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Protein-Independent Lead Permeation Through Myelin Lipid Liposomes

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SUMMARY

We have investigated the permeability of protein-free myelin lipid liposomes to inorganic lead by using the fluorescent probes fura-2, oxonol V, pyranine, and carboxyfluorescein. Inorganic lead readily crossed the lipid bilayer, as detected with fura-2, to an extent that depended on the external pH and the total nominal lead concentration in the assay medium. Lead entry generated an internally positive transmembrane potential, which could be detected by oxonol V fluorescence quenching, and dissipated a transmembrane pH gradient by alkalinization of the intravesicular space, as measured with pyranine. These results cannot be explained by lead-mediated nonspecific damage to membrane lipids, based on the following results: 1) lead exposure did not increase carboxyfluorescein leakage from liposomes, 2) it did not increase the permeability of the lipid bilayer to glucose or KCl, 3) it did not generate peroxidation products in contact with myelin lipids, and 4) it did not induce chemical hydrolysis or modification of any myelin lipid class. We conclude that the principal molecular mechanism of lead permeation through a pure lipid bilayer is the passive diffusion of $Pb(OH)^+$. We discuss the toxicological relevance of these findings for cells in general and for myelin in particular and suggest that this mechanism might contribute significantly to the total lead entry into the cells.

Lead is recognized as an environmental and occupational hazard that has a significant impact on the health and development of many biological species. Several organs and tissues, such as kidney, liver, blood, bone, and reproductive organs, are the targets of lead toxicity (1). Disorders in the central and peripheral nervous systems, such as lead encephalopathy and lead neuropathy, are also well documented (2). It has been reported that neuroglia (astroglia and oligodendroglia) are primary targets for lead toxicity in the central nervous system, which might affect neuronal function (3, 4). Both oligodendrocytes (the cells that synthesize the myelin sheaths) and astrocytes are very prone to lead injury. Oligodendrocyte damage induces a severe myelination deficit.

Fundamental mechanisms of lead toxicity seem to be related to the ability of this metal to interact with proteins (functional groups of enzymes, high affinity metal-binding proteins, etc.) (5) and Ca^{2+}-regulated processes (6). Inside the cells, lead disturbs several cellular functions, such as mitochondrial respiration (1), and alters cellular Ca^{2+} homeostasis by interaction with a number of Ca^{2+}-dependent effector mechanisms, such as calmodulin, protein kinase C, and neurotransmitter release (2, 6, 7). Although the mechanism of lead entry into cells is not fully understood, several groups have reported that Pb^{2+} might reach the cytoplasm through voltage-gated calcium channels (8, 9).

Other toxic heavy metals are able to cross pure lipid bilayers by diffusion, a property that might contribute to the total metal permeation into the cells (10, 11). However, inorganic lead permeation through lipid bilayers has not been investigated thus far. These precedents prompted us to investigate the molecular mechanisms and properties of lead permeation across the membrane of liposomes prepared with protein-free total myelin lipids, making use of several fluorescent probes. Lipid vesicles and liposomes are widely recognized tools for the study of the mechanisms of membrane permeability. We have chosen myelin lipids as a substrate because previous work from our laboratory revealed myelin lipids, with low permeability to cations and polar molecules (12, 13), to be a substrate of choice for investigation of the alteration of membrane permeability induced by heavy metals (14). In addition, the lipid component accounts for 75–80% of the total myelin dry weight, and the involvement of inorganic lead in several myelin lipid synthesis alterations has been recently documented (3). Furthermore, these membranes are primary targets for lead neurotoxicity (1).

We report in this paper protein-independent lead entry

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ABBREVIATIONS: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MLVs, multilamellar vesicles; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPTLC, high performance thin layer chromatography; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

across the myelin lipid bilayer. Lead permeation is electrogenic and dissipates a transmembrane pH gradient by alkalinization of the acidic compartments. These effects cannot be explained in terms of lead-induced lipid peroxidation, membrane fusion, or chemical alteration of myelin membrane lipids. From the results reported here we conclude that the main lead species that permeates the lipid bilayer appears to be $Pb(OH)^+$. The biological and toxicological relevance of these findings is discussed.

