Erythrocyte Osmotic Fragility in the Presence of Lead or Mercury[1] (37064)

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It has been known for many years that heavy metals effect red cell fragility in a complex manner. A number of investigators have reported that erythrocytes from lead poisoned individuals have an increased mechanical, but decreased osmotic fragility (1, 2). The precise mechanism of this unusual finding still remains to be explained (3). There seems to be general agreement that the chronic anemia associated with mercury poisoning is due to the direct effect of mercury on the red cell (4, 5). The anemia associated with lead poisoning, however, is variable and may range from mild to severe, or may be absent (1–3). This suggests a complex interaction between lead and the red cell membrane. The ability of lead or mercury to combine with sulfhydryl groups in the cell membrane has been suggested as one mechanism leading to red cell destruction. Although it is known that lead and mercury combine more readily with red cell than with plasma proteins, we have little information on how much metal ion is required to cause erythrocyte destruction either *in vitro* or *in vivo*. Quantitative information on the level of lead or mercury required to cause osmotic hemolysis in normal human erythrocytes may help explain the mechanism of the anemia observed in poisoning with these metals.

Because of the tendency of both lead and mercury to combine with proteins in a complex manner, it has been difficult to obtain good *in vivo* data of red cell fragility. *In vitro* studies of erythrocyte fragility have been hampered by the relative insensitivity of conventional techniques (using tubes containing salt concentrations of decreasing osmolarity) to distinguish minor cell populations, and to allow studies of the rate of hemolysis (6). With the recording Fragilograph (7), we found it possible to obtain reproducible erythrocyte fragility curves in the presence of either lead or mercury. The effect on erythrocyte fragility of incubating normal human red cells with known quantities of lead or mercury are reported below.

Methods and Materials. Blood samples from apparently healthy individuals were collected in 10 ml Vacutainer tubes containing EDTA. The tubes were centrifuged at 1050*g* for 15 min in a refrigerated centrifuge, and plasma and buffy coat were removed and discarded. The erythrocytes were washed twice with buffered, physiologic saline, then resuspended in an equal volume of isotonic saline, pooled, and recentrifuged. Each experiment involved the pooled red cells from five or six individuals and all samples were stored in an ice bath until incubation.

Incubation mixtures for osmotic fragility studies were prepared by making a 1:1 dilution of the washed packed erythrocytes with a solution of either lead acetate or mercuric chloride in Veronal-buffered isotonic saline containing 1% glucose. The hematocrits of the experimental and control samples, respectively, were adjusted to about 45%. The final volume of each test sample was 2 ml, and the amount of lead or mercury was adjusted to final concentrations between 10^{-4} to 10^{-6} *M*. Samples were tested immediately after resuspension of the erythrocytes and following 1, 2, or 24 hr of incubation at 37° in a shaking water bath. Aseptic techniques were not used in preparing the erythrocyte suspensions, but all solutions, containers, and closures were sterile at the start of each ex-

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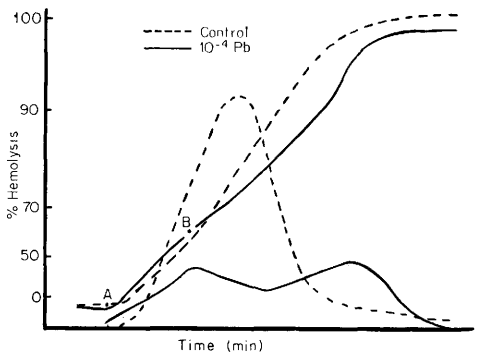

FIG. 1. Biphasic curves found with pooled erythrocytes incubated with 10^{-4} *M* Pb. (A) the threshold hemolysis point, and (B) 50% hemolysis as determined from a nomogram adjusted to the sample hematocrit. The salt concentration at points (A) and (B) were determined from the NaCl calibration of the individual Fragilograph membranes (8). (- -) Normal control readings.

periment.

Osmotic fragility was studied with a Kalmedic Fragilograph (Model D-2) immediately after the addition of 25 μl of the appropriate erythrocyte suspension to 0.75 ml of 0.9% NaCl buffered with Veronal to pH 7.3. The instrument was set to record the simultaneous cumulative and derivative curves from a calibrated membrane chamber maintained at 25° with a circulating water bath (8). The salt concentration for threshold (zero) hemolysis was obtained from the point where the slope of the derivative curve intersected the base line. The 50% lysis salt concentration was determined from the Fragilograph nomogram adjusted to the sample hematocrit. The microhematocrits, obtained from the Fragilograph nomogram, were checked with an International hematocrit centrifuge.

