Inhibition of Carbon Dioxide Fixation by Lead Acetate in Rat Liver Mitochondria

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These studies were undertaken to determine the mechanism by which intravenously administered lead salts inhibit hepatic gluconeogenesis. Within 1 h after the intravenous administration of lead acetate (10 mg), there is 97% inhibition of CO₂ fixation in isolated rat liver mitochondria. This effect is concentration-dependent. The induction of phosphoenolpyruvate carboxykinase activity observed with starvation was also inhibited by intravenously administered lead acetate, but the activities of pyruvate kinase, glucose 6-phosphate dehydrogenase and pyruvate carboxylase were unaffected, as was the oxidation of palmitate and palmitoyl-CoA by mitochondria from Pb²⁺-treated animals. The addition of reduced glutathione to mitochondria from Pb²⁺-treated animals had no effect on the inhibited CO₂ fixation. ATP concentrations in mitochondria from Pb²⁺-treated animals are decreased and the dose-response relationships for the effect of Pb²⁺ on CO₂ fixation and ATP concentrations correspond. We conclude that the decrease in mitochondrial ATP in Pb²⁺-treated animals is probably responsible for the marked inhibition of CO₂ fixation, and hence the impairment of gluconeogenesis from alanine, lactate and pyruvate observed by others.

Lead salts induce several biochemical and morphological hepatic alterations, including the inhibition of several hepatic enzymes (Waltrach, 1964; Teras & Kakhn, 1967; Sroczynski *et al.*, 1968; Vallee & Ulmer, 1972; Walton, 1973). Studies have indicated that the intravenous injection of lead acetate inhibits hepatic gluconeogenesis (Filkins, 1973; Cornell & Filkins, 1974), as well as the starvation-induced increases in the activity of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (Rippe & Berry, 1973). Cornell & Filkins (1974) have demonstrated in isolated hepatocytes that the administration of lead acetate induces the greatest impairment in gluconeogenesis with alanine, lactate and pyruvate as substrates, with less inhibition from fructose. The present studies were undertaken to assess the site or sites of inhibition of these early steps in gluconeogenesis by lead salts. We have therefore evaluated two obligatory steps in gluconeogenesis from alanine, pyruvate and lactate to yield phosphoenolpyruvate: that of CO₂ fixation by pyruvate in rat liver mitochondria and the reversible conversion of the product oxaloacetate into phosphoenolpyruvate by cytosolic phosphoenolpyruvate carboxykinase.

Experimental

Male Sprague-Dawley rats (200 g) fed *ad libitum* were anaesthetized with diethyl ether and injected via the femoral vein with lead acetate or equimolar

quantities of acetate as sodium acetate. The rats were thereafter deprived of food and at the specified times were anaesthetized with ether and the livers perfused via the portal vein with 20 ml of 0.154 M-NaCl. Rats were killed and the livers removed, then minced and homogenized in a solution containing D-mannitol (220 mM), sucrose (70 mM) and Hepes* (2 mM), pH 7.4. Mitochondria were prepared by a modification of the method of Schnaitman & Greenawalt (1968) as previously described (Amatruda *et al.*, 1975). All cytosolic enzyme analyses were performed on the supernatant fraction of a 15 min centrifugation at 4°C and 27000g. CO₂ fixation in isolated mitochondria and phosphoenolpyruvate carboxykinase activity were assayed immediately, and the remainder of the 27000g supernatant fraction was frozen at -20°C for assay of pyruvate kinase (EC 2.7.1.40) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity within 1 week, during which time there was no loss of activity. Mitochondrial CO₂ fixation was assayed by the method of Adam & Haynes (1969) and phosphoenolpyruvate carboxykinase activity by the method of Chang & Lane (1966). Pyruvate carboxylase (EC 6.4.1.1) activity was measured as described by Adam & Haynes (1969), after the disruption of mitochondria with a Polytron homo-

* Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; ATPase, adenosine triphosphatase (EC 3.6.1.3); Bistris, 2-[bis-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol.

