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Effect of paraoxon on erythrocyte metabolism as measured by oxygen uptake *in vitro*

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Summary

1. Oxygen consumption *in vitro* and persistence in the general circulation of rabbit erythrocytes treated with the cholinesterase inhibitor paraoxon were determined.

2. Paraoxon *in vitro* reduced oxygen consumption below a measureable level within 2 hours. By contrast, the metabolic inhibitor N-ethylmaleimide (NEM) produced complete inhibition within 15 minutes.

3. Erythrocytes from rabbits orally dosed with parathion also exhibited marked depression of oxygen consumption.

4. Glutathione (GSH) restored oxygen uptake to pretreatment levels within 15 min in erythrocytes previously inhibited with NEM or paraoxon.

5. Erythrocytes treated with NEM were rapidly removed from the general circulation while paraoxon treated cells were removed at a rate comparable to untreated cells.

Introduction

While the action of anticholinesterase compounds has been studied extensively in the nervous system, in the blood the observations have been confined to their effects on cholinesterase activity primarily as an indication of the degree of exposure to them.

There are reports in the literature suggesting that anticholinesterase compounds may affect the erythrocyte itself. Some investigators have reported changes in red cell membrane permeability to Na^+ and K^+ following cholinesterase inhibition (Greig, Faulkner & Mayberry, 1953 ; Holland & Greig, 1950 ; Ludwig, Greig & Peterson, 1951) and in addition, considerable evidence has been presented indicating that the permeability of the cell membrane is dependent on cell metabolism (Bartlett & Marlow, 1953 ; Danowski, 1941 ; Dawson & Widdas, 1963 ; Gabrio, Finch & Huennekens, 1956 ; Harris, 1941 ; Jacob & Jandl, 1962b ; Prankerd, 1955 ; Prankerd & Altman, 1954). In view of these relationships, it was of interest to investigate the possibility of an alteration of cell metabolism by an anticholinesterase compound. This would be of particular importance if an impaired metabolism was responsible for the observation of Guthrie, Santolucito & Caruolo (1967) that transfused, paraoxon-inhibited (red blood cells) (RBCs) were removed from the rabbit circulation more rapidly than non-inhibited RBCs.

The purpose of this study was (1) to determine the O_2 consumption of the rabbit erythrocyte before and after exposure to paraoxon as a measure of metabolism and

(2) to elucidate the cause of the apparent shorter persistence of the paraoxon treated erythrocyte in the general circulation.

Methods

Blood for *in vitro* studies was obtained from mature New Zealand white rabbits by cardiac puncture. The blood was immediately diluted with cold medium and centrifuged. The erythrocyte suspending medium contained per litre: NaCl, 0·9 g; KCl, 0·2 g; $CaCl_2$, 0·2 g; $MgCl_2$, 0·05 g; glucose, 1 g; N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid (HEPES), 1 g. The erythrocytes were washed twice and finally resuspended in the medium to approximately the original blood volume. The final suspension was saturated with oxygen at one atmosphere before respirometry. In the first part of the experiment, the oxygen uptake was measured before and after exposure to paraoxon. In the second part, the oxygen uptake of cells from rabbits previously dosed orally with parathion was measured.

The RBC count, mean cell volume (MCV), and haematocrit (HCT) were determined at the time of pipetting into the reaction flask using a model B Coulter Counter. Oxygen consumption was measured at a bath temperature of 38° C with the Gilson Differential Recording Respirometer. Each reaction flask received 2 ml of resuspended cells, 1·5 ml of medium, and 0·5 ml of 20% KOH in the centre well. Erythrocyte cholinesterase activity was determined by gas chromatography using 3,3-dimethyl butyl acetate as substrate (Cranmer & Peoples, 1971).

The agents to be added were prepared so that the addition of 1 ml to the reaction flask resulted in a final concentration of 2×10^{-3}M paraoxon, 2×10^{-3}M glutathione (GSH), and 3×10^{-3}M N-ethylmaleimide (NEM).

