Effects of Lead on the Lipid Composition of Micrococcus luteus Cells

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Micrococcus luteus cells cultivated in medium containing lead salts exhibited a sequence of changes in the quantity of total cellular lipids with essentially no changes from normal cellular yields. The lipid composition of cells cultivated one to four times was moderately decreased (phase I) whereas that of cells cultivated five to six times was reduced by as much as 50% (phase II). Cells cultivated more than six times in lead-containing media had progressively greater quantities of lipid (phase III) approaching that found in control cells. These cells with reestablished lipid contents showed no further effects from more prolonged exposure to lead salts. Chromatographic studies of total lipids of cells of each lipid phase revealed relatively complete lipid compositions. These results indicated that lead is apparently affecting a common biochemical parameter in the biosynthesis of lipids of lipid phase II cells. Changes in the relative quantities of individual components were observed in both the nonpolar and polar lipids in each lipid phase. The most notable changes were the decrease in aliphatic hydrocarbons with concomitant increases in the diglycerides and components identified as a complex family of ketones. Microscopy examinations of control and lead-treated cells revealed electron dense inclusion bodies in membrane fragments in only lead-treated cells.

Previous studies in our laboratory (30, 31) have demonstrated that virtually all of the lead taken up by the bacterial cells was immobilized in the cellular envelope and associated largely with the cellular membrane. Investigations into the mechanisms of lead's immobilization and manifestation of toxicity in bacterial cells are completely lacking. Since specific lipids have been reported to form stable complexes with lead, usually under specific experimental conditions (11, 20, 38), it appears possible that the lipids of the membrane could have a role in the immobilization of lead and be a source of relatively small increments of lead transport to other parts of the cell. To determine the extent that lipids are involved, this paper describes the effects of lead on the total and individual membrane lipids of the natural skin bacterium Micrococcus luteus. The total lipid composition of the cells will be shown to be directly influenced by the presence of lead whereas essentially no differences are observed in culture yields. An accompanying paper describes in vitro studies on the interaction between specific lipids and lead.

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MATERIALS AND METHODS

Culturing conditions. M. luteus ATCC 533 (14, 21) was cultivated at 27°C by the shake culture method in flasks containing Trypticase soy broth (BBL) with and without lead salts. Lead bromide or lead nitrate salts (0.6 mg/ml) were introduced into respective flasks containing 100 or 200 ml of culture medium. This direct exposure method, as opposed to the indirect method of introducing lead via dialysis membranes (30), appeared to have no observable effect on the cultivation of the cells. After autoclaving, only about 20% of the total lead remained solubilized or dispersed in the growth medium. The capability of bacteria to readily abstract additional lead from water-insoluble precipitates has been previously acknowledged (30). 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 cold 0.05 M phosphate buffer (pH 7.6) (10) and recentrifuged. A 5-ml suspension of 0.1 ml of wet-packed cells of the harvested and washed control (no lead) or lead-treated cells was used as the inoculum for the respec-
tive next cultivation. This procedure was repeated until significant variations between the control and lead-treated cells were observed. In this manuscript, the cell preparations are identified by the number of consecutive cultivations or number of growth cycles. Changes in cell sensitivity to lead were continuously monitored by the impregnated disk technique of Novick and Roth (22). As the test organism passed through various states of sensitivity to lead, the lead cells, as well as the simultaneously cultivated control cells, were cultivated for one growth cycle in 100 ml of Trypticase soy broth (30). The medium contained 0.6 mg of lead acetate per ml and 200 μCi of [2-14C]acetate or 500 μCi of [32P]orthophosphate. The cultures were harvested in early stationary phase of growth by centrifugation and washed as described. Portions of centrifuged cells were dried by lyophilization, and specific yields were determined gravimetrically. Cellular dry weights were corrected for precipitated lead by determining the amounts of lead by atomic absorption spectrometry (30). Both wet-packed and dried cells were extracted for lipid contents.