genizer (PT-10) at maximum speed for 4×15 s homogenizations. Glucose 6-phosphate dehydrogenase activity was assayed as described by Glock & McLean (1953) as modified by Yagil *et al.* (1974), except that all assays were done in the presence of 0.1M-MgCl₂. Pyruvate kinase was assayed at 366nm and 30°C as described by Weber *et al.* (1965). Fatty acid oxidation with [1-¹⁴C]palmitate and [1-¹⁴C]-palmitoyl-CoA as substrates was measured as water-soluble material as previously described (Amatruda *et al.*, 1975). Mitochondrial ATP concentration was measured on neutralized HClO₄ extracts of mitochondria isolated from sodium acetate and lead acetate-treated rats in a Zeiss PMQ II spectrophotometer by the hexokinase method as described by Estabrook *et al.* (1967). Mitochondrial ATPase (EC 3.6.1.3) activity was measured by titration with 1mM-NaOH at 30°C by using a pH-stat method (Chance & Nishimura, 1967). For this assay mitochondria were suspended in a solution containing D-mannitol (220mM) and sucrose (70mM). The reaction mixture contained 2.5mM-ATP, 3mM-MgCl₂ and 0.1–1.0mg of mitochondrial protein in a final volume of 2.0ml. The hydrolysis of ATP was measured as H⁺ production by maintaining the reaction mixture at a constant pH of 8.0 after the addition of ATP. The reaction was carried out in a London Radiometer Recording Titration System operated in the pH-stat mode (The London Co., Copenhagen, Denmark). Protein was measured by the method of Jacobs *et al.* (1956).

[1-¹⁴C]Palmitate (54mCi/mmol), [1-¹⁴C]palmitoyl-CoA (56mCi/mmol) and NaH¹⁴CO₃ (50mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Acetyl phosphate and phosphotransacetylase (EC 2.3.1.8) were purchased from Sigma, St. Louis, MO, U.S.A., reduced glutathione was from Boehringer Mannheim, Indianapolis, IN, U.S.A., and ATP, Bistris and D-L-carnitine were from Aldrich, Milwaukee, WI, U.S.A. Lead acetate was purchased from Fischer Scientific Co., Pittsburgh, PA, U.S.A. All other reagents, enzymes and cofactors were purchased from Calbiochem, San Diego, CA, U.S.A.

Results and Discussion

Animals treated with 10mg of lead acetate/rat intravenously exhibit profound and prolonged suppression of CO₂ fixation by isolated mitochondria (Fig. 1). At 1h after Pb²⁺ administration there is greater than 97% inhibition of CO₂ fixation. In mitochondria isolated 4h after Pb²⁺ administration there is 79% inhibition of CO₂ fixation compared with control mitochondria. CO₂ fixation in mitochondria from control animals is enhanced after 24h of starvation and at this time there is 88% inhibition of CO₂ fixation in mitochondria from Pb²⁺-treated

Fig. 1. Inhibition of CO₂ fixation in isolated liver mitochondria from rats injected intravenously with lead acetate. Lead-treated rats (○) were injected with 10mg of lead acetate via the femoral vein, and control (●) animals with an equimolar amount of acetate as sodium acetate. At the times indicated the rats were killed and CO₂ fixation was assessed as described in the Experimental section. The values represent the mean $\pm$ S.E.M. of duplicate determinations from four to seven separate experiments at each time indicated.

Fig. 2. Dose-dependence of the effects of intravenously administered lead acetate on CO₂ fixation in rat liver mitochondria.

Rats were injected via the femoral vein with the indicated amount of lead acetate (○) or an equimolar amount of acetate as sodium acetate (●) and killed 1h after injection. CO₂ fixation was assessed as described in the Experimental section. The values represent the mean $\pm$ S.E.M. of duplicate determinations from four separate experiments at each concentration.

animals. This effect is concentration-dependent (Fig. 2). In mitochondria isolated 1h after the intravenous administration of 0.1mg or 1mg of lead acetate/rat, there is no inhibition of CO₂ fixation. A 5mg dose of lead acetate, however, inhibits CO₂ fixation by 77%. As shown above, when given to four additional animals 10mg of lead acetate again

Table 1. *Phosphoenolpyruvate carboxykinase, pyruvate kinase and glucose 6-phosphate dehydrogenase activity after intravenous administration of lead acetate*

Animals were injected intravenously with lead acetate (10 mg/rat) or the molar equivalent of acetate as sodium acetate, killed at the indicated times and the activities of phosphoenolpyruvate carboxykinase, pyruvate kinase and glucose 6-phosphate dehydrogenase were determined as described in the Experimental section. The values represent the mean $\pm$ S.E.M. of duplicate determinations from three (pyruvate kinase) or four (phosphoenolpyruvate carboxykinase and glucose 6-phosphate dehydrogenase) separate experiments.

