Decay of red cell cholinesterase activity with cell age

In a series of papers, HOLLAND, GREIG and others[1,2] have presented evidence in favour of the view that erythrocyte acetylcholinesterase (AChE) is concerned with the maintenance of the normal permeability properties of the cell. Thus, they have shown that if red cells are suspended in a bicarbonate medium, the addition of acetylcholine to the system reduces ion transfer and subsequent haemolysis. The effect of acetylcholine is reversed by the addition of AChE inhibitors[2]. From these and other results the authors conclude that the active metabolism of acetylcholine by the cell enzyme is conducive to the preservation of the normal cation balance and that migration of ions in the direction of the concentration gradients occurs when the AChE is inactive due to lack of substrate or the presence of inhibitors. In view of these findings it is of interest to investigate any decay of the AChE activity with increasing age of cells.

The AChE activity of intact human erythrocytes was determined manometrically; the method was based on that given by ALDRIDGE[3]. Two suspension media were employed, the first containing NaCl, 0.15 *M*; $MgCl_2$ 0.035 *M*; and $NaHCO_3$ 0.0312 *M*, and the second differing from this in having the $MgCl_2$ replaced by an isotonically equivalent amount of NaCl. These solutions, which buffer at pH 7.7 when equilibrated with an atmosphere containing 5% CO_2, were stored at 3° C. Human erythrocytes were prepared for experiment by removing the plasma from citrated whole blood, and then washing the separated cells three times with the appropriate suspension medium by centrifugation. The cells were then spun down at a standard speed for a standard time (2000 r.p.m. for 10 minutes) to obtain a reproducible state of packing before making up to the required suspension density with buffer. After placing the cell suspensions in the manometer vessels, acetylcholine (measured from a micrometer syringe) was added to give a final concentration of $5.5 \cdot 10^{-3}$ *M*. The vessels were fixed in position in the thermostat at 37° C and their contents gassed with an atmosphere of 5% CO_2 and 95% air for ten minutes. A further ten minutes was allowed for equilibration before sealing off the manometers. Readings of the gas output were then taken at 10 minute intervals.

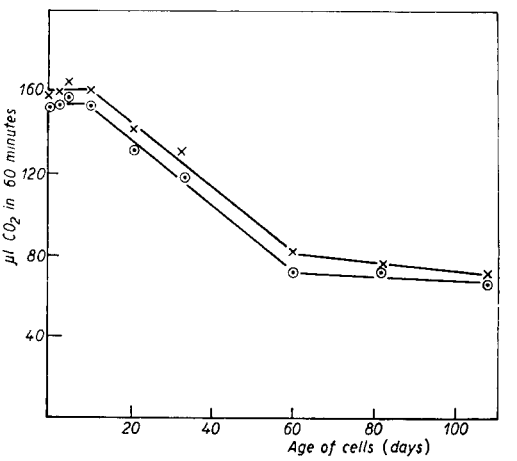

Fig. 1. Decay of erythrocyte acetylcholinesterase activity with storage of cells; 0.3% cell suspension, $5.5 \cdot 10^{-3}$ *M* acetylcholine. × magnesium present; ⊙ magnesium absent.

For the present investigation the conditions of storage of the red cells were chosen to be those usually employed in the blood banks. A sample of human blood was taken and treated with glucose citrate. It was immediately divided into nine separate samples, and each stored under sterile conditions at 2–3° C. For each experiment one of these samples was opened and divided into two portions for the preparation of suspensions with and without magnesium. The AChE activity of each suspension was then determined in the manner described, and the results shown in the figure were obtained. Each point in the figure represents the mean of at least three independent determinations, with a total scatter of less than 3%. The esterase activity is seen to remain effectively constant for the first ten days and then to fall more or less linearly to approximately 50% of its original value in a time of the order of 60 days; and then it falls only slightly during the succeeding 50 days. When the blood sample for the determination at 60 days was centrifuged a slight pigmentation of the plasma was observed, indicating the onset of haemolysis. Haemolysis was further advanced in the two samples opened later. In view of the finding of HOLLAND and GREIG that permeability effects were obtained with AChE inhibitors only in concentrations which produce not less than 50% inhibition[4], it is interesting that the onset of haemolysis occurs when the AChE activity has decayed to about 50% of its original value. The sudden change in the rate of enzyme decay corresponds to the onset of haemolysis (as nearly as can be ascertained from the present observations) but it is uncertain if these two are causally related. One possible explanation of this sudden change in the rate of decay would be the existence of more than one acetylcholine hydrolysing mechanism, not all of which lose their activity during the length of time of these experiments.

