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Lead Toxicity and Phosphate Deficiency in *Chlamydomonas*

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ABSTRACT

The addition of lead salts to phosphate-containing *Chlamydomonas reinhardtii* media caused precipitation of $Pb_3(PO_4)_2$, effectively removing phosphate from solution. The effect of Pb^{2+} on growth of *Chlamydomonas* in liquid cultures depended strictly on the ratio of the equivalents of Pb^{2+} to phosphate present. When the amount of Pb^{2+} approached equivalency with phosphate, cell growth was initially slow as cells adhered to the surface of the precipitated $Pb_3(PO_4)_2$. Later, cells grew at a normal rate, spread throughout the solution, and reached the same densities obtained in the absence of Pb^{2+}. Cells did not survive when the amount of Pb^{2+} in the culture exceeded the equivalents of phosphate.

Elemental analysis showed that in the presence of equivalent Pb^{2+} and phosphate, considerable Pb^{2+} remained in solution. The concentration of dissolved Pb^{2+} did not vary significantly when the amount of Pb^{2+} added to the culture was increased slightly, from an amount which permitted growth to an amount which completely prevented growth. The concentration of phosphate was decreased to an undetectable level when the amount of Pb^{2+} approached equivalency with phosphate.

In the presence of the chelating agent nitrilotriacetic acid, higher concentrations of Pb^{2+} remained in phosphate-containing media. The chelated Pb^{2+} did not retard the growth of *Chlamydomonas*.

It appears that Pb^{2+} is not toxic to *Chlamydomonas*, but kills cells by depriving them of phosphate.

Previous studies have shown that Pb^{2+} adversely affects the growth of plants (2, 16). However, the study of lead toxicity is complicated in vascular plants since insoluble Pb^{2+} salts tend to deposit in the conducting xylem and may interfere with transport (4, 7). For this reason, unicellular algae may be more useful for studies of Pb^{2+} toxicity at the cellular and subcellular levels. A number of reports have shown that Pb^{2+} affects the growth of aquatic algae (9, 14). Lead deposits have been seen in and on algal cells in cultures containing Pb^{2+} (11, 12). Relatively few reports have demonstrated specific physiological effects of lead on growing algal cultures (10, 15), and these reports have not demonstrated any specific causes for the effects.

The purpose of this study is to demonstrate under what conditions Pb^{2+} adversely affects liquid cultures of *Chlamydomonas*, and to determine the mechanism whereby the cultures are affected.

MATERIALS AND METHODS

Algal Growth and Sampling. *Chlamydomonas reinhardtii* wild type strain 137 C(+) was grown photoheterotrophically in 500-ml conical culture flasks similar to the method of Gorman and Levine (6). Suspensions containing 250 ml were slowly agitated at approximately 28 C. Light, supplied continuously by Sylvania F40CW Lifeline fluorescent lamps, gave an intensity of 50 $\mu E \cdot m^{-2} sec^{-1}$ at the flask surface. Composition of the culture media (6) is listed in Table I. The phosphate concentration was varied as indicated in the text. Lead, as $Pb(NO_3)_2$, and other components were added as indicated. The pH of the medium remained at approximately 7.3 during exponential growth, and increased gradually to about 8.0 as cell density reached maximum.

In experiments using low concentrations of phosphate, all glassware used in the preparation of culture media was washed with 3 N HNO_3 and extensively rinsed with double-distilled H_2O. Cells were rinsed by centrifugation through phosphate-free media prior to inoculation into low phosphate flasks. Cell cultures were grown and maintained axenically.

