Effects of Fluorinated Dinitrobenzenes on Erythrocyte Membrane Acetylcholinesterase

Among the few enzymes recognized in the human erythrocyte membrane, acetylcholinesterase (ACHE) is one of the most investigated. Previous studies have indicated that this enzyme appears to be located at the outer surface of the membrane[1,2]. BERG et al.[3] have recently reported that treatment of human red cells at alkaline pH with 1-fluoro-2,4-dinitrobenzene (FDNB) and 1,5-difluoro-2,4-dinitrobenzene (DFDNB) produced modifications of the erythrocyte membrane which were characteristic for each reagent. While FDNB caused cells to become permeable to sodium and potassium and to lyse in isotonic buffer, erythrocytes treated with DFDNB became rigid, did not lyse in water and could not be broken by ultrasonication[3]. Both reagents react by displacement of fluorine, with free amino, tyrosyl, histidyl or sulfhydryl groups to form stable derivatives. FDNB reacts with one such group to yield dinitrophenyl (DNP) derivatives and DFDNB reacts with 2 groups, provided they are 5 Å apart, to form dinitrophenylene cross-links[4]. When we measured the ACHE activity of erythrocytes treated with FDNB and DFDNB we found that each reagent affected the enzyme in a characteristically distinct manner. This observation prompted an investigation of the factors influencing the effects of these substances on red cell membrane ACHE. Additional indications for conducting these studies constitute the demonstration that FDNB irreversibly inhibits glucose transfer in intact erythrocytes[5] and the increasing use of red cells conjugated with FDNB and DFDNB for the detection of anti-DNP antibodies[6,7].

Blood obtained from normal adult individuals and from new-born infants was centrifuged and the plasma and buffy coat were removed by suction. The erythrocytes were washed twice with 20 vol. of ice-cold 0.15 *M* NaCl and 5 times with 20 vol. of chilled 0.1 *M* sodium-potassium phosphate buffer, pH 8.0. After the last centrifugation the red cells were adjusted to a 50% suspension with phosphate buffer. Five per cent stock solutions of FDNB and DFDNB in methanol were prepared daily and kept at 4 °C in a tightly closed container. The effects of FDNB and of DFDNB on red cell ACHE was investigated by adding appropriate amounts of the reagents to 0.5% cell suspensions in 0.1 *M* phosphate buffer, pH 8.0. Methanol was added to the controls. Following incubation the erythrocytes were washed 5 times with 20 vol. of buffer and adjusted to a 50% suspension after the last centrifugation. ACHE activity was measured at 25 °C on replicate 0.1% cell suspensions in 0.1 *M* phosphate buffer, pH 8.0 using acetylthiocholine iodide as substrate and 5:5'-dithiobis-(2-nitrobenzoic acid) as color reagent[8]. Thermal inactivation of ACHE was investigated at 56 °C using a thermostatically controlled water-bath constant to ± 0.5 °C. The remaining enzyme activity was subsequently measured at 25 °C. Further details are indicated in Figure 2.

Incubation at 25 °C of a 0.5% erythrocyte suspension with 5×10^{-4} *M* FDNB at pH 8.0, followed by 5 washings of the cells, caused a slow, time-dependent loss of ACHE activity, while treatment with DFDNB under the same conditions resulted in a rapid reduction of enzyme activity (Figure 1A). No loss of ACHE activity was noted in controls incubated with methanol. Treatment of cells under identical conditions with up to 5×10^{-2} *M* NaF did not cause reduction of ACHE activity; this finding ruled out the possibility that the loss in enzyme activity noted was due to fluoride ions. From experiments in which different concentrations of FDNB and DFDNB were added to complete assay systems and initial rates measured, it was possible to infer that the reduction in enzyme activity was not caused by interference with the ACHE assay. This possibility was also ruled out by