Experimental Procedures

Materials

Water used throughout was obtained from a Milli-Q water purification system (Millipore). CHELEX-100 chelating resin, EGTA, nigericin, acridine orange, HEPES, MES, 2-thiobarbituric acid, and potassium gluconate were from Sigma. Fura-2 and valinomycin were from Calbiochem. EDTA, precoated silica gel 60 HPTLC plates, $Pb(NO_3)_2$, KCl, Tris, $CaCl_2$, and $HgCl_2$ were purchased from Merck. The fluorescent probes 6-carboxyfluorescein and oxonol V [bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol] were from Molecular Probes. Sephadex G-25, G-50, and G-75 were from Pharmacia. Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate), Triton X-100, $TlCl_3 \cdot 4H_2O$, triethyl lead chloride, and $FeSO_4 \cdot 7H_2O$ were obtained from Serva, BDH, Aldrich, Alfa, and Probus, respectively.

Buffers and Liposomes

Total myelin lipids were extracted from bovine brain white matter with tetrahydrofuran, as described previously (12). Lyophilized lipids were dissolved in chloroform in a Pyrex glass tube, and the solution was evaporated to dryness. Unless indicated otherwise, dry lipid film was hydrated with 10 mM HEPES-Tris, 100 mM KCl, pH 7.4, by vortexing for 10 min, to prepare MLVs. All buffers used were treated with CHELEX-100 chelating resin before use, to sequester contaminating trace metals.

Fluorescence Assays

Lead. All fluorescence assays were carried out at 25°, with continuous stirring, in a Shimadzu RF-5001 PC spectrofluorometer, using a 3-ml quartz cuvette. Permeability of liposomal membranes to Pb^{2+} was monitored with fura-2. MLVs (40 mg of lipid/ml) were prepared in buffer (10 mM HEPES-Tris, 100 mM KCl, 1.5 mM EGTA-Tris, pH 7.4, 40 μM fura-2). EGTA was included in the buffer to sequester endogenous lipid calcium. Nontrapped fura-2 was removed by gel filtration through Sephadex G-50, which was equilibrated and eluted with the same buffer lacking EGTA and fura-2. For these experiments 50 μl of fura-2-loaded MLVs were added to 2 ml of 10 mM HEPES-Tris, 100 mM KCl, pH 7.4. After stabilization of the base-line, a specific amount of $Pb(NO_3)_2$ was added to the cuvette. Fluorescence of fura-2-loaded MLVs was recorded in ratio mode at 340 nm and 380 nm (excitation) and 510 nm (emission). Slits for excitation and emission wavelengths were set at 5 nm. The following ratios of fluorescence were measured: 1) the 340-nm/380-nm fluorescence ratio at saturating Pb^{2+} concentration, with lysis of MLVs with Triton X-100 (0.15% final concentration) at the end of the experiment (R_{max}); 2) the 340-nm/380-nm fluorescence ratio of fura-2 in its free form (EDTA was added afterward) (R_{min}); and 3) the fluorescence ratio at 380 nm between the free form and the lead-saturated form of fura-2 (Sf_2/Sb_2). The intraliposomal Pb^{2+} concentration ($[Pb^{2+}]_i$) was calculated from these ratios according to the equation $[Pb^{2+}]_i = K_d \times [(R - R_{min})/(R_{max} - R)] \times (Sf_2/Sb_2)$. The dissociation constant of the Pb^{2+}/fura-2 complex, K_d, was assumed to be 4.2×10^{-12} M (15). R corresponded to the 340-nm/380-nm fluorescence ratio of the test sample.

Membrane potential. Liposomal transmembrane potential was monitored after the addition of lead by using the fluorescent probe oxonol V (16). This probe responds to the generation of an internally positive transmembrane potential with the quenching of its fluorescence. MLVs prepared in 10 mM HEPES-Tris, 100 mM KCl, pH 7.4, were diluted into the same buffer containing 5 μM oxonol V, at a final lipid concentration of 50 μg/ml. After stabilization of the base-line, aliquots of a stock solution of $Pb(NO_3)_2$ or $CaCl_2$ were added and the changes in fluorescence intensity were recorded. Measurements were carried out with an excitation wavelength of 580 nm and an emission wavelength of 640 nm. Slits for excitation and emission wavelengths were set at 5 and 15 nm, respectively.

