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Radiophosphate ($^{32}PO_4$) Incorporation into Phosphatidic Acid of Lead-Poisoned and Normal Red Cells (38799)

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Current evidence suggests that lead produces a variety of biochemical, structural and physiologic changes in the developing and mature red cell. Among these are abnormalities in heme synthesis, globin synthesis, potassium exchange, and iron transport (1). The cell membrane may also be affected since it contains large quantities of phosphatide which is particularly sensitive to heavy metals. A defect in the membrane may in turn affect various properties of the cell (1). In a preliminary study we had shown that a membrane lipid abnormality does occur which consists of a decrease in the incorporation of radioactive phosphate ($^{32}PO_4$) into the membrane phosphatide, phosphatidic acid. This was demonstrated in the erythrocytes of humans and rabbits with plumbism (2). The present study is a further description of radiophosphate incorporation into phosphatidic acid of the red cells of lead-intoxicated humans and rabbits and compares the degree of incorporation to survival of the cells, to their osmotic sensitivity as well as evaluating incorporation of $^{32}PO_4$ into phosphatidic acid of the normal erythrocyte.

Methods. Seven patients and seven rabbits with plumbism were studied (Table I). Complete studies on four of the patients (R. Y., N. N., R. P., F. M.) have been previously described (3). The remaining three patients (S. H., E. H., B. G.) had also been exposed to high concentrations of lead and had plumbism. Lead poisoning in rabbits was induced by the subcutaneous injection of a 4% solution of lead acetate (400 mg/kg body weight). Lead intoxication in humans was determined by clinical data and by analyses for lead in blood, urine, and bone (3). In rabbits, the diagnosis was confirmed by blood lead analyses. Routine blood studies were obtained by standard techniques (4). Red cell osmotic fragility was studied with a Fragiligraph[1] at 21° ± 0.5° C on fresh samples of blood (Na_2 ethylenediaminetetraacetic acid 1 mg per 1 ml blood or on defibrinated blood). Erythrocyte survival was measured with ^{51}Cr and $DF^{32}P$ labelling techniques (5).

Measurement of the incorporation of ^{32}P-labeled phosphate into red cells was obtained by incubating fresh whole blood with a solution containing 15 μCi $Na_2H^{32}PO_4$ (high specific activity) and 1 mg glucose per 1 ml packed erythrocytes as noted earlier (6). Radioactivity was measured with a thin-window, gas flow counter. Radiolabeled phosphatide activity was determined by scraping the silica gel spot containing the phosphatide into a planchet or by cutting the paper chromatogram into equal sections and placing them in planchets. Sufficient total counts were obtained to give a standard error of less than 1%. Determinations were made in triplicate or quadruplicate. Lipid analyses, phosphatide separation, and phosphorus analyses as well as preparation of red cell-rich fractions, and young and old red cell populations were obtained by methods described previously (6, 7). The following metabolic inhibitors were used in *in vitro* studies: sodium fluoride (NaF), iodoacetate (HIAA), lead acetate ($Pb(CH_3COO)_2$), *p*-hydroxy mercuribenzoate (PMB), *N*-ethylmaleionide (NEM), ouabain, mercuric chloride ($HgCl_2$) (1, 8, 9). Other substances used were trypsin (10) and roentgen irradiation (48,000 rad) (11). The various inhibitors and other substances were used in concentrations known to have marked metabolic effects or until prelytic levels were reached.

[1] Kalmedic Instruments, Inc. 425 Park Ave., New York, NY.

TABLE I. BLOOD AND BONE ANALYSES OF LEAD-POISONED PATIENTS AND RABBITS

	Blood lead (mg/100 g)	Bone lead (mg/100 g)	Hematocrit (%)	Reticulocyte count (%)	Red cell survival $T_{1/2}$ (days)
Patients					
R. Y.	0.19	35.0	28	8	—
N. N.	0.12	14.7	42	3	21[a]
R. P.	0.13	9.9	42	2	41[a]
F. M.	0.10	5.9	42	2	24[a]
S. H.	0.12	—	30	7	—
E. H.	0.29	40.0	36	2	21[b]
B. G.	0.11	11.8	42	1	35[b]
Normal	<0.08	<4.0	38–45	<2	45–55[a] 25–33[b]
Rabbits					
1	0.25	—	27	6	6[c]
2	0.20	—	31	7	6
3	0.13	—	24	8	5
4	0.15	—	29	8	5
5	0.30	—	27	11	4
6	0.19	—	34	7	8
7	—	—	31	8	—
Normal	<0.05[d]		38–42	<2	10–14

[a] Diisopropylfluoro-^{32}phosphate-labeled red cells.

