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Distribution of lead-203 in human peripheral blood in vitro

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ABSTRACT In-vitro experiments using ^{203}Pb were performed to identify the lead binding components in human peripheral blood. The distribution of lead in plasma, in the red cell membrane, and within the red cell was also investigated. Studies of the distribution of ^{203}Pb in whole blood showed that at a lead concentration of 2·45 μmol/l (50 μg/100 ml) about 94% of lead had been incorporated by the erythrocytes and 6% remained in the plasma. After extraction of lipid by a methanol/chloroform mixture, about 75% of the lead was found to be associated with the protein fraction. The lipid contained about 21% of the ^{203}Pb, the remainder being in the aqueous plasma. SDS polyacrylamide gel electrophoresis of blood plasma showed that almost 90% of the ^{203}Pb was present in the albumin fraction; the remainder was likely to be associated with high molecular weight globulins. Several binding sites were identified on the erythrocyte membrane. The high molecular weight component, about 130 000-230 000, was the most important ^{203}Pb binding site. Chemical modification of membrane proteins suggested that the carboxyl groups are the major ligand responsible for most of the lead binding. SH groups of the membrane may have a minor role, but amino groups did not appear to affect the lead binding. The binding of lead to erythrocytes was not confined to membranes, over 80% of lead in blood penetrates into erythrocytes and binds to intracellular components. Gel chromatography of the haemolysate showed that over 90% of the ^{203}Pb was attached to the haemoglobin molecule.

The mechanism by which lead transfers from the external to the internal environment is not fully understood, but it is generally agreed that this metal is transported by the peripheral blood.[1 2]

Numerous studies on the uptake and binding of lead with peripheral blood have not succeeded in showing clearly either the mechanism of interaction or the binding site. Mortensen and Kellog[3] suggested that lead is bound to the red blood cell or to the plasma, and only a small amount is in a free ionised form. Clarkson and Kench[4] showed that lead has a high affinity for the erythrocytes.

The precise lead binding site in the red cell, however, is still unknown. On the one hand, several investigators consider that the most likely sites of fixation are in the cell membrane.[5] Reddi[6] proposed that there may be two components in the red cell responsible for the lead uptake; one is protein and the other a non-protein, probably a phenolic compound. This hypothesis has been discounted by Clarkson and Kench,[4] who suggested that lead exists in the blood as a peptised lead phosphate-sol, the groups of which are probably aggregated on the surface of the cell to form a larger particulate. On the other hand, recent studies by Barltrop and Smith[7 8] using tracer ^{203}Pb do not confirm that lead has an affinity to the red blood cell membrane. These authors separated the ^{203}Pb-containing fraction of RBC by sephadex gel filtration and ultracentrifugation. Lead was found attached to the intracellular constituents rather than the stromal membrane, and to a molecule similar in size to the haemoglobin polypeptide. White,[9] however, and Selhi and White[10] believe that the attachment takes place in the erythrocyte membrane, which results in alteration of membrane protein conformation.

The identification of specific lead binding sites in the blood is hindered by experimental difficulties such as the necessity to detect and to measure trace amounts of lead in microsamples of subcellular

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components and complex separation procedures. It was hoped, by using the tracer ^{203}Pb to (*a*) study the distribution of lead in peripheral blood, and (*b*) identify the lead binding site(s) in various blood constituents.

Materials and methods

CHEMICALS AND RADIOCHEMICALS

Tris-(hydroxylmethyl)-aminomethane was obtained from Hopkins and William (Searle) Co. Molecular weight markers for PAGE and gel filtration calibration were purchased from BDH Ltd; acrylamide and NN′—methylene-bis-acrylamide from Bio-Rad Lab. Sephadex G-75 was purchased from Pharmacia (UK) Ltd; TEMED, DTT, and ammonium-persulphate were reagent grade from Sigma (London). Protein standard, bovine serum albumin was obtained from BDH Ltd.

Commercially available enzymes and chemicals to identify the lead binding groups were obtained from Sigma (London).

^{203}Pb was prepared in a cyclotron (MRC unit, London) by bombardment of a thallium target and was carrier free.

Blood was obtained from volunteers by venepuncture, with heparin as anticoagulant.

