PRELIMINARY NOTES

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The effect of proteolytic enzymes on acetylcholinesterase activity, the sodium pump and choline transport in human erythrocytes

In the course of a study of the choline transport system in human erythrocytes[1] an attempt was made to remove the acetylcholinesterase activity of these cells by digestion with proteolytic enzymes. It was found that such a treatment will inactivate the cholinesterase activity almost completely without reducing choline transport or the activity of the sodium pump. This suggests that treatment with proteolytic enzymes might be a useful tool in the study of transport mechanisms.

Bank blood, usually 3–7 days old, was washed several times with a solution containing 147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM Tris to give pH 7.4 at 37°. The cells were incubated with and without proteolytic enzyme at 37°; the hematocrit was around 10 %. After the incubation the erythrocytes were treated as follows.

Cells used for the measurement of cholinesterase activity were washed in isotonic saline; 0.5 ml of packed cells was then lysed in 45 ml of a solution containing 1 mM $MgCl_2$; 5 ml of 0.1 M acetylcholine were added and the pH adjusted to 7.8. The rate of hydrolysis of acetylcholine was measured by titrating with 5 mM NaOH at constant pH. After determining the rate of hydrolysis in this way over three 10-min periods the light absorption of a suitably diluted aliquot of the solution was measured at 540 nm and the cholinesterase activity related to the concentration of erythrocytes.

Cells used for the measurement of sodium and choline fluxes were washed in the buffer. Choline influx was measured by incubating the cells in buffer containing [^{14}C]choline and various concentrations of unlabelled choline[1]. The activity of the sodium pump was examined by incubating the cells for 3 or 5 h in the presence and in the absence of ouabain (10 μg/ml) in a buffer containing 10 mM K^+ and 0.2 % glucose. The intracellular Na^+ concentrations were measured at the beginning and at the end of the incubation period with a flame spectrophotometer after the cells had been washed in isotonic $MgCl_2$ and lysed.

The data shown in Fig. 1 indicate that the acetylcholinesterase of erythrocytes can be inactivated by treatment with pronase, trypsin or chymotrypsin; prolonged treatment removes virtually all the enzyme activity. The hemolysis was always less than 5 % and there was no significant difference between the treated cells and the control. When aliquots from each batch of enzyme treated cells and from the controls were incubated for 1 h in buffer containing 2 μM [^{14}C]choline the uptake of radioactivity by the treated cells was always identical to that found with the control. In three experiments cells incubated for 16 h with chymotrypsin were used to determine choline influx at various concentrations of choline so that the maximum flux and the choline concentration giving half maximum flux could be calculated. The average values for the control cells were 0.25 μmole/l cells per min for the maximum flux

and 6.8 μM for the apparent Michaelis constant; the corresponding values for chymotrypsin treated cells were 0.28 μmole/l cells per min and 6.5 μM.

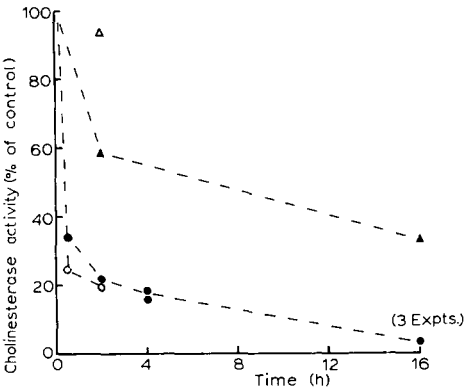

Fig. 1. Inactivation of the acetylcholinesterase of human erythrocytes by proteolytic enzymes. The cells were incubated for the time indicated on the abscissa with pronase, 100 μg/ml (○); chymotrypsin, 300 μg/ml (●); trypsin, 50 μg/ml (△); and trypsin, 200 μg/ml (▲). The cholinesterase activity is expressed as percentage of the activity found with control cells that were incubated without enzyme. The absolute values for the controls averaged 960 μmoles acetylcholine hydrolyzed per 100 ml cells per min and there was no significant difference between controls incubated in buffer for various times.

