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Uptake of Lead by Human Erythrocytes *in vitro*

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In the course of extensive studies on the effect of lead on erythrocytes *in vitro*, Aub, Fairhall, Minot & Reznikoff (1926) observed that whereas the cells became more fragile towards mechanical trauma they were more resistant to rupture by hypotonic solutions. Their experiments were made mainly on washed cells suspended in phosphate-free Ringer solutions, since the injurious effects were most manifest in such media.

It was postulated that precipitation of lead triphosphate took place on the erythrocyte membrane by reaction of lead salt with inorganic phosphate groups, with release of hydrochloric acid, and that points of localized acidity were responsible for increased fragility. Lead in whole blood was much less injurious than in phosphate-free Ringer suspensions, the difference being directly related to the inorganic phosphate content of the plasma. Any damage that was observed in cells in whole blood presumably arose by a simultaneous reaction of lead with the phosphate groups of the cell surface, which is largely dominated by such groups (Furchgott & Ponder, 1941). It follows that small quantities of lead added to whole blood should remain in the plasma as colloidal lead phosphate, whereas greater quantities would increase the fraction bound to the cells.

Certain aspects of this hypothesis have been discounted. Bischoff, Maxwell, Evans & Nuzum (1928) observed that lead salts of weak acids, e.g. lead glycerophosphate, produced changes in the erythrocyte similar to those with lead chloride, although no mineral acid was released. Bambach, Kehoe & Logan (1942) and Mortensen & Kellogg (1944) observed a marked uptake of lead by erythrocytes even at low blood levels. The latter workers observed that small quantities of the radioisotope of lead were taken up rapidly by dog erythrocytes both *in vivo* and *in vitro*, from 90 % in 1 min. to a final level of 98 % at the end of 15 min. The process was reversible, and markedly dependent on temperature. At concentrations greater than 1 mg. of Pb^{2+} ions/100 g. of blood, the cells may be saturated and, at the maximum level, may contain only 35 % of the lead present.

The present work was undertaken to obtain more information on the interaction of lead and erythrocytes when brought into mutual contact. Our observations indicate that addition of lead salts to suspensions of erythrocytes in plasma, in isotonic sodium chloride or in Krebs–Ringer bicarbonate solutions results in the immediate formation of peptized lead phosphate sol. Combination with the cell occurs by coagulation or flocculation of the peptized sol on the cell surface.

EXPERIMENTAL

Materials

Blood from healthy young men was collected from the antecubital vein into a 0·9 % NaCl solution containing 1 g. of glucose/100 ml. and anticoagulant [2 mg. of heparin B.P. (Evans Medical Supplies Ltd., Liverpool) or 3 mg. of an ammonium oxalate–potassium oxalate mixture/10 ml. of blood]. The blood was used immediately after collection or stored at 4° and used within 48 hr.

The NaCl solutions used in this work were rendered lead-free by passage through a column of sulphonated polystyrene ion-exchange resin, Zeo-Karb 225 (The Permutit Co. Ltd., London, W. 4), in the sodium cycle. The solutions in the Krebs–Ringer bicarbonate mixture (Umbreit, Burris & Stauffer, 1951) were made lead-free in a similar manner, with the ion-exchange column prepared in the appropriate cycle.

The apparatus was of Pyrex glassware which was cleaned with boiling HNO_3 and thoroughly washed with doubly

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distilled water. Where Pyrex was unobtainable soda glass was coated with silicone (Repelcote: 2% solution of a methylchlorosilane in carbon tetrachloride; Hopkin and Williams Ltd.) and cleaned with HNO_3.

Methods

Determination of lead. The organic constituents in the blood specimen were removed by a wet-oxidation technique. The blood was added to a 100 ml. Erlenmeyer flask containing 1 ml. of H_2SO_4 (sp.gr. 1·84), 2 ml. of HNO_3 (sp.gr. 1·42) and 0·5 ml. of $HClO_4$ (70%, w/v). These reagents were obtained free from lead. The mixture was maintained at 100° until the solid constituents were dispersed and a pale-yellow solution was obtained. The temperature was carefully raised until white fumes appeared. After cooling, 1 ml. of HNO_3 was added, the mixture diluted with approx. 40 ml. of doubly-distilled water and heated until H_2SO_4 alone remained. This procedure was found sufficient to remove all organic substances in quantities of blood up to 5 ml. Unless this method was carefully followed, frothing and 'bumping' of the oxidation mixture occurred, which caused extensive loss of material from the flask.

Analysis for lead was then carried out by the dithizone 'reversion technique' of Irving & Butler (1953). Two precautions, in addition to those stated by the authors, were found necessary. First, it was found that the A.R. CCl_4 solvent contained a substance which destroyed dithizone and which could not be removed by distillation. This interfering substance could be removed, however, by washing the CCl_4 with 1% neutralized hydroxylamine solution. Secondly, as a further and necessary insurance against any destruction of dithizone, 0·5 m. of 20% neutralized hydroxylamine was added to the second ammoniacal-citrate extracting solution.