DETERMINATION OF RADIOACTIVITY AND OF LEAD IN BLOOD

Determinations of radioactivity and of lead in blood were carried out by the methods described by Ong and Lee in the companion paper.[11]

IDENTIFICATION OF LEAD IN PLASMA

The approach to plasma protein electrophoresis was based on that described by Tombs and Akroyd.[12] Electrophoresis was carried out with a current of 5 mA along each tube and at a voltage gradient of about 7 V/cm until the marking dye reached a preset mark about 0·5 cm from the end of the gel.

The gel was stained overnight with 0·25% Coomassie blue in methanol, acetic acid, and water (50:10:40 by volume). Destaining was carried out with methanol, acetic acid, and water as above for 48 hours, with three changes of wash solution. After destaining, the gels were scanned with a Beckman model 26 double beam UV spectrophotometer with access to a transverse scanner.

PREPARATION OF ERYTHROCYTE MEMBRANES

Erythrocyte membranes were prepared by the methods described in the companion paper.[11]

ENZYMIC MODIFICATION OF ERYTHROCYTE MEMBRANE

Membranes containing ^{203}Pb were incubated with trypsin, phospholipase-C, and neuraminidase in 10 mM Tris-HCl buffer (pH 7·0) according to the methods of Duffy and Schwarz.[13]

The mixture contained 0·1 mg of enzyme protein per mg of membrane protein in a total volume of 1 ml. The reaction was terminated by the addition of 5 ml of ice-cold histidine imidazole buffer (pH 7·0), and the membranes were centrifuged at 6000 g for 20 minutes at 4°C. The membranes were then washed twice more with this buffer, and the radioactivity was measured by gammaspectrometry. The soluble fraction was subjected to overnight dialysis in a high salt content, and the radioactivity of free lead was determined.

CHEMICAL MODIFICATION OF ERYTHROCYTE MEMBRANES

Chemical reagents were used to identify the principal lead binding molecule(s) in the membrane by the methods described by Ong and Lee.[11]

MOLECULAR GROUP RESPONSIBLE FOR LEAD BINDING IN THE ERYTHROCYTE MEMBRANE

The proteins of the human erythrocyte membrane were separated by polyacrylamide gel electrophoresis as described by Fairbanks *et al*[14] in 1% sodium dodecyl sulphate (SDS).

Solubilisation of red cell membrane before electrophoresis was achieved by adding 0·5 ml of a solution containing 3% SDS, 0·1% mercaptoethanol, 0·1 M Tris-HCl at pH 7·6 to 0·5 ml of the membrane suspension, and the solution was incubated at 37°C for 30 minutes. Suspensions containing 50-70 μg of the membrane proteins with 2% SDS, 6% sucrose, and Bromophenol blue were applied for electrophoresis. They were introduced by gravity flow through a 20 μl Dummond microcap and discharged gently on to the top of the gel.

Electrophoresis was performed with the current at 8 mA along each tube. The running time was about 90 minutes. The electrophoresis process was carried out in an apparatus that could run six columns simultaneously. The position of the tracking dye was marked in each gel by pricking it with a fine syringe needle dipped with Indian ink. Duplicate gels, unstained, were sliced and applied for radioactivity screening.

Commercially available proteins of known molecular weight were used as markers for molecular weight calibration of the gel. The protein standards used were as follows: bovine serum albumin, human gammaglobulin, ovalbumin, IgG, cytochrome-C, and pepsin.

EXTRACTION OF PROTEIN AND LIPID

Lipids and proteins were extracted from whole blood, plasma, and erythrocyte according to the method described by Ong and Lee.[11]

IDENTIFICATION OF HAEMOGLOBIN ON G-75

Samples of haemolysate were separated by gel filtration chromatography on Sephadex G-75. The basic procedure was similar to that of Takeda *et al.*[15]

After incubation with 10 μCi of ^{203}Pb, the erythrocytes were washed twice with saline buffer and lysed with six volumes of double distilled water. The stroma was removed by centrifugation at 30 000 g, and the supernatant was then converted to cyanhaemoglobin by adding 50 μmol of sodium cyanide. The lysate was then dialysed against 500 volumes of 0·1 M Tris-HCl buffer, pH 8·1, at 5°C, containing 50 μmol of CN^-, with two changes overnight. The Sephadex column (40 × 18 cm), which had been calibrated with globulin, albumin, and pepsin, was equilibrated with the same buffer overnight. Thirty mg of the lysate were layered on to the column and eluted with 0·1 M Tris-HCl buffer.