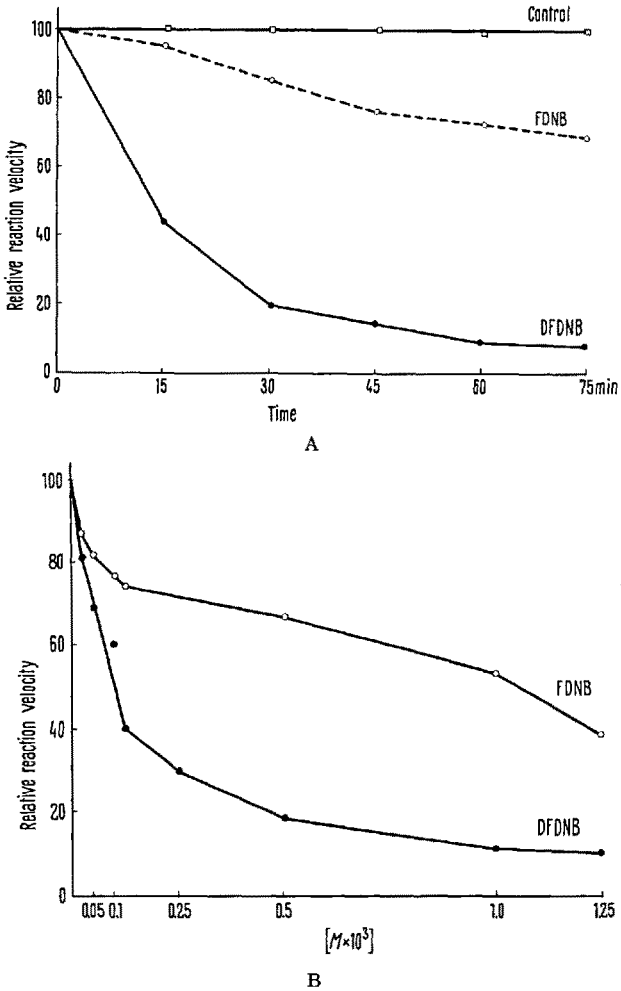

Fig. 1. Effect of time and concentration on inactivation of erythrocyte ACHE by FDNB and DFDNB. (A) At 4 °C, 0.02 ml of a 5% solution of reagents in methanol was added to replicate tubes containing 10 ml of a 0.5% cell suspension in 0.1 *M* phosphate buffer, pH 8.0. Methanol was added to controls. After thorough mixing, tubes were incubated at 25 °C. At the time indicated, 2 tubes of each set were withdrawn, the cells washed 5 times with 0.1 *M* phosphate buffer, pH 8.0. ACHE activity was measured as indicated in text and results related to controls. (B) Same as (A) except that varying amounts of reagents and methanol were used and all tubes were incubated for 30 min.

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finding no decrease in enzyme activity when intact erythrocytes were preincubated with and then assayed in phosphate buffer previously used to wash FDNB- or DFDNB-treated cells.

Incubation of red cells with increasing amounts of FDNB and DFDNB also revealed distinct patterns of ACHE inactivation (Figure 1B). While the rate was accelerated at relatively low concentrations, 31% and 80% of the enzyme was inactivated by $5 \times 10^{-4} M$ FDNB and DFDNB, respectively. In the controls treated with increasing amounts of methanol no reduction was observed. When cells were exposed simultaneously to both reagents, an additive effect was seen. Incubation at different temperatures also revealed greater inactivation by DFDNB than by FDNB. At 4 °C DFDNB destroyed 50% of the activity, while FDNB caused only a 10% loss, At 37 °C, 96% was inactivated by DFDNB and 38% by FDNB. Repeated washing of cells exposed to the reagents did not restore enzyme activity. The enzyme of ACHE-deficient erythrocytes from new-born infants with ABO-hemolytic disease[8] and of cells separated by density into young and aged populations[9] was more affected by DFDNB than by FDNB. Red cell integrity was not necessary for the inactivating effects of FDNB and DFDNB, because the enzyme of cell-free membrane preparations[1] behaved like that of intact erythrocytes.

