The effect of lead on the red cell membrane

H. S. SELHI
Ph.D.

J. M. WHITE
M.D.

Department of Haematology, Royal Postgraduate Medical School, Du Cane Road, London

Summary

Conformational changes have been shown in the proteins of the red blood cell membrane, induced by lead poisoning. Evidence that the change in spatial arrangement of proteins is believed to be responsible for inhibition of the sodium (Na^+)/potassium (K^+) ATPase of the RBC membrane is presented.

Introduction

Many theories have been postulated for the interaction of lead with cell membranes (Hoogeveen, 1970; Aub *et al.*, 1926; Maxwell and Bischoff, 1929; Clarkson and Kench, 1958). However, the mode of interaction and the resultant changes in cell membrane structure have not been investigated in depth. Lead also causes inhibition of the Na^+/K^+ ATPase pump of the erythrocyte (Hasan *et al.*, 1967a; Hasan, Vikko and Hernberg, 1967b; Hernberg, Vikko and Hasan, 1967; Secchi and Alessio, 1969). The enzyme is sparsely distributed over the cell membrane as judged by its ouabain binding characteristics (Gardner and Coulon, 1972). Although it is known that the membrane-bound enzyme is inhibited, it is not known exactly which step in the complex mechanism of enzymic reaction is affected. Clearly, two modes of studying the cell membrane are available, namely, the study of changes in gross membrane lipoprotein structure, and the study of microscopic changes in the cell membrane as judged by alteration of enzymic activity. The former study was carried out by selective iodination of exposed tyrosine residues (Phillips and Morrison, 1971) and the latter by studying the high energy intermediates formed for the Na^+/K^+ ATPase reaction.

Materials and methods

All reagents used were of the highest purity commercially available. ($\gamma^{32}P$) ATP and $Na^{125}I$ were purchased from the Radiochemical Centre, Amersham, England; lactoperoxidase was purchased from Sigma, England; ^{203}Pb, as the chloride, was obtained from the MRC Cyclotron Unit, Hammersmith Hospital. Unlabelled lead was used in its acetate form.

Preparation of washed cells

Freshly drawn blood (20 ml) from normal subjects was added to 1 ml of 0·2 M Na_2 EDTA. The volume was made up to 50 ml with cold isotonic saline and the sample centrifuged at 2000 g for 5 min at 4°C. The supernatant and the white cells were aspirated and the cells resuspended in saline and washed three times by centrifugation.

Preparation of ghosts

The method of Dodge, Mitchell and Hanahan (1963) was used.

Concentration-dependent binding of lead

Saline washed RBC were treated with Pb^{2+} at levels of 0, 25, 50, 100, 150, 200, 300 and 500 μg/100 ml saline at 37°C for 30 min. The ratio of packed cells to the lead solution was 1 : 50 v/v. Tracer amounts of ^{203}Pb (2–5 μCi) per sample were included in the lead solution. Incubation was terminated by cooling the cells on an ice bath for 3 min. The cells were sedimented at 4°C and washed by centrifugation, using saline (five washes). The sample at this stage was referred to as the washed lead treated cells. These cells were lysed in 10 ml of 5% Triton X-100 and the level of radioactivity determined using the Packard Tricarb γ-spectrometer. The experiment was repeated using ghost preparation in place of RBC. 18,000 *g* force was used for sedimentation of the ghosts, which were freeze-thawed before use.

Labelling with ^{125}I

The method used was essentially that of Phillips and Morrison (1971). Washed lead treated cells were iodinated by suspension of 0·5 ml packed cells in 1 ml labelling solution. Labelling of ghosts was carried out after treatment with lead and saline washing of the membranes. The ghosts were freeze-fractured before use.

SDS gel electrophoresis

Membrane samples were solubilized in sodium dodecyl sulphate and electrophoresed on 5% gels as described by Fairbanks, Steck and Wallach (1971).

The gel was fractionated into slices of equal thickness (approx. 1 mm) and the radioactivity in each specimen counted.

Protein determination

The method of Lowry et al. (1951) was used for the determination of protein.