The haemoglobin fraction was scanned at 495 or 539 nm or both. Fractions absorbing at 280 nm for protein were also recorded. All fractions were determined for radioactivity.

Results

DISTRIBUTION OF ^{203}Pb IN BLOOD

The percentage distribution of ^{203}Pb in plasma, haemolysate, and stromal membrane is shown in fig 1. A high proportion of lead (80%) had penetrated the erythrocyte membrane to bind with the non-stromal fraction, whereas only a small amount (14%) of the ^{203}Pb was attached to the membrane fraction. Blood plasma contained an even smaller amount of the ^{203}Pb (about 6%).

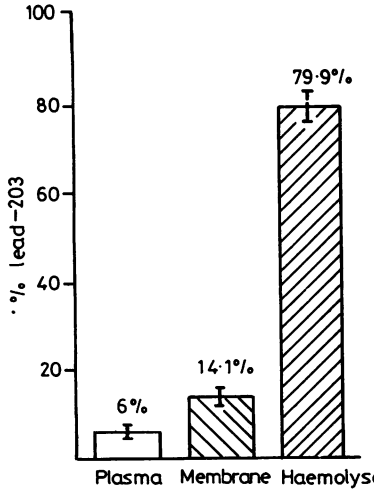

Fig 1 *Percentage distribution of ^{203}Pb distribution in peripheral blood.*

Table 1 *Distribution of bound ^{203}Pb in whole blood*

Fraction	*μmol/l ± SEM**	*% of lead in the fraction**
Incubated with 2·45 μmol/l of $^{203}PbCl_2$		
Protein	1·63 ± 0·07	74·4 ± 4·15
Lipid	0·48 ± 0·01	21·9 ± 1·09
Aqueous	0·08 ± 0·01	3·7 ± 0·04
Incubated with 4·85 μmol/l of $^{203}PbCl_2$		
Protein	3·46 ± 0·12	72·8 ± 3·25
Lipid	1·12 ± 0·02	23·6 ± 1·20
Aqueous	0·17 ± 0·01	3·6 ± 0·05

*Means of three experiments each in duplicate.

DISTRIBUTION OF ^{203}Pb IN WHOLE BLOOD AFTER EXTRACTION OF LIPID

Table 1 shows the results of ^{203}Pb distribution at molecular level. The protein fraction contained most (74%) of the total lead present. About 22% of the ^{203}Pb was found in the organic (lipid-containing) phase and about 3% was found unassociated, probably in free ionic form.

The experiment was repeated with 4·85 μmol/l of ^{203}Pb in the incorporating phase. The results (table 1) indicate that an increase of the lead concentration in the incorporating phase did not appear to alter the binding percentage significantly.

MOLECULAR GROUPS RESPONSIBLE FOR ^{203}Pb BINDING IN THE PLASMA

A preliminary investigation was undertaken to detect the primary distribution of lead in the plasma using the methods of Turner and Hulme.[16] The plasma was arbitrarily classified into albumin and the globulins. The results of three experiments, each in duplicate on the ^{203}Pb distribution in those two protein subgroups, are summarised in table 2. A large proportion (88·2%) of the labelled material was in the albumin.

The finding that only 12% of ^{203}Pb was attached to the globulins is interesting, as it is generally assumed

Table 2 *Distribution of lead in the plasma*

Fraction	*nmol/g of protein (Mean ± SEM)*	*% of lead in the fraction*
Albumin	157·42 ± 25·2	88·2
Globulin	20·38 ± 1·4	11·8

Recovery of radioactivity was 87% and 82%, respectively.

