyte surface have not been elucidated, several pertinent observations have been described. Thus, it has been shown that whereas formaldehyde treatment suppresses red cell agglutinability by ABO and MN specific antibodies without affecting antibody uptake (2, 3); both the agglutinability by and the absorption of Rh-specific antibodies are decreased or abolished (3). These observations indicate that the effect of formaldehyde is directed toward the agglutinogens at the red cell membrane and not against the antibodies. Absorption and elution of influenza virus hemagglutinating activity of formaldehyde-treated red cells is indistinguishable from that of normal erythrocytes (4), suggesting that some components of the red cell surface are unaltered by exposure to the aldehyde. Formaldehyde has been used in the study of the electrokinetic behavior of erythrocytes (5), but no investigation of its effects on a specific red cell membrane constitutent has been made.

Among the few enzymes recognized in the human erythrocyte membrane, acetylcholinesterase (ACHE) appears to be located at the outer surface of the cell with its active site(s) oriented in an outward direction (6, 7). Since ACHE activity is reduced in newborns affected with ABO hemolytic disease (8), and inasmuch as blood-group specific antigenic receptors also are located at the red cell surface (9), we have investigated the effect of formaldehyde on ACHE in intact erythrocytes and in hemoglobin-free membranes. An additional indication for conducting these experiments is the recent demonstration that formaldehyde can be useful in the delineation of genetically determined variants of human serum cholinesterase (10).

Materials and Methods. Blood from normal adult individuals was centrifuged, and the plasma and buffy coat were removed by suction. The erythrocytes were washed three times with 20 vol of ice-cold physiological saline or with 0.1 *M* sodium–potassium phosphate buffer, pH 7.0 or 8.0. After the last centrifugation an approximate 50% red cell suspension was prepared and used immediately. Hemoglobin-free membranes were prepared by osmotically induced hemolysis and stored at 4° as a 50% suspension. A commercial 37% formaldehyde solution, stabilized with 10–15% methanol, was appropriately diluted in saline or phosphate buffer. Unless otherwise indicated, to 1 vol of a 50% cell suspension, 4 vol of a correspondingly diluted formaldehyde solution were added and incubated at 37°. Cells treated with methanol-containing solutions and with buffer alone served as controls. At the end of the incubation period, erythrocytes were washed thrice with 100 vol of the diluent and after the last centrifugation adjusted to a 50% suspension. ACHE activity was measured at 412 mμ on replicate 0.1% suspensions in 0.1 *M* phosphate buffer, pH 8.0, using acetylthiocholine as substrate and 5:5′-dithiobis-(2-nitrobenzoic acid) as color reagent (8). Specific activity

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TABLE I. Effect of Formaldehyde Concentration on ACHE Activity.[a]

Formaldehyde (%)	Physiological saline		Phosphate buffer, pH 7.0		Phosphate buffer, pH 8.0	
	Specific activity	% Activity remaining	Specific activity	% Activity remaining	Specific activity	% Activity remaining
None	175	100.0	158	100.0	165	100.0
0.19	174	99.4	156	98.7	140	84.9
0.25	175	100.0	152	96.2	137	83.0
0.30	174	99.4	155	98.1	135	81.8
0.37	163	93.1	153	96.9	116	70.3
0.49	157	89.7	152	96.2	90	54.5
0.62	149	81.1	137	86.7	67	40.6
0.74	149	81.1	118	74.7	43	26.1
0.93	130	73.7	95	60.1	29	17.5
1.23	108	61.7	87	55.1	28	16.9
1.48	94	53.7	86	54.4	19	11.5

[a] To replicate 0.2 ml of 50% red cell suspensions in the indicated diluents, 0.8 ml of formaldehyde solution was added. After incubation at 37° for 60 min, cells were washed three times with 100 vol of the same diluents and after the last centrifugation adjusted to a 50% hematocrit. Moderate hemolysis was observed during the washing. Residual ACHE activity was measured as indicated in text and related to controls.