There are many reports that some divalent cations are activators of AChE (see *e.g.*[5]). It is seen from the figure that the presence of magnesium in the buffer has very little effect upon the enzyme activity in the present system. This may be due to the presence of other activators and does not necessarily imply that magnesium is not an activator of the red cell enzyme. The addition

of a divalent cation does not, however, appear to be of importance in studies of this kind with intact red cells.

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The aconitase of *Aspergillus niger**

JACOBSOHN *et al.*[1] suggested that aconitase may consist of two enzymes, one catalyzing the reaction citrate $\rightleftharpoons$ *cis*-aconitate and the other *cis*-aconitate $\rightleftharpoons$ *iso*citrate. BUCHANAN AND ANFINSEN[2], however, obtained a 23-fold purification of aconitase with a relatively constant ratio of citric acid to *iso*citric acid being formed from *cis*-aconitic acid throughout the process. In contrast, RACKER[3] reported that an *iso*citrase-citrase ratio in crude heart extracts of 2.1, changed to 7.5 upon purification of the preparations. In his review of the literature on aconitase, OCHOA[4] concluded that the evidence to date suggested that only one enzyme was responsible for the equilibrium between citrate, *cis*-aconitate and *iso*citrate.

Preliminary studies with extracts of *Aspergillus niger* indicate that the aconitase system in this microorganism is made up of at least two enzymes. In the experiments undertaken citric acid was determined by a modification of the method of NATELSON *et al.*[5] and *d-iso*citric acid by the *iso*citric dehydrogenase method of OCHOA[6]. The protein content of the purified enzyme extracts was calculated from the optical density observed at 280 and 260 mμ in a model DU spectrophotometer. All enzyme fractionations were carried out in the cold. Crude enzyme extracts were prepared by sonic disintegration of the washed mycelium from 24-hour cultures of *Aspergillus niger* (72–4) grown on a rotary shaker in the medium of MARTIN[7]. 20 mg of cysteine were added, prior to disintegration, for each 100 ml of mycelial suspension. The crude sonicate was centrifuged at 14,000 r.p.m. and solid ammonium sulfate added to the supernatant to give 0.25 saturation. After centrifugation, the sediment was discarded and ammonium sulfate added to the supernatant to 0.75 saturation. The precipitate, after centrifugation, was suspended in *M*/100 phosphate buffer (pH 7.4), the pH adjusted to 7.4, and the solution dialyzed against the same buffer to which about 5 mg of cysteine per liter had been added. A protamine sulfate solution (20 mg/ml) was added to the dialysate until the ratio of absorption at 280 to that at 260 mμ reached 0.63. The mixture was then dialyzed as previously. After dialysis, the ratio at 280:260 mμ was 0.82. The dialysate was then treated with solid ammonium sulfate to give three successive fractions with 0.42, 0.55 and 0.75 saturation respectively. Each precipitate was suspended in buffer and dialyzed. The ratio of citric acid to *iso*citric acid as produced by the enzyme complex was determined on each of the fractions as follows: 0.05 *M* tris buffer (pH 7.4), enzyme solution, 1.5 μmoles *cis*-aconitate and water to 1.0 ml volume were incubated in a small pyrex test tube at 30° C in a water bath. The reaction was started by the addition of the *cis*-aconitate after the mixture had reached 30° C. 0.5 ml aliquots were removed at the desired times, usually 10 and 20 or 20 and 40 minutes and each was added to 0.15 volumes of 0.4 *M* acetate buffer (pH 3.8). The mixture was heated in boiling water to precipitate the protein, cooled and then centrifuged. The supernatant was adjusted to pH 7.4 with KOH. The citric acid and *iso*citric acid content of the supernatant was determined. The results for the 20 minute incubation period are given in Table I. A reaction mixture minus *cis*-aconitate served as an endogenous control and all results are corrected in this respect.

Since the ratio of citric acid to *iso*citric acid formed by the enzyme solution, decreased markedly as the degree of saturation with ammonium sulfate was increased, further fractionation was undertaken. The solution of the 0.55–0.75 fraction was brought to 0.60 and then 0.75 saturation with solid ammonium sulfate, the respective precipitates were centrifuged, suspended in buffer and dialyzed. The ability to form citric and *iso*citric acids from *cis*-aconitate was then determined on each fraction. As may be seen in Table I, the ratio of citric to *iso*citric acids formed by the new fractions was lower

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