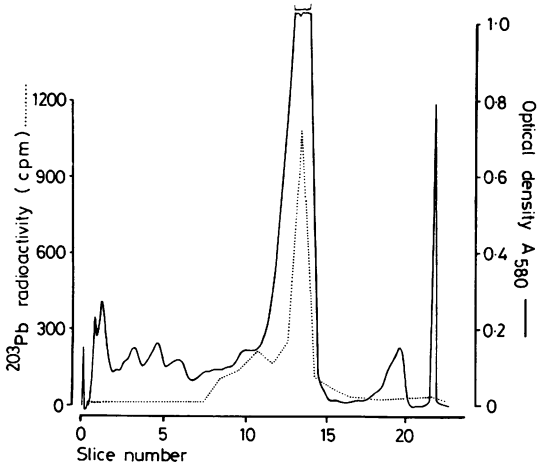

Fig 2 *Densitometric scan of plasma polypeptide after electrophoresis.*

that lead does not appear to be associated with these large protein molecules, although a decreased antibody formation in experimental animals exposed to lead has been reported.[17]

Despite the simplicity of techniques, polyacrylamide gel electrophoresis offered the possibility of separating the closely related constituents of plasma into discrete protein molecules. Figure 2 is a densitometric scan of the Coomassie-blue stained plasma polypeptides on a gel after electrophoresis in SDS. Several distinct components are observed. The main component is the albumin of molecular weight, about 68 000, which constitutes about 70% of the total plasma protein.[18] Various globulin polypeptides known to have higher molecular weight (ranging from 80 000 to 160 000) appear before the albumin fraction.

Accompanying the electrophoretogram of plasma protein is the ^{203}Pb radioactivity spectrum of the gel. Apparently lead is concentrated in the albumin fraction. No lead was found in the heavy molecular weight globulins. A trace amount of ^{203}Pb, however, was detected just before the albumin peak. This ^{203}Pb association with gammaglobulin molecule is not surprising, as table 2 has shown that a small amount of lead was attached to the globulin fraction. Interestingly, Cember *et al*,[19] who investigated the distribution of mercury in human plasma, found that a trace amount of mercury was first attached to the gammaglobulin and gradually transferred into albumin, which became the prominent binding fraction.

A confirmation experiment was performed on freshly prepared plasma. After ensuring that the plasma was haemoglobin free by scanning at 495 nm, 5 μCi of $^{203}PbCl_2$ was introduced. Electrophoresis was carried out in an identical manner. The results obtained were reproducible and essentially similar to that shown in fig 2.

Table 3 *Effect of enzymes on the binding of ^{203}Pb*

Enzymes	*nmol of Pb bound per mg of membrane protein Mean ± SEM*		*Decrease in bound ^{203}Pb (% untreated)*
	Control	*Treated*	
Trypsin	178·3 ± 17·6	92·2 ± 10·7	48·3 ± 4·7
Phospholipase −C	163·5 ± 18·2	138·3 ± 11·9	15·4 ± 2·8
Neuraminidase	152·3 ± 16·4	149·5 ± 15·5	1·8 ± 0·2

Mean radioactivity recovery was approximately 81%.

EFFECTS OF ENZYMES ON ^{203}Pb BINDING TO RBC MEMBRANE

Table 3 shows the effects of commercially available enzymes on ^{203}Pb binding in the erythrocyte membrane. All results are of three experiments, each in duplicate. Trypsin, which hydrolysed about 45% of the membrane protein,[20] displaced about 50% of the bound ^{203}Pb. Phospholipase-C, which removed 69% of the phospholipid from the membrane, released only about 15% of the bound ^{203}Pb. Incubation of the membrane with neuraminidase for one hour at 60°C, although cleaving 84% of the sialic acid residues from the membrane, had no significant effect on the ^{203}Pb release.

Subsequent analysis of the supernatant by dialysis showed that most of the ^{203}Pb in the soluble fraction was dialysable, suggesting that it did not have an affinity for the enzymes. The radioactivity recovered was 84%, 78%, and 79% for trypsin, phospholipase-C, and neuraminidase, respectively.

Although the enzymes were not very specific in their action on the membrane, trypsin also released 23% of the phospholipid and 61% of the sialic acid during hydrolysis, and phospholipase-C released about 16% of the membrane protein. These results are compatible with the suggestion that most of the firmly bound ^{203}Pb is associated with membrane protein and to a lesser extent with phospholipid; sialic acid may play only a minor part in the binding of ^{203}Pb.