Precipitation of lead from various solutions. Lead chloride (5 μg. of Pb^{2+} ions) in 0·1 ml. of 0·16 M-NaCl was added to 3 ml. of each of the following solutions maintained at 20° in Pyrex test tubes: plasma; plasma dialysed for 24 hr. against 3 l. of Krebs–Ringer bicarbonate solution; 0·16 M-NaCl; 0·16 M-NaCl exposed for 1 hr. to thrice-washed erythrocytes; Krebs–Ringer bicarbonate solution; Krebs–Ringer bicarbonate solution exposed to thrice-washed erythrocytes for 1 hr.; Krebs–Ringer bicarbonate solution containing arginine, lysine, glutamic acid, histidine, glycine, cystine, glutamine, serine, threonine, glucose, citric acid, α-oxoglutaric acid, lactic acid and urea, in concentrations equivalent to those quoted for plasma (Spector, 1956) and, in addition, 1 mg. of ammonium oxalate–potassium oxalate mixture and approx. 1 mg. of heparin (Evans Medical Supplies Ltd.). After allowing the solutions to stand for 20 min. after addition of lead, they were centrifuged at 4500 *g* in a MSE (Measuring and Scientific Equipment Co., Ltd.) medium centrifuge for 3 min., 2 ml. of the supernatant was removed and this portion and that remaining were analysed for lead. The rate of precipitation of lead added to Krebs–Ringer bicarbonate solution was followed. Lead (5 μg. as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl) was added to a series of tubes containing 3 ml. of Krebs–Ringer bicarbonate solution at 20°. After standing for various times and centrifuging at 4500 *g* for 3 min., 2 ml. of the supernatant was carefully removed in a graduated pipette and lead estimated in the sample and in the residue. Values were corrected to give the lead in 3·1 ml. of solution, which, subtracted from the total amount of lead found, provided a measure of the amount of lead precipitated.

Removal of lead by non-specific adsorbents. Weighed quantities of alumina (γ Al_2O_3 for chromatographic-adsorption analysis, British Drug Houses Ltd., London), fuller's earth (British Drug Houses Ltd., for adsorption purposes) and charcoal (British Drug Houses Ltd., activated decolorizing powder) were washed by repeated suspension in doubly-distilled water in glass-stoppered 10 ml. Pyrex test tubes. Plasma (3 ml.) was added to each, the mixture shaken for 20 min. and the plasma adjusted to pH 7·3 with dilute HCl. Lead (5 μg. as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl) was added, the mixture shaken for 20 min. at 20°, centrifuged and the plasma removed for lead analysis. Next, 3 ml. of plasma was exposed to each of the adsorbents as described and 5 μg. of lead was added after removal of the adsorbents. The plasma was allowed to stand for 20 min. at 20°, then centrifuged and 2 ml. of clear supernatant was removed for lead analysis. The residue was likewise analysed.

Uptake of lead by human erythrocytes in the presence of other metal salts and organic bases. Solutions of various cations and organic bases were prepared in 0·16 M-NaCl containing 0·01 M-barbiturate buffer, pH 6·8. At this pH the uranyl ion [$(UO_2)^{2+}$] is unstable and hence the quantity of free $(UO_2)^{2+}$ ions is very much less than that calculated from the weight of uranium present.

Buffered metal-ion solution (3 ml.) was added to 2 ml. of thrice-washed, packed erythrocytes and the suspension brought to 20° in a Warburg bath. Lead (4 μg.) was added as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl and the system allowed to equilibrate for 15 min. at 20°. After centrifuging at 4500 *g* for 3 min. the supernatant and cells were separated and their lead content was determined.

Uptake of lead by human erythrocytes in suspensions buffered at various pH *values.* Buffered solutions were prepared in the range pH 5–6 with 0·1 M-acetate buffer and in the range pH 6·0–6·6 with 0·02 M-citric acid–sodium phosphate buffer.

Thrice-washed, packed erythrocytes (2 ml.) from fresh heparinized blood were suspended in 3 ml. of each of the buffer solutions contained in 10 ml. glass-stoppered Pyrex test tubes. Below pH 6·4, addition of erythrocytes to the buffer caused a gradual raising of pH. Repeated washing with the buffer solution was necessary before a constant pH was obtained. The tubes were then immersed in a water bath at 20° and allowed to stand for 1 hr. Lead (4 μg.) was added as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl and the tubes were gently agitated in the water bath for a further 20 min. After centrifuging at 4500 *g* for 3 min. the supernatant and centrifugate were separated and their lead content was estimated.