Time after lead acetate (h)	Lead acetate (mg/rat)	Phosphoenolpyruvate carboxykinase activity (nmol of HCO ₃ ⁻ /min per mg of cytosolic protein)		Pyruvate kinase activity (nmol of NADH/ min per mg of cytosolic protein)		Glucose 6-phosphate dehydrogenase activity (nmol of NADP ⁺ /min per mg of cytosolic protein)		
		...	0	10	0	10	0	10
0			14.2 ± 2.3	—	240 ± 11	—	7.0 ± 1.1	—
1			11.6 ± 1.7	13.7 ± 2.3	285 ± 43	227 ± 12	8.4 ± 1.8	6.1 ± 0.8
4			15.8 ± 2.0	15.0 ± 0.9	248 ± 35	229 ± 25	10.9 ± 4.1	8.0 ± 2.2
24			27.2 ± 4.2	17.3 ± 2.0	198 ± 4	203 ± 15	9.0 ± 2.2	10.7 ± 1.3

inhibited CO₂ fixation by 97% (Fig. 2). These data indicate that the earliest step in the gluconeogenesis from pyruvate is progressively inhibited by intravenous lead acetate at doses from 1 to 10 mg per animal.

The second component of the dicarboxylic acid shuttle (Utter, 1963) is that of the synthesis of phosphoenolpyruvate from oxaloacetate by phosphoenolpyruvate carboxykinase. After an intravenous injection of 10 mg of lead acetate there was no effect on phosphoenolpyruvate carboxykinase activity for 4 h (Table 1). As previously reported, however, Pb²⁺ did prevent the induction of phosphoenolpyruvate carboxykinase activity after deprivation of food for 24 h (Rippe & Berry, 1973). In comparison with Figs. 1 and 2, however, it is clear that the inhibition of CO₂ fixation by Pb²⁺ is greater than the inhibition of phosphoenolpyruvate carboxykinase activity and thus may represent the primary site for the inhibition of production of phosphoenolpyruvate from pyruvate in Pb²⁺-treated animals.

Decreased phosphoenolpyruvate formation from pyruvate could result from an enhancement of the reverse reaction, which is catalysed by pyruvate kinase. The activity of this enzyme, however, is unaffected by Pb²⁺ (Table 1). As previously demonstrated (Krebs & Eggleston, 1965), we observed starvation-induced decreases in pyruvate kinase activity after 24 h of food deprivation. This decrease in activity of pyruvate kinase in conjunction with the enhanced activities of phosphoenolpyruvate carboxykinase and CO₂ fixation will increase the flux of pyruvate to phosphoenolpyruvate with starvation. Pb²⁺ had no effect on the starvation-induced changes in pyruvate kinase activity.

Glucose 6-phosphate dehydrogenase activity (Table 1) was also investigated to establish the specificity of the observed Pb²⁺ effects. However, lead acetate

injected *in vivo* had no significant effects on the activity of this enzyme.

