Mitochondrial Oxidative Phosphorylation: Interaction of Lead and Inorganic Phosphate[1] (35252)

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Several of the physiological and biochemical defects observed in the liver, kidney, and erythropoietic tissue of lead poisoned animals have been associated with altered mitochondrial structure and function (1–3). These observations suggested a direct effect of lead on the functional integrity of mitochondria. Reticulocytes incubated with 100 μM lead acetate (PbAc) for 3 hr were found to have a significant inhibition in O_2 uptake. Exposure of actively respiring reticulocyte mitochondria to the same concentration of lead caused an immediate drop in oxidative activity (4, 5). Our preliminary studies with beef heart mitochondria indicated that the presence of inorganic phosphate in the incubation medium interfered with the interaction of lead with the mitochondrial membrane (6, 7). These findings, and the scarcity of information on the specific effects of lead on mitochondrial respiration, prompted an investigation of the dose–response relationship between lead concentration and mitochondrial oxidative phosphorylation in the presence and absence of exogenous inorganic phosphate.

Materials and Methods. Beef heart mitochondria were prepared by Nagarse digestion in a solution containing 250 mM sucrose with 1.0 mM ethyleneglycol-bis(*beta*-aminoethyl ether)-*N*,*N*′tetraacetic acid (EGTA), and buffered at pH 7.0 with tris(hydroxymethyl)aminomethane (Tris) (8). The mitochondrial samples were washed twice with Tris–sucrose solution to avert chelation of EGTA with the lead ion. The incubation medium utilized for the respiratory measurements contained 250 mM sucrose, 10 mM K_2HPO_4 (P_i), and 10 mM sodium succinate as substrate, adjusted to pH 7.4 with Tris–chloride (Tris–Cl). Oxygen uptake was recorded with a Yellow Springs Instrument Company Model 53 oxygen electrode system in 4 ml of air-equilibrated medium (9, 10). Respiratory measurements were made at 25°, immediately on addition of 2.5 mg of mitochondrial protein to the reaction chamber. The initial slopes of the oxygen electrode tracing were used to determine control values for succinate oxidation (State 4), adenosine diphosphate-stimulated respiration (State 3), and adenosine diphosphate to oxygen (ADP:O) ratios. State 3 respiration was initiated by addition of 0.5 μmole of ADP to the mitochondria respiring in the succinate medium. The respiratory control ratio (RCR) was calculated as State 3/State 4. Respiratory control ratios were used as a measure of the functional integrity of the mitochondria, and ADP:O ratios as an index of phosphorylative capacity (11). Control values were compared with the respiration of the same mitochondrial sample after exposure to 1 to 100 μM PbAc. Additions of ADP and PbAc to the reaction chamber were made in 10 μl quantities with a Hamilton constant rate CR 700 syringe. Mitochondrial respiration was again measured in the same medium as described, except that exogenous inorganic phosphate was omitted. During the course of respiratory measurements, lead acetate, inorganic phosphate, and adenosine diphosphate were introduced into the reaction chamber in the following order: P_i, PbAc, ADP; and PbAc, P_i,

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TABLE I. Heart Mitochondrial Oxidative Phosphorylation as a Function of Lead Acetate (PbAc) Concentration.

Mitochondria (2.5 mg of protein) were suspended in 250 m*M* sucrose, 10 m*M* K_2HPO_4, and 10 m*M* sodium succinate (pH 7.4 with Tris–Cl). Succinate oxidation (State 4) and ADP-stimulated respiration (State 3) were determined (μg-atoms O_2/mg of protein/hr at 25°). Total incubation period was 6–8 min. Values indicate the mean of 14 determinations.

	Respiration rate							
	State 4		State 3		ADP/O ratio		Respiratory control ratio	
PbAc (μM)	Control	PbAc	Control	PbAc	Control	PbAc	Control	PbAc
1	4.71	4.73	13.6	13.7	1.12	1.10	3.44	3.50
5	4.28	3.86[a]	13.0	12.9	1.17	1.17	3.14	3.07
10	4.75	4.05[a]	12.0	10.6[a]	2.45	2.24	2.89	2.62
50	5.02	4.35[a]	14.4	12.5[a]	1.62	1.60	2.83	2.62
100	5.10	4.50[a]	14.9	13.5[a]	1.87	1.83	2.93	2.81

[a] Significantly different from control $p < 0.01$ by Student's t test.