Effect of chelating agents on uptake of lead. The chelating agents chosen for study were the disodium salt of ethylenediaminetetra-acetic acid (EDTA), sodium hexametaphosphate and glutathione, whose active lead-binding groups correspond to carboxylate, phosphate and sulphydryl respectively. A mM-solution of each reagent was prepared in 0·1 M-barbiturate buffer, pH 6·8, and the molarity of the solution adjusted to 0·16 M with NaCl.

In the first experiment, 2 ml. of thrice-washed, packed erythrocytes was suspended in 3 ml. of solution and allowed to stand for 1 hr. at 20°. Lead (4 μg.) as $PbCl_2$ was

then added and the system allowed to equilibrate in a Warburg bath at 20° for 15 min. After centrifuging at 4500 *g* for 3 min. the supernatant and cells were separated and the lead content was measured.

In the second experiment, a saline suspension containing the equivalent of 2 ml. of packed erythrocytes in 2 ml. of 0·16M-NaCl was allowed to stand for 1 hr. at 20°. Lead (4 μg.) was then added, the system allowed to equilibrate for 15 min. and, after centrifuging at 4500 *g* for 3 min., the supernatant was removed by pipette. The centrifuged cells were resuspended in 3 ml. of chelating agent solution and allowed to stand for 15 min. at 20°. The lead content was then estimated in the cells and saline by the usual procedure.

Dialysis studies. Lead (10 μg.) as $PbCl_2$ in 0·2 ml. of 0·16M-NaCl was added separately to two suspensions, each 6 ml., of whole oxalated blood. The suspensions were allowed to equilibrate at 20° for 0·5 hr. or for 18 hr. before dialysis against 1 l. of Krebs–Ringer bicarbonate solution containing mM-EDTA for 24 hr. The dialysis was carried out with Visking cellophan sacs, of diameter 3·8 cm., which had previously been washed free of glycerol and other impurities. After completion of dialysis the contents of the sacs were removed, their volumes measured and lead was estimated in the cells and plasma after separation by centrifuging at 4500 *g* for 3 min.

Kinetic studies of uptake of lead by human erythrocytes. A series of experiments was carried out to obtain information on the kinetics of uptake of lead by erythrocytes when suspended variously in plasma, Krebs–Ringer bicarbonate solution and NaCl solution in the presence of heparin or oxalate as anticoagulant.

Pyrex test tubes (10 ml.) containing samples of fresh heparinized blood (5 ml.) were attached by their ground-glass joints to the holders of a Warburg apparatus and immersed in the water bath maintained at 20±0·05°. After equilibration of temperature, 0·1 ml. of 0·16M-NaCl containing 4 μg. of lead as $PbCl_2$ was added, the contents of the tube were mixed and the time was noted. The tube was allowed to stand in the bath for a measured time before centrifuging at 4500 *g* for 3 min. The uptake of lead by the cells in the blood was presumed to have ceased 30 sec. after the start of the centrifuge. After centrifuging, the supernatant plasma was removed by graduated pipette, the volume noted and the plasma transferred to a 100 ml. Erlenmeyer flask for determination of lead. Similarly, the lead content of the residual cells was determined after removal from the tube with doubly-distilled water.

Kinetic studies were made at 10° and 20° with heparinized whole blood, at 20° with oxalated whole blood and finally at 20° with 3 ml. of oxalated plasma containing the equivalent of 0·3 ml. of packed erythrocytes. The rate of uptake of lead at 37° was very rapid and precluded accurate study at that temperature.

Suspensions of erythrocytes in NaCl solution were prepared. A volume (2 ml.) from well below the top of a column of packed cells, obtained by centrifuging whole heparinized blood, was transferred to a 10 ml. glass-stoppered Pyrex test tube and washed three times by suspension in 0·16M-NaCl. A volume (3 ml.) of 0·16M-NaCl was then added and the tubes were allowed to equilibrate for at least 1 hr. in a Warburg water bath at 20°. This allowed the diffusible substances from the erythrocytes to reach equilibrium concentrations in the saline. Exactly the same procedure was then followed as for whole blood. A similar kinetic run was carried out at 20° for a suspension of 0·5 ml. of packed erythrocytes in 5 ml. of NaCl solution. A kinetic study with identical procedure as for NaCl solution was made on a suspension containing the equivalent of 0·3 ml. of packed erythrocytes in 3 ml. of Krebs–Ringer bicarbonate solution.

RESULTS

The reversion analytical procedure was found to give excellent linearity over a range of amounts of lead from 0 to 7 μg. Recoveries of lead added to whole blood were obtained by analysis of lead in the plasma and erythrocytes separated by centrifuging at 4500 *g* for 3 min. From timed trials it was judged that the erythrocytes were deposited and thereby removed from contact with lead 0·5 min. after the start of the centrifuge. In the range studied, the recoveries of lead (Table 1) were excellent, and demonstrated a marked predilection of lead towards the cells. The standard deviation of the values is 1%, which as applied to a 5 μg. quantity of lead is equivalent to a variation of ± 0·05 μg.