Total hemoglobin and oxyhemoglobin of selected samples were compared to determine the presence of methemoglobin. Each sample was centrifuged, the supernate was aspirated, diluted with phosphate buffered saline (pH 7.4), and oxyhemoglobin was determined spectrophotometrically at 575 nm. Total hemoglobin was determined by the absorbance at 555 nm after addition of sodium hydrosulfite to the supernate.

Eight pooled erythrocyte samples were used for the lead studies, and nine for the mercury studies. Each experiment consisted of one control and four experimental samples each with a different concentration of the heavy metal. The mean and standard deviation were calculated for each set of data, and statistical significance was tested using Student's *t* test.

Results. The normal osmotic fragility curves, simultaneously recorded as cumulative and derivative curves were considerably modified by the presence of lead (Fig. 1). Biphasic curves of this type indicate the presence of two populations of erythrocytes—one osmotically fragile and the other osmotically resistant (8).

Hematocrits increased slightly after the 24 hr incubation of control erythrocytes (Fig. 2), and salt concentrations for threshold hemolysis showed a small shift from 0.40 to 0.45% NaCl. The salt concentration for 50% hemolysis, however, was not significantly changed after 24 hr of incubation of normal red cells in our medium. In contrast, erythrocytes incubated for 1 hr in the isotonic medium containing 10^{-4} *M* Pb (2000 μg Pb/100 ml) showed a significant decrease in hematocrit ($p < .001$), but there was no further decrease in hematocrit with 24 hr of incubation (Fig. 2). A decrease in hematocrit became apparent after 24 hr incubation with 0.3×10^{-4} *M* Pb (800 μg Pb/100 ml), but did not occur with 10^{-5} or 10^{-6} *M* Pb (200

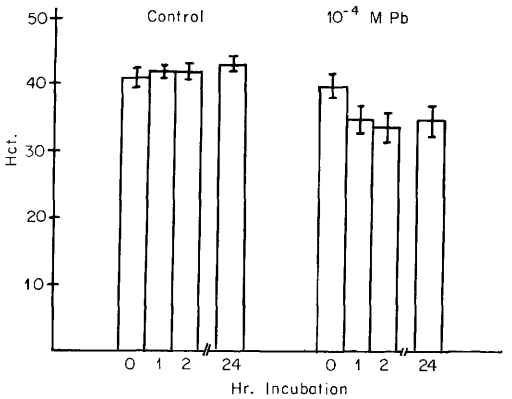

FIG. 2. Microhematocrits of control erythrocytes and erythrocytes incubated with 10^{-4} *M* Pb for different time periods. Bars show the standard deviation.

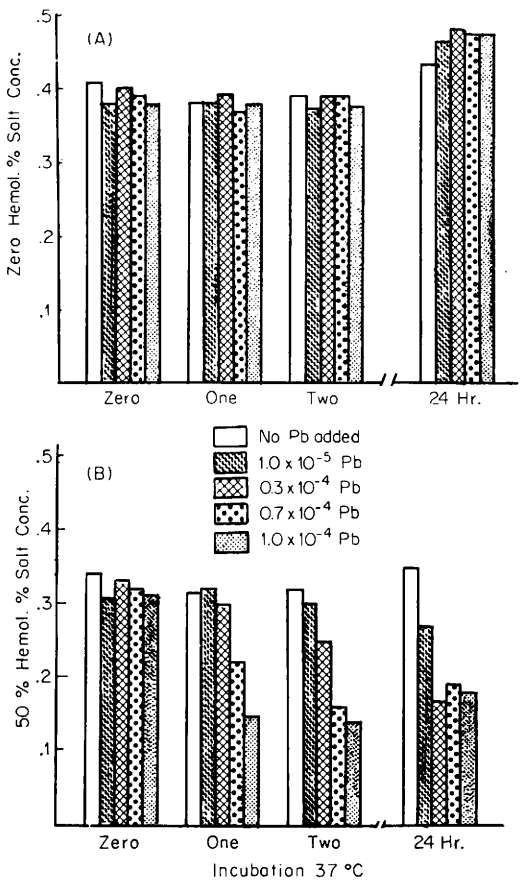

FIG. 3A. Salt concentration for threshold (zero) hemolysis of control and lead incubated erythrocytes. (B) Salt concentration for 50% hemolysis of control and lead incubated erythrocytes: A decreased salt concentration indicates increased resistance to osmotic lysis.

or 20 μg Pb/100 ml). The decrease in hematocrit after incubation of erythrocytes with 10^{-4} *M* Pb for 1 or 2 hr was not accompanied by visual evidence of hemolysis, but after 24 hr incubation with 10^{-4} *M* Pb the supernate was pink.

The salt concentration for threshold hemolysis with cells incubated 1 or 2 hr in isotonic saline containing lead was about the same as control cells (Fig. 3A). After 24 hr incubation, however, the salt concentration for threshold hemolysis for all samples containing lead were significantly higher than the controls ($p < .001$). The supernatant fluid of samples incubated with lead visually showed about the same slight hemolysis as the incubated control samples.