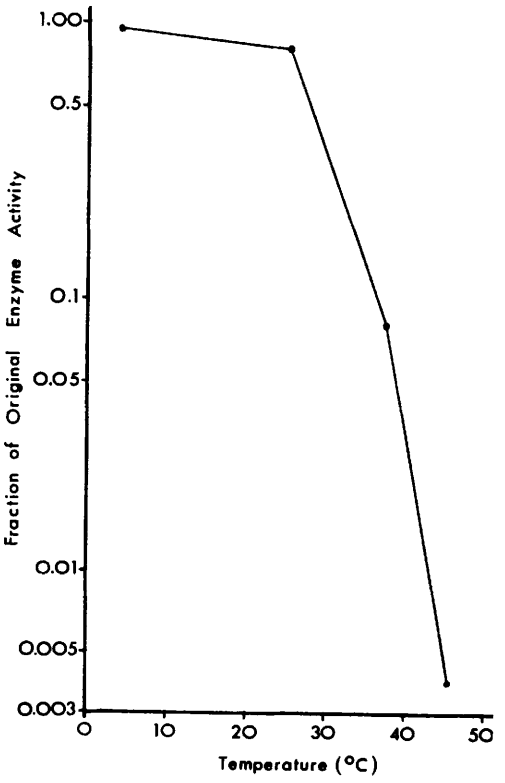

FIG. 1. Effect of temperature on ACHE inactivation by formaldehyde. At 4°, 0.8 ml of a 1.23% formaldehyde solution in phosphate buffer, pH 8.0, were added to 0.2 ml of a 50% membrane suspension in the same buffer. After incubation for 60 min at the temperatures indicated, membranes were washed thrice with 100 vol of chilled buffer. Residual enzyme activity was related to controls incubated without formaldehyde.

was expressed as ΔOD/min/mg of hemoglobin, the latter measured at 540 mμ as cyanmethemoglobin. When red cell membranes were used, enzyme activity was related to the protein concentration determined according to Lowry *et al.* (11).

Results. Incubation of whole erythrocytes with formaldehyde at 37° caused a concentration-dependent loss of ACHE activity. Whereas the effect was relatively discrete in saline or in phosphate buffer, pH 7.0, treatment at pH 8.0 resulted in a substantial reduction in activity (Table I). At this pH, approximately 17 and 83% of the enzyme activity were destroyed with solutions containing 0.30 and 0.93% of formaldehyde, respectively. No loss of ACHE activity was noted in controls incubated with methanol. From experiments in which different concentrations of formaldehyde were added to complete assay systems and initial rates measured, it was possible to infer that the reduction in ACHE activity was not due to interference of the aldehyde with the enzyme assay. Re-

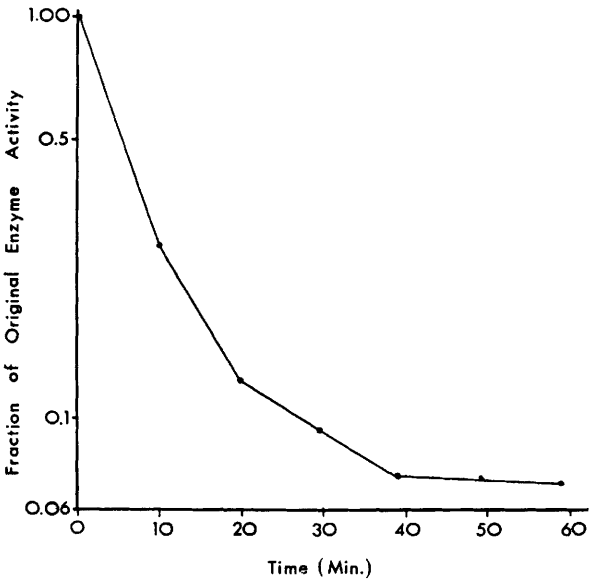

FIG. 2. Effect of time on ACHE inactivation by formaldehyde. At 4°, to replicate 0.2 ml of a 50% membrane suspension, 0.8 ml of formaldehyde (1.23%) in 0.1 *M* phosphate buffer, pH 8.0, were added; tubes were incubated at 37° for time indicated, and the membranes were immediately washed three times with 100 vol of ice-cold buffer and stored at 4°. ACHE activity was measured simultaneously as indicated in text and residual activity related to controls.

peated washing of formaldehyde-treated cells did not restore ACHE activity, and none was detected in the supernatant liquid after incubation with formaldehyde. No differences in ACHE inactivation were noted when erythrocytes from individuals of different blood groups or cells from patients with diverse hemolytic diseases were tested. Essentially the same patterns of enzyme inactivation were seen with hemoglobin-free membrane preparations.