[b] ^{51}Cr-labeled red cells.

[c] All rabbits had ^{51}Cr-labeled red cell survival studies.

[d] Five normal rabbits.

Results. Blood lead levels in lead-poisoned patients and in lead-poisoned rabbits, and bone lead concentrations in patients were increased (Table I). Red cell survival as measured by ^{51}Cr or DF^{32}P labeling of red cells was normal in one patient (B. G.) and decreased in four patients (N. N., R. P., F. M., E. H.) (Table I). The remaining two patients (R. Y., S. H.) had other evidence of hemolysis. All lead-poisoned rabbits had shortened red cell survival. Red cell osmotic fragility in all the lead-poisoned rabbits (Nos. 2, 3, 4, 5, 6, 7) and humans in whom it was measured (E. H., B. G.) was abnormal. In the rabbits, all of which had hemolytic anemia, and in the human who was hemolyzing (E. H.), the curves showed heterogeneous populations of red cells with increased, normal, and decreased resistance to osmotic lysis (Fig. 1). The salt concentrations at which 50% lysis occurred in this group of rabbits and in the patient was normal. In the lead-poisoned patient with normal red cell life span (B. G.), the population of cells was homogeneous showing cells with marked resistance to osmotic lysis (Fig. 2). Concentrations of red cell lipids including total lipid, phospholipid, and cholesterol were within normal limits in both humans and rabbits with increased lead levels (Table II).

The incorporation of ^{32}P-phosphate in the normal and lead-poisoned cells of humans and rabbits occurred predominantly into a single phosphatide which had been characterized as phosphatidic acid (12). The

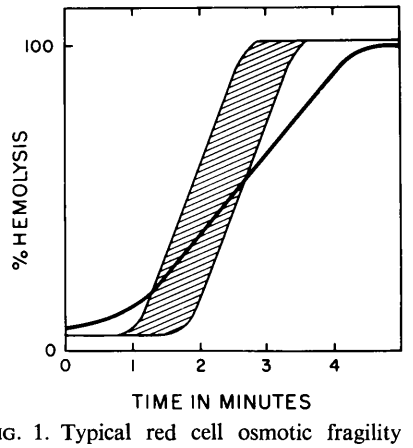

FIG. 1. Typical red cell osmotic fragility curve in a patient (E. H.) with plumbism and hemolysis. The cross-hatched area represents the normal range.

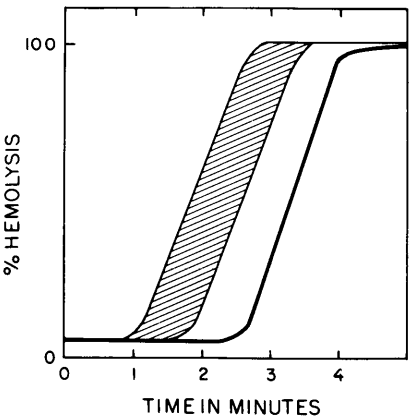

FIG. 2. Typical red cell osmotic fragility curve in a patient (B. G.) with plumbism but without hemolysis. The cross-hatched area represents the normal range.

incorporation into red cell phosphatidic acid of the seven lead-intoxicated humans and rabbits was significantly less than that observed in normals ($P < 0.01$) (Table III).

The enzyme inhibitors, HIAA and NaF, when added to normal blood in *in vitro* studies, altered incorporation of ^{32}P-phosphate into red cell phosphatidic acid. HIAA caused a decrease in incorporation of ^{32}P-phosphate into red cell phosphatidic acid at low concentrations which became gradually more severe as concentrations were increased (Fig. 3). In contrast, low concentrations of NaF showed an increased incorporation of ^{32}P-phosphate into phosphatidic acid prior to a decrease in incorporation as concentrations were increased (Fig. 3). Similar results were obtained in *in vitro* studies using increasing concentra- of lead ($Pb(CH_2OO)_2$) (Fig. 4). The sulfhydryl inhibitors, PMB and NEM, as well as the proteolytic enzyme trypsin in low concentrations inhibited radiophosphate uptake into red cell phosphatidic acid (Table IV). $HgCl_2$, and X-ray showed no effect on the incorporation of ^{32}P-phosphate into red cell phosphatidic acid. Uptakes in young and old red cell populations were similar.