Measurement of Specific Growth Rates. Five- or 10-ml aliquots of the culture suspension were removed periodically from growing cultures and Chl concentrations measured. From these measurements, exponential growth rates were determined. During exponential growth ln Chl concentration was calculated as a function of time, and the best straight line determined by linear regression of the data points. At least four data points were used for each regression. The coefficient of determination (r^2) was greater than 0.99 in nearly all experiments. The specific growth rate (k), in hr^{-1}, was determined as $(\ln A - \ln A_o)t^{-1}$, where A and A_o represent the two Chl concentrations at the end and beginning of any time interval, t, along the regression line. The standard error (S_k) of the regression coefficient k was virtually always less than 0.006 hr^{-1}. For each experimental treatment duplicate flasks were prepared. The two calculated k values seldom varied by more than 5% for identical treatments, and were averaged to give a final value of k for a particular treatment.

Growth rates were also determined by measuring the increase in total protein or packed cell volume in some experiments. Rates were identical to rates determined using Chl measurements.

Chl was determined by the method of Arnon (1).

Lead and Phosphate Analyses. Pb^{2+} concentration in solution was determined in aliquots of the culture by first centrifuging to pellet the cells and $Pb_3(PO_4)_2$ precipitate. The remaining clear solution was acidified with HNO_3 and analyzed with a Perkin-Elmer model 603 atomic absorption spectrometer.

Phosphate was determined in aliquots of the same solution by the method of Simon and Boltz (13), through formation and extraction of phosphomolybdate which was analyzed for Mo by atomic absorption.

RESULTS

When Pb^{2+} was added to *Chlamydomonas* culture medium at any concentration, a white precipitate of $Pb_3(PO_4)_2$ formed immediately. Table IIa shows the effect of adding various amounts on Pb^{2+} to culture flasks containing 1.07×10^{-3} M phosphate in normal growth medium. Pb^{2+} was seen to have very little effect on the growth rate of *Chlamydomonas* until the ratio of the equivalents of Pb^{2+} to phosphate exceeded 1.0. A 50% excess of Pb^{2+} over this ratio completely retarded growth and the cells eventually died. Table IIb shows that considerably less Pb^{2+} was required to inhibit culture growth in medium which was identical except for the presence of much less phosphate. In this case, too, inhibition occurred precisely when the equivalents of Pb^{2+} and

Table I. Composition of Chlamydomonas growth medium.

Compound	Moles/liter	Compound	Moles/liter
NH_4Cl	7.48×10^{-3}	$ZnSO_4$	7.65×10^{-5}
$CaCl_2$	3.40×10^{-4}	H_3BO_3	1.84×10^{-4}
$MgSO_4$	4.06×10^{-4}	$MnCl_2$	2.58×10^{-5}
Tris	2.00×10^{-2}	$FeSO_4$	1.80×10^{-5}
CH_3COOH	1.74×10^{-2}	$CoCl_2$	6.73×10^{-6}
Na_2HPO	6.45×10^{-4}	$CuSO_4$	4.65×10^{-6}
NaH_2PO_4	4.20×10^{-4}	$(NH_4)_6Mo_7O_{24}$	8.90×10^{-7}
Na_2EDTA	1.34×10^{-4}		

Table II. Effect of lead on growth of Chlamydomonas at different phosphate concentrations.

Growth rates were determined by measuring the increase in Chl concentration with time. In each experiment k was calculated and expressed as percent of the k value with no added Pb^{++}.

a. Cultures in 1.07×10^{-3} M phosphate. The k in the control (0 Pb^{++}) was 0.103 hr^{-1}.

	(Moles Pb^{++} added per liter culture) $\times 10^3$				
	0	0.4	0.8	1.6	2.4
Ratio $[Pb^{++}]/[Phosphate]$	0	0.25	0.50	1.00	1.50
Growth Rate (% of control)	100	101	93	96	0

b. Cultures in 2.1×10^{-5} M phosphate. The k in the control (0 Pb^{++}) was 0.094 hr^{-1}.

	(Moles of Pb^{++} added per liter culture) $\times 10^5$					
	0	0.8	1.6	3.2	4.8	8.0
Ratio $[Pb^{++}]/[Phosphate]$	0	0.25	0.50	1.00	1.50	2.50
% of Control Rate	100	102	99	88	63	0

phosphate in the flask were equal. At various phosphate concentrations, inhibition of cell growth always occurs at the point where the equivalents of Pb^{2+} equal the equivalents of phosphate in the culture. In all of these experiments in which Pb^{2+} was present, a precipitate was present, so the concentration of Pb^{2+} and phosphate in solution was considerably less than if all had dissolved.