Microscopy examinations of cells. Washed, lyophilized cells representative of cultures subjected to increasing periods of lead exposure were reconstituted in distilled water and applied in dilute suspension onto small mica squares. Specimens were coated with gold and examined on a Hitachi HHS-2R scanning electron microscope.

The isolation of cellular membrane, digested cell wall, and cytoplasm of control and lead-treated cells was accomplished according to previously described procedures (30). The remaining 0.6 mg of lead acetate was extracted by sonication of aqueous suspension. Carbon-stabilized, collodion-coated grids were prepared according to the method described by Dowell (8). A drop of the membrane suspension in distilled water was placed on a coated grid and allowed to stand for several minutes. The grid was drawn off with filter paper and dispersed with liquid allowed to evaporate. A very thin coat of carbon was evaporated over the unstained, fixed membrane preparations. Electron microscopy examinations were performed on a Hitachi HU-12 equipped for high contrast microscopy.

Lipid extraction and column fractionation. Fresh cell preparations were suspended in 0.05 M phosphate buffer, pH 7.6, and extracted by the method of Bligh and Dyer (6). The extracted cell debris was then reextracted in 0.1 M acetate buffer (pH 5.0) as described by Bertsch et al. (5). These combined extracts are referred to in this paper as the total lipid fraction. The lipid-extracted cell debris remaining was refluxed in 2.5% methanolic-hydrochloride for 1 h and extracted four times with large volumes of petroleum ether to remove any bound lipid material. The growth medium as well as the cell washes were extracted for lipid materials by partitioning with chloroform. To prevent loss of lipid-lead complexes that may exist with modified lipid properties, the lipid extracts were not passed through Sephadex G-25 columns to remove possible uncomplexed lead salts. Total lipids were fractionated on heat-activated silicic acid columns (Unisil, 325 mesh, weight ratio of silicic acid to sample 30:1). Eluting solvents were hexane, benzene, chloroform, acetone, and methanol (21). About 99% of the phospholipid phosphorus eluted in the methanol fraction (for descriptions of lipid composition of M. luteus see references 12, 16, 21, 23, 33).

Total lipids were also dissolved in a minimum of chloroform and the solution was diluted with 10 volumes of acetone and kept at 0°C overnight. After centrifugation to remove the precipitated polar lipids, the supernatant liquid containing the nonpolar lipids (including the yellow carotenoid pigments) was brought to dryness in vacuo; these lipids were stored in acetone at 0°C. The precipitated polar lipids and column-fractionated polar lipids were decylated by mild alkaline methanolysis and chromatographed on columns (6 mm by 81 cm) of Dowex 1-8x (200 to 400 mesh) in the formate form and eluted with an ammonium formate-sodium borate gradient as previously described (26).

Alkaline hydrolysis. Polar lipid components were decylated by mild alkaline methanolysis according to the procedure described by Tornabene and Ogg (35). Glycero phosphat esters were recovered from the water-methanol layer. Fatty acids were recovered from the chloroform layer.

The nonpolar lipids containing the carotenoids that were stored in cold acetone were saponified in 10% methanolic-KOH at room temperature for 2 h. The reaction was stopped by addition of 0.9% saline solution. The nonpolar lipids were extracted with large volumes of diethyl ether for the quantitation of carotenoids as previously described (17).

Thin-layer chromatography. Total and column-fractionated lipids were routinely analyzed on silicic acid-coated thin-layer plates in solvent mixtures: (A) diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, by volume) as the first solvent and hexane-diethyl ether (96:4, by volume) as the second solvent for the separation of nonpolar lipids (9); and (B) chloroform-acetone-methanol-acetic acid-water (55:20:10:5, by volume) for separation of polar lipids (15). Components were visualized by iodine vapors or radioautography. Decylated water-soluble products were separated on cellulose thin-layer chromatographic plates (Eastman Chromatograms 6064, Rochester, N.Y.) with solvents of 3.8 M ethylenediaminetetraacetate and 0.7 M NH4HCO3 in 90 mM NH4OH containing 67% by volume, ethanol in the first dimension and isobutyric acid-water-concentrated NH4OH (66:33:1, by volume) in the second dimension as previously described (26, 29). The compounds were detected by radioautography.