TABLE II. RED CELL LIPID CONCENTRATIONS IN LEAD-POISONED PATIENTS AND RABBITS.

	Total lipid (mg × 10^{-12}/ RBC)	Total phospholipid (mg × 10^{-12}/ RBC)	Total cholesterol (mg × 10^{-12}/ RBC)
Patients			
Mean ± 1 SD[a]	583±83	309±37	123±9
Range	510–713	246–340	110–132
No.	5	5	4
Normal			
Mean ± 1 SD	479±85	300±36	112±9
Rabbits			
Mean ± 1 SD	395±79	200±27	99±20
Range	267–463	162–229	67–121
No.	7	7	7
Normal			
Mean ± 1 SD	445±87	256±84	122±24

[a] Standard deviation.

TABLE III. ^{32}P-PHOSPHATE INCORPORATION INTO RED CELL PHOSPHATIDIC ACID OF LEAD-POISONED PATIENTS AND RABBITS.[a]

	PAP[b] (cpm × 10^6/1 mg)		PAP[b] (cpm × 10^6/1 mg)
Patients		Rabbits	
Mean ± 1 SD[c]	4.47 ±0.64	Mean ± 1 SD	5.46 ±2.43
Range	3.50–5.28	Range	2.28–8.44
No.	7	No.	6
Normal		Normal	
Mean ± 1 SD	10.69 ±4.19	Mean ± 1 SD	12.90 ±5.42
Range	4.61–25.53	Range	5.11–22.25
No.	44	No.	16
P value	<0.01	*P* value	<0.01

[a] Three-hour 20 min incubation period of ^{32}P-Phosphate with Red Cells.

[b] Phosphatidic acid phosphorus.

[c] Standard deviation.

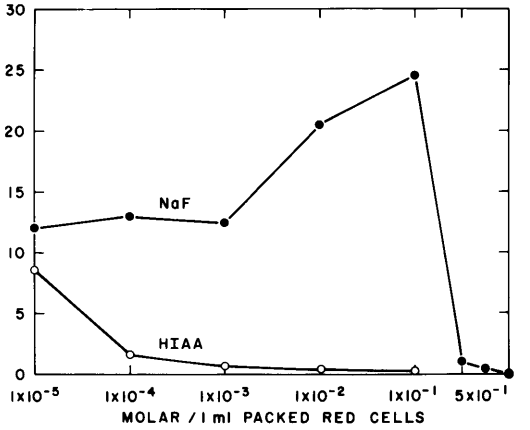

FIG. 3. The effect of various concentrations of HIAA and NaF on ^{32}P-phosphate incorporation into red cell phosphatidic acid. Each point represents the mean of six experiments. * Phosphatidic acid phosphorus.

Discussion. The incorporation of radiophosphate into phosphatidic acid of the red cells of humans and rabbits with lead poisoning was regularly decreased. Phosphatides are structural components of most cell membranes, and certain of these phosphatides appear to be metabolically active and allied to various cell functions (9) which would suggest that a metabolic defect might affect cell survival. Although the precise relationship and effect of the present

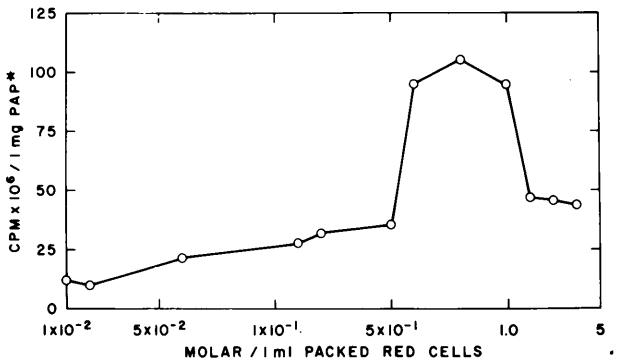

FIG. 4. The effect of various concentrations of lead acetate on ^{32}P-phosphate incorporation into red cell phosphatidic acid. Each point represents the mean of four experiments. * Phosphatidic acid phosphorus.

finding upon red cell life span of lead-poisoned cells is not known, it is unlikely that a direct relationship exists since the decrease in radiophosphate incorporation into phosphatidic acid occurred whether or not hemolysis was present and since the actual turnover of phosphatidic acid is quantitatively not significant (12). These findings suggest that the decrease in phosphatidic acid labeling represents a defect which, per se, does not affect red cell survival, but might be one of several factors influencing red cell survival. Red cell longevity is not affected by the lead burden in the body, as assessed by the measurement of bone lead levels, since a direct relation between the two does not occur, nor by cell lipid levels since they were normal.