When $Pb(NO_3)_2$ was replaced by $Pb(OCOCH_3)_2$ in the cultures, growth was retarded exactly as with $Pb(NO_3)_2$, with complete inhibition seen when Pb^{2+} reached equivalency with phosphate. The results did not differ when an equivalent amount of nitrate as $NaNO_3$ was also added to the culture. The results do not reflect any effect of nitrate on the culture.

At Pb^{2+}/phosphate equivalency ratio less than 1.0, the cells did not immediately go into suspension following inoculation, but adhered to the precipitate at the bottom of the flask. This did not prevent cell growth although initial growth rate was very slow. As culture growth continued, a deep green layer was seen to develop on the surface of the precipitate. Eventually cells went into free suspension, and the cell concentration then began to increase exponentially in the flask. Growth rates were measured during this exponential period. The lag which occurred in the presence of Pb^{2+} may be seen in Figure 1. Cultures containing no Pb^{2+} (top curve) were well into exponential growth when the first measurements were taken 48 hr after inoculation. Cultures grown in the presence of Pb^{2+} (middle and bottom curves) experienced a lag which in these experiments extended to about 56 hr following inoculation. A comparison of the middle and top curves shows that in the presence of Pb^{2+} equal to 95% of the phosphate equivalents, cultures eventually increased in density as rapidly as cultures containing no Pb^{2+}. In both treatments the calculated k during exponential growth was 0.092 hr^{-1}. The maximum cell density attained was also virtually identical in control flasks and in flasks containing Pb^{2+} equivalent to 95% of the phosphate present. When the Pb^{2+} content was increased approximately 10% to 105% of the phosphate equivalents, cell growth was noticeably retarded (bottom curve of Fig. 1). The maximum k obtained was only 0.070 hr^{-1}, and maximum cell density reached was considerably less than in cultures containing only slightly less Pb^{2+}. In other experiments a 20% increase in Pb^{2+} over phosphate equivalents was shown to decrease the growth to less than half of the control value while a 50% increase completely inhibited growth.

When cell growth had reached a stationary phase, the solution was centrifuged to remove the precipitate and suspended cells, and analyzed for lead and phosphate. Table III shows the Pb^{2+} and phosphate analyses, and is most easily interpreted by direct comparison with Figure 1. Although cell growth rate was the same as with no Pb^{2+} when the Pb^{2+} content was 95% of the equivalents of phosphate, the concentration of Pb^{2+} in solution did increase, as seen by comparing experiments 1 and 2 in Table III. Increasing the Pb^{2+} content another 10% considerably decreased cell growth but the concentration of Pb^{2+} in solution was virtually unchanged (experiment 4 in Table III). Therefore, the growth rate changes appreciably under conditions in which the Pb^{2+} concentration in solution changed very slightly, if at all, as the variation among experiments 2, 3, and 4 was within experimental error.

In contrast, the phosphate was depleted to a concentration below the level of detection when the Pb^{2+} content was 95% of the equivalents of phosphate. Although the sensitivity of the analysis precluded detection of any change in phosphate concentration as more Pb^{2+} was added to the flask, it is reasonable to assume that phosphate was further depleted.

