Interaction of Lead and Bacterial Lipids

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Studies on the interaction of lead with lipid components indicate that individual lipids do not provide specific stable binding sites for lead, but that natural membrane lipid mixtures may simply provide an environment suitable for nucleation of lead.

Micrococcus luteus cells have the capacity to grow in a culture medium containing substantial quantities of lead salts. Not only are these cells able to grow in the presence of lead, but cell yields, respiration rate, and protein synthesis remain largely unaffected as compared with that of control cells growing in the absence of lead salts (10, 14, 15). One marked difference between control cells and lead-exposed cells, however, is the very significant drop in the free lipid contents in the cell membranes of the lead-exposed cell (10). This observation, coupled with the observation that lead is not found in the cytoplasm but rather is immobilized in the membrane fraction (14, 15), prompted us to consider that the lead may be immobilized by binding with specific lipids of the membrane. Such an interaction could prevent the lipids from being extracted by lipid solvents and, thus, account for the observed drop in the free lipids.

This paper describes the study that was conducted in an attempt to determine the extent to which established lipids of the membranes of M. luteus are involved in the specific site of interaction with lead. Interactions between lead and specific membrane lipids and lipid mixtures were examined by placing lipid-impregnated paper disks in contact with lead-containing solutions. Lead retention by specific membrane lipid fractions in the disk experiments are discussed in terms of compositional factors associated with lead retention.

MATERIALS AND METHODS

Culturing conditions. M. luteus ATCC 533 (9, 16, 17) was cultivated at 27°C by the shake culture method in flasks containing Trypticase soy broth (BBL). The cells were harvested from the broth preparations by centrifugation when the cells reached their early stationary phase of growth (48 h). The cells were washed twice with a saline solution previously described (14) and recentrifuged.

Lipid extraction and column fractionation. Washed cells were immediately suspended in saline solution and extracted by the method of Bligh and Dyer (2) for the removal of total free lipids. Virtually all of the lipids of this organism are membrane associated. The total lipid extract was then transferred to a column containing heat-activated silicic acid (Unisil, 325 mesh, weight ratio of silicic acid to sample 30:1). The lipids were fractionated with the following eluting solvents: hexane, benzene, chloroform, acetone, and methanol, to remove nonpolar lipids, glycolipids, and phospholipids, respectively. About 99% of the phospholipid phosphorus was in the methanol eluate (for descriptions of lipid compositions of M. luteus cells, see references 5, 7, 9, 10, 13, 16). Carotenoid pigments were present in all eluates except hexane.

Analysis of lipid contents. The lipid contents were routinely analyzed on silicic acid-coated thin-layer plates in solvent mixtures of (A) diethyl ether, benzene, ethyl alcohol, and acetic acid (40:50:2:0.2, by volume) as the first solvent; hexane and diethyl ether (96:4, by volume) as the second solvent for the separation of nonpolar lipids (3); and (B) chloroform, acetone, methanol, acetic acid, and water (50:20:10:10:5, by volume) for separation of polar lipids (6). Components were visualized by exposure to iodine vapors. Isolated components were scraped from the plates and the components were eluted with a mixture of chloroform, methanol, and water (10:5:1, by volume). Membrane hydrocarbons were isolated from the lipid extracts chromatographically in hexane and identified as described elsewhere (9, 16, 17).

Preparation of lipid-impregnated membrane disks. Total as well as fractionated and purified M. luteus lipids from the control cultures were studied for their chemical affinities for lead ions. The major lipid components of M. luteus are acyclic hydrocarbons (9, 16, 17), pigments (7), aminolipids (5), and phospholipids (5, 10). The following commercially prepared lipids (Supelco, Inc., Supelco Park, Pa.) were also included in this study: diphasphatidyl glycerol, phosphatidyl glycerol, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, tripalmitate, trioleate, tetradecane, and methylated as well as unmethylated, saturated, and unsaturated fatty acids.
Between 100 and 150 mg of total, fractionated, or pure lipids were applied in chloroform to each 47-
mm fibrous, membrane prefilter disk (Millipore Corp.). Disks not containing lipids and those contain-
ing tetracosane, an n-alkane, were used as controls. The disks were prepared in duplicate for each experiment. The disks were taken just to dryness under a stream of nitrogen followed by drying in vacuo over potassium hydroxide for 10 to 15 min. One disk was submerged in a small petri dish (50 mm) containing 10 ml of either lead bromide, lead nitrate, or lead acetate (0.1 to 0.4 mg of lead salt per ml of degassed, unbuffered distilled water at a pH between 6 and 7). The second disk was then added directly on top of the first one, permitting a liquid layer to remain between them. The two disks in the solution were allowed to stand overnight at room temperature. The pH of the solution remained constant throughout the experiment. The disks were then removed, placed on a sintered glass filter, and washed by filtration with 200 ml of water (greater quantities of water were ineffective in removing additional lead). The disks were dried as before and inserted into a membrane filter holder (Millipore Corp.) and washed successively with 200 ml each of chloroform, acetone, methanol, methanol-water (9:10, by volume), and water. The eluates were examined for lead and changes in properties of lipid components.

