Inactivation of Erythrocyte Membrane Acetylcholinesterase by Tetraphenylboron

Sodium tetraphenylboron (TPB) forms insoluble salts with potassium, ammonium and choline ions. Dilute solutions dissociate cells[1], but cells thus treated are unable to carry out normal protein synthesis[2], do not incorporate ^{14}C-acetate into lipids[3] and show ultrastructural alterations[4]. TPB uncouples oxidative phosphorylation[5], prevents the light-induced uptake of monovalent cations by chloroplasts[6], but stimulates the labeling of phospholipids in goldfish brain preparations[7] and duplicates certain pharmacological actions of acetylcholine[8]. Other studies have indicated that TPB inhibits the Mg^{2+}-dependent ATPase of thyroid cell membranes as well as other enzymes[9]. However, the effect on the ATP-ase was partially reversed by dialysis[9]. In this report we show that exposing human erythrocytes to TPB results in the irreversible inactivation of acetylcholinesterase (ACHE), an enzyme located at the outer surface of the cell membrane[10,11].

Venous blood from adult individuals was collected with EDTA. The erythrocytes were washed 3 times with 20 volume of ice-cold 0.15 *M* NaCl solution. The supernatant and buffy coat were removed by suction. A 50% (v/v) erythrocyte suspension was prepared after the last centrifugation and used immediately. When the influence of pH was investigated, the cells were washed with and suspended in the appropriate 0.1 *M* sodium phosphate buffer. Hemoglobin-free membranes were prepared by osmotically induced hemolysis[12]. A 10^{-2} *M* stock solution of TPB in 0.15 *M* NaCl was prepared daily. Unless otherwise indicated, to one volume of a 50% erythrocyte suspension, 20 volume of an appropriately diluted TPB solution were added and incubated at 37 °C. Erythrocytes treated with saline alone served as controls. After incubation the cells were washed 4 times with 40 volume of chilled 0.15 *M* NaCl and adjusted to a 25% suspension after the last centrifugation. ACHE activity was measured at 25 °C on duplicate 0.1% cell suspensions in 0.1 *M* sodium-potassium phosphate buffer, pH 8.0 using acetylthiocholine as substrate and 5:5′-dithiobis (2-nitrobenzoic acid) as color reagent[13]. Specific activity was expressed as Δ A/min/mg hemoglobin. The latter was determined at 540 nm as cyanmethemoglobin. Further details are indicated in Tables II and III.

Incubation of erythrocytes with TPB at 37 °C caused a concentration-dependent loss of ACHE activity (Table I). Whereas the effect was moderate at concentrations below 1.5×10^{-4} *M*, approximately 60% of the activity was lost at 6×10^{-4} *M*. The action of higher concentrations of TPB could not be tested with intact cells because of overt hemolysis either during incubation or during the subsequent washings of the erythrocytes. However,

Table I. Effect of TPB on erythrocyte ACHE activity

TPB ($M \times 10^{-4}$)	ACHE Specific activity	Percent remaining
6	53	39
4	71	53
3	83	61
2	102	76
1.5	108	80
1.0	126	93
0.75	130	96
None	135	100

To duplicate 0.5 ml of a 50% suspension of thrice-washed erythrocytes, 10 ml of TPB in 0.15 *M* NaCl were added. TPB was omitted from the controls. After 30 min incubation at 37 °C the cells were washed 4 times with 0.15 *M* NaCl and adjusted to a 25% suspension. ACHE activity was measured as indicated in text.

Table II. Effect of TPB on ACHE reaction

TPB *M*	ΔA/412 nm
10^{-3}	precipitation
5×10^{-4}	precipitation
10^{-4}	0.404
5×10^{-5}	0.391
10^{-5}	0.410
5×10^{-6}	0.398
None	0.410

To 1 ml of a 1% suspension of erythrocyte membranes in 0.1 *M Tris*-HCl, pH 8.0, 9 ml of different concentrations of TPB in the same buffer were added. ACHE activity was measured as indicated in text.

Table III. Effect of cell concentration on ACHE inactivation by TPB

Red cells (ml)	(TPB)[a]/(PCV)	ACHE activity
0.1	160	70
0.2	80	81
0.4	40	83
0.5	32	86
0.7	23	102
1.0	16	105
1.5	11	116
2.0	8	130
3.0	5.3	136
	Control	144

8 ml of TPB (3×10^{-4} *M*) in 0.15 *M* NaCl were added to duplicate tubes containing various amounts of a 50% cell suspension (range: 0.1 to 3.0 ml). Following incubation at 37 °C for 30 min, erythrocytes were washed 4 times and enzyme activity determined as indicated in text.
[a] Ratio volume of TPB: packed cell volume.

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red cell integrity was not necessary for inactivation because the enzyme of cell-free membrane preparations behaved like that of intact erythrocytes. Furthermore, the percent reduction in ACHE activity in membranes prepared from TPB-treated cells was the same as that observed with whole erythrocytes. The possibility that the effect on ACHE was due to the interaction of TPB with components of the assay system was ruled out by mixing experiments and by adding increasing amounts of the agent to complete assay systems. Enzyme activity in mixtures of various proportions of treated cells and controls was additive, indicating absence of inhibition. Membrane preparations suspended in 0.1 *M* *tris*-HCl, pH 8.0 were used to assess the effect on the enzyme reaction. No inhibition of ACHE activity was noted with up to 10^{-4} *M* TPB (Table II). However, considerable precipitation occurred at higher concentrations.