The salt concentration for 50% hemolysis decreased significantly ($p < .001$) after 1 hr of incubation with 0.7 or 1.0 $\times$ 10^{-4} *M* Pb, after 2 hr with 0.3 $\times$ 10^{-4} *M* Pb, and after 24 hr with 10^{-5} *M* Pb (Fig. 3B). The striking characteristic of the hemolysis, as seen in the Fragilograph recordings of cells incubated with lead, was the biphasic shape of the derivative curve (Fig 1). The biphasic curve was apparent after 1 hr of incubation with 10^{-4} *M* Pb, but did not appear with 10^{-5} *M* Pb until the cells have been incubated for 24 hr. Another interesting observation was that the cumulative curves of lead incubated samples failed to reach the 100% hemolysis line, suggesting that complete lysis did not occur. Samples of lead incubated cells were withdrawn from the dialysis chamber at the end of each run and, when examined microscopically, showed numerous crenated unhemolyzed cells. Dialysis had reduced the salt concentrations of these solutions to less than 0.01% NaCl and yet complete hemolysis had not occurred. In a few experiments, lead incubated cells were added directly to distilled water and these cells failed to show complete lysis after several hours.

In contrast to the biphasic derivative curve of samples incubated with lead, red cells incubated with mercury were monophasic. Fragilograph curves of mercury incubated samples were similar in shape to those of control samples after incubation for 1 or 2 hr, but were displaced to the left after incubation for 24 hr (Fig. 4). Thus, some lysis of fragile cells had occurred prior to our testing procedure. The microhematocrits of erythrocytes incubated with 10^{-4} *M* Hg were about the same as those of the control samples.

Although the supernatant fluid of control and lead treated samples showed only slight visual evidence of hemolysis after incubation for 24 hr, the supernatant fluid from samples incubated with 10^{-4} *M* Hg had considerable free hemoglobin. The free hemoglobin released into the incubation media of 24 hr control and mercury treated cell samples was measured, and calculations showed 0.3 and

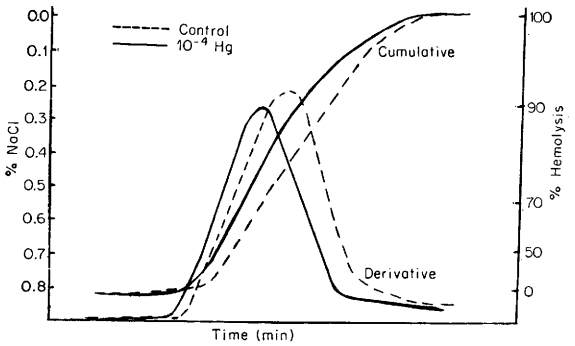

FIG. 4. Shift in cumulative and derivative curves on incubation of erythrocytes at 37° with 10^{-4} *M* Hg. (--) Normal control recordings.

2.06% hemolysis, respectively. Total hemoglobin and oxyhemoglobin concentrations done on samples after incubation for 24 hr, indicated that neither the control samples, nor samples with the different concentrations of lead or mercury, had detectable amounts of methemoglobin, *i.e.,* there were no differences found between the concentrations of total hemoglobin and oxyhemoglobin.

Discussion. The lowest concentration of lead that we studied (10^{-5} *M*) is equivalent to a blood level of about 200 μg/100 ml. This is approximately five times the 40 μg/100 ml level which is considered as the upper limit of normal by Chisolm (9). The pattern of osmotic fragility we found with 10^{-5} to 10^{-4} *M* Pb, however, was comparable to that found in patients with lead poisoning. Harris and Greenberg (10) found that erythrocytes from patients with plumbism had normal osmotic fragility when tested immediately, but became highly resistant to osmotic stress after incubation for 24 hr. Our Fragilograph studies showed that there were really two populations of red cells, one susceptible to osmotic lysis and the other resistant to osmotic lysis, and that the osmotically resistant population became dominant on incubation. The early hemolytic effect described in some cases of lead poisoning may occur in those individuals with a relatively large population of osmotically fragile erythrocytes. The presence of a second population of red cells highly resistant to osmotic stress, especially after incubation with lead has suggested a modified osmotic fragility test which may be useful in the diagnosis of lead poisoning (11).