Analytical procedures. The eluates from the lipid-containing membrane disks (Millipore Corp.) were analyzed by spectrophotometric methods. Samples were analyzed for lead by dissolution in concentrated nitric acid followed by atomic absorption spectrometric analysis with a Varian Techtron AA-5 spectrophotometer. Visible and ultraviolet absorption spectra were recorded for each lipid fraction with a dual beam Perkin-Elmer 120 automatic spectrophotometer according to previously described procedures (11). Infrared spectra of isolated components were taken in thin films in carbon tetrachloride with a 257 Perkin-Elmer infrared spectrophotometer. Phosphorus determinations were made according to the colorimetric procedure of Allen (1). The lipid contents of each eluate from the lipid-impregnated disk were analyzed by standard chromatographic procedures described above.

RESULTS

The technique of using lipid-impregnated membrane disks was chosen for this study because of the experimental accessibility of the preparations and for ease of studying the interactions with relatively large amounts of both lipids and lead solutions. There were no significant differences in the results obtained in this phase of the study on the basis of different lead compounds employed; therefore, only the data using lead bromide are presented.

The results in Table 1 show that 4.5 × 10^4 ng (0.4% of total) of lead was retained by two control disks containing a total of 250 mg of tetracosane after water washes of the disk. Virtually all of the tetracosane was removed in the chloroform washing of the disks; methanol, and in some cases methanol-water washings, removed most of the lead retained by these impregnated disks. Comparable retention of lead and its elution from the disks was also observed in the control disks containing no lipids. With disks containing membrane lipids

| Table 1. Distribution of lead in different solvent eluates of membrane disks impregnated with the total or fractional lipids of M. luteus and exposed to a lead bromide solution* |
| Eluate | Controls | Total cellular-free lipid | Total non-phospholipids | Total phospholipids |
|--------|----------|---------------------------|------------------------|--------------------|
|        | Pb ng | Pb Pb | Pb | Pb | Pb | Pb | Pb | Pb | Pb |
| (10^10) | (% | (10^10) | (%) | (μg) | (%) | (10^10) | (%) | (μg) | (%) |
| Chloroform | 0.1 | 2.2 | 25.4 | 23.6 | 4,440 | 82 | 25.5 | 18 ChCl₄ | 18 ChCl₄ |
| Acetone | 0.4 | 8.9 | 39.6 | 36.8 | 96 | 1.8 | 5 | 6.3 | 1.4 |
| Methanol | 2.5 | 55.6 | 33.0 | 30.7 | 80 | 1.5 | 35 | 43.9 | 10 |
| Methanol-water | 0.7 | 15.6 | 3.3 | 3.1 | 9 | 0.2 | 12 | 15.1 | 0.8 |
| Disk (extracted) | 0.8 | 17.8 | 6.3 | 5.9 | 770 | 14.2 | 2.2 | 2.8 | 0.9 |

*Control consisted of 250 mg of tetracosane applied to two membrane disks; 250 mg of the total cellular-free lipid sample and 300 mg each of the combined nonphospholipids and total phospholipids were prepared in the same manner. All experiments were prepared in multiples of a 10 ml of lead solution consisting of 1,200 μg of Pb/ml of water. The data presented are an average of five experiments. Eluates consist of 200 ml of each solvent used to elute the lead and lipids from the disks. Pb was determined by atomic absorption spectrophotometry; phosphorus (P₁) was determined colorimetrically according to procedure of Allen (1).

* Values represent amounts of Pb that were resolubilized in 30 ml of chloroform or water, respectively, after the chloroform eluate was taken to dryness.
that were not treated with lead, about 90% of the lipids were eluted in the chloroform wash with complete lipid recovery after the methanol wash. For two lead-treated disks containing a total of 250 mg of extracted membrane lipids, $1.1 \times 10^5$ ng (9.0% of total) of lead was retained after water washes of the disks. Successive chloroform, acetone, and methanol washes of these disks contained nearly equal portions of lead (Table 1), even though the majority of the membrane lipids (82% lipid phosphorous) were removed in the chloroform wash. The quantity of lead retained, corrected for all controls, was 0.43 mg of lead/mg of lipid. This datum clearly indicates that the total membrane lipids are much more effective (nearly 25 times) at retaining lead than the pure tetracosane.

The recovered membrane lipids were unaltered chromatographically and relatively complete lipid compositions were detected in each of the chloroform, acetone, and methanol washes by thin-layer chromatographic procedures (for descriptions of representative chromatograms see reference 10). There were some moderate shifts in relative proportions of the diphasphatidyl glycerol and phosphatidyl glycerol and the 1,3- and 1,2-diglyceride. The increased quantities of phosphatidyl glycerol and 1,2-diglyceride in lipid solvent eluates accompanied by the detection of free phosphorus in the reaction mixture and water washes of disks indicate that 10 to 13% of the lipids are possibly degraded or altered. This is further supported by the presence of about 14% of the phospholipid phosphorus on the disks after all washes (Table 1). Small quantities of carotenoid pigments were also visibly retained. Methanolic-hydrochloride washings of these disks followed by a water wash were required to elute the remaining lipid content and lead. Trace amounts of hydrolyzed lipids, fatty acids, and acyclic hydrocarbons were detected in this methanolic-hydrochloride wash, indicating that there was an occurrence of bound or altered lipids in these lead-exposed disks.