ADP. To study the effects of lead on uncoupled mitochondria, dinitrophenol-stimulated respiration was measured in an incubation medium (pH 7.4) containing 250 m*M* sucrose, 10 m*M* sodium succinate, and 0.1 m*M* 2,4-dinitrophenol (DNP). No exogenous inorganic phosphate was added to this system, but uncoupled mitochondria were exposed to 0.01 to 100 μM PbAc.

Results. Comparison of control and lead-treated mitochondria during State 4 respiration indicated that 5 to 100 μM PbAc inhibited oxidative activity only 10 to 15 % (Table I). There was some inhibition of State 3 respiration on exposure to 10, 50, or 100 μM PbAc, but little or no change in State 3 respiration was observed with lower PbAc concentrations. We found that lead had no significant effect on the ADP:O ratios, and that 5 to 100 μM PbAc lowered RCR values only slightly. Goyer *et al.* (2) have reported large changes in the O_2 uptake, RCR, and ADP:O ratio of kidney mitochondria isolated from lead treated rats. Thus, the small magnitude of respiratory inhibition by lead, which we found, and the absence of significant changes in RCR and ADP:O ratios (Table I) in these tightly coupled beef heart mitochondria were unexpected findings (6).

It was apparent that some component in our incubation medium was reducing the inhibitory effect of lead ion. In the absence of exogenous P_i, 2.5 μM PbAc produced a 15% inhibition of State 4 respiration; 50 μM PbAc caused a 55% inhibition and 200 μM PbAc completely abolished mitochondrial oxygen consumption (Fig. 1a). Addition of 10 μmoles of P_i to mitochondria exposed to 2.5 to 50 μM PbAc caused respiration to return to approximately control State 4 values; O_2 uptake of mitochondria exposed to 100 μM PbAc increased to only about 50% of controls. When respiration had been completely abolished by 200 μM PbAc the addition of 10 μmoles of P_i produced only a slight increase in O_2 uptake. Comparable responses were obtained when the chelating agent, EDTA (0.5 μmoles), was added instead of P_i. ADP had no stimulatory effect on the respiration of mitochondria treated with 200 μM PbAc. Mitochondria exposed to 100 μM PbAc showed a depressed rate of State 3 respiration and a slightly lower ADP:O ratio as compared to controls. Lower lead concentrations (2.5 to 50 μM) had little or no effect on State 3 respiration, ADP:O, or RCR ratios.

It was evident that exogenous P_i added to the mitochondrial suspension prior to the addition of PbAc reduced the inhibitory effects of lead (Fig. 1b). Since the presence of P_i interfered with the action of lead ion on mitochondria, attempts to interpret the effect of lead by studies of ADP:O ratios could be misleading. Based on these observations, the effect of lead was studied with mitochondria uncoupled by the addition of DNP.