In order to determine the rate of removal of erythrocytes from the circulation, 10 ml blood were removed from the rabbit, washed, and incubated with ^{51}Cr for 30 minutes. The cells were then washed to remove free chromium ion and resuspended. Following incubation for 2 h with either NEM, paraoxon, or no treatment, 5 ml of the washed resuspended cells were reinjected into the donor. Blood samples (1·0 ml) were taken at 10 min intervals, then hourly for the first 6 h and then at 24, 48, and 72 hours. Radioactivity was determined in a liquid scintillation counter and expressed as counts per ml of packed cells. All values are expressed as means $\pm$S.D. and differences tested for significance by Student's *t*-test.

Results

Comparison of the mean cell volume (MCV) and haematocrit (HCT) of freshly drawn blood with washed and resuspended erythrocytes revealed that no significant change in these values was attributable to the medium after 30 min (Table 1). The oxygen consumption of non-treated, washed erythrocytes, based on 115 determinations using three female and two male rabbits, was $((1{\cdot}5 \pm 0{\cdot}2) \times 10^{-15}$ L/rbc)/hour. Day-to-day variation in erythrocyte oxygen uptake for a given animal was as great as the variation between animals.

Erythrocytes were treated with a known metabolic inhibitor, NEM at a concentration of 3×10^{-3}M in order to verify that the measured oxygen uptake was associated with metabolic processes. Oxygen consumption (twenty-nine determinations) of erythrocytes 15 min after introduction of NEM was not measurable but could be

established by the addition of GSH to a final concentration of 2×10^{-3}M. Comparison of pre-NEM oxygen uptake with that obtained following NEM plus GSH revealed an insignificant difference of $((0{\cdot}2 \pm 0{\cdot}2) \times 10^{-15}$ L/rbc)/h. Addition of GSH to non-inhibited cells did not alter oxygen consumption.

The effect of paraoxon was examined after it had been established that oxygen consumption could be abolished by NEM and restored by GSH. After the oxygen uptake of untreated erythrocytes was determined, paraoxon was introduced into the reaction vessel to a concentration of 2×10^{-3}M and the measurement continued. A decreased rate of uptake was evident within 30 min of the addition of paraoxon and by 2 h uptake was abolished. The addition of GSH to 2×10^{-3}M at this time, restored oxygen uptake to the pre-paraoxon levels within 15 min, the difference between pre-paraoxon and paraoxon plus GSH (23 determinations) being $((0{\cdot}3 \pm 0{\cdot}6 \times 10^{-15})$ L/rbc)/h and non-significant. At this paraoxon concentration, there was no measurable acetylcholinesterase activity in the erythrocytes before or after addition of GSH.

To test the effect of paraoxon on the integrity of the erythrocyte membrane, eight reaction vessels were prepared from one pooled blood sample and paraoxon added to four of them. They were then incubated for 4 h and the MCV $\pm$S.D. for treated and non-treated samples was $90{\cdot}3 \pm 0{\cdot}2$ μm^3 and $91{\cdot}4 \pm 0{\cdot}9$ μm^3, respectively. Both treated and non-treated cells had increased their MCV by 15% during the 4 hours.

Oral dosing of rabbits with parathion also resulted in depressed oxygen uptake of erythrocytes *in vitro*. In four animals given 10 mg parathion/kg orally and tested at the time of appearance of severe clinical signs of toxicity, erythrocyte acetylcholinesterase activity was undetectable. At this time oxygen uptake of erythrocytes was inhibited but could be restored by the addition of GSH (Table 2).

The disappearance rate from the rabbit circulation was quite different for NEM treated and paraoxon treated ^{51}Cr labelled erythrocytes. After injection of labelled cells treated with NEM, the specific activity of the 48 h blood sample was 6% of the 15 min sample; for paraoxon and non-treated cells, this value was 70%.