In pursuit of defining specific interactions between lead and individual lipid components, membrane lipid subfractions and pure lipid components were studied in the same manner. The results obtained from membrane disks impregnated with either the total combined nonphospholipids or phospholipids obtained from silicic acid columns containing total free membrane lipids are given also in Table 1. The lead retained by 300 mg of total nonphospholipids and 300 mg of phospholipids was $7.97 \times 10^4$ ng (6.5% of total) and $5.49 \times 10^4$ ng (4.5% of total), respectively. The corrected lead retentions for the total nonphospholipids and phospholipids are 0.26 and 0.18 mg of lead per mg of lipid, respectively. These values are some 40 to 60% lower than the retention of lead by total cellular lipids. The elution profile of the lead clearly demonstrates that the retention of lead is not dependent on the lipid phosphorus content. In a manner similar to the total lipid disk experiments, substantially greater quantities of lead were retained by membrane-mixed, lipid-treated disks as compared to the control disks containing tetracosane. There are moderate unexplainable differences between the elution profiles of disks containing total cellular free lipids and those containing lipid subfractions. Approximately equal portions of lead were eluted in chloroform, acetone, and methanol washes from total membrane lipid impregnated disks; the chloroform, methanol, methanol-water eluates and the chloroform, methanol eluates removed the lead from disks impregnated with total nonphospholipids and phospholipids, respectively. An affinity of lead for phospholipids, however, appears apparent by the elution of 76% of the lead and 93.5% of the lipid phosphate in chloroform eluate of total phospholipid-impregnated disks.

Reduction in volume of chloroform washes of disks containing lipid subfractions (Table 1) from 200 ml to approximately 50 ml by evaporation at 40°C under a stream of purified nitrogen resulted in a heavy precipitation of materials. The chloroform washes were taken to dryness in vacuo. Solubilization studies on the residues revealed that 18,000 ng and 40,000 ng of lead of the nonphospholipids and phospholipids, respectively, were resolubilized in 30 ml of chloroform. The solid residue was soluble in distilled water. Similar insolubility problems were encountered during concentrations of other washes. The data demonstrated a definite increase in the solubilization of lead in a solvent-containing lipid mixture. Ultraviolet, visible, and infrared spectrophotometric analyses (as well as nuclear magnetic resonance analyses of a few preparations) of these chloroform-soluble preparations provided no interpretable data that could support the formation of a coordination complex between lead and lipid components. The spectra of the lipid components were the same with and without lead treatments. Chromatographically the lipid components were the same as those of the controls.
Individual microbial lipids and pure commercial lipids were placed on separate membrane disks and studied in the same manner. In none of these experiments was there significant retention of lead or increased solubilization of lead in organic solvents. The results from these experiments were identical to the tetracosane controls. Random mixing of the pure lipids showed increased retention of lead but the amounts retained were not comparable to the natural mixtures of lipids obtained from bacterial cells.

**DISCUSSION**

Studies with lipid-impregnated disks in contact with lead solutions demonstrated an appreciable retention of lead by complex lipid mixtures. A decrease in the retention of lead was observed by lipid contents that were less than natural mixtures. None of the individual free lipids isolated from extracts of intact cells nor pure commercial lipids were found to be effective in the retention of lead. These results, in part, should not be unexpected since zwitterionic lipids (phosphatidyl choline, phosphatidyl ethanolamine, etc.) should not strongly interact with cationic metals. Phospholipids common to *M. luteus* extracts are only moderately anionic lipids and apparently ineffective in producing detectable quantities of plumbated lipid components. Because phosphate groups and individual membrane lipids are amenable to interactions with lead (4, 12, 18), it was expected that stable, specific, lead-lipid formations would be found in the lipid extracts. Our principal conclusion is that this expectation was essentially wrong. Although the mechanisms responsible for the results observed are somewhat obscure, the results indicate that there is lead binding to lipids but that the nature of the interaction is rather non-specific. The stability of lead in lipid milieu has been established whereas spectrophotometry and chromatography of solubilized lipid-lead preparations provided no evidence for the occurrence of stable ionic or covalent bonds between lead and lipid components. These results are therefore explainable on the combination of strong affinity of lead to associate with any chemical group capable of associating with hydrogen ions and the combination of electronic and steric factors present in lipid mixtures. This constitutes a proposal of a type of macromolecular complexing phenomenon. This proposal may provide some basis for explaining the apparent casual relationship that occurs between cells, specific cellular components, and lead.

Previous studies in mammalian systems suggest that lead-protein binding contributes to a tertiary structure or aggregation of protein units to form inclusion bodies (8). The report has virtually excluded lipid involvement. The data presented here show that there is a definite relationship between the lipid contents of cellular membranes and lead. The nature of this relationship suggests that the lipids could also have a role in the formation of inclusion bodies and the transport of lead.

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