The effect of formaldehyde on ACHE activity was strikingly dependent on the temperature of incubation. Thus, while formaldehyde treatment with phosphate buffer, pH 8.0 at 4 and 25° resulted in relatively small reduction, incubation at 45° caused the loss of more than 99% of the enzyme activity (Fig. 1). Similarly, ACHE inactivation was also time-dependent (Fig. 2). The initial rapid loss of enzyme activity, followed by a slower rate indicated saturation of the membrane preparations by formaldehyde, suggesting that inactivation was also dependent on enzyme concentration. When this possibility was tested it was found that the reduction in ACHE activity was inversely proportional to the amount of cells present when the concentration and volume of formaldehyde were kept constant. Inactivation of ACHE was not associated with changes in substrate specificity, K_m, pH profile, and effect of enzyme inhibitors.

Discussion. The foregoing experimental results have demonstrated that treatment of human erythrocytes with formaldehyde under conditions used in sensitization procedures caused inactivation of the cell membrane ACHE. The failure to restore enzyme activity by repeated washing indicated that this effect was irreversible. This observation contrasts the reported regeneration of agglutinability by ABO and MN specific antibodies after extensive washing of formaldehyde-treated erythrocytes (3). In experiments in which the effect of formaldehyde pretreatment of $Rh_0(D)$ positive red cells on the binding of ^{131}I anti-$Rh_0(D)$ was assessed, it was found that the reduction in antibody uptake was dependent on the concentration of aldehyde used (12). In the present study we found that ACHE inactivation was not

only dependent on formaldehyde concentration, but also on pH, temperature, and time of incubation.

Formaldehyde is known to react with positive amino groups of protein molecules with the formation of mono- and dimethylol derivatives. However, no positively charged groups could be detected by electrophoresing erythrocytes pretreated with formaldehyde (5). These findings suggest that such groups may lie deeper within the cellular peripheral zone than sialic acid (5), which is responsible for the electrokinetic behavior of erythrocytes in an electrolyte medium. Removal of sialic acid-containing mucoids by proteases, but not the removal of sialic acid by neuraminidase is also associated with the loss in ACHE activity (6). Although formaldehyde has been used for a long time as a tissue fixative and as a tool in hemagglutination procedures, the work reported here represents the first instance in which an irreversible effect on a specific enzymatic constituent of the human erythrocyte membrane could be demonstrated.

Summary. Treatment of human erythrocytes with formaldehyde caused inactivation of the surface-located acetylcholinesterase. The enzyme of whole cells and of hemoglobin-free membranes behaved alike upon exposure to the aldehyde. The effect was irreversible and depended on concentration, pH, time, and temperature.

1. Grossgebauer, K., Modde, H., and Hartmann, D., Arch. Hyg. Bakt. **147**, 524 (1963).

2. Moskowitz, M., and Carb, S., Nature **180**, 1049 (1957).

3. Májsky, A., Z. Immunitaetsforschung **118**, 73 (1959).

4. Flick, J. A., Proc. Soc. Exp. Biol. Med. **68**, 448 (1948).

5. Weiss, L., Bello, J., and Cudney, T. L., Int. J. Cancer **3**, 795 (1968).

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

7. Firkin, B. G., and Wiley, J. S., *in* "Progress in Hematology" (E. B. Brown and C. V. Moore, eds.), Vol. 5, p. 26. Grune & Stratton, New York (1966).

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

9. Ceppellini, R., and Landy, M., J. Exp. Med. **117**, 321 (1963).

10. Whittaker, M., Clin. Chim. Acta **26**, 141 (1969).

11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

12. Kaufman, B., and Masouredis, S. P., J. Immunol. **91**, 233 (1963).

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