Effect of lead on transmembrane pH gradients. Transmembrane pH gradients across liposomal membranes were monitored with the pH-sensitive probe pyranine (17). MLVs were prepared in the buffer indicated in the figure legends, containing 0.5 mM pyranine, at a final lipid concentration of 40 mg/ml. Extraliposomal pyranine was then removed by chromatography through Sephadex G-25, using the minispin column method of Fry *et al.* (18). Pyranine-containing liposomes were further diluted 100-fold into the external assay medium. Changes in fluorescence were tested after the addition of $Pb(NO_3)_2$, $TlCl_3$, or triethyl lead. Measurements were carried out in ratio mode at 450 nm and 400 nm (excitation) and 510 nm (emission). Slits were set at 3 nm. For calibration, pyranine-containing MLVs made up in 10 mM MES-Tris, 100 mM KCl, pH 4.76, were diluted 100-fold into 10 mM HEPES-Tris/100 mM KCl buffers of increasing pH values (from 5.48 to 7.43) in the presence of the K^+/H^+-exchanger ionophore nigericin. This ionophore dissipates the initial transmembrane pH gradient, so the internal pH equals the external pH. Further addition of Triton X-100 did not induce any fluorescence modification. By plotting the logarithm of the 450-nm/400-nm fluorescence ratio versus pH, a calibration line was obtained (19).

Membrane fusion. To investigate possible effects of lead on interlamellar interaction and membrane fusion, liposomes were loaded with carboxyfluorescein. Briefly, MLVs were prepared in 10 mM HEPES-Tris, 100 mM carboxyfluorescein, pH 7.4. Nontrapped fluorescent probe was removed by gel filtration of liposomes through a Sephadex G-75 minicolumn, which was equilibrated and eluted with the same buffer lacking carboxyfluorescein. Aliquots of this suspension were diluted 500-fold into the external buffer, and the evolution of the base-line was recorded. Aliquots of $Pb(NO_3)_2$ were then added. At the end of the experiments, Triton X-100 was added to break liposomes and liberate the dye. Measurements were carried out at 490 nm (excitation) and 550 nm (emission). Slits were set at 1.5 nm.

Effect of Lead on the Chemical Integrity of Myelin Lipids

To investigate possible chemical alterations of myelin lipid classes as a consequence of lead exposure, MLVs (20 mg of lipid/ml) were incubated in 10 mM HEPES-Tris, pH 7.4, with either $Pb(NO_3)_2$ or $HgCl_2$ at a final concentration of 200 μM, for 4.25 hr at room temperature. Total lipids were then extracted with 19 volumes of chloroform/methanol (2:1, v/v) (20). After partitioning with 0.2 volumes of 0.1 M KCl, the upper phases were discarded and the lower organic phases, containing total lipids, were evaporated to dryness with a nitrogen stream and redissolved in chloroform/methanol (2:1, v/v). Lipid composition of samples and controls was analyzed by two-dimensional HPTLC on 10- × 10-cm precoated silica gel HPTLC plates (Merck). The solvents used were methyl acetate/*n*-propanol/chloroform/methanol/0.25% aqueous potassium chloride (25:25:25:10:9, by volume) for the first dimension and chloroform/methanol/acetone/acetic acid/water (75:15:30:15:7.5, by volume) for the second dimension. Lipids were visualized with the copper acetate reagent (13).

Results

The first set of experiments was designed to investigate the entry of lead into MLVs at identical intra- and extravesicular

pH values, making use of fura-2. This probe was successfully used to detect intracellular lead in previous reports (9, 15). Results depicted in Fig. 1 clearly indicate a dose-dependent increase in intraliposomal lead as the total nominal lead concentration in the extraliposomal compartment is raised. These results indicate a protein-independent liposomal membrane permeability to lead.

At the pH and ionic composition used in the experiments of Fig. 1, several lead species are present in solution, such as $PbCl_2$, $Pb(OH)^+$, $Pb(OH)_2$, $PbCl_3^-$, and Pb^{2+}, which may contribute to the passive diffusion of lead through the bilayer. To investigate whether lead entry is electrogenic, that is, whether the main lead species that crosses the membrane carries charges, we performed the experiments shown in Fig. 2. The fluorescent dye oxonol V responds to the generation of an internally positive membrane potential with quenching of its fluorescence. Addition of lead to the assay medium containing liposomes and oxonol V resulted in a significant fluorescence quenching, not detectable when lead was replaced by equimolar Ca^{2+} (Fig. 2). On the other hand, lead did not quench oxonol V fluorescence in the absence of liposomes (data not shown). These results indicate that the main lead species crossing the membrane must be positively charged, because lead permeation appears to be electrogenic.