Discussion

The oxygen uptake values of untreated erythrocytes observed in this experiment are consistent with those reported by others (Long, 1961). This is interpreted as

TABLE 1. *Comparison of mean cell volume and haematocrit of freshly drawn rabbit erythrocytes with cells which have been separated from plasma, washed, and reconstituted to the original volume with incubation medium*

	No. of samples	Mean cell volume (μm^3) mean $\pm$S.D.	Haematocrit % mean $\pm$S.D.
Erythrocytes in plasma	5	$92{\cdot}0 \pm 2{\cdot}4$	$51{\cdot}3 \pm 2{\cdot}4$
Erythrocytes in medium	20	$91{\cdot}6 \pm 5{\cdot}5$	$53{\cdot}0 \pm 4{\cdot}9$

TABLE 2. *O_2 consumption of erythrocytes from rabbits given a lethal oral dose of parathion, before and after* in vitro *addition of glutathione*

Rabbit	Replicates flasks	O_2 Consumption (L/rbc)/h Before mean $\pm$S.D.	After mean $\pm$S.D.
6	5	$0{\cdot}0 \pm 0{\cdot}0 \times 10^{-15}$	$1{\cdot}4 \pm 0{\cdot}4 \times 10^{-15}$
4	6	$0{\cdot}8 \pm 0{\cdot}2 \times 10^{-15}$	$1{\cdot}6 \pm 0{\cdot}3 \times 10^{-15}$
2	4	$0{\cdot}5 \pm 0{\cdot}1 \times 10^{-15}$	$1{\cdot}8 \pm 0{\cdot}5 \times 10^{-15}$
3	5	$0{\cdot}0 \pm 0{\cdot}0 \times 10^{-15}$	$1{\cdot}1 \pm 0{\cdot}2 \times 10^{-15}$

indicating that the respirometry procedures used did not unduly disturb normal RBC metabolism. NEM enters erythrocytes and completely blocks intracellular GSH at doses which begin to diminish glycolysis (Jacob & Jandl, 1962a). In our experiment, this concentration of NEM abolished oxygen uptake within 15 min of its addition. Since GSH penetrates the membrane poorly, if at all (Jacob & Jandl, 1962a), the rapid restoration of oxygen uptake mediated by this compound in NEM-inhibited cells suggests utilization of free oxygen primarily by membrane-bound enzyme systems. In contrast to the rapid inhibition by NEM, paraoxon completely inhibited oxygen uptake only after 2 hours. The implications of this time course are that a different step in the metabolic sequence was blocked by paraoxon compared to NEM, or that paraoxon gained access to critical groups more slowly. In either case, the rapid restoration of oxygen uptake in paraoxon-inhibited erythrocytes following the addition of GSH leads to the conclusion that paraoxon, as well as inhibiting the esterase, may either block −SH groups essential in the transfer of electrons to free oxygen or inactivate some other component in the electron transfer system for which GSH can act as substitute.

Participation of the acetyl cholinesterase–acetylcholine (AChE–ACh) system in cation distribution across the erythrocyte membrane has received considerable attention. Lost potassium can be replaced against a concentration gradient in both dog and human erythrocytes, when cells having AChE activity are incubated with ACh (Greig *et al.*, 1953). AChE activity does not, however, seem to be related to the rate of potassium leakage from human erythrocytes stored at 25° C (Goodman, Marrone & Squire, 1955). Prankerd (1956) questions the functional significance of this enzyme in ion transport because of the low rate of synthesis of its substrate. In our experiment the stability of the MCV of cells treated with paraoxon and incubated for 4 h suggests that no net change in cellular osmolarity occurred.