There was a possibility that some of the added lead might have formed a precipitate with inorganic phosphate in the plasma (invisible owing to the minute amounts of lead used) and was carried down with the cells on centrifuging and consequently included in values for uptake of lead by the cells. A similar result could arise with several of the other suspending media, many of

Table 1. *Recoveries of added lead from 5 ml. of whole blood calculated from analyses of plasma and cells after separation by centrifuging*

Lead added (μg.)	Lead found (μg.)			Added lead recovered (μg.)	Recovery (%)
	Plasma	Cells	Total		
0·00	0·08	1·67	1·75	—	—
0·63	0·12	2·27	2·39	0·64	101
1·25	0·14	2·85	2·99	1·24	99
1·87	0·13	3·48	3·61	1·86	100
2·50	0·15	4·11	4·26	2·51	100
3·12	0·14	4·79	4·93	3·18	102
4·38	0·14	6·01	6·15	4·40	100

Table 2. *Deposition of lead from various media*

After the addition of 0·1 ml. of 0·16 M-NaCl containing the equivalent of 5 μg. of lead the system was allowed to stand for 20 min. at 20°. Precipitated lead was removed by centrifuging at 4500 *g* for 3 min.

	Suspension medium					
	Oxalated plasma	0·16 M-NaCl	0·16 M-NaCl exposed to erythrocytes	Krebs–Ringer bicarbonate soln.	Krebs–Ringer bicarbonate soln. after exposure to erythrocytes	Krebs–Ringer bicarbonate soln. containing heparin, oxalate and small organic molecules
pH of medium after addition of $PbCl_2$	7·31	5·50	6·80	7·39	7·39	7·39
Percentage of added lead precipitated	7·3	0·0	10·0	84	20	84

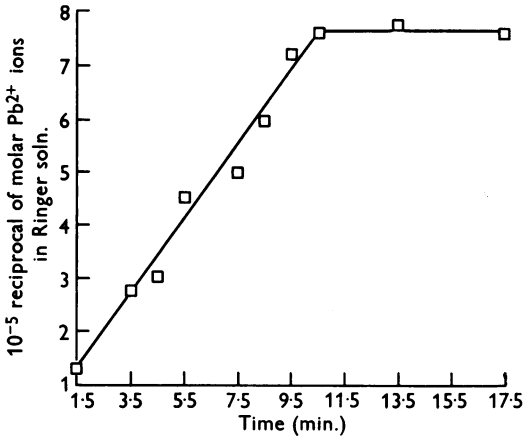

Fig. 1. Precipitation of lead from Krebs–Ringer bicarbonate solution at pH 7·39 and 20°. Results are plotted as a test for second-order kinetics with respect to lead.

Table 3. *Removal of added lead from plasma by non-specific adsorbents or from plasma after exposure to the same adsorbents*

For experimental details see text.

	% of added lead removed by centrifuging	
Adsorbent used	Suspension of adsorbent in plasma	Plasma after exposure to adsorbent
Chromatographic alumina (2 g.)	97	7·1
Activated charcoal (2 g.)	98	6·8
Fuller's earth (2 g.)	96	7·0

which contained inorganic phosphate at pH 6·8–7·4. In Krebs–Ringer bicarbonate solution (Table 2) 84 % of the lead added was precipitated in this manner. The rate of precipitation was measured (Fig. 1) and plotted as a test for second-order kinetics, which apply to the uptake of lead by erythrocytes. A fairly close agreement with second-order kinetics was apparent, but it was difficult to assess the exact value of the time of cessation of the precipitation since this would continue during centrifuging. Deposition of already-formed precipitate was assessed to require 1·5 min., and the precipitate measured after centrifuging for 3 min. was deemed to represent that formed until 1·5 min. after start of the centrifuge.

After exposure of the Krebs–Ringer bicarbonate solution to erythrocytes much less lead was removed, and in plasma, the ionic content of which was similar to that of Krebs–Ringer bicarbonate solution, 7·3 % only of the added lead was precipitated. Lead added was, however, readily removed by non-specific adsorbents (Table 3). Moreover, the plasma after exposure to such adsorbents still retained its property of stabilizing lead, confirming that the non-specific adsorbent was directly responsible for removal of the heavy metal.