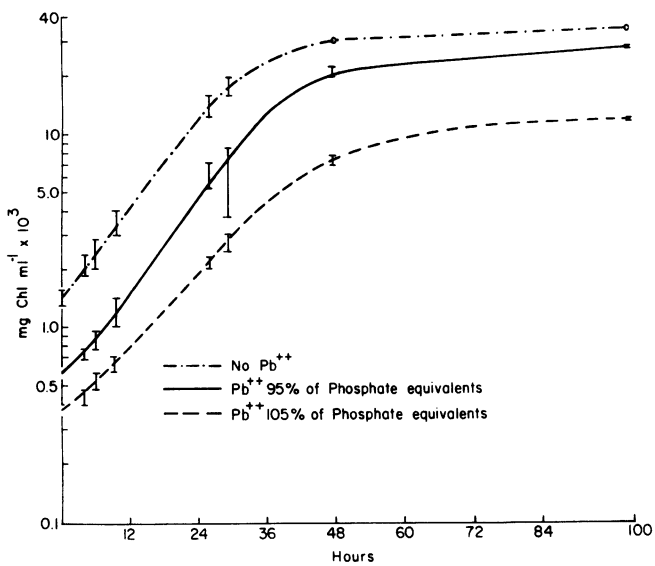

FIG. 1. Rate of increase in Chl concentration of *Chlamydomonas* in standard growth medium in the presence of various amounts of lead. Each flask was inoculated with cells equivalent to 8.0×10^{-6} mg of Chl/ml 48 hr prior to time zero. Error bars represent variation seen in values measured in duplicate flasks.

Table III. Analysis of Pb^{++} and phosphate composition of Chlamydomonas growth media containing various concentrations of Pb^{++}.

In all but the last experiment, cells were grown up to at least 1×10^{-2} mg/ml Chl, then centrifuged from solution along with the lead phosphate precipitate. The remaining clear solution was then used for analysis. The last experiment represents a flask not inoculated with algae. Each flask contained 1.67×10^{-3} M phosphate. The % of Pb^{++} equivalents is calculated with respect to the equivalents of phosphate. See Fig. 1 for growth curves of cells in cultures of Expts. 2 and 4.

Expt. No.	Pb^{++} Added	M Pb^{++} in Solution	M Phosphate in Solution
1.	50% Equivalents	3.2×10^{-5}	2.20×10^{-6}
2.	95% Equivalents	7.6×10^{-5}	$<0.3 \times 10^{-6}$
3.	100% Equivalents	7.8×10^{-5}	$<0.3 \times 10^{-6}$
4.	105% Equivalents	7.7×10^{-5}	$<0.3 \times 10^{-6}$
5.	100% Equivalents, no cell inoculation	4.0×10^{-5}	3.07×10^{-6}

Experiment 5 in Table III shows that when the solution of standard media containing equivalent amounts of Pb^{2+} and phosphate was separated from the precipitate in flasks containing no inoculum, less Pb^{2+} was present and considerably more phosphate was seen. A comparison with experiments 2, 3, and 4 indicates that actively growing cells removed the available phosphate from solution and increased the Pb^{2+} concentration by removing the counterion.

The above results indicate that *Chlamydomonas* is very effective in acquiring phosphate from dilute solutions. This is confirmed in Table IV. Cultures grew exponentially at a maximum rate in the presence of 1.1×10^{-5} M phosphate. Growth rates could not be measured accurately at phosphate concentrations much below this level because the initial rate of growth rapidly decreased as phosphate was depleted. In other experiments (data not shown) cells which were washed free of external phosphate and inoculated into phosphate-free medium were shown to double two or three times before growth ceased, indicating that the internal phosphate concentration of the cell may be considerably diluted before cell growth stops. If Pb^{2+} were affecting cell growth by removing phosphate from the media only, cell growth should not immediately cease when the culture is exposed to an excess of Pb^{2+}. Exponentially growing cultures were removed by centrifugation from standard media and transferred to media which were identical except for the presence of Pb^{2+} at a Pb^{2+}/phosphate equivalency ratio of 1.5. Total cell content in the flask as measured by Chl concentration increased slightly, and did not begin declining for more than 24 hr after inoculation into the Pb^{2+}-containing medium. When cells were killed by other methods such as brief exposure to 50 C heat, Chl concentration immediately began decreasing. This suggests that Pb^{2+} may not be causing immediate death of cells. Even at the rather heavy inoculation of cells used (0.25 mg of Chl into 250-ml flasks), virtually all cells adhered to $Pb_3(PO_4)_2$ precipitate.