Among the positively charged lead species present in solution under the conditions used in Figs. 1 and 2, Pb^{2+}, $Pb(OH)^+$, and $PbCl^+$ are predominant. To discriminate which of these contributes more decisively to the total lead permeation, we carried out the experiments depicted in Figs. 3 and 4, using the pH-sensitive probe pyranine. When trapped inside the liposomes, pyranine monitors intravesicular pH. If cationic $Pb(OH)^+$ was the main permeating species, then the addition of lead should dissipate a pH gradient (internally acidic) pre-established across the liposomal membranes. In Fig. 3, pyranine-containing liposomes prepared in 10 mM MES-Tris, 100 mM KCl, pH 4.73, were diluted 100-fold into the external buffer (10 mM HEPES-Tris, 100 mM KCl, pH

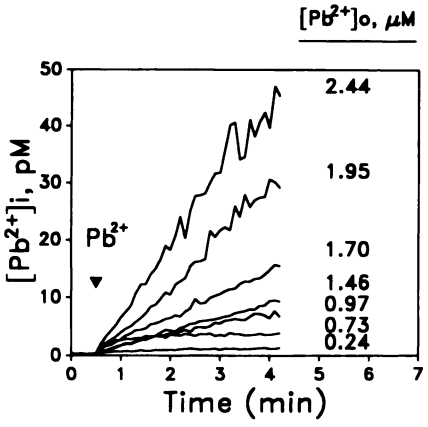

Fig. 1. Lead permeation through myelin lipid MLVs. MLVs were prepared in 10 mM HEPES-Tris, 100 mM KCl, 1.5 mM EGTA-Tris, pH 7.4, containing 40 μM fura-2. Nonencapsulated fura-2 was removed by gel filtration through Sephadex G-50 that was equilibrated and eluted with external buffer (10 mM HEPES-Tris, 100 mM KCl, pH 7.4). At time 0, 50-μl aliquots of fura-2-containing MLVs were added to the cuvette, which contained 2 ml of the external buffer. At the time indicated (*arrowhead*), aliquots from a stock solution of 5 mM $Pb(NO_3)_2$ in water were added to give the final lead concentrations shown (*numbers on the right*). The final lipid concentration in the assay medium was 0.5 mg/ml.

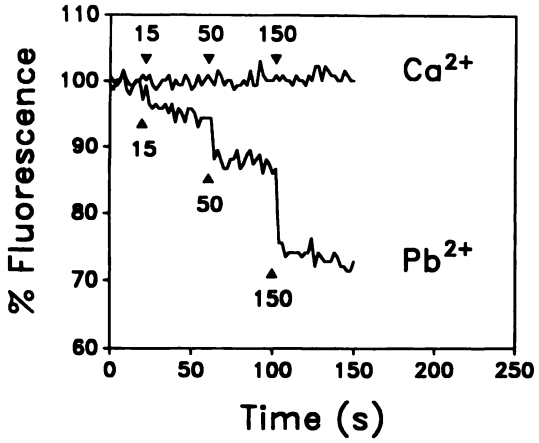

Fig. 2. Transmembrane potential generated by lead permeation. MLVs were prepared in buffer (10 mM HEPES-Tris, 100 mM KCl, pH 7.4). Aliquots of 5 μl were added to the cuvette containing 2 ml of the same buffer with 5 μM oxonol V. After base-line stabilization, equimolar amounts of either $CaCl_2$ or $Pb(NO_3)_2$ were added (*arrowheads*). *Numbers*, nmol of each compound. The final lipid concentration in the assay medium was 50 μg/ml.