No interference with attachment of lead to human erythrocytes in buffered NaCl suspension is brought about by any of a number of other metal salts and organic bases [K^+, Ca^{2+} ions up to 0·16 M; Mg^{2+} ions, acetylcholine chloride, methylene blue, quinine, up to 10 mM; Cu^{2+}, Hg^{2+}, $(UO_2)^{2+}$, Zn^{2+}, Al^{3+}, $(Co[NH_3]_5,H_2O)^{3+}$, Te^{4+} ions up to mM]. With an initial lead concentration (immediately after addition of lead chloride) of 7 μM, all competing species were present in great excess, having molar concentrations of 150–10 000 times that of lead. The uptake of lead was very much inhibited by an increased H^+ ion concentration in the medium (Fig. 2): a pH change from 6·6 to 6·0 had a profound effect.

It is clear that chelating agents such as EDTA and sodium hexametaphosphate largely prevent the passage of lead on to the erythrocytes, but lead already attached can be withdrawn only very

slowly even by powerful chelating agents of the metal (Table 4).

Dialysis studies (Table 5) confirmed that added lead was removable from the cells after contact with lead for 18 hr. Nevertheless, the lead of the cells as obtained from the donor could not be removed by dialysis against EDTA.

The data collected on the kinetics of uptake of lead by erythrocytes are presented in Figs. 3–6. Removal of lead by the cells, as computed from concentration in the suspending media, agreed well with second-order criteria in all the experiments, except when the medium was Krebs–Ringer bicarbonate solution (Fig. 4). The specific velocity constant was very much influenced by the composition of the suspending solution. Thus for whole heparinized blood $k_{20°} = 5{\cdot}61 \times 10^4$ min.$^{-1}$ (Fig. 5), and for a 0·9% NaCl suspension of cells from the same blood specimen $k_{20°} = 29{\cdot}0 \times 10^4$ min.$^{-1}$ (Fig. 3). It is also apparent that the reaction velocity was approximately proportional to the total volume of cells present both in saline and in whole blood. In saline (Fig. 3) the reaction rates are 29×10^4 min.$^{-1}$ and $7{\cdot}8 \times 10^4$ min.$^{-1}$, a ratio of 3·7, corresponding to a cell volume ratio of 4·0. In oxalated blood (Figs. 5 and 6) the specific-reaction rates, $k_{20°}$, are $4{\cdot}5 \times 10^4$ and $0{\cdot}55 \times 10^4$ respectively, giving a ratio 8·2 similar to the cell volume ratio 7·8. This relationship between velocity constant and cell volume did not hold closely when oxalated blood was compared with heparinized blood (Fig. 5), the ratio of the constants being 1·25 as compared with a cell volume ratio of 0·91. The cells were from different specimens of blood, and this may explain the apparent anomaly. The two linear curves in Fig. 5 demonstrate that the velocity of uptake of lead was dependent on temperature:

$$k_{20°}/k_{10°} = 5{\cdot}61 \times 10^4/2{\cdot}16 \times 10^4,$$

giving Q_{10} of 2·6 and an activation energy of 15 800 cal. mole^{-1}.

Erythrocytes (0·3 ml.) in whole oxalated blood treated with 5 μg. of lead at 20° (Fig. 6) reached a saturation level in about 60 min., equivalent to 11·4 μg. of lead/ml. of packed cells, which compares

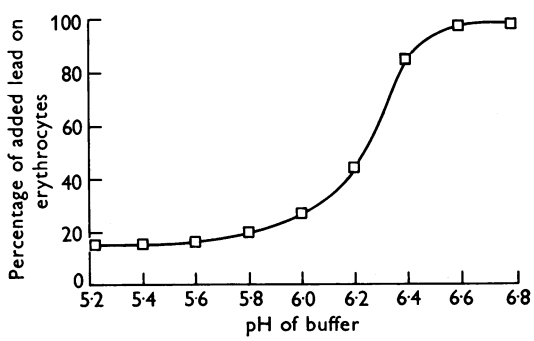

Fig. 2. Uptake of lead by human erythrocytes in buffered-saline suspension, as a function of pH. Packed erythrocytes (2 ml.) were suspended in 3 ml. of saline buffer solution and were allowed to stand for 20 min. after the addition of 5 μg. of lead.

Table 4. *Distribution of* Pb^{2+} *ions between human erythrocytes and chelating agents*

Expt. 1: packed cells (2 ml.) were added to 3 ml. of 0·16 M-NaCl containing the chelating agent, final concentration mM. Pb^{2+} ions (4 μg.) were added and the suspension was allowed to stand at 20° for 15 min. Expt. 2: 4 μg. of Pb^{2+} ions were allowed to equilibrate for 15 min. with 2 ml. of packed cells suspended in 5 ml. of NaCl soln. The leaded cells were separated and suspended for 15 min. in 3 ml. of 0·16 M-NaCl containing the chelating agent, concentration mM.