Radiophosphate was primarily incorporated into phosphatidic acid of the human and rabbit red cell. Although other studies have shown that other phosphatides, e.g., phosphatidyl serine in human red cells (13) and inositide in swine and beef red cells (14), are labeled, the present finding is similar to that of Reed (12) which corroborated our earlier observation (6). The differences in phosphatide labeling between studies may be related to differences in preparatory methods of the cells or in incubation techniques. Radiophosphate incorporation is SH dependent as shown by the marked inhibitory effect of the sulfhydril inhibitors, PMB, NEM, HIAA, and occurs at the cell surface as demonstrated by the inhibitory effect of trypsin upon the surface. Although the glycolytic inhibitor, NaF,

TABLE IV. THE EFFECT OF VARIOUS SUBSTANCES ON THE INCORPORATION OF ^{32}P-PHOSPHATE INTO PHOSPHATIDIC ACID OF NORMAL HUMAN RED CELLS.

Substance	Packed RBC (molar/1 ml)	PAP[a] (cpm × 10^6/1 mg)
PMB	4.5×10^{-3}	3.83[b]
NEM	4.5×10^{-3}	1.17
Trypsin	1×10^{-7}[c]	0.25
Normal		10.69 ±4.19[d]

[a] Phosphatidic acid phosphorus.
[b] Mean of four experiments.
[c] Grams/100 ml RBC.
[d] Standard deviation.

markedly decreased radiophosphate incorporation into phosphatidic acid, the inhibition probably occurred by actions other than the effect on glycolysis (15), since glycolysis, per se, does not affect labeling of red cell phosphatidic acid (12). This concept is reinforced by the effects which various concentrations of NaF have upon the labeling of phosphatidic acid by radiophosphate. The effects follow the Schultz-Arendt rule in which increased activity (radiophosphate incorporation) occurs at low concentrations of inhibitor with decreased activity at higher levels and suggests that the NaF effect is multiple. The effects of lead ($Pb(CH_2OO)_2$) on radiophosphate uptake showed results similar to those observed with NaF when measured *in vitro* with various concentrations of lead. Radiophosphate labeling of phosphatidic acid in the red cell is not ouabain sensitive

and thus is not related to cation transport as it is in the salt glands of birds (9). The absence of a ouabain affect has been shown previously (12).

The osmotic fragility studies of the red cells of the lead-poisoned humans and rabbits regularly showed the predictable osmotically resistant cell populations. When hemolysis was present, however, populations with increased fragility were also observed. No relationship between the level of radiophosphate incorporation and the types of osmotic population of cells was observed. The cause of the osmotically fragile populations of red cells is not known. Aub *et al.* (16) have shown that the red cells of lead poisoning have increased mechanical fragility and have suggested that the cells become more susceptible to trauma during passage through capillaries with subsequent hemolysis. With the increased susceptibility of these cells to trauma and membrane loss, one might predict that a population of osmotically sensitive cells would be formed as described for the red cell in other anemias (17).

It was of interest that the decreased incorporation of labeled phosphate into phosphatidic acid in the red cells of the lead-poisoned humans and rabbits was opposite to that described for a young cell population (12) or in the osmotically fragile red cells of patients with hereditary spherocytosis (13). This would imply, at least for phosphatidic acid, that a different type of membrane kinetics may exist in the red cell of lead poisoning.

Summary. Radiophosphate incorporation into phosphatidic acid is decreased in the red cells of lead-poisoned humans and rabbits. The decrease in activity is not related to the length of red cell survival nor to the differences in osmotic fragility observed in the lead-poisoned cells. Incorporation of radiophosphate into phosphatidic acid of normal red cells is sulfhydryl dependent and occurs at the cell surface. Lipid composition of the red cell membrane of the lead-poisoned humans and rabbits is normal.

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