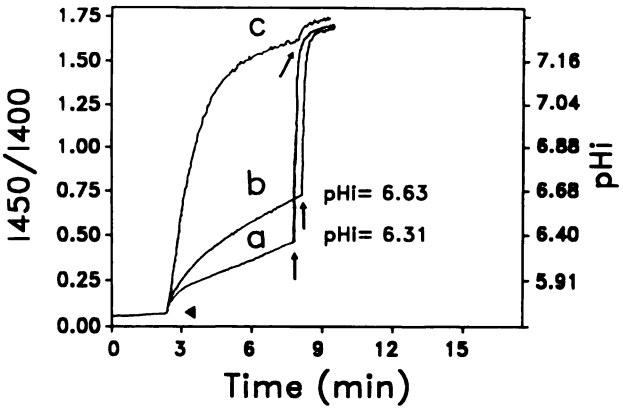

Fig. 3. Dissipation of a transmembrane pH gradient by lead entry. MLVs were prepared in 10 mM MES-Tris, 100 mM KCl, 0.5 mM pyranine, pH 4.76. Nonentrapped pyranine was removed by gel filtration as described in Experimental Procedures. At time 0, 20 μl of pyranine-containing MLVs were diluted 100-fold into the external buffer (10 mM HEPES-Tris, 100 mM KCl, pH 7.31). At the time indicated (*arrowhead*), the following compounds were added: 8 μM $Pb(NO_3)_2$ (*trace a*), 26 μM $Pb(NO_3)_2$ (*trace b*), or 0.3 μM $TlCl_3$ (*trace c*). At the end of the experiments, 4 μM nigericin was added (*arrows*) to equalize internal and external pH values.

7.31) (transmembrane pH gradient = 2.58 pH units) and the 450-nm/400-nm fluorescence ratio was recorded. At this acidic intraliposomal pH (pH 4.73) this ratio is minimal. Addition of micromolar concentrations of $Pb(NO_3)_2$ resulted in a slow but significant increase of this fluorescence ratio, which reflects intraliposomal alkalinization. Thus, a total nominal lead concentration of 26 μM raised the internal pH from 4.73 to 6.63 in about 6 min (Fig. 3). Voltage-clamping of the membrane potential near 0, achieved by the inclusion of 0.4 μM valinomycin under the same experimental conditions, resulted in a greater intraliposomal alkalinization (data not shown), thus confirming the electrogenic nature of lead entry. In comparison, the addition of 0.2 μM Tl^{3+} caused a much faster neutralization of internal pH as a result of a Tl^{3+}-mediated Cl^-/OH^- exchange across the bilayer (14).

Because previous reports have pointed out the participa-

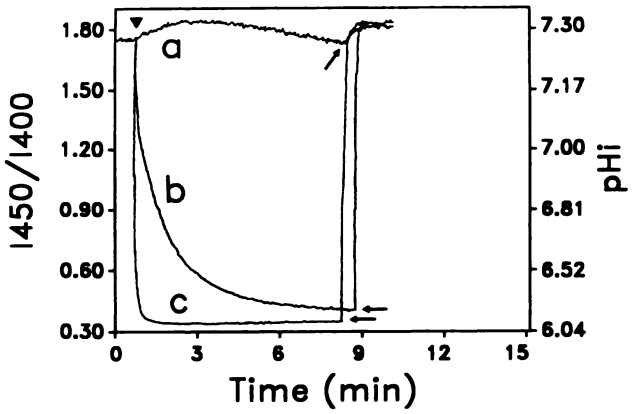

Fig. 4. Lack of mediation by inorganic lead of a Cl^-/OH^- exchange process through the lipid bilayer. MLVs were prepared in 10 mM HEPES-Tris, 100 mM potassium gluconate, 0.5 mM pyranine, pH 7.31. Nonencapsulated fluorescent dye was removed by gel filtration, as detailed in Experimental Procedures. At time 0, 20-μl aliquots of MLVs were diluted 100-fold into the external medium (10 mM HEPES-Tris, 100 mM KCl, pH 7.31), to establish a inwardly directed transmembrane chloride gradient. At the time indicated (*arrowhead*), the following compounds were added: 26 μM $Pb(NO_3)_2$ (*trace a*), 0.3 μM triethyl lead (*trace b*), or 0.2 μM $TlCl_3$ (*trace c*). At the end of the experiments, 0.15% Triton X-100 was added (*arrows*).

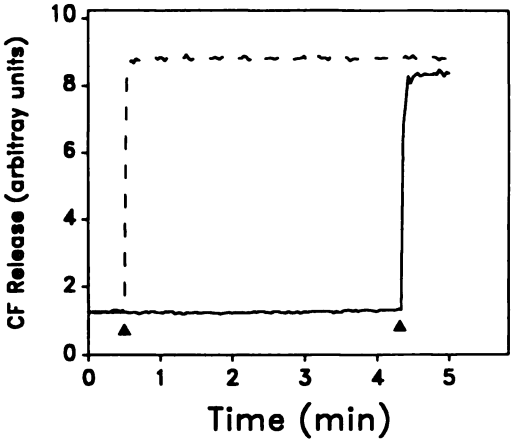

Fig. 5. Lack of induction by inorganic lead of increased leaking of MLVs. Liposomes were prepared in 10 mM HEPES-Tris, 100 mM carboxyfluorescein (*CF*), 100 mM KCl, pH 7.4. External dye was removed by gel filtration through Sephadex G-75. At time 0, aliquots of the filtered liposomes were diluted 600-fold into the external medium (10 mM HEPES-Tris, 100 mM KCl, pH 7.4). At the time indicated (*first arrowhead*), 120 μM $Pb(NO_3)_2$ (*solid line*) or 0.15% Triton X-100 (*dashed line*) was added. At the end of the experiment with $Pb(NO_3)_2$, 0.15% Triton X-100 was added (*second arrowhead*).