Carotenoid preparations were separated on silica gel-coated plates in a solvent system of chloroform-methanol (95:5, by volume) (27) or 10% ethanol in petroleum ether (boiling point, 30°C) (19). Spots were visualized by scanning with an ultraviolet lamp.

Analytical methods. The dry weight of the extracted lipid was obtained by gravimetric analysis. Fatty acid methyl esters were prepared by esterification with 2.5% methanolic-hydrochloride (13). Hydro-
carbons and derivatized lipids were analyzed on an F and M 5750 gas chromatograph equipped with dual flame ionization detectors. Chromatographic analyses were carried out on a stainless-steel column (93 m by 0.075 cm) coated with Igepal C0990 (21, 34).

Visible and ultraviolet absorption spectra of the total lipids in 15% acetone in petroleum ether or in ethanol were recorded with a dual beam Perkin-Elmer 120 automatic spectrophotometer. The quantitation of the total cellular carotenoid pigment content was calculated assuming an E1%1 cm of 3 × 10^4 at 468 nm (17). Infrared spectra of isolated components were taken in thin films in carbon tetrachloride with a 257 Perkin-Elmer infrared spectrophotometer. Samples were analyzed for lead by dissolution in concentrated nitric acid followed by atomic absorption spectrometric analyses with a Varian Techtron AA-5 spectrophotometer. Phosphorus determinations were made by the colorimetric method of Allen (3). Protein determinations were made by the method of Lowry et al (18). Oxygen consumption of washed cell preparations was determined with a Clark oxygen electrode.

Measurement of radioactivity. Radioautographs were made with Kodak no-screen X-ray film. Samples were assayed for radioactivity in a Beckman LS-133 Scintillation system with a mixture of Aquasol (New England Nuclear)-acetic acid-water (435:40:25, by volume) as the scintillation fluid and on aluminum planchets monitored with a thin-end window Geiger-Müller counter. Radioactive samples of the deacylated lipids recovered from the Dowex-1 column were dried in separate scintillation vials in a 60°C oven overnight and suspended in the above described scintillation fluid and counted.

RESULTS

Effects of lead on total cellular lipids. Total lipid extractions were obtained from cells by employing both neutral and acidified pH solvent systems. The two separate extraction procedures were performed to remove all lipid-soluble materials while preventing any disruption of lead-lipid complexes that may be formed, and which could be removed intact with a neutral lipid extraction. The distribution of 32P and 14C activity in various lipid extracts of control and lead-treated cells as well as the growth medium are shown in Table 1. The convenient and reproducible neutral pH extraction of Bligh-Dyer was consistently more effective in removing lipids from lead-treated cells of consecutive growth cycles 1 to 7 than from control cells, indicating the possibility of the lipids of the lead-treated cells being more readily accessible to the neutral extracting solvents. In lead-treated cells after the 7th consecutive growth cycle, the efficiency of lipid removal by the neutral pH extraction procedure was comparable to that observed for the control cells. The acidified pH solvent extraction procedure effectively removed all the remaining lipid-soluble materials from the cells as indicated by the trace amounts of fatty acid esters detected in methanolic-hydrochloride hydrolysates. This more firmly bound free lipid accounted for only 0.3 to 7.0% of the total fraction. Extraction of lipids from intact lead cells with solvents containing ethylene diaminetetraacetic acid produced no major differences in the quantity of lipids obtained. Only traces of 14C- and 32P-labeled lipids were detected in the growth medium indicating that the bacterial cells were not leaking significant quantities of membrane lipids.