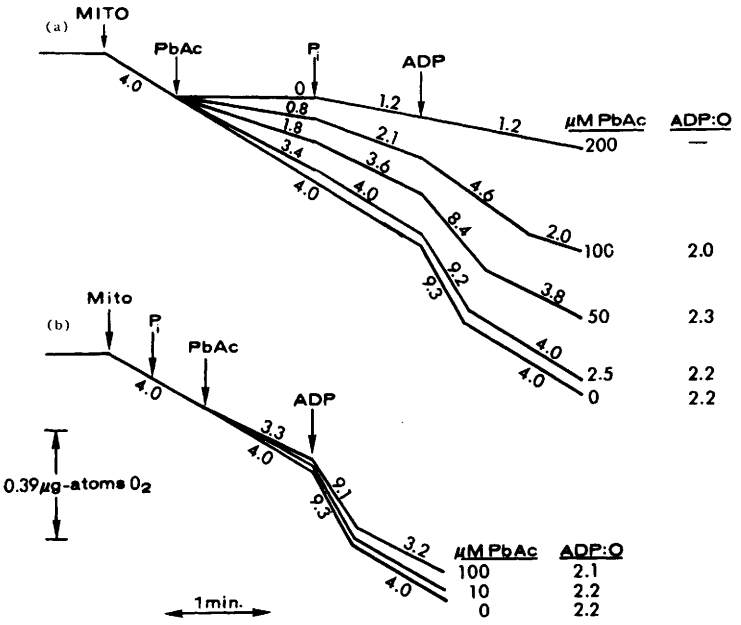

FIG. 1. Effect of lead acetate (PbAc) on mitochrondrial respiration when PbAc was added (a) before, and (b) after the addition of inorganic phosphate (P_i). Beef heart mitochondria (2.5 mg of protein) were suspended in 250 m*M* sucrose and 10 m*M* sodium succinate solution; pH 7.4 with Tris–Cl. The mean oxygen uptake of three measurements is indicated on the tracings μg-atoms O_2/mg of protein/hr at 25°). Addition of 0.5 μmole of adenosine diphosphate (ADP), 10 μmoles of P_i, and PbAc is indicated by arrows. PbAc concentrations and ADP:O ratios are listed at the right of each tracing.

Measurement of control DNP-stimulated respiration during the first 2 min averaged about 5.5 μg-atoms O_2/mg of protein/hr (Fig. 2). In the absence of PbAc, or in the presence of very low concentrations of PbAc (0.01 to 0.05 μ*M*), DNP-stimulated respiration increased approximately 6% during the third and fourth min of measurement. PbAc concentrations of 0.1 and 0.5 μ*M* caused little change from control respiratory values, but higher PbAc concentrations produced a significant depression of mitochondrial respiration. DNP-stimulated respiration fell to approximately State 4 levels when exposed to 5 or 10 μ*M* PbAc, and dropped below State 4 values when 50 or 100 μ*M* PbAc was added to the incubation mixture. A semilog plot of the inhibition of DNP-stimulated mitochondrial respiration and the PbAc concentration indicates an exponential relationship which was not evident in our oxidative phosphorylation studies with coupled mitochondria (Fig. 2).

To demonstrate that inorganic phosphate had the ability to reduce the respiratory in-

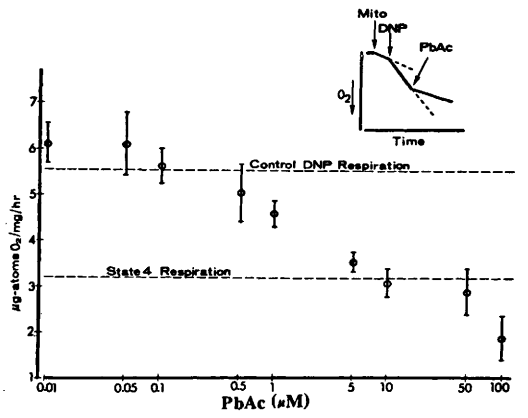

FIG. 2. Dinitrophenol (DNP)-stimulated respiration versus lead acetate (PbAc) concentration. Mitochondria (2.5 mg of protein) were suspended in a medium containing 250 m*M* sucrose and 10 m*M* sodium succinate, at pH 7.4 and 25°. Oxygen uptake (ordinate) was stimulated with 0.1 m*M* DNP. PbAc concentrations are plotted along the abscissa on a semilog scale. Each point (O) represents the mean of six determinations of DNP respiration with PbAc; (---) succinate oxidation (State 4) without lead; bars indicate ± standard error; insert (top, right) shows a typical oxygen electrode tracing.