In a series of experiments in which lead acetate injected *in vivo* inhibited mitochondrial CO₂ fixation by 77% (results not shown), fatty acid oxidation in the same mitochondrial preparations in the presence of ATP was unchanged from control values. This was investigated with both palmitate and palmitoyl-CoA as substrates because of possible effects of Pb²⁺ on their interconversion. Rat liver mitochondria were isolated 1 h after the intravenous injection of 5 mg of lead acetate per rat or an equimolar concentration of acetate as sodium acetate (controls). Fatty acid oxidation from 0.4 μ g of [1-¹⁴C]palmitate/ml and 0.5 μ g of [1-¹⁴C]palmitoyl-CoA/ml was measured as previously described (Amatruda *et al.*, 1975). Under these conditions mitochondria from control animals oxidize 9.0 $\pm$ 1.5 ng of [1-¹⁴C]palmitate and 22.3 $\pm$ 1.7 ng of [1-¹⁴C]palmitoyl-CoA per 5 min per mg of mitochondrial protein. Mitochondria from Pb²⁺-treated animals oxidize 10.3 $\pm$ 3.5 ng of [1-¹⁴C]palmitate and 25.9 $\pm$ 6.6 ng of [1-¹⁴C]palmitoyl-CoA per 5 min per mg of mitochondrial protein. The values represent the mean $\pm$ S.E.M. of duplicate determinations from three separate experiments. These data again point to an apparent specificity of the Pb²⁺ on CO₂ fixation.

To determine if the inhibition of CO₂ fixation by Pb²⁺ is due to direct inhibition of pyruvate carboxylase activity, we evaluated pyruvate carboxylase activity in addition to CO₂ fixation in parallel experiments with the same mitochondrial preparations. At 1 h after the intravenous injection of 10 mg of lead acetate, CO₂ fixation was inhibited by 100, 98 and 100% in three separate experiments, but there was no inhibition of pyruvate carboxylase activity in two of these studies and only 8.9% inhibition in the third. The addition of reduced glutathione (1 mM),

an agent that binds Pb^{2+} , to mitochondria from the Pb^{2+} -treated animals had no effect on the inhibition of CO_2 fixation by lead acetate injected *in vivo*.

Intramitochondrial ATP is required for gluconeogenesis from C_3 precursors. Although the addition of ATP to intact mitochondria from Pb^{2+} -treated animals had no effect on the inhibition of CO_2 fixation, the ATP concentrations in mitochondria from Pb^{2+} -treated rats were decreased in a dose-dependent fashion (Table 2). Animals treated with 10 mg of lead acetate demonstrated an 84% decrease in mitochondrial ATP content. Further, the dose-response relationships for the effect of Pb^{2+} on CO_2 fixation and on ATP concentrations correspond (Fig. 3). Such changes in intramitochondrial ATP could explain the impaired gluconeogenesis previously observed both *in vivo* and in isolated hepatocytes after intravenous administration of lead acetate (Filkins, 1973; Cornell & Filkins, 1974). A close relationship between ATP concentrations and gluconeogenesis from lactate was observed by Wilkening *et al.* (1975) in the isolated perfused liver using ethionine to lower intrahepatic ATP concentrations. These effects of ethionine on inhibition of gluconeogenesis might also be exerted at the pyruvate-carboxylation step by virtue of its ability to decrease intrahepatic ATP.

Decreased cytochrome content of kidney mitochondria from Pb^{2+} -poisoned rats (Rhyne & Goyer, 1971), as well as a decrease in the microsomal cyto-

Fig. 3. Decreased CO_2 fixation ($\circ$) and ATP concentration ($\bullet$) in isolated rat liver mitochondria from animals treated with lead acetate

The values for CO_2 fixation are the same as those reported in Fig. 2 and those for ATP concentration are plotted from the data in Table 3.

Table 3. ATPase activity in mitochondria from rats treated with lead acetate

Animals were injected via the femoral vein with lead acetate (10 mg) or its molar equivalent of sodium acetate (control). After 1 h the animals were killed and liver mitochondria prepared and assayed for ATPase activity as described in the Experimental section. Mitochondria were disrupted as described in the legend to Table 2. This experiment is representative of five similar experiments.

Table 2. Effect of lead acetate on mitochondrial ATP concentration

Animals were killed 1 h after receiving intravenous lead acetate at the indicated dosage or an equimolar concentration of acetate as sodium acetate. Mitochondria were isolated as described in the Experimental section and disrupted with a Polytron homogenizer (PT-10) at maximum speed for 4×15 s homogenizations. Mitochondrial ATP concentration was then measured on neutralized $HClO_4$ extracts as described in the Experimental section. Since the intravenous injection of sodium acetate had no effect on mitochondrial ATP concentration, the ATP concentration of mitochondria from animals not treated with lead represents the mean $\pm$ S.E.M. of all rats treated with sodium acetate. The values represent the mean $\pm$ S.E.M. of duplicate determinations of four to six separate experiments at each concentration.