The amounts of lead found in the lipid extract of cells grown in lead-containing media were on the order of 0.005 to 0.03 μg of lead per mg of lipid. Between 15 to 23% of the lead abstracted by the cells was in the methanolic-water phase of the lipid extracts. In the lipid-extracted cell debris, 75 to 82% of the lead abstracted by the cells was detected. Repetitive washing of the extracted cell debris with various solvents or

| Extraction of lipids                  | Controls | No. of growth cycles in medium containing lead |
|--------------------------------------|----------|-----------------------------------------------|
|                                      | 1-4      | 5-7                                           | 8-10                                           |
|                                      | 14C  | 32P  | 14C  | 32P  | 14C  | 32P  | 14C  | 32P  |
| Neutral Bligh-Dyer                   | 80.5 | 93.7 | 99.3 | 95.7 | 98.4 | 96.1 | 79.8 | 93.0 |
| Acidified Bligh-Dyer                 | 5.8  | 6.2  | 0.3  | 4.1  | 0.5  | 3.8  | 4.8  | 6.9  |
| Chloroform partition of culture      | 0.2  | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | 0.8  | <0.1 |
| medium                               |        |      |      |      |      |      |      |      |
| Petroleum ether partition of metha-  | 13.5 | 0.4  | 0.9  | 14.6 |      |      |      |      |
| nolic-hydrochloride hydrolysate      |        |      |      |      |      |      |      |      |

*The assay of radioactivity from 14C- and 32P-labeled cells were carried out using a Beckman LS-133 scintillation system. Results presented for control and lead-exposed cells are an average of three separate experiments. The results are shown as percentage of total lipid-soluble counts per minute.*
tris(hydroxymethyl)aminomethane buffer containing 5 mg of ethylenediaminetetraacetate per ml of neutral pH was ineffective in eluting additional lead. Reduction of sulfhydryl groups of extracted cell debris with parachloryl-mercuriophenylsulfonic acid and washing with tris-(hydroxymethyl)aminomethane buffer had little effect on the retention of lead. The mechanisms by which lead is contained in this fraction remain obscure.

Figure 1 shows the total lipid contents for lead-treated and control cells. The results presented are those for Pb(NO$_3$)$_2$-treated cells unless otherwise stated. PbBr$_2$ and Pb(NO$_3$)$_2$ salts in the growth media had the same effect on _M. luteus_ cells; however, the response to PbBr$_2$ was slower. The yields of cells grown in the presence of lead fluctuated somewhat at times but on the average there were no significant differences between the yields of lead-treated and control (non-lead-treated) cells. The effects of lead on the cellular lipids has consistently resulted in three describable phases. Phase I is that area where the lipid content of lead-treated cells from one to four consecutive cultivations remains somewhat lower than that of control cells (Fig. 1); phase II is that area represented by the fifth to sixth consecutive cultivations that shows the significant drop in lipid content to more than one-half that of control cells; and phase III is that area after the sixth harvest that is indicated by lipid contents that are comparable to the amounts obtained for phase I cells. These phases correspond with observable changes in pigmentation of the intact cells. Phase I cells often showed a linear decrease in pigment content over the first four harvests whereas in some runs the loss of carotenoid pigmentation occurred after the initial exposure to lead. The pigmentation in phase II occurred after the initial exposure to lead. The pigmentation in phase II cells was always reduced to just a visibly detectable yellowish tint in contrast to the rich, deeply yellow pigmented control cells. Due to this inconsistency, the loss in pigmentation could not be used as a visible indicator for the occurrence of phase II. However, in phase III cells the bright yellow pigmentation was reestablished. The cellular pigmentation remained visibly stable and comparable to the control preparations over the next 30 days (harvest 22), at which time the experiment was terminated. The lipid profile presented in Fig. 1 and just described is readily reproducible; however, it must be pointed out that the occurrence of each phase may vary from two to three consecutive growth cycles depending on the experimental conditions. Phase II rarely lasted more than two growth cycles and its initiation and duration was not exactly predictable.