	Expt. 1		Expt. 2	
Chelating agent	% of added Pb^{2+} ions in cells	% of added Pb^{2+} ions in chelate solution	% of added Pb^{2+} ions in cells	% of added Pb^{2+} ions in chelate solution
Ethylenediaminetetra-acetic acid	9	91	74	26
Sodium hexametaphosphate	8	92	91	9
Glutathione	48	52	95	5

Table 5. *Removal of lead from erythrocytes by dialysis*

Whole oxalated blood (6 ml.) containing the equivalent of 3 ml. of packed erythrocytes was equilibrated at 20° for different times with 8·33 μg. of lead as $PbCl_2$. The blood specimen was contained in a Visking cellophan sac and dialysed for 24 hr. against 1 l. of Krebs–Ringer bicarbonate solution containing mM-ethylenediaminetetra-acetic acid.

Time of equilibration of lead with whole blood (hr.)	Quantity of lead added (μg.)	Lead found before dialysis (μg.)				Lead found after dialysis (μg.)	
		Plasma	Cells	Total	Pb found in excess of quantity added	Plasma	Cells
0·5	8·33	0·72	9·71	10·43	2·10	0·45	2·26
18·0	8·33	0·38	10·22	10·60	2·27	0·00	2·21

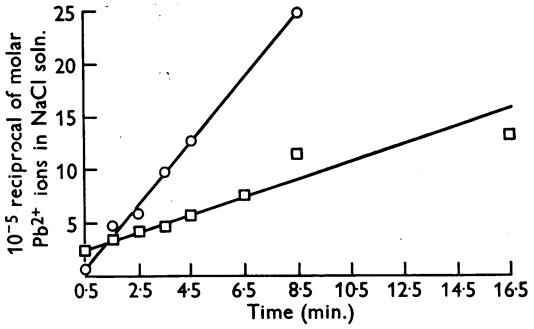

Fig. 3. Uptake of lead by human erythrocytes in 5 ml. of NaCl suspension at 20° containing the equivalent of 0·5 (□) or 2 (○) ml. of packed erythrocytes.

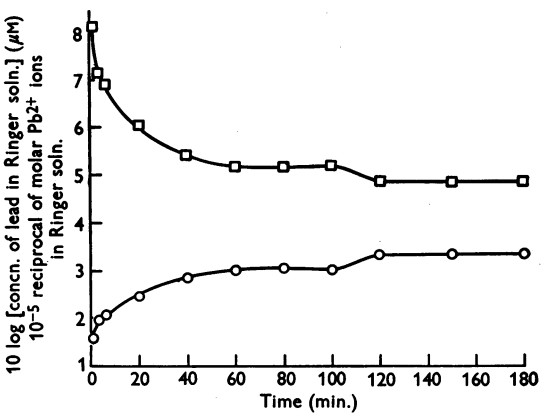

Fig. 4. Uptake of lead by human erythrocytes, equivalent to 0·3 ml. of packed cells suspended in 3 ml. of Krebs–Ringer bicarbonate solution at 20°. Results are plotted for 10 log (Pb^{2+} ions in Ringer) μM (□) and for 10^{-5} ($1/Pb^{2+}$ ions in Ringer) (M^{-1}) (○).

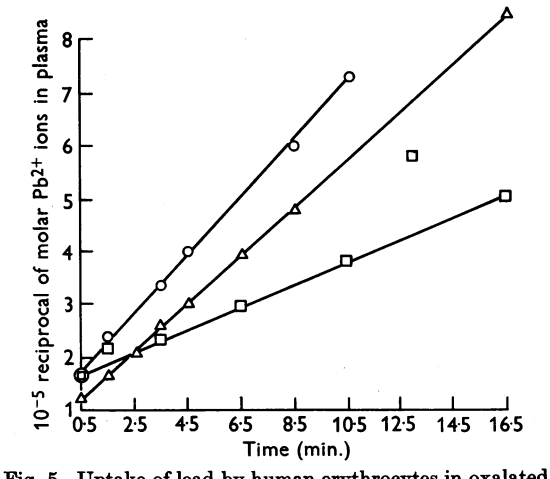

Fig. 5. Uptake of lead by human erythrocytes in oxalated whole blood at 20° (△) and in whole heparinized blood at 10° (□) and 20° (○). Results are plotted as a test for second-order kinetics with respect to lead.

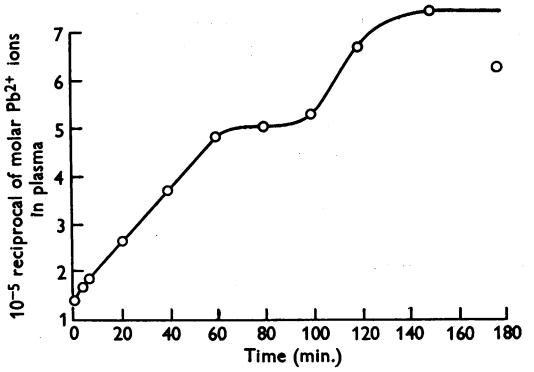

Fig. 6. Uptake of lead by human erythrocytes in whole oxalated blood at 20°, containing the equivalent of 0·3 ml. of packed erythrocytes in 3 ml. of plasma. Results are plotted for 10^{-5} (reciprocal of the molar concentration of lead in plasma).