Dose of lead acetate (mg/rat)	ATP concn. (nmol/mg of mitochondrial protein)
0	1.03 ± 0.08
1	1.07 ± 0.23
2	0.99 ± 0.20
5	0.29 ± 0.04
10	0.16 ± 0.04

Mitochondrial preparation	ATPase activity (nmol of H^+ released/min per mg of mitochondrial protein)	
	Control	Lead acetate
Intact	10.0	18.9
Intact + 2,4-dinitrophenol (0.3 mM)	52.9	71.5
Disrupted	34.2	51.9
Disrupted + 2,4-dinitrophenol (0.3 mM)	90	371.0

chrome *P*-450 content and activity 24 h after intravenous administration of Pb^{2+} salts (Scoppa *et al.*, 1973; Alvares *et al.*, 1972) point to haemoproteins as a locus of Pb^{2+} toxicity. The decrease in ATP concentration within 1 h of lead acetate administration (Table 2), however, renders it unlikely that a decrease in cytochrome content could explain our findings. Further, the demonstration that fatty acid oxidation is unimpaired in mitochondria from Pb^{2+} -treated animals renders it also unlikely that a functional impairment in mitochondrial haemoproteins exists. This is confirmed by our observation that with succinate as substrate there is no difference in the

ADP/O ratio or the respiratory-control ratio in mitochondria isolated 1 h after the intravenous injection of 10 mg of lead acetate as compared with mitochondria isolated 1 h after the intravenous injection of equimolar quantities of sodium acetate. ADP/O ratios are 1.8 ± 0.1 , and respiratory-control ratios are 3–7 in these mitochondria. This is in contrast with the findings of Teras & Kakhn (1967), who observed decreased oxygen consumption and oxidative phosphorylation in rabbit mitochondria 2 h after the intravenous administration of lead acetate.

These studies lead us to conclude that the inhibition of gluconeogenesis by Pb²⁺ occurs at the level of CO₂ fixation in mitochondria through a dose-related decrease in mitochondrial ATP concentrations. The specific mechanism by which lead acetate administration decreases mitochondrial ATP is as yet unknown. We have investigated the possibility that mitochondrial ATPase activity may be affected by lead. The ATPase activity of mitochondria is believed to represent reversal of the reaction that normally synthesizes ATP from ADP and P_i. We therefore decided to examine ATPase activity in mitochondria with low ATP contents from Pb²⁺-treated animals. As illustrated in Table 3, ATPase activity in intact mitochondria from Pb²⁺-treated rats is increased almost twofold over that of mitochondria from control animals. Similarly, ATPase activity is enhanced in disrupted mitochondria from Pb²⁺-treated animals. In the presence of 2,4-dinitrophenol both intact and disrupted mitochondria from Pb²⁺-treated animals exhibit enhanced ATPase activity. This enhancement is additive to that of a maximally effective concentration of 2,4-dinitrophenol (Myers & Slater, 1957). It is therefore likely that ATPase activity in whole and disrupted mitochondria from Pb²⁺-treated animals is enhanced by a mechanism different from that of 2,4-dinitrophenol. Further, a direct effect of Pb²⁺ on ATPase activity cannot be excluded. Whether this enhancement of ATPase activity is related to the decreased ATP concentrations in mitochondria from Pb²⁺-treated animals, however, remains to be demonstrated.

Although we have not investigated other tissues, the present studies may give insight to the mechanism of Pb²⁺ toxicity in other tissues, including the kidney and central nervous system. Multiple effects of Pb²⁺ have been demonstrated in the kidney. These include partial uncoupling of mitochondria and alterations in mitochondrial ultrastructure (Goyer *et al.*, 1968; Goyer & Krall, 1969). A possible

defect in ATP generation could explain these mitochondrial alterations and would lead to renal-tubular dysfunction for processes that are energy-dependent, e.g., transport of amino acids in the proximal tubule.

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