**Effects of lead on cells of lipid phases.** Microscopy examination of lead-treated cells from lipid phase II showed evidence of intracytoplasmic leakage. Attempts to prepare protoplasts of lead-treated phase II cells by the lysozyme treatment previously described (30) were not always successful; the protoplasts often lysed after brief lysozyme treatments. The results suggested an osmotically sensitive cellu- lar condition. No irregularities were observed in the control cells. Light and electron microscopy examinations of intact micrococcal cells demonstrated no observable irregularities between the cell surfaces (cell walls) of the treated and untreated cells. These observations suggest that the unstable osmotic condition of the protoplasts of lipid phase II cells after removal of the cell walls, and the leakage of intracellular materials from intact lead-treated cells, are due to instability of the cytoplasmic membrane. These findings are supported by the fact that cellular immobilization of lead appears to be largely associated with cell membranes (30). Electron microscopy examinations of membrane preparations of control and lead-treated cells of lipid phase II are shown in Fig. 2A–C. The membrane preparation from control cells (Fig. 2A) reveals the rupture of the cytoplasmic membrane, some membrane enfolding, and granular material interpreted to be cytoplasmic and cell wall debris. The micrographs reveal electron dense aggregates in or on membrane fragments of lead-treated cells only (Fig. 2B–C); X-ray microanalysis shows these aggregates to

![Fig. 1. Changes in total lipid content of control (---) and lead-treated (-----) M. luteus during consecutive growth in medium containing lead salts.](image-url)
FIG. 2. Electron micrographs of representative unstained and unfixed membrane subfractions from (A) control cells and (B, C) cells exposed to lead bromide.
be amorphous complexes that contain lead. Lead-treated cells from lipid phases I and III contained lead inclusion bodies; however, the protoplast and membrane preparations were more structurally intact than that of lipid phase II. It has not yet been established whether or not there are localized interactions between these electron dense aggregates and membranes as indicated by the micrographs and if the inclusions are directly responsible for the fragmentation of the membrane. However, the noticeable feature in virtually all membrane preparations from lead-treated cells of lipid phase II was the marked lack of structural consistency of the membranes (Fig. 2B-C).

Monitoring of cell sensitivity to lead by the impregnated disk method of Novick and Roth (22) revealed that the average size of the zones of inhibition for lipid phase I cells were comparable to those obtained for control cells (2.3 cm). The zones of inhibition for lipid phase II cells fluctuated somewhat but the average zone was slightly larger (2.8 cm) than that of control cells. The toxicity of lead became more variable than the control cells in all preparations of cells in lipid phase III. Zones of inhibition varied from no zone of inhibition to those sensitivity levels that were consistently obtained for control cells. Perhaps the inconsistencies in the disk method are based on the fact that when lead-treated cells are cultivated in lead-free media a recovery of the cells is observed; the seemingly less stable unpigmented cells of lipid phase II are restored to apparent normal states after the second consecutive cultivation. Restoration of the cells was determined by osmotic stability of protoplast preparations, spectral analyses of carotenoid content, and electron microscopy examination of cell membrane preparations.

Quantitation of the protein content of membrane subfractions of control and lead-treated cells showed that the two preparations were not significantly different. Proteins comprised 38.6 to 44.2%, by weight, of the membrane. Similarly little if any differences were observed in the rate of oxygen consumption by control and lead-treated cells.