The low solubility of $Pb_3(PO_4)_2$ (approximately 2.5×10^{-7} M in H_2O at pH 7.3) (5) greatly restricts the concentration of dissolved Pb^{2+} in media containing excess phosphate. NTA,[1] a Pb^{2+}-chelating agent, was added to culture media to prevent the precipitation of Pb^{2+} salts. Table V shows the growth rates of *Chlamydomonas* in the presence of various concentrations of NTA with and without added Pb^{2+}. A molar ratio of 2:1 for NTA to Pb^{2+} was used since greater relative amounts of Pb^{2+} did not prevent $Pb_3(PO_4)_2$ precipitation. NTA in the absence of Pb^{2+} is seen to inhibit cell growth at 3.2×10^{-3} M concentration. Pb^{2+} appeared to have no effect since no more inhibition is seen in the presence of Pb^{2+} than in its absence at any NTA concentration. Note that the level of Pb^{2+} in solution is about 100 times higher than that measured in the absence of NTA (Table III).

Table IV. Growth rate of Chlamydomonas on various concentrations of inorganic phosphate.

Growth rates were determined by measuring the increase in Chl concentrations with time. The k value for cells in 1.07×10^{-3} M phosphate was defined as the 100% rate, which represented 0.089 hr^{-1}. Other values are expressed as % of that control rate.

	Phosphate Concentration (M) X 10^4						
	10.7	1.67	0.53	0.21	0.11	0.053	0
k (% of Control)	100	96	102	99	100	60	0

Table V. Effect of NTA and Pb^{++} on the growth rate of Chlamydomonas.

Growth rates were determined by measuring the increase in Chl concentrations with time. In the control experiment with no NTA, k was 0.108 hr^{-1}, and is defined here as the 100% rate of growth. Other values shown represent percentages of this value. All experiments were performed using growth medium containing 2.1×10^{-5} M inorganic phosphate.

	Molarity of NTA X 10^3			
	0	1.6	3.2	6.4
Without Pb^{++}	100	100	96	78
With [NTA]/[Pb^{++}] = 2.0	100	105	95	79

DISCUSSION

The results presented above indicate that Pb^{2+} prevents the growth of *Chlamydomonas* in liquid culture when the equivalents of Pb^{2+} exceed the equivalents of phosphate in the growth media. The low solubility of $Pb_3(PO_4)_2$ and the measured low phosphate concentrations in growth media containing Pb^{2+} suggest two possible explanations. The cells may not be able to sequester enough phosphate from media containing excess Pb^{2+} because the Pb^{2+} effectively removes virtually all free phosphate from solution. The presence of phosphate in solution could provide an effective means of keeping the Pb^{2+} level below the lethal concentration until the available phosphate has all precipitated; excess Pb^{2+} would then remain in solution, increasing the concentration to a lethal value. The available evidence appears to support the former hypothesis, as summarized below.

a. Increasing the Pb^{2+}/phosphate equivalency ratio from 0.95 to 1.05 considerably decreases growth rate although ratios less than 0.95 do not decrease cell growth rate or maximum culture density attainable (Table II and Fig. 1). An increase in Pb^{2+}/phosphate equivalency from 0.95 to 1.05 hardly increases the concentration of Pb^{2+} which occurs in solution (Table III). Decreased cell growth is difficult to account for by an increase in Pb^{2+} concentration exposed to the cells. The concentration of phosphate in solution when equivalent Pb^{2+} and phosphate are present is very low (3×10^{-6} M, Table III), and becomes even lower ($<3.0 \times 10^{-7}$ M) when the viable cells are inoculated into the flask. This very low concentration of phosphate is less than cells normally need for optimum growth in standard media containing no Pb^{2+} (Table IV). The low phosphate concentration is probably exaggerated due to cells taking up virtually all available phosphate. This is also consistent with the observation that in the presence of equivalent Pb^{2+} and phosphate, the Pb^{2+} concentration in solution rises following inoculation of flasks with viable cells (experiments 3 and 5 in Table III). The removal of phosphate would drive more Pb^{2+} into solution by effectively depleting the counterion since all other Pb^{2+} salts are more soluble than $Pb_3(PO_4)_2$.