well with the value quoted by Bambach *et al.* (1942) of 0·55 mg. of lead/100 g. of blood. After approx. 90 min. a further smaller uptake of lead occurred. A similar situation arose when 0·3 ml. of cells was suspended in Krebs–Ringer bicarbonate solution (Fig. 4). Also the two curves exhibit saturation phenomena after about 60 min. With the Krebs–Ringer bicarbonate suspension, however, the experimental values do not obey second-order criteria and much less lead is taken up by the cells.

DISCUSSION

In agreement with the findings of Bambach *et al.* (1942) and Mortensen & Kellogg (1944) the present investigations have shown that when 5 μg. of lead as lead chloride is added to 5 ml. of whole blood, over 95% is rapidly attached to the cells. It is difficult to see how such a distribution of lead could occur if the metal was taking part in the reaction as the bivalent cation Pb^{2+}, since plasma components such as glycerophosphate, phospholipids and proteins contain groups such as phosphate, sulphydryl and carboxyl, all of which can bind ionic lead and thereby prevent its uptake by the cell. Likewise if attachment to the cell involved reaction of Pb^{2+} ions with such groups on the cell membrane, competitive inhibition of uptake of lead would be expected from cations of similar properties. Yet Table 4 demonstrates that none of a wide variety of potential competitors tested showed a significant effect. Clearly lead must be operative in some other form or combination, as a metal complex or chelate with organic plasma components or as a dispersed colloidal metal salt.

Aub *et al.* (1926) emphasized the importance of inorganic phosphate for the stability of lead salts in whole blood and saline suspensions. The work of Millet & Jowett (1929) and Maxwell & Bischoff

(1929) demonstrated that under the conditions of ionic strength and pH of plasma, lead triphosphate would tend to be precipitated when the concentration of lead was raised to levels above approximately 0·1 μM. Lead present in plasma after equilibration with cells is of a concentration of about the same order, namely 0·5 μM. Goldberg, Ashenbrucker, Cartwright & Wintrobe (1956) have observed that the inhibition of haem synthesis *in vitro* by lead in this range of concentration was less in phosphate buffer than in 2-amino-2-hydroxymethylpropane-1:3-diol buffer. Table 2 records the quantity of lead precipitated when amounts of lead equivalent to a concentration of about 5 μM are added to various solutions. Almost complete precipitation of lead, presumably as the triphosphate salt, occurred in Krebs–Ringer bicarbonate solution, which has an inorganic phosphate concentration identical with the value quoted for serum (Power, 1953). Yet in plasma, 0·16 M-sodium chloride at pH 6·8 or Krebs–Ringer bicarbonate solution exposed to erythrocytes for 1 hr., very little precipitation of lead was observed. It seems probable that the addition of lead salts to these solutions will result first in the formation of lead triphosphate. Normally this would coagulate or flocculate until precipitation occurred. But the second stage is prevented by a 'peptization' process whereby organic substances in the plasma, or originating from the cell (Lovelock, 1954), are adsorbed on the surface of the particles of the lead phosphate sol and prevent further coagulation, a common phenomenon when insoluble metal-salt sols are formed in the presence of such substances as agar, gelatin or citric acid (Packter & Matalon, 1954). Such metal-salt sols, other than at their equivalence point, carry electrical charges and may be expected to be removed from the suspending media by non-specific adsorbents. Such was the case for lead in plasma (Table 3). The removal of lead from plasma by cells might be a similar adsorption mechanism.

The importance of the sol form of lead phosphate was demonstrated by the effect of pH on the cell interaction (Fig. 2). As the pH of the supporting medium fell from 6·6 to 6·0, uptake of lead by the cells was markedly reduced. It is in this range that the highly insoluble lead triphosphate is transformed into the more soluble lead monohydrogen phosphate, whereby the sol nature of the lead phosphate would be destroyed. The charge on the lead triphosphate sol is determined by the ratio of Pb^{2+} ions to PO_4^{3-} ions in the medium. In the presence of excess of phosphate, as in plasma and Krebs–Ringer bicarbonate solution, the sol would bear a negative charge. In saline suspensions of thrice-washed erythrocytes, the concentration of free phosphate would be very low so that the net charge on the lead sol could well be reversed. This would account for the more rapid reaction with the negatively charged erythrocyte in saline as compared with Krebs–Ringer bicarbonate solution or plasma (Figs. 3–5). These results imply that the protective action of plasma and of other solutions containing excess of phosphate is due, not to competition for available Pb^{2+} ions, as proposed by Aub and his colleagues, but to the fact that the approaching lead phosphate sol carries a negative charge in such a medium. Changes in sedimentation rate and fragility of the cells have been discussed elsewhere in terms of this concept (Clarkson & Kench, 1958).