STUDY OF GROUPS ASSOCIATED WITH LEAD BINDING

Ong and Lee[11] present evidence that carboxyl groups are responsible for the binding of about 68% of ^{203}Pb in the erythrocyte membrane and the thiol (SH) groups for about 18%. The amino groups did not appear to bind a significant amount of ^{203}Pb.

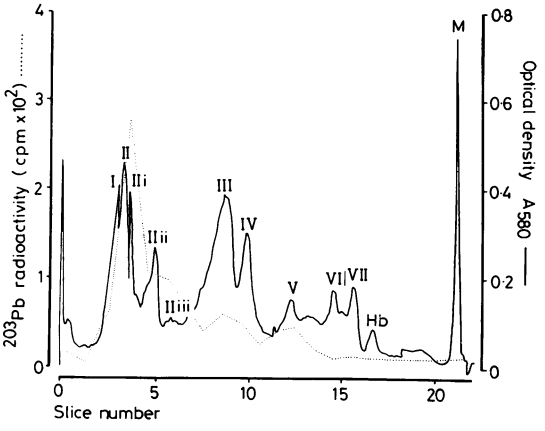

Fig 3 *Distribution of ^{203}Pb in the erythrocyte membrane.*

DISTRIBUTION OF ^{203}Pb IN THE ERYTHROCYTE MEMBRANE

The erythrocyte membrane has been shown to carry about 14% of blood lead (fig 1). Attempts were made to define more closely the principal binding groups in the stromal membrane by electrophoresis.

The erythrocyte membranes were prepared as described in the methods section. The membrane (ghosts) pellet was then washed three times to remove the cations loosely associated with the membrane, leaving those ^{203}Pb ions that were firmly bound. The labelled stromal membranes were then dissolved in an excess of SDS, and the polypeptides were denatured and separated in polyacrylamide gel according to the methods of Fairbanks *et al.*[14]

Figure 3 shows the densitometric scan of Coomassie-blue stained ghosts polypeptide after electrophoresis in SDS. The protein profile consists of several well-resolved bands and is arbitrarily labelled I-VII according to Steck.[21] The configuration of these protein groups is molecular weight dependent and is in descending order from left to right.

The ^{203}Pb distribution shows two notable features:

(1) The prominent radioactivity peak was apparently associated with high molecular weight polypeptide II and its subordinate components. These polypeptides are known to have a molecular weight of about 130 000 to 230 000.

(2) Trace amounts of radioactive lead were recovered in polypeptides III and IV. There was also some in polypeptide V.

ASSOCIATION OF LEAD WITH INTRACELLULAR CONSTITUENT

Figure 4 is the chromatogram of haemolysate separated by G-75 and shows (*a*) the elution characteristics of the protein band with the calibrated graph suggest a mean molecular weight of 67 000, which is virtually that of haemoglobin polypeptide, and (*b*) the radioactive peak together with the protein and haemoglobin all appeared on the same fractions (fraction 68 to 73).

The radioactive labelled material lost in the chromatography column is only a small amount (8%) in comparison to the binding in the protein fraction; it is reasonable therefore to conclude that haemoglobin is the principal molecule interacting with the ^{203}Pb.

A similar result was obtained with haemolysate, which had been in direct contact with ^{203}Pb in vitro for 60 minutes, suggesting that the organised red cell plays little part in determining binding site and that the initial binding is probably of a chemical or physical type rather than through a complex of biological sequences.

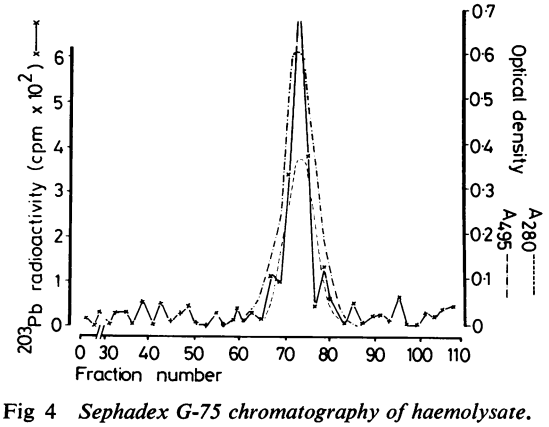

Fig 4 *Sephadex G-75 chromatography of haemolysate.*

Discussion

This study was designed to investigate the distribution of lead in human peripheral blood, and to identify possible lead binding sites. Peripheral blood is the first tissue after absorption to receive lead absorbed either from the gastrointestinal tract or respiratory system en route to other organs.[22] The actual kinetics of lead distribution are complicated, but it is generally presumed to be transported by the blood.[1 2]