Our control samples became slightly more susceptible to osmotic lysis after incubation for 24 hr, as indicated by the increase in salt concentration for threshold hemolysis (Fig. 3A). This was anticipated because Young and co-workers (12) have reported that the addition of glucose to the incubation medium does not completely prevent autohemolysis of normal erythrocytes. The slight hemolysis we observed in control samples after 24 hr of incubation (0.3%) was within the expected limits reported by Young *et al.* (12). However, the decreased hematocrit we found with red cell samples incubated with lead was probably due partially to cell shrinkage and partially to hemolysis of the osmotically fragile cells. We found numerous crenated cells in the samples incubated with lead in agreement with Passow, Rothstein and Clarkson (4) who indicated that crenated cells were readily produced when erythrocytes were incubated with lead. The number of crenated cells found was based on the concentration of lead used and duration of the incubation. Passow, Rothstein and Clarkson (12) showed that lead caused K^+ to leak from red cells and suggested that this occurred without a corresponding uptake of either Na^+ or water. Thus, the supposedly isotonic incubation medium we used, in effect, became hypertonic to the lead treated cells causing them to crenate.

The appearance of numerous crenated RBC in the lead incubated samples occurred at the same time that the biphasic derivative curves were found on the Fragilograph recordings (Fig. 1). The appearance of a second population of cells in the derivative curve

was coupled with the shift to the right of the cumulative curve, and a decrease in the salt concentration for 50% hemolysis. This indicated that cells in the second peak were highly resistant to hemolysis. It can also be inferred that the first peak showed a smaller population of osmotically fragile erythrocytes. The time required for development of the osmotically resistant population was dependent on the concentration of lead in the incubation medium and the duration of incubation. It occurred more readily in 10^{-4} *M* Pb and was delayed in 3×10^{-4} *M* Pb and did not occur after incubation to 10^{-5} *M* Pb.

The dual effect of lead on red cell fragility, noted above, may be explained by the complex interaction of lead with the erythrocyte. Lead apparently interferes not only with erythrocyte ion and water content, but with intracellular enzyme activity and hemoglobin synthesis as well (13). The formation of relatively insoluble lead–phosphate and lead–protein complexes at the cell surface act to inhibit passive transport (4). The inhibition of membrane sulfhydryl containing and possibly other enzymes depresses active transport (3), and lead interaction with the cell contents further influences the ability of the erythrocyte to maintain its integrity.

The salt concentration for 50% hemolysis in the Fragilograph (0.34%) was lower than that usually obtained for normal red cells by conventional methods (0.41%). This is probably due to two factors: Fragilograph determinations were recorded from a chamber with a continuously changing salt concentration, and the determinations were made in a Veronal-buffered NaCl solution which may provide some protection against osmotic hemolysis. There is some evidence for a correlation between osmotic hemolysis and erythrocyte age. It has been reported that young erythrocytes are more resistant to osmotic stress (14), and this may be related to the higher membrane phospholipid concentration of young compared to older red cells (15). Thus, there is the possibility that the first cells to hemolyze are the older cells, and that the more resistant population is made up of younger erythrocytes.

Mercury incubated red cells, unlike lead, showed a single peak in the osmotic fragility derivative curve (Fig. 4). The salt concentration for zero and 50% hemolysis, after incubation with mercury, was significantly higher than controls, and indicated an overall increase in osmotic fragility. During incubation with 10^{-4} *M* Hg there was a 5-fold increase in hemoglobin release indicating that lysis of some cells had occurred prior to our testing procedure. Mercury like lead, inhibits active transport, and causes a loss of potassium from erythrocytes. Unlike lead, in the presence of mercury, the loss of potassium is followed by an uptake of both Na^+ and H_2O leading to an osmotic lysis (4). Mercury binds avidly to sulfhydryl groups inhibiting both membrane mediated active transport and intracellular metabolism in a way that is undoubtedly different from lead. Both lead and mercury are sulfhydryl binding agents, but they apparently have different affinities for SH groups (4). This was manifest by the general hemolytic effect of mercury on erythrocytes, while lead was more selective and caused hemolysis of some cells and prevented the osmotic hemolysis of a significant population of erythrocytes after the osmotically fragile cells had lysed.

Summary. The osmotic fragility of human erythrocytes was analyzed with a recording Fragilograph after incubation with known amounts of lead or mercury. Incubation with lead produced two populations of red cells, one osmotically fragile and the other osmotically resistant. Mercury (10^{-4} to 10^{-6} *M* Hg), unlike lead, caused an overall increase in osmotic fragility. With lead an osmotically resistant population of erythrocytes developed after 1 hr incubation with 0.7 to 1.0×10^{-4} *M* Pb (1500 to 2000 μg Pb/100 ml), after 2 hr with 0.3×10^{-4} *M* Pb (800 μg Pb/100 ml), but did not appear until 24 hr incubation with 10^{-5} *M* Pb (200 μg Pb/100 ml). The gradual development of an osmotically resistant population of erythrocytes with lead, but not with mercury, indicates different modes of interaction of these two ions with the red cell membrane and contents.

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