**Nonpolar lipids.** The nonpolar lipids of cells cultivated in medium containing lead are shown in Fig. 3-5 and Table 2. The nonpolar lipids chromatographed on silicic acid-coated plates in nonpolar lipid solvent A revealed that the same distribution of lipid components occurs in both the neutral pH lipid extract and the acidified lipid extract (Fig. 3). The removal of small amounts of more tightly retained nonpolar lipids in the acidified extracting solvent is in

![Image](image-url)

**Fig. 3.** Radioautograms of **C-labeled total lipids from control and lead-treated M. luteus after separation on silica gel G-coated thin-layer plates. Chromatograms were developed in solvent system A. Spots were also visualized by exposure to iodine vapors. HC, Aliphatic hydrocarbons; KE, ketones; TG, triglyceride; 1,3 DG, 1,3-diglyceride; 1,2 DG, 1,2-diglyceride; FA, fatty acids; MG, monoglyceride; PL, polar lipids.
accord with previous findings (1, 28). Four major components were visualized in both control and lead-treated cells. The distribution of $^{14}$C among these components is described in Table 2. The most pronounced difference in the nonpolar lipids of lead-treated cells was seen in the reduction of the aliphatic hydrocarbons (Table 2). The hydrocarbons decreased by more than 98% in lipid phase II cells, whereas a decrease of 78 and 66% were recorded for lipid phase I and III cells, respectively. The distribution and relative proportions of the individual hydrocarbon components obtained from cells in each of the lipid phases were unaltered (for description and identification of the hydrocarbons see references 21, 33, 34). Components identified as a family of ketones were found in only minute quantities in the control cells (1, 36); the quantities of these ketones increased sharply in lead-treated cells. The exact identities of these ketones and their metabolic role in \textit{M. luteus} is under investigation and will be reported elsewhere. The relative proportions of the diglycerides changed in each of the lipid phases with the 1,2-diglycerides becoming more predominant in extracts from cells of lipid phase III. Only trace quantities of monoglyceride were detected. In all repetitive experiments with control cells, no significant variations in the relative quantities of the individual lipids were observed in the lipid extracts.

Spectrophotometric analyses of the total non-

![Fig. 4. Visible spectra of total carotenoid content of control (2.6 mg of lipids/ml, solid line), lead nitrate-treated (7.8 mg of lipids/ml, dotted line), and lead bromide-treated (5.9 mg of lipids/ml, dashed line) cells in 15% acetone in petroleum ether. Extracts were from the fifth consecutive complete growth cycle.](image)

![Fig. 5. Ultraviolet spectra of total lipid content of control (1.2 mg of lipids/ml solid line), lead nitrate-treated (1.4 mg of lipids/ml, dotted line), and lead bromide-treated (1.5 mg of lipids/ml, dashed line) cells in ethanol. Extracts were from cells harvested from the fifth consecutive complete growth cycle.](image)

| Component           | Control | Lipid phases* |
|---------------------|---------|---------------|
|                     | I       | II            | III           |
| Hydrocarbons        | 73.0    | 15.6          | 1.3           |
| Ketones             | 2.5     | 14.4          | 11.4          |
| 1,3-Diglycerides    | 16.4    | 35.8          | 39.8          |
| 1,2-Diglycerides    | 9.7     | 34.0          | 47.4          |
| Monoglycerides      | <1.0    | <1.0          | <1.0          |

*The nonpolar lipids from $^{14}$C-labeled cells were fractionated on a silicic acid column followed by silica gel G-coated thin-layer plates in solvent system A. Components visualized with iodine vapors were scraped from the plate directly into scintillation vials and counted; values are expressed as percentage of total counts per minute.

*For description of lipid phases, see Fig. 2.
polar lipid fractions confirmed that the carotenoid pigments absorbing in the visible spectrum were either absent or greatly reduced in quantities (Fig. 4) in cells cultivated in lead-containing media. The carotenoid content of cells isolated from lipid phase II (Fig. 1) was consistently 91 to 98% lower than that found in the control cells. The slower response of PbBr₂-exposed cells is evident in the absorbance of the relative spectra.

To determine if the reduced absorbance was due to an alteration of the carotenoids by the presence of trace quantities of lead in the lipid extract, the carotenoid fraction was extracted free of lead by partitioning in a solution of methanol-chloroform-water containing 0.01% ethylenediaminetetraacetate. Re-examination of the pigment contents showed no measurable differences in their spectral properties.