tion of some inorganic cations (Hg^{2+}, Cu^+, and Tl^{3+}) in Cl^-/OH^- exchange mechanisms, we performed the experiments shown in Fig. 4 to investigate whether the effects of lead in Fig. 3 could be attributed to this mechanism. In these experiments an inwardly directed chloride gradient across the membrane of pyranine-containing MLVs was imposed for internal pH = external pH = 7.31. Any substance catalyzing a Cl^-/OH^- exchange should promote the efflux of OH^- from the intraliposomal compartment at the expense of the Cl^- influx, resulting in intraliposomal acidification. Under those conditions, both Tl^{3+} and triethyl lead produced rapid intravesicular acidification. Conversely, inorganic lead at much higher concentrations failed to alter the internal pH significantly (Fig. 4). Experiments equivalent to those shown in Fig. 3 but carried out in the absence of chloride ions also revealed a lead-mediated intraliposomal alkalinization (data not shown). All of these results support the idea that lead-mediated neutralization of acidic compartments is not due to a Cl^-/OH^- exchange. Similar results were achieved by using acridine orange instead of pyranine as a pH probe (data not shown).

The experiments summarized in Fig. 3 could be explained if a cationic hydroxyl species of lead, such as $Pb(OH)^+$, was the main permeating form of lead. Additionally, these effects could also be explained if lead exposure induced nonspecific damage of the membrane bilayer, which in turn would increase the proton permeability of liposomes, which is very low for this kind of lipid extract (13). Such nonspecific damage might be derived from alteration of membrane lipid packing by the interaction of lead with negative lipids, lipid peroxidation, or the generation of hydrolysis products such as lysophospholipids and free fatty acids. To investigate the contribution of all of these phenomena, we planned the following experiments.

In Fig. 5 we investigated the effect of lead on the leaking of liposomes. MLVs were loaded with a high (100 mM) concentration of carboxyfluorescein and filtered through Sephadex G-75 to remove external dye. At such a high concentration, carboxyfluorescein fluorescence is self-quenched. Leakage of the entrapped dye into the external buffer results in dilution and enhancement of fluorescence. As can be seen in Fig. 5, lead concentrations as high as 120 μM did not induce carboxyfluorescein leakage. Addition of Triton X-100 produced the release of entrapped dye, yielding maximal fluorescence. Light-scattering experiments (data not shown) revealed a nonsignificant alteration by lead of liposomal membrane permeability to glucose or KCl. On the other hand, inorganic lead concentrations as high as 450 μM failed to generate lipid peroxidation products in contact with liposomes, as determined by the thiobarbituric acid assay (21) (data not shown).

As a final test for lead-mediated nonspecific damage of myelin membrane lipids, we analyzed the chemical stability of myelin lipids, after exposure to lead, by two-dimensional HPTLC. Previous results indicated a specific interaction of Hg^{2+} with myelin PE plasmalogen, resulting in its hydrolysis and the generation of the lysophospholipid, so we considered it interesting to test this possibility for Pb^{2+}. As depicted in Fig. 6, lead exposure did not alter the mobility of any spot, in comparison with control. Additional spots or reductions of any lipid species, with respect to controls, were not detected. Conversely, Hg^{2+} exposure markedly reduced the spot corresponding to PE and generated a new spot overlapping with PI (Fig. 6, *arrows*). In summary, these results indicate that lead does not induce nonspecific damage of myelin membrane lipids.

To further confirm the participation of $Pb(OH)^+$ in total lead permeation across the lipid bilayer, we performed the experiments depicted in Fig. 7. Conditions were identical to those used in Fig. 1, but the pH of the external buffer was varied from 5.00 to 8.00 and a fixed amount of lead (1 μM final concentration) was added at the beginning of the experiments. As expected, lead permeation was inhibited at low pH values and accelerated at higher pH values.