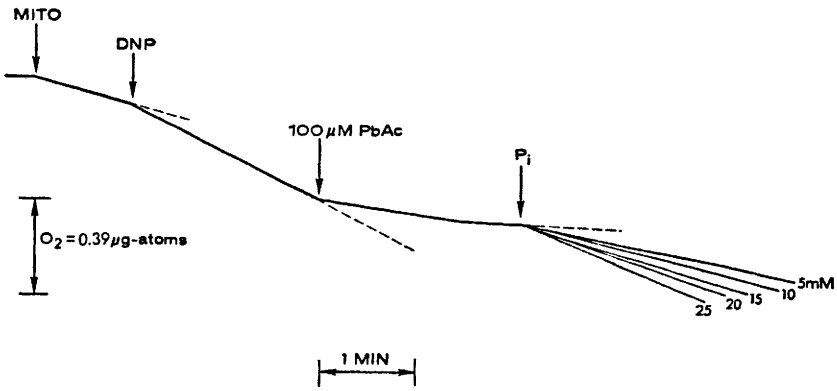

FIG. 3. Diminution of lead-inhibited, dinitrophenol-stimulated respiration of beef heart mitochondria by addition of 5 to 25 m*M* K_2HPO_4 (P_i). Mitochondria (2.5 mg of protein) were stimulated with 2,4-dinitrophenol (0.1 m*M* DNP); P_i was adjusted to pH 7.4 with Tris–Cl prior to addition. Shown schematically, are the results of five determinations at pH 7.4 and 25°.

hibition caused by lead, DNP-stimulated mitochondria were first inhibited with 100 μM PbAc, and then titrated with increasing concentrations of inorganic phosphate (Fig. 3). Addition of 5 m*M* K_2HPO_4 produced a slight reduction of the lead-induced respiratory inhibition. Inhibition of the DNP-stimulated respiration was diminished further by subsequent increases in exogenous P_i concentration (10 to 20 m*M*). A P_i concentration of 25 m*M* K_2HPO_4 was able to completely abolish the respiratory inhibition caused by 100 μM PbAc.

Discussion. This investigation, and the recent report by Koeppe and Miller (12) confirms our earlier observation (5, 6) that exogenous P_i has a protective effect on the susceptibility of mitochondria to lead. In our study, mitochondria exposed to relatively high lead levels (50 to 100 μM PbAc), *in vitro*, showed little or no alteration in function, if exogenous inorganic phosphate was present before exposure to lead. In the absence of exogenous P_i, however, even low PbAc concentrations (2.5 to 50 μM) caused a marked inhibition of succinate oxidation, and ADP-stimulated respiration. The addition of P_i to mitochondria whose respiration was inhibited with lead (2.5 to 100 μM PbAc), partially released the respiratory inhibition; this inhibition was not reversible, however, when higher lead levels were used (200 μM). Although 100 μM PbAc inhibited mitochondrial succinate oxidation, we found that the same lead concentration had little effect on oxygen uptake when *N,N,N',N'*-tetramethylphenylenediamine (TMPD)-ascorbate was used as substrate. Since TMPD-ascorbate is oxidized by the cytochrome oxidase system of mitochondria, the absence of an inhibitory effect by lead suggests that this is not a primary site of lead action.

Our data indicate that inorganic phosphate probably combines with lead ion and thereby prevents lead interaction with the mitochondrial membrane. Apparently, the association of lead ion with P_i is much stronger than the interaction of lead with its site of action on mitochondria. Thus, the predominant effects of lead on mitochondria will be determined, in part, by the composition of the suspending medium, especially the anionic species. In most tissues the intracellular concentration of P_i is probably sufficient to thwart the effects of relatively low concentrations of lead on mitochondria. However, we found that once the lead ion interacted with the coupled mitochondria, lead's deleterious effects on respiration were not completely reversible. In uncoupled (DNP-stimulated) mitochondria addition of P_i could reverse the respiratory inhibition, but this required relatively high P_i concentrations. In all cases the extent of lead action was concentration dependent. Similar findings of decreased respiration and uncoupled oxidative phosphorylation, in liver mitochondria (1) and kidney mitochondria (2) isolated from lead poisoned animals, have

been reported.

Unpublished studies by Hwang, Scott, and Brierley, have recently established that lead, like zinc, mercurials, and certain other heavy metals (13–15), induces energy-linked accumulation of potassium acetate and passive osmotic swelling of heart mitochondria in isotonic potassium chloride. It should be noted that the effects of zinc on isolated mitochondria are enhanced by the presence of P_i, even though zinc phosphate salts form under these conditions (14). The zinc phosphate appears to be adsorbed by the mitochondria in a form which permits rapid interaction with binding sites in the membrane. The present results clearly establish that lead phosphate is not able to fulfill this role, *in vitro*.

These observations may have physiological relevance to the mode and time course of lead poisoning in man. Apparently, inhibition of mitochondrial respiration requires relatively high concentrations of lead, or conditions which can increase free ionic lead, such as decreased exogenous inorganic phosphate concentration.

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