The chromatographic separation of total carotenoid pigments obtained from saponification of acetone-soluble nonpolar lipids (4, 19) showed near identical patterns between control and lead-treated cells with the exception of a minor change in the Rᵣ value of only one minor component.

Spectral analyses in the ultraviolet region shows components absorbing between 325 to 224 nm (Fig. 5), characteristic of napthoquinone (vitamin K) structures (24, 32). The vitamin K components were neither quantitatively nor qualitatively altered in the course of this study.

There was no evidence for stable lipid-lead complexes formed under these experimental conditions. This conclusion was based on the similarities in the chromatographic separation of lipids from control and lead-exposed cells and the failure to detect by atomic absorption spectroscopy quantities of lead in isolated lipid components.

**Phospholipids.** The ¹⁴C- and ³²P-labeled polar lipids were fractionated from total lipids by cold acetone precipitation and columns of silicic acid to enhance the resolution of the lipid components by thin-layer chromatography. Essentially all of the ³²P-labeled lipids were recovered in the cold acetone precipitates and in the methanol eluates of silicic acid columns. No significant quantity of ¹⁴C activity was detected in the acetone eluate demonstrating the absence of a significant glycolipid fraction. The separation of the phospholipids obtained from culture medium and from neutral and acidified pH extracts of control and lead-treated cells is shown in Fig. 6. The components were identified by comparing their Rᵣ values to those of authentic standards and by two-dimensional chromatography of the deacylated water-soluble products as described previously (29). The relative proportions of the total deacylated water-soluble products of phospholipids of cells obtained in each of the three lipid phases are presented in Table 3. The phospholipids consisted of two major and two minor phospholipids in all fractions studied. The two major phospholipids were identified as diphosphatidyl glycerol and phosphatidyl glycerol comprising 18 to 25% and 75 to 82% of the total phosho-

![Fig. 6. Radioautograms of ³²P-labeled phospholipids from control and lead-treated M. luteus cells after separation over silica gel G-coated thin-layer plates. Chromatograms were developed in solvent system B. Spots were also visualized by exposure to iodine vapors. PA, Phosphatidic acid; DPG, diphosphatidyl glycerol; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PC, phosphatidyl choline; PI, phosphatidyl inositol.](image-url)
TABLE 3. Distribution of \(^{32}P\) activity among the major phospholipids of control and lead-exposed M. luteus

| Ester of deacylated lipid | Control | Lipid phases* |
|----------------------------|---------|---------------|
|                            | I       | II            | III           |
| GPG                        | 75.9    | 76.6          | 84.0          | 81.5          |
| GPPG                       | 24.6    | 23.2          | 14.6          | 18.0          |
| GPI                        | <1.0    | <1.0          | <1.0          | <1.0          |
| GP                         | <1.0    | <1.0          | <1.0          | <1.0          |

*The assay of radioactivity was carried out in a Beckman scintillation system and results are expressed as a percentage of total counts per minute. Various lipid components were fractionated on a Dowex-1 anion exchange column. GPG, Glycerolphosphorylglycerol; GPPG, diglycerolphosphorylglycerol; GPL, glycerolphosphorylimositol; GP, glycerophosphate.

* For description of Lipid phases, see Fig. 2.

lipid fractions, respectively. The two minor phospholipids identified as phosphatidyl inositol and phosphatidic acid were found in trace quantities in all cellular preparations (Table 3). Phosphatidyl glycerol was the only major phospholipid isolated from the growth medium. However, the quantities isolated were quite low comprising less than 0.1% of the total phospholipid. Quantitatively, the phospholipid compositions of control and lead-treated cells were not significantly different with the exception of a 9.0% decrease in the diglycerolphosphorylglycerol content of cells of lipid phases II and III (Table 3). The diglycerolphosphorylglycerol content in cells from lipid phase III was static at about 18% of the total phospholipids, some 6% lower than that of the control cells.