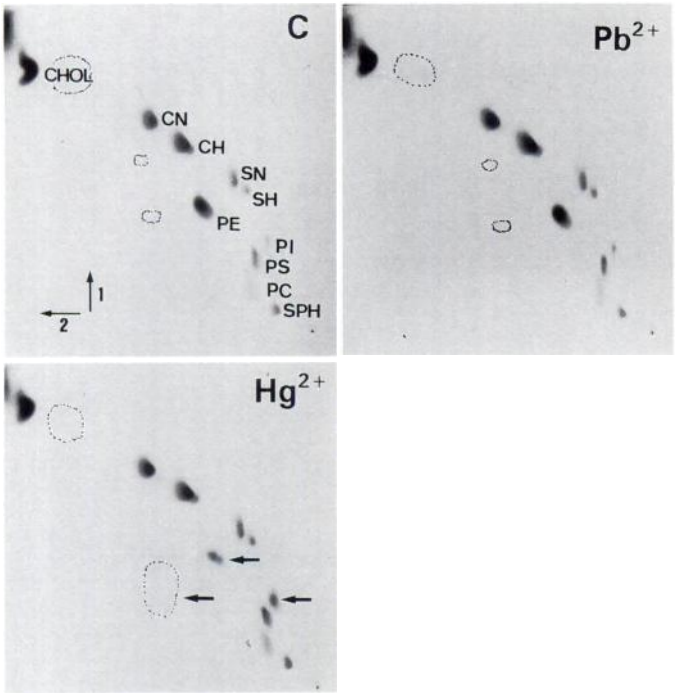

Fig. 6. Lack of induction by inorganic lead of a chemical alteration of any myelin lipid class. MLVs prepared in 10 mM HEPES-Tris, pH 7.4, were incubated with either 200 μM $Pb(NO_3)_2$ (Pb^{2+}) or 200 μM $HgCl_2$ (Hg^{2+}), at a final lipid concentration of 20 mg/ml, for 4.25 hr at room temperature. Total lipids were then extracted from each sample and analyzed by two-dimensional HPTLC as described in Experimental Procedures. In the control sample (*C*), metals were replaced by an equivalent volume of buffer. *CHOL*, cholesterol; *CN*, nonhydroxylated cerebrosides; *CH*, hydroxylated cerebrosides; *SN*, nonhydroxylated sulfatides; *SH*, hydroxylated sulfatides; *PS*, phosphatidylserine; *PC*, phosphatidylcholine; *SPH*, sphingomyelin. *Arrows in the lower plate*, differences with respect to the control plate. *Areas surrounded by dotted lines*, minor lipid classes present in the original plates and lost in the photographs.

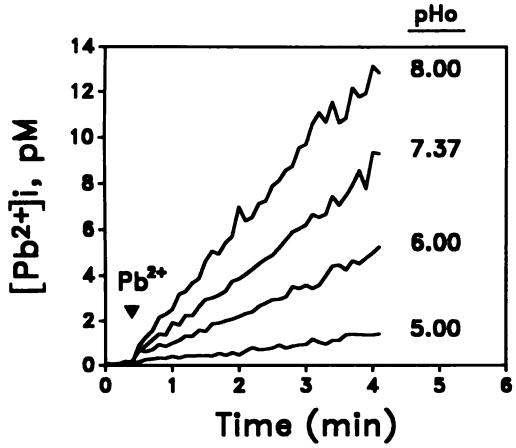

Fig. 7. Effect of external pH on lead permeation across the liposomal membrane. MLVs were prepared in 10 mM HEPES-Tris, 100 mM KCl, 1.5 mM EGTA-Tris, 40 μM fura-2, pH 7.4. Nonencapsulated fura-2 was removed as described in the legend to Fig. 1. At time 0, 50-μl aliquots of fura-2-containing MLVs were added to the cuvette, which contained 2 ml of 10 mM MES-Tris buffer (pH 5.00 or pH 6.00) or 10 mM HEPES-Tris buffer (pH 7.37 or pH 8.00), each containing 100 mM KCl. At the time indicated (*arrowhead*), the same amount of lead [final concentration, 1 μM $Pb(NO_3)_2$] was added in all cases.

Discussion

This paper reports a protein-independent downhill permeation of inorganic lead species through the membranes of myelin lipid liposomes. The intraliposomal lead concentration was clearly dependent on the total nominal lead concentration present in the assay medium (Fig. 1). The fact that lead permeation generates an internally positive transmembrane potential (Fig. 2) implies that some of the lead species that cross the membrane are positively charged.