Labelling of erythrocyte ghosts with ($\gamma^{32}P$)

The method is a modification of Post et al. (1965). All labelling experiments were performed at 0°C. Ghost preparation (0·8–1 mg protein) was added to 50 mmol/l tris buffer pH 7·4, 100 mmol/l NaCl and 2 mmol/l MgCl$_2$. Labelling was initiated by adding 0·05 mmol/l ($\gamma^{32}P$) ATP and the reaction quenched 5 sec later with 5 ml ice-cold 5% TCA, containing 1 mmol/l unlabelled ATP and Pi. The suspension was filtered through a filter (pore size 0·8 μm). Each filter was washed four times with 5 ml of TCA-ATP-Pi solution. The precipitate was counted in 2π geometry using a liquid scintillation technique. Experiments were repeated using 100 mmol/l KCl in place of NaCl. An arbitrary standard of 100% intermediate formation is taken for untreated ghosts in the presence of 100 mmol/l NaCl. All other values are expressed as a percentage of this.

Results
Lead binding

Figure 1 shows the amount of lead bound to red cells and to freeze-thawed ghosts. Lead binding to
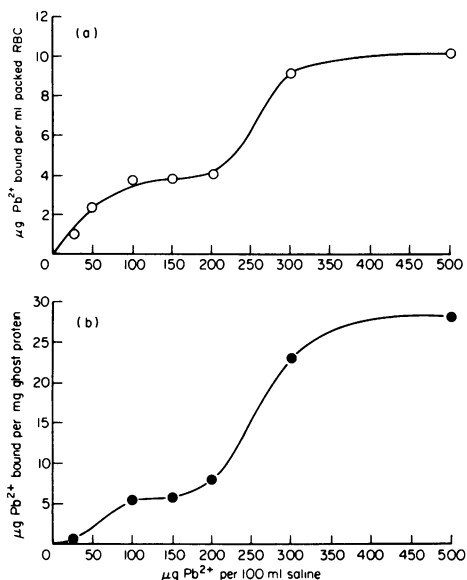
saline washed erythrocytes showed a biphasic reaction with increasing concentrations of lead. This pattern is consistent for ghost membranes prepared from the cells by the method of Dodge et al. (1963). As shown in the figure, the half maximal saturation concentrations of lead for the two binding sites A and B were approximately 60 and 240 μg/100 ml saline (approx. μmol/l and 12·0 μmol/l Pb^{2+}).

Iodination experiment

Control experiments without the lead-treated red blood cells showed that one type of molecular weight protein was predominantly labelled when lactoperoxidase was present outside the intact RBC. Using lead-treated RBC the amount of labelling decreased sharply. Lead at 25 μg/100 ml saline showed a large decrease in ^{125}I binding and the amount of ^{125}I bound at elevated levels of lead showed that at 500 μg/100 ml saline the ^{125}I was approximately 3% of control value (Fig. 2). Proteins iodinated with ^{125}I have a molecular weight range of
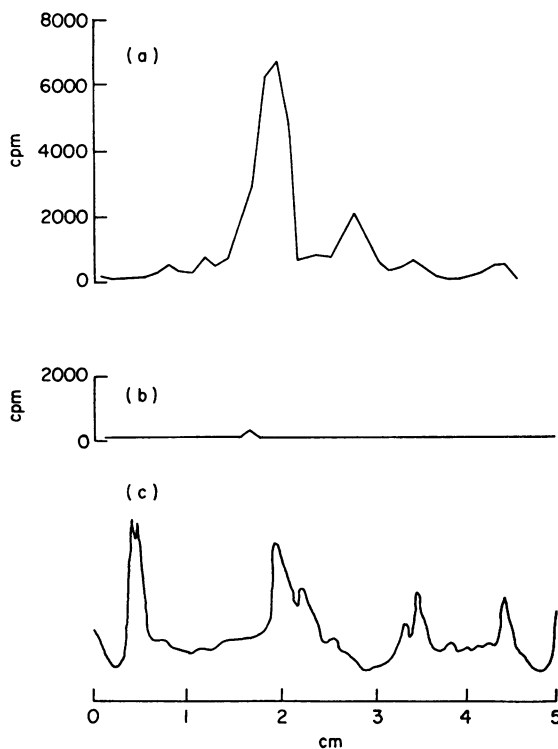
FIG. 2. ^{125}I-labelling of erythrocytes. The cpm shows the counts due to ^{125}I. (a) ^{125}I-labelling of normal cells; (b) ^{125}I-labelling at 500 μg Pb^{2+} per 100 ml saline; (c) the SDS gel electrophoretic pattern after Coomassie blue staining with left-hand side negative. A gradual decrease in ^{125}I-binding is noted as the Pb^{2+} level is increased. Only the control and final concentration used is shown.