Quantitatively, the ratio of phospholipids to nonpolar lipids remained constant at approximately 3:1 for both control and lead-treated cell preparations. The most significant difference in the phospholipids, and thus the total lipids, of lead-treated cells is that the quantity of lipids of lipid phases I, II, and III cells was on the order of 11.2, 58, and 11.2% lower than that of control cells, respectively.

**DISCUSSION**

The effects of lead on *M. luteus* described in this report are, in part, similar to those previously reported (30) in that lead in a culture medium in concentrations approaching solubility limits had no detectable effects on over-all growth rate and viability. These findings are further supported in this study by the closeness of the values of respiration rates, cellular membrane protein, and uniformity and thickness of cell walls of control and lead-treated cells. The most unusual features detected for the effects of lead on these bacteria are the changes in their total lipid contents. Initially, exposure to lead resulted in a moderate drop in the lipid content with a noticeable decrease in cellular pigmentation. A second phase was marked by some 50% decrease in the lipid content with little detection of pigmentation; this phase of the cells is relatively short rarely lasting more than two consecutive cultivations. The cells of this second lipid phase showed evidence of membrane instability suggesting a relationship to the drop in total membrane free lipids. These cells then proceeded into a third phase by reestablishing their lipid and pigment contents and showing no further effects from lead on more prolonged exposure.

The basis for the recovery of the cells comprising the third phase has not yet been determined; however studies are currently underway to test the possibility of genetic resistant factors and the selection of a lead-resistant population. The results of these studies will be reported elsewhere.

The reduction in the total lipid content per cell weight appears to be the result of less lipid formation by the cells since no significant lipid content was detected in either the lipid extracted cell debris or the culture media. That cells may contain a reduced lipid content in their membranes without affecting cell viability and yield is supported by the work of Salton and Schmitt (25) and White (39), who showed that many lipids present in normal cells are not necessary for a functional membrane system in bacteria. The depressed lipid contents contained nearly equal quantities of nonpolar and polar lipids, which is comparable to the ratios found in control cells. The lipid compositions were essentially complete, with the individual lipids showing varying degrees of quantitative changes. In the nonpolar lipid fraction the accumulation of a family of ketones and an increase in the diglyceride content with a concomitant decrease in hydrocarbon content (Table 2) infers a relationship between these lipids and hydrocarbon synthesis. Apparently the inhibitory effect of lead on the hydrocarbon synthesis is not unique since Albro and Dittmer (2) have previously reported that the content of iron ions in cell-free extracts of this same test organism has inhibiting effects on the formation of hydrocarbons. In the polar lipid fraction, the relative intensities of the lipid components were also not greatly altered. The effect that lead has on the ratio of diphasphatidyl glycerol and
phosphatidyl glycerol in the lead-exposed cells may be related to cardiolipin synthetase which has previously been reported to be responsible for shifts in ratios of these two phospholipids in *M. luteus* (7), or simply the results of partial lipid degradation. The fact that lead promotes the reduction of total lipids, with the maintenance of relatively complete lipid compositions, suggests that at least one of the biosynthetic target sites of lead is a common biochemical parameter involved in biosynthesis of lipids.

None of the individual free lipids extracted from intact cells were found to be associated with lead. The datum was somewhat expected since the exposure to lead resulted in reduction of the total lipid composition. The absences of functionally specific plumbated lipids are supported by an accompanying report (37) that demonstrates that only a mixture of cellular lipids have sufficient properties for retaining substantial amounts of lead. In view of these results that have established relationships between lead and cellular lipids, it appears possible that the membrane lipids could provide the environment suitable for nucleation of the observed lead-containing aggregates. This proposal could have relevancy to mammalian systems where lead inclusion bodies are common in lead-exposed cells and where the uptake and retention of lead has been attributed totally to cellular proteins (20). The exact chemical nature of the lead-containing aggregates and their biological significance in *M. luteus* are under investigation.

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