At the pH values and lead and chloride concentrations used in the experiments of Figs. 1 and 2 (100 mM KCl, pH 7.4), the dominant Pb(II) species present in solution is $Pb(OH)^+$ (22), but other minor species carrying positive charge (i.e., Pb^{2+} and $PbCl^+$) might also contribute to the electrogenicity of lead permeation. Nevertheless, because inorganic lead dissipates a pre-established pH gradient across the membrane, by alkalinization of the internal acidic compartment (Fig. 3), the involvement of $Pb(OH)^+$ is more possible. Thus, external $Pb(OH)^+$ would cross the lipid bilayer by passive diffusion, reaching the internal acidic space and there reacting with H^+ to yield Pb^{2+} and H_2O. The whole process would lead to the dissipation of the initial pH gradient and the accumulation of lead inside the liposomes. This interpretation regards lead permeation as a consequence of a downhill passive diffusion of $Pb(OH)^+$ through the membrane lipids. However, an increase in the membrane permeability might be derived from a direct interaction of Pb(II) species with membrane lipid components, namely the interaction with the polar head groups of negatively charged myelin lipids (sulfatides, phosphatidylserine, phosphatidic acid, and PI). This interaction, which has been extensively studied for other divalent cations such as Ca^{2+} (23), would alter the membrane lipid packing from a bilayer state to nonlamellar structures, resulting in an increase of membrane permeability. If so, results depicted in Fig. 3 could be derived simply from the fact that exposure to lead increases the H^+/OH^- permeability of myelin lipids, which is normally very low (13). Moreover, other heavy metals, such as Fe^{2+} or Cu^+, may catalyze a Fenton-type lipid peroxidation reaction, and alteration of membrane permeability as a result of lipid peroxidation is well documented (24, 25). On the other hand, other toxic metals such as Hg^{2+} interact with myelin PE plasmalogen, leading to its hydrolysis and thus liberating lyso-PE and free fatty acids (26), which in turn may nonspecifically increase the membrane permeability.

To explore all of these possibilities, we performed the experiments of Figs. 5 and 6. Lead exposure did not produce membrane fusion (Fig. 5) and did not increase the membrane permeability to glucose or KCl (data not shown). Furthermore, lead itself did not generate peroxidation products in contact with myelin lipids, in contrast to Fe^{2+} (data not shown). In addition, lead exposure did not promote the chemical hydrolysis or alteration of any myelin lipid, in contrast to Hg^{2+} (Fig. 6) (26). Thus, it appears that the principal mechanism of lead permeation through the liposomal membranes is the passive diffusion of $Pb(OH)^+$. In agreement with this, lead entry was depressed at low external pH values and accelerated at higher pH values (Fig. 7). Interestingly, a recent report has suggested a similar mechanism for lead transport across the blood/brain barrier (27).

The results shown in this paper are relevant to the inter-

pretation of some effects of lead on intracellular functions. Numerous papers have reported the blockade of voltage-activated calcium channels by lead (28–30). In addition to this effect, several findings point out that lead readily crosses the channel and gains access to the cytoplasm (8, 9). However, significant lead entry into chromaffin cells occurs readily under nondepolarizing conditions, i.e., when calcium channels are closed (8, 9). Thus, additional mechanisms not yet reported must contribute to the total lead entry into the cells. Results shown in this paper might represent one of them.

With respect to myelin, inorganic lead exposure induces a severe myelination deficit (31). Although most of the experimental data on lead encephalopathy and peripheral neuropathy point to a loss of myelin as a consequence of secondary demyelination, early experiments showed that segmental demyelination was a predominant change in rats (1). It is important to note that the multilamellar myelin arrangement is stabilized by the electrostatic interactions of divalent cations with negative lipids at the intraperiod lines. Furthermore, several myelin-associated enzymes are activated by Ca^{2+}. One of them, calpain II, is a neutral protease that is able to degrade some myelin proteins that are critical for stabilization at the major dense line, such as myelin basic protein (32, 33). Perhaps lead, in addition to producing primary damage to other cellular targets such as oligodendrocytes or neurons, might also interfere with these Ca^{2+}-regulated processes essential for myelin sheath stabilization. It is worthwhile to note that a significant amount of lead was detected in the central nervous system myelin. Of the intrinsic trace metals in isolated myelin, levels of lead were highest on a weight basis and the second in importance, after zinc, on a molar basis (34).

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