Jacob & Jandl (1962b) demonstrated rapid removal from the circulation of transfused erythrocytes previously treated with sulphydryl blocking agents. We were able to confirm the shorter existence in the circulation of NEM treated erythrocytes. However, paraoxon did not contribute to a more rapid removal of inhibited RBC, suggesting that significant sulphydryl inhibition by this agent did not occur. The discrepancy between this observation and that of Guthrie *et al.* (1967) in which replacement of 50–60% of a rabbit's normal erythrocytes with paraoxon-inhibited erythrocytes resulted in a marked decrease in the RBC count by 18 h is not readily explainable. It may be that, unlike NEM treated cells, paraoxon treated cells, when sufficient numbers are present, trigger a non-selective removal of erythrocytes from the circulation.

REFERENCES

BARTLETT, G. R. & MARLOW, A. A. (1953). Erythrocyte carbohydrate metabolism; 1. The flow of C^{14} glucose carbon into lactic acid, carbon dioxide, cell polymers, and carbohydrate intermediate pool. *J. Lab. clin. Med.*, **42**, 178–187.

CRANMER, M. F. & PEOPLES, A. (1971). A sensitive gas chromatographic method for human cholinesterase determination. *J. Chromat.*, **57**, 365–371.

DANOWSKI, T. S. (1941). The transfer of potassium across the human blood cell membrane. *J. biol. Chem.*, **139**, 693–705.

DAWSON, A. C. & WIDDAS, W. F. (1963). Inhibition of the glucose permeability of human erythrocytes by N-ethyl maleimide. *J. Physiol., Lond.*, **168**, 644–659.

GABRIO, B. W., FINCH, C. A. & HUENNEKENS, F. M. (1956). Erythrocyte preservation: A topic in molecular biochemistry. *J. Hematol.*, **11**, 103–113.

GOODMAN, J. R., MARRONE, L. H. & SQUIRE, M. C. (1955). Effect of *in vivo* inhibition of cholinesterase on potassium diffusion from the human red cell. *Am. J. Physiol.*, **180**, 118–120.

GREIG, M. E., FAULKNER, J. S. & MAYBERRY, T. C. (1953). Studies on permeability. IX. Replacement of potassium in erythrocytes during cholinesterase activity. *Archs Biochem.*, **43**, 39–47.

GUTHRIE, F. E., SANTOLUCITO, J. A. & CARUOLO, E. V. (1967). Transfusion of paraoxon-treated homologous erythrocytes into rabbits. *Bull. Environ. Contam. Toxicol.*, **2**, 220–227.

HARRIS, J. E. (1941). The influence of the metabolism of human erythrocytes on their potassium content. *J. biol. Chem.*, **141**, 579–595.

HOLLAND, W. C. & GREIG, M. E. (1950). Studies on the permeability of erythrocytes. III. The effect of physostigmine and acetylcholine on the permeability of dog, cat and rabbit erythrocytes to sodium potassium. *Am. J. Physiol.*, **162**, 610–615.

JACOB, H. S. & JANDL, J. H. (1962a). Effect of sulfhydryl inhibition on red blood cells. I. Mechanism of hemolysis. *J. clin. Invest.*, **41**, 779–792.

JACOB, H. S. & JANDL, J. H. (1962b). Effect of sulfhydryl inhibition on red blood cells. II. Studies *in vivo*. *J. clin. Invest.*, **41**, 1514–1523.

LONG, CYRIL, Ed. (1961). *Biochemist's Handbook*, p. 797. London: E. & F. N. Spon. Ltd.

LUDWIG, P. E., GREIG, M. E. & PETERSON, S. W. (1951). Studies on permeability. V. The effects of acetylcholine and physostigmine on the permeability of human erythrocytes to sodium and potassium. *Biochem. Biophysics.*, **30**, 241–250.

PRANKERD, T. A. J. (1955). The metabolism of the human erythrocyte. A review. *Br. J. Haemat.*, **1**, 131–145.

PRANKERD, T. A. J. (1956). The activity of enzymes in metabolism and transport in the red cell. *Int. Rev. Cytol.*, **5**, 279–301.

PRANKERD, T. A. J. & ALTMAN, K. I. (1954). A study of the metabolism of phosphorus in mammalian red cells. *Biochem. J.*, **58**, 622–633.

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