The ouabain sensitive Na^+ efflux from cells treated for 4 h with chymotrypsin was examined in two experiments, one carried out in duplicates. The results (Table I) show that the ouabain sensitive Na^+ efflux from these cells is virtually the same as that from control cells. However, the chymotrypsin treated cells have a slightly higher internal Na^+ at the end of the incubation periods, suggesting that the enzyme might increase the leak permeability to Na^+.

When cells treated with chymotrypsin for 4 h were added to incomplete rhesus (D) antibody, agglutination occurred. Chymotrypsin is therefore similar to other proteolytic enzymes in rendering erythrocytes agglutinable by incomplete antibodies and to explain this phenomenon it has been suggested that the enzymes remove certain superficial structures from the cell surface[2].

TABLE I

OUABAIN SENSITIVE NET Na^+ FLUXES IN CONTROL AND CHYMOTRYPSIN TREATED CELLS

A and A′ are duplicates; the cells were pre-incubated with and without chymotrypsin for 4 h before the net Na^+ fluxes during 5 h were measured. B: 4-h pre-incubation with and without chymotrypsin; net Na^+ fluxes during 3 h.

Cells	*Intracellular Na^+ (mmoles/l cells)*			*Ouabain-sensitive Na^+ loss (mmoles/l cells)*
	At the beginning of the incubation	*At the end of the incubation*		
		Without ouabain	*With ouabain*	
A control	15.8	11.3	16.5	5.2
A chymotrypsin	16.1	13.7	18.8	5.1
A′ control	15.8	10.9	18.0	7.1
A′ chymotrypsin	16.1	13.8	19.6	5.8
B control	11.0	9.6	12.7	3.1
B chymotrypsin	12.3	11.7	15.3	3.6

The results described indicate that the acetylcholinesterase of erythrocytes is readily accessible to proteolytic enzymes. The failure of these enzymes to affect choline transport and the sodium pump even after prolonged incubation suggests that the outward facing parts of these transport mechanisms are either not accessible or resistant to the enzymes used. MARCHESI AND PALADE[3] found that the (Na^+-K^+)-activated ATPase in the ghosts of guinea-pig erythrocytes is rapidly inactivated by trypsin but that this inactivation can be largely prevented by preincubating the ghosts with ATP and Mg^{2+}. The experiments reported here were done with intact cells containing ATP; the sodium pump may therefore have been protected and it is possible that, had ATP not been present, the pump might have been affected by chymotrypsin. The view that a protein is part of the choline transport system is supported by the observation that the transport of choline can be inhibited by *p*-chloromercuriphenylsulfonic acid, presumably because this compound blocks sulfhydryl groups. Since pronase, a rather nonspecific enzyme, does not interfere with choline transport it appears that the outward facing part of this transport system is resistant or not readily accessible to the proteolytic enzymes. There is some similarity between the results described here and the observation made by ALBUQUERQUE *et al.*[4] that at the neuromuscular junction proteolytic enzymes will inactivate cholinesterase without reducing the acetylcholine sensitivity. These authors conclude that the receptor protein is either resistant to denaturation or that its hydrolysis does not interfere with its receptor function.

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Active Na^+ transport in isolated frog gastric mucosa during hypoxia

The potential difference between the mucosal and serosal sides of the gastric mucosa, with the mucosa negative, has been extensively studied, but the nature of this potential difference is still a matter for debate. HOGBEN[1–3] demonstrated that in the isolated frog mucosa the short-circuit current could account for the net Cl^- transport *minus* the H^+ transport. He therefore concluded that the electrogenic process for the mucosal potential was an active Cl^- transport from the serosal to the mucosal side. He did not, however, find any evidence for active transport of Na^+. In mammals, however, the situation has been reported to be different. CUMMINS AND VAUGHAN[4–6] found in isolated rat stomach walls a definite active Na^+ transport from the mucosal to the serosal side. They concluded that there was a species difference between rat