The decreasing availability of phosphate could account for decreased culture growth as the amount of Pb^{2+} is changed from 0.95 to 1.05 of the equivalents of phosphate, although the concentration of phosphate in solution was below the level of detection. One might expect a significant change in phosphate concentration over this equivalency range since the phosphate concentration is decreased by at least a factor of 9 (Table IV) as the Pb^{2+}/phosphate equivalency ratio increases by approximately a factor of 2 from 0.5 to 0.95. A further 10% increase in this ratio should therefore further decrease the phosphate concentration significantly.

b. EDTA is extensively used in algal culture media to prevent precipitation of essential minerals. The EDTA chelate of Pb^{2+} is exceedingly stable ($\log K_a = 18.2$) (8), and equivalent amounts of EDTA and Pb^{2+} prevent precipitation. However, 1.6×10^{-3} M EDTA was toxic to *Chlamydomonas*. The NTA-lead chelate is much less stable ($\log K_a = 11.8$), but at a NTA-Pb^{2+} concentration ratio of 2:1, no precipitation occurred in phosphate-containing media. Furthermore, NTA was not as toxic to cells as EDTA. The Pb^{2+} in the presence of NTA (Table V) indicated no toxicity at any concentration tested since decreased growth at high concentration of Pb^{2+}-NTA was not greater than in the presence of NTA alone. While chelating agents permit an increase in total solubility of lead salts in phosphate-containing media, the concentration of free Pb^{2+} is still subject to the equilibrium given by:

$$\frac{[Pb_3(PO_4)_2]}{[Pb^{2+}]^3 [PO_4^{3-}]^2}$$

[1] Abbreviation: NTA: nitrilotriacetic acid.

so that it is effectively impossible to increase the concentration of free Pb^{2+} in phosphate-containing media at neutral pH.

c. That cells grow well on $Pb_3(PO_4)_2$ precipitate indicates that they are growing where Pb^{2+} concentration is highest. The reason why dilute cell cultures adhere exclusively to this surface is unknown, but this is where the phosphate concentration is also highest in the flask, which should confer a selective advantage for cell growth in a phosphate-deficient media. The adhesion of cells to the insoluble precipitate was not attributable to a specific effect of PP^{2+}. When all of the phosphate of the media was added as the insoluble $Ca_3(PO_4)_2$ in the absence of Pb^{2+}, cells initially grew slowly on the precipitate, but eventually began accumulating in free suspension and growing exponentially. The appearance of the culture flasks and growth rates in the presence of $Ca_3(PO_4)_2$ were very similar to those in $Pb_3(PO_4)_2$.

These results suggest that the effect of Pb^{2+} on algal growth is an indirect one, whereby $Pb_3(PO_4)_2$ precipitation induces a phosphate deficiency. The possibility of a threshold effect of Pb^{2+} occurring at an extremely narrow concentration range cannot be completely excluded, but the data indicate that possibility is unlikely. Previous observations have been reported which have shown that the concentration of available phosphate and other minerals has a significant effect on the sensitivity of plants to Pb^{2+} (3, 7, 16). A recent report has specifically dealt with the problem of $Pb_3(PO_4)_2$ insolubility in algal culture media (9). That study notes the effect of Pb^{2+} on the very limited growth of green algae in phosphate-free media. In other experiments glycerol phosphate was substituted for Pi to avoid precipitation of $Pb_3(PO_4)_2$. The results of adding Pb^{2+} to phosphate-free media were similar to observations reported here. Addition of Pb^{2+} to glycerol phosphate medium decreased growth in liquid cultures and on agar plates, indicating a direct toxic effect of Pb^{2+}.

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