Enzyme inactivation was irreversible, repeated washing of TPB-treated cells did not restore ACHE activity and none was detected in the supernatant liquid after incubation with TPB. Prolonged dialysis of hemolysates and of membrane preparations did not reverse enzyme inactivation. Reduction in activity was also noted when whole blood was exposed to TPB. Additional information was obtained by studying other parameters which influence enzyme inactivation. Thus, the effect of TPB was dependent on temperature. With 3×10^{-4} *M*, 12% of the activity was lost at 4 °C as compared with a reduction of 39% and 52% at 37 °C and 45 °C, respectively. Essentially the same proportion of activity was destroyed when cells were incubated with TPB dissolved in 0.1 *M* sodium phosphate buffers adjusted to pH's varying between 6.5 and 8.5. Inactivation was a relatively fast process and the percent of activity lost did not significantly increase with the length of incubation; e.g., with 2.4×10^{-4} *M* and at 37 °C, 30% of the activity was destroyed after 10 min and 35% after 180 min. The action of TPB was related to the amount of erythrocyte in the incubation mixture. Thus, reduction in ACHE activity was inversely proportional to the amount of cells present when the concentration and volume of TPB were kept constant (Table III).

Our results show that the exposure of human erythrocytes to TPB resulted in the reduction of ACHE activity. That this loss represents irreversible enzyme inactivation was substantiated by the failure to augment or restore ACHE activity by repeated washing or prolonged dialysis of TPB-treated cells or membranes. These findings resemble the effect seen with other ACHE-inactivating agents[10,14,15] but contrast the partial restoration of Mg^{2+}-ATPase by dialysis of thyroid cell membrane preparations treated with TPB[9]. Several additional lines of evidence indicate that this agent altered the enzyme rather than interacted with components of the assay system. These include: 1. The absence of inhibition when ACHE activity was measured in the presence of TPB; 2. Enzyme activity was assayed on 0.1% dilutions of cells or membranes and any remaining TPB would have been diluted by the same factor and 3. ACHE activity in mixtures of treated cells and controls was additive.

Since ACHE does not require potassium for activity, the present results cannot be attributed to chelation. Although the hemolysis noted at concentrations greater than 6×10^{-4} *M* is probably due to the lipid solubility of TPB[16], it appears unlikely that enzyme inactivation is related to this characteristic, since ACHE activity is not affected by hypotonic lysis or by hemolytic, polyene antibiotics such as amphotericin B and filipin[14]. A more likely possibility is that TPB reacts directly with the ACHE of the red cell membrane causing molecular changes which render the enzyme inactive. Such an interpretation would be in accord with the notion of a TPB-protein interaction, as has been postulated to explain the inhibition of other enzymes by this agent[9]. Although the present investigation was restricted to a constituent of the erythrocyte membrane, the study of the effects of TPB on intracellular components could yield additional information which may explain the unusual alterations observed when other types of cells and their organelles are exposed to this substance[17].

Zusammenfassung. Menschliche Erythrozyten wurden mit Tetraphenylbor (TPB) behandelt, wobei die TPB-Konzentrationen über 6×10^{-4} *M* hämolytisch waren. Die Acetylcholinesterase der Erythrozytenmembranen wurde durch nicht-hämolytische TPB-Konzentrationen irreversibel, temperatur- und konzentrationsabhängig inaktiviert.

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Activation of Subtilisin by Luteotropic and Luteinizing Hormone

The aim of the present paper is to provide information about a specific effect exerted by mammalian peptide hormones on the action of subtilisin. The rate of the hydrolysis of *N*-L-leucyl-2-naphthylamine catalyzed by subtilisin was 10–11 times higher in the presence of sheep luteotropic hormone (0.014 m*M*) and 7–8 times in the presence of equine luteinizing hormone (0.011 m*M*), than without any hormones. No other enzyme-hormone system investigated in this laboratory was found to display this type of effect, which was termed competitive activation.

Luteinizing hormone and luteotropic hormone, as well as *N*-L-leucyl-2-naphtylamine, were obtained from Mann Research Laboratories, Inc. (New York, N.Y., USA). Subtilisin of *Bacillus subtilis* was purchased from Sigma Chemical Company (St. Louis, Mo., USA). All other reagents were products of E. Merck AG (Darmstadt, Germany). The assay of subtilisin was in principle carried out with *N*-L-leucyl-2-naphthylamine as substrate, as described earlier[1]. It may be mentioned, however, that all reactions were performed in 0.025 *M* borate buffer, pH 8.0, at 30 °C. The amount of subtilisin was 30 µg in all reaction mixtures (0.6 ml)[1], unless otherwise mentioned. The experimental data were treated by a numerical method which involved regression analysis of weighted data by the method of least squares. Details about the computer calculations and about the use of Hanes' equation in the determination of the kinetic parameters have been