The kinetic studies of uptake of lead by human erythrocytes (Figs. 3–6) emphasize, however, that the process is not a simple adsorption of the phosphate sol on the cell surface. The reaction is markedly influenced by temperature, having a Q_{10} of 2·8 and an activation energy of 15 800 cal. mole^{-1}. This is typical of a chemical reaction rather than an adsorptive process. Mortensen & Kellogg (1944) first noted the influence of temperature on uptake of lead by erythrocytes and arrived at a similar conclusion. All the kinetic data closely obey second-order reactive criteria for the lead taking part, which again discounts a simple adsorption process. The values obtained by Mortensen & Kellogg for uptake of lead by dog erythrocytes and plotted for first-order kinetics do not obey linearity at 20°. If these values are plotted for second-order requirements, excellent linearity is obtained. An examination of the curve in Fig. 6, for cells present in only small quantity, reveals that the cells do not enter directly into the reaction with lead. Even at points very close to the cessation of uptake of lead, second-order characteristics are still obeyed. Such could arise only from the collision of two lead atoms with the cell surface always in excess and not a determinant of the observed kinetics. Nevertheless, the cells markedly affect the reaction rate and do so in a regular fashion, as outlined in the Results section. It seems likely that such second-order kinetics refer to coagulation of the peptized lead sol (Packter & Matalon, 1954), with the cell surface acting as a catalyst to the reaction; in other words, uptake of lead occurs by the combination of the peptized lead phosphate sol particles on the surface of the cell to form a precipitate or larger particulate form of lead phosphate. The cell surface, being largely dominated by phosphate groups, would provide an ideal surface for such an aggregation. Once the coagulation of lead phosphate sol on the cell surface has been completed, the lead salt will have properties similar to those of the usual precipitate, namely that the reverse reaction of dispersion of the coagulate is slow. This is borne out in Table 4. Although chelating agents such as EDTA, hexametaphosphate and glutathione will bind ionic lead quite strongly and

effectively prevent uptake by the cell, they remove the metal salt only very slowly once it is attached to the cell. Lead phosphate added to cells could, however, be removed by prolonged dialysis against Krebs–Ringer bicarbonate solution containing mM-EDTA (Table 5). Since Foreman & Trujillo (1954) have shown that EDTA cannot enter the erythrocyte, it is clear that the lead removed by the chelating agent must be on the surface of the cell and remain there for at least 24 hr. after the initial uptake. Lead originally present in the cells when the blood specimen was collected could not be removed by this treatment and was presumably located intracellularly. Such intracellular lead, responsible during maturation of the erythrocyte for inhibition of several stages in biosynthesis of haem, is probably in a form other than phosphate, since a peptized phosphate sol would hardly provide sufficient Pb^{2+} ions for inactivation of enzyme.

SUMMARY

1. A study is described of the uptake of lead *in vitro* by human erythrocytes, including experiments on the form of lead in plasma and the kinetics of lead-binding.

2. Lead appears to exist in plasma as a peptized lead phosphate sol, which is rapidly aggregated in a second-order reaction to particulate form on the surface of the erythrocytes. More than 95% of small quantities of added lead are rapidly attached to the cells.

3. No competitive inhibition of uptake of lead was observed with any of a number of potential competitors tested, including other heavy metals, Cu^{2+}, Hg^{2+}, $(UO_2)^{2+}$ and Tl^{4+} ions.

4. Chelating agents such as ethylenediaminetetraacetic acid, hexametaphosphate and glutathione remove lead attached to erythrocytes only slowly *in vitro*; intracellular lead was not withdrawn.

5. It is suggested that the protective effect of plasma and of other solutions containing excess of phosphate is due to a charge effect on the lead phosphate sol, leading to additional negative charge on the erythrocyte.

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Manometric Studies of Bracken [*Pteridium aquilinum* (L.) Kuhn] Thiaminase

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Bracken thiaminase catalyses the fission of the methylene–quaternary nitrogen bond of thiamine with transfer of the pyrimidine moiety to an amine acceptor (see, for example, Fujita, Okamoto & Nose, 1955; Kenten, 1957) according to the general equation

$$P\text{–}CH_2\text{–}T^+ + R\text{–}NH_2 \rightarrow P\text{–}CH_2\text{–}NHR + T + H^+, \quad (1)$$

where P and T stand for the pyrimidine and thiazole components of thiamine. Therefore it should be possible to study the transfer reaction of thiaminase manometrically in bicarbonate–carbon dioxide buffer by measuring the carbon dioxide output which would accompany the release of the H^+ ion. In the present work the suitability of the manometric method for studying the action of bracken thiaminase has been examined.