FIG. 1. Lead binding to (a), saline washed erythrocytes; (b), ghost preparation.

100,000 to 90,000. Proteins with high molecular weight (100,000 to approx. 250,000) and those with low molecular weight (90,000 to approx. 15,000) are not significantly labelled with ^{125}I. Results obtained from freeze-thawed ghosts were entirely different. Control cells without lead treatment gave ghost preparations which were iodinated giving two distinct peaks in the SDS gel electrophoresis in the molecular weight region of 100,000 to 50,000. As the level of lead was increased for treatment of cells, the ghost preparation gave progressively larger numbers of ^{125}I-labelled peaks. The pattern of labelling showed a gradual increase in new peaks appearing in the 100,000 to 10,000 molecular weight region of the SDS gel. The labelling of protein in the high molecular weight region, although significant, remained constant at a fixed level. No prominent peak of activity could be found in this region. (Fig. 3).
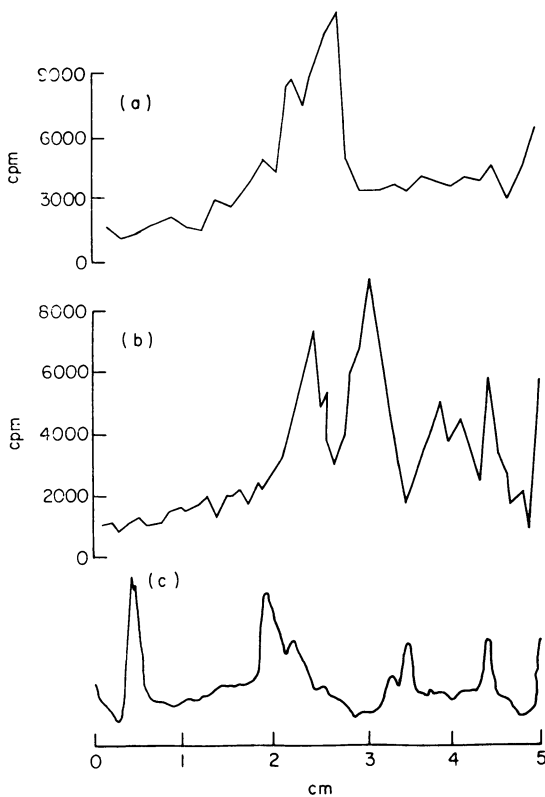
High energy intermediate formation

It was found that high energy intermediates formed in the presence of Na^+ and K^+ were raised in the lead treated ghosts as compared with normal ghosts. However, the $(\gamma\text{-}^{32}P)$ of $AT^{32}P$ was incorporated to a greater extent in the presence of K^+ than Na^+ between 25 and 150 $\mu gPb^{2+}/100$ ml saline. For lead values greater than this, the intermediates formed in the presence of Na^+ and K^+ were both stimulated to the same extent, i.e. 200% of control value (Figs. 4 and 5).
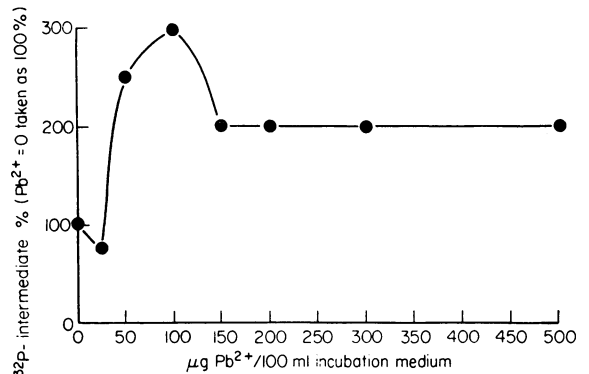
FIG. 3. ^{125}I-labelling of ghost membrane preparation. As in Fig. 2 the cmp shows the counts due to ^{125}I. Only the control (a) and 100 μg Pb/100 ml saline (b) are shown. (c) is that of SDS gel electrophoresis with Coomassie blue staining to locate the protein bands. The left-hand side is positive with respect to the right-hand side.

FIG. 4. Formation of high energy ^{32}P intermediate at 0°C in the presence of 100 mmol/l Na^+ with varying concentration of Pb^{2+} used for binding to the membrane preparation. Preincubation at 37°C for 30 min with Pb^{2+}.
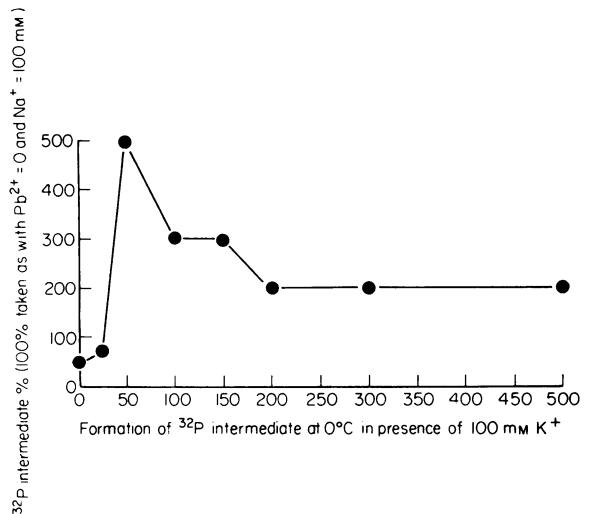
FIG. 5. Formation of high energy intermediate in the presence of 100 mmol/l K^+. The x-axis shows the level of Pb^{2+} used in $\mu g/100$ ml saline. Preincubation at 37°C for 30 min with Pb^{2+}.
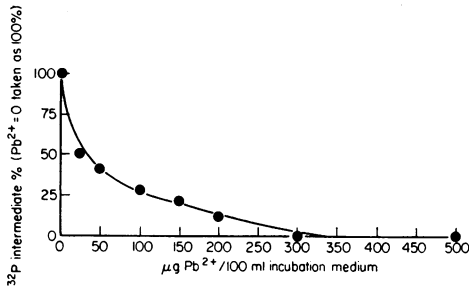
FIG. 6. High energy intermediate formed in the presence of 'free' Pb^{2+}. The total intermediate is taken as the difference between intermediate formation in the presence of 100 mmol/l Na^+ and 100 mmol/l K^+. The value of 100 mmol/l Na^+ with Pb = 0 is arbitrarily taken as 100%. Incubation at 0°C for 30 sec with Pb^{2+}.

When lead was added 30 sec before addition of radioactive ATP, the difference between Na^+ stimulated intermediate and K^+ stimulated intermediate formation showed an exponential decrease (Fig. 6).

Discussion

The lead binding curves of Fig. 1 show that there may be two types of site on or in the erythrocyte with different affinities for lead. However, on further investigation it was found that the two sites did not differ markedly in their binding affinities for lead. That the two types of site were located on the cell membrane and not attributable to intracellular binding was demonstrated by using RBC ghosts, which were free of intracellular inclusions and haemoglobin. The ghosts had been freeze-thawed to expose both surfaces of the membrane to lead. Therefore, compartmentalization of lead can be ruled out. Although the amount of lead bound to ghosts is greater than that bound to the whole cells, the pattern remains unchanged. On this account, the binding of lead to whole cells most probably occurs at the cell membrane surface exclusively (preliminary studies have shown that the intracellular components of the red blood cells are able to elute lead bound on the membrane on haemolysis. The observed intracellular binding of lead is a complex phenomenon due to this exchange mechanism).
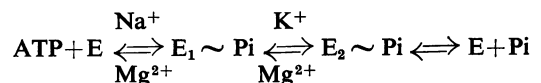
The most satisfactory explanation is that the lead at low concentration induces conformational changes in proteins of the membrane, exposing new groups for lead binding. For the detection of such changes, use was made of the specific interaction of iodine with tyrosine (and to a lesser extent, with histidine) groups of membrane proteins. This reaction is catalysed by lactoperoxidase at room temperature and at isotonic strength. Since lactoperoxidase is of high molecular weight, and cannot penetrate the the intact cell membrane by passive diffusion, the labelling of the membrane with ^{125}I occurs at the surface only. As shown in the results, the number of groups which are susceptible to iodination is greatly reduced for whole blood cells which have been treated with increasing amounts of lead. Such behaviour can be explained by conformational change in the protein components of the cell membrane which may be induced by the various levels of lead used.

Labelling of the whole cell from the inside only was attempted by entrapping the lactoperoxidases in RBC by use of reversible haemolysis. Results were difficult to interpret owing to increased resistance of the lead-treated cells to haemolysis.

Using the freeze-thawed ghost preparation, the amount of ^{125}I bound to the membrane increases with increasing levels of Pb^{2+}. The SDS gel electrophoresis shows that new peaks of radioactivity continuously appear as the level of lead is raised. Although the pattern of ^{125}I-labelling for both the whole cell and the ghost membrane is different, both suggest that conformational change in protein components of the RBC is induced by lead.

Clearly, the study shows that gross membrane structure is changed in the presence of lead. However, it does not tell us how this gross change affects the environment of enzymes located in the cell membrane. Does the change in conformation described lead to the well known inhibition of the Na^+/K^+ cation pump or is there a direct interaction of lead with such enzyme systems causing inhibition? The Na^+/K^+, Mg^{2+} dependent adenosine triphosphatase was taken as the model for detection of microscopic changes in the membrane. In simplified form, the reaction may be stated as going through two well defined conformational changes in the presence of Na^+ and K^+.

$$ATP+E \underset{Mg^{2+}}{\overset{Na^+}{\Longleftrightarrow}} E_1 \sim Pi \underset{Mg^{2+}}{\overset{K^+}{\Longleftrightarrow}} E_2 \sim Pi \Longleftrightarrow E+Pi$$

The enzyme (ATPase) is represented as E, the inorganic phosphate as Pi. It is interesting to note that the change in level of $E_1 \sim Pi$ and $E_2 \sim Pi$ complex at 180–200 µg/100 ml corresponds to the beginning of the second part of the biphasic response discussed earlier.

It is concluded that since both intermediates formed in the presence of Na^+ and K^+ are raised, the inhibition of the enzyme is at the terminal step, i.e. the breakdown of $E_2 \sim Pi$ to free enzyme and Pi is inhibited in ghosts which have been treated with lead. Allowing lead to interact with a ghost preparation gave a significant increase in optical density at 280 nm after about 10 min. This optical density

increase is interpreted as the unfolding of membrane protein chains which expose increasing amounts of of amino acid residues to the aqueous, non-membranous phase. Experiments were carried out in which lead was introduced to the membrane preparation at 0°C for 30 sec before the addition of radioactive ATP. Under these conditions little conformational change should be detectable. It was noted that both intermediates decreased with increasing concentrations of lead. The amount of intermediate formed in the presence of Na^+ and K^+ showed an exponential decrease with increasing lead. This type of reaction is typical of cation inhibition of the Na^+/K^+ ATPase, e.g. Ca^{2+} inhibition (Tobin *et al.*, 1973).

It is apparent that in the presence of free lead (where no conformational change has apparently taken place), the first step in the reaction scheme is the site of inhibition. This is in contradiction to the bound lead effect when conformational change has taken place and excess lead is removed. The possibility exists that the free lead is also interacting with ATP. If this is correct then the exponential decrease can be explained as a decrease in substrate concentration, i.e. ATP.

What does this work suggest in terms of the known membrane structure? Steck, Fairbanks and Wallach (1971) carried out studies of membrane ghosts using various hydrolytic enzymes. They proposed from their studies that components I and II (the high molecular weight components of approx. 250,000) are situated at the cell exterior and exposed to the aqueous phase. Our findings using ^{125}I-labelling, which would specifically label the hydrophylic tyrosine residues, show that for the whole blood cells these components are relatively inert to labelling even after most of the cell membrane has changed its conformation. Using the freeze-thawed ghosts, although the high molecular weight protein is significantly labelled, this appears to be only a background type of labelling without any distinct peak in this region.

The ^{125}I-labelling experiments suggest that the components I and II are probably not accessible to lactoperoxidase catalysed iodination. Alternatively, under the conditions used, lead is unable to induce observable changes in the pattern of labelling. It appears that both components I and II may be localized at some part of the membrane continuum, which is probably not available to the aqueous phase. A probable explanation could also be that components I and II are buried in the membrane, being covered either by phospholipids or other proteins. Since both components I and II (spectrin or tektin A) are easily removed from the membrane by the gentle procedure of exposing the membrane to chelating agents, it is probable that even after

freeze-fracturing the ghosts the membrane is capable of forming vesicles which are impermeable to the high molecular weight lactoperoxidase. If this is correct then all the iodination takes place at one surface only and the pattern of iodination is that of single-sided membrane conformational change(s). If this is not true, then spectrin has either very few groups which are susceptible to iodination, or, as stated previously, it is protected from iodination by other macromolecules.

References

AUB, J.C., FAIRHALL, L.T., MINOT, A.S. & RETZNIKOFF, P. (1926) *Lead Poisoning.* Williams and Wilkins Company, Baltimore.

CLARKSON, T.W. & KENCH, J.E. (1958) Uptake of lead by human erythrocytes *in vitro. Biochemical Journal,* 69, 432.

DODGE, J.T., MITCHELL, C. & HANAHAN, D.J. (1963) The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Archives of Biochemistry and Biophysics,* 100, 119.

FAIRBANKS, G., STECK, T.L. & WALLACH, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry,* 10, 2606.

GARDNER, J.D. & COULON, T.P. (1972) The effect of sodium and potassium on ouabain binding by human erythrocytes. *The Journal of General Physiology,* 60, 609.

HASAN, J., HERNBERG, S., METSALA, P. VIKKO, V. (1967a) Enhanced potassium loss of blood cells from men exposed to lead. *Archives of Environmental Health,* 14, 309.

HASAN, J., VIKKO, V. & HERNBERG, S. (1967b) Deficient red cell membrane Na^+ and K^+ ATPase in lead poisoning. *Archives of Environmental Health,* 14, 313.

HERNBERG, S., VIKKO, V. & HASAN, J. (1967) Red cell ATPases in workers exposed to inorganic lead. *Archives of Environmental Health,* 14, 319.

HOOGEVEEN, J.T. (1970) Thermoconductometric investigation of phosphatidylcholine in aqueous tertiary butanol solution in absence and presence of metal ions. In: *Effects of Metals on Cells, Subcellular Elements and Macromolecules.* Eds. J. Maniloff, J. R. Coleman, M. W. Miller. Thomas, Springfield, Illinois. p. 207.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry,* 193, 265.

MAXWELL, L.C. & BISCHOFF, F. (1929) The reaction of lead with the constituents of the erythrocytes. *Journal of Pharmacology and Experimental Therapeutics,* 37, 413.

PHILLIPS, D.R. & MORRISON, M. (1971) Exposed protein on the intact human erythrocyte. *Biochemistry,* 10, 1766.

POST, R.L., KUME, S., TOBIN, T., ORCUTT, B. & SEN, A.K. (1965) Flexibility of an active centre in sodium plus potassium adenosine triphosphatase. *The Journal of General Physiology,* 54, 306 S.

SECCHI, G.C. & ALESSIO, L. (1969) Ricerche sul meccanismo d'inibizione della Na^+/K^+-ATPasi eritrocitaria ad opera del piombo. *Medicina del Lavoro,* 60, 670.

STECK, T.L., FAIRBANKS, G. & WALLACH, D.F.H. (1971) Disposition of the major peptides in the isolated erythrocyte membrane: proteolytic dissection. *Biochemistry,* 10, 2617.

TOBIN, T., AKERA, T., BASKIN, S.I. & BRODY, T.M. (1973) Calcium ion and sodium- and potassium-dependent adenosine triphosphatase: its mechanism of inhibition and identification of the E_1 P intermediate. *Molecular Pharmacology,* 9, 336.