The high proportion of ^{203}Pb found in the erythrocytes confirms the in-vitro findings reported by Clarkson and Kench[4] and the limited uptake of lead by the plasma is in accordance with the in-vivo observations of Baloh.[23]

Very little is known about lead in plasma. It has been suggested that it comprises two fractions, a diffusible metabolically active fraction and a complex protein bound fraction.[24] Gurd and Murray,[25] however, showed that virtually all the lead in the plasma was associated with the protein fraction. The present study using electrophoresis suggests that ^{203}Pb is bound to plasma albumin and only a trace fraction is associated with high molecular weight globulins. There is no evidence from the present observations whether the minor fraction associated with the globulins is an active fraction, and that with albumin is a stable protein complex.

The distribution of ^{203}Pb in erythrocyte membranes (fig 3) suggested that the binding of this metal at the membrane is heterogeneous and included many groups. According to Steck,[21] polypeptide II is a large molecule and is adherent to the inner surface of the red cell membrane. This polypeptide is known to have a molecular size of about 130 000 to 230 000.

Although the bindings of ^{203}Pb to polypeptides III and IV are less than that to polypeptide II, various sources available have suggested that the polypeptide band III might be concerned in the interaction with lead. This polypeptide has a molecular weight of about 82 000 to 85 000 and represents about 20% of the total membrane protein[26] and is known to have a high content of protein sulphydryl (SH) groups.[27] Therefore the binding of ^{203}Pb to this band is not surprising, as lead has been shown to react with sulphydryl groups. Furthermore, it has been suggested that this polypeptide spans and penetrates the membrane, and thus may participate in ion transportation.[21] Recently, this polypeptide was reported to contain a phosphorylated intermediate of Na^+/K^+—ATPase.[28] If this is the case it may in part explain the mechanism by which lead inhibits Na^+/K^+—stimulated adenosine triphosphatase.[29 30]

In the present investigation membrane proteins were separated by polyacrylamide gel electrophoresis, and the results suggested that ^{203}Pb is attached to high molecular weight polypeptides (MWt 130 000-230 000). This finding confirms our earlier observation, when erythrocyte membrane proteins were separated by gel chromatography.[11]

Use of group specific reagents suggested that the binding of ^{203}Pb in red blood cell membrane is heterogeneous and that the binding sites are multiple. Carboxyl groups have been shown[11] to be those that interact most with the lead. This observation may be compared with that of Duffy and Schwarz,[13] who showed that carboxyl groups are concerned in the formation of the intermediate of Na^+/K^+—ATPase. If lead combines with the carboxyl group at the active site of Na^+/K^+—ATPase the phosphorylation activity of the enzymes would be prevented, as noted above. Other groups may also be concerned in ^{203}Pb binding; inhibition of the membrane SH groups with PCMB has suggested that sulphydryl groups may have a minor role.

Examination of blood components at the molecular level indicated that lead has a strong affinity for plasma and erythrocyte protein (table 2). Only an insignificant amount of ^{203}Pb was recovered from the lipid fraction and less than 6% was in free ionic form. These results confirmed our other findings[11] and are markedly consistent with in-vivo results reported by Beattie *et al.*[31]

The distribution of ^{203}Pb in erythrocytes helps to explain the long controversy of lead binding at the stromal membrane[4 10] and at the cytoplasm.[7 8] These groups of investigators did not apparently differentiate these two constituents. The present investigation shows that erythrocyte haemolysate has a strong affinity for ^{203}Pb. This confirms the result of Bruenger *et al*,[32] who showed that ^{210}Pb interacted with an intracellular constituent and only a small amount is attached to the membrane of red blood cells.

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