Inactivation of ACHE was not associated with changes in substrate specificity, pH profile, Km, and effects of inhibitors. However, it was accompanied by striking changes in the thermostability of the enzyme. Residual ACHE activity following treatment with DFDNB was more heat resistant than the enzyme of membranes treated with FDNB and of controls (Figure 2). Similar results were obtained when partially heat-inactivated ACHE preparations[10] were exposed to the reagents, washed with buffer and then incubated at 56 °C. These observations indicated that DFDNB did not preferentially inactivate a more heat-labile ACHE species and suggested that the formation of a cross-linked dinitrophenylene derivative had conferred greater stability to the residual enzyme activity. To substantiate this contention, cell membranes pretreated with FDNB and DFDNB were exposed to 5% triton X-100 in $8 M$ urea[11]. Solubilization and complete loss of ACHE activity was seen with the controls and with membranes exposed to FDNB. No solubilization and minimal reduction of the residual enzyme activity was observed with the DFDNB-treated membranes. Additionally, incubation of controls and FDNB-treated preparations with 1 mg/ml crystalline papain[1] resulted in complete inactivation of ACHE, while no further reduction in activity was noted with membranes pretreated with DFDNB.

Our observations on the effects of FDNB and DFDNB on erythrocyte ACHE complement the findings of BERG et al.[3]. These authors correlated the fragility of FDNB-treated red cells with the osmotic imbalance created by the increased permeability to Na^+ and K^+ and ascribed the resistance to lysis of DFDNB-treated erythrocytes to the formation of dinitrophenylene cross-links which would be strong enough to withstand an equilibrium osmotic pressure computed at about 0.6 atm. The ACHE inactivation by FDNB and DFDNB resembles the effects of FDNB on glutamate dehydrogenase[12] and on fructose-1,6-diphosphatase[13,14]. However, in contrast to rabbit liver fructose diphosphatase[14], ACHE activity was not stimulated by low concentrations of FDNB or DFDNB, nor did the reagents cause alterations usually associated with modifications of an allosteric site. This work represents another instance where we could show that substances which modify the membrane of the human erythrocyte, specifically alter the properties of the surface located ACHE[15].

Zusammenfassung. Die Acetylcholinesterase der Erythrozytenmembranen wurde mit 1,5-Difluor-2,4-dinitrobenzol stärker inaktiviert als mit 1-Fluor-2,4-dinitrobenzol. Nach Difluordinitrobenzol-Behandlung war die restliche enzymatische Aktivität hitze- und harnstoffunempfindlich. Die Inaktivierung des Fermentes war konzentrations-, zeit- und temperaturabhängig.

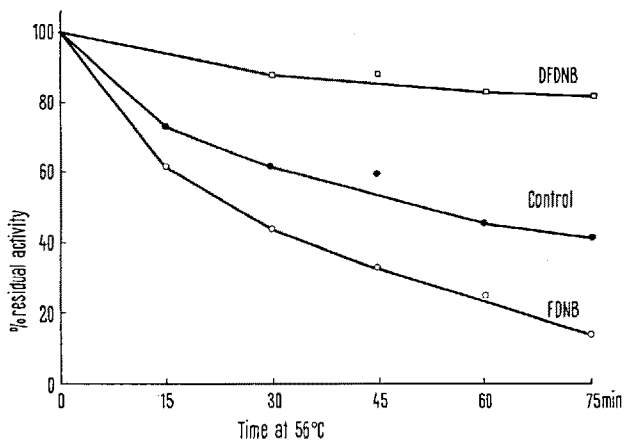

Fig. 2. Effect of time on thermostability of ACHE. Erythrocyte membranes prepared by osmotic lysis were treated for 30 min with $5 \times 10^{-4} M$ FDNB and DFDNB at pH 8.0 and 25 °C. Methanol was added to controls. After 5 washings with $0.1 M$ phosphate buffer, pH 8.0, a 5% membrane suspension in $0.001 M$ phosphate buffer, pH 8.0 was prepared. Replicate 0.2 ml aliquots were incubated at 56 °C for time indicated, immediately diluted with 10 ml of ice-cold $0.1 M$ phosphate buffer, pH 8.0 and stored at 4 °C. Enzyme activity was measured simultaneously as indicated in text